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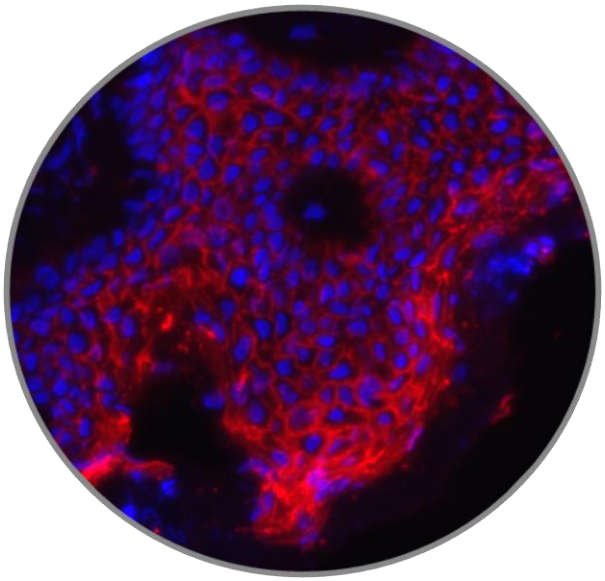

PhD thesis

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Allergic Contact Dermatitis to para-phenylenediamine and the immunology involved

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Allergic contact dermatitis to para-phenylenediamine and the immunology involved

This thesis is the result of a scientific collaboration between

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This PhD thesis is based on the following 2 manuscripts:

The manuscripts will be referred to using their roman numerals throughout the thesis.

I. Skin barrier changes upon exposure to p-phenylenediamine in hair dye exposed individuals with and without development of allergic contact dermatitis

II. Subclinical up-regulation of immune related genes in skin from non-allergic hairdressers exposed to p-Phenylenediamine

Further publications in connection to this thesis:

~ Hand eczema among hairdressing apprentices in Denmark following a nationwide prospective intervention programme: 6-year follow-up.

~ Non-oxidative hair dye products on the European market: What do they contain?

Preface

This thesis was made possible by the financial support from the Danish Hairdressers' and Beauticians' Union, the Danish Hairdresser Association, the Danish Working Environment Research fund and Aage Bangs foundation which is gratefully acknowledge.
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Sanne Steengaard Meisser
Davos, September 2019

Abbreviations

ACD:	Allergic contact dermatitis (ACD)
AD:	Atopic dermatitis
APC:	Antigen presenting cell
AMP:	Antimicrobial protein
AD:	Atopic dermatitis
CLMP:	CAR-like membrane protein
CLDN:	Claudin gene
CTLA4;	Cytotoxic T-lymphocyte-associated protein 4
FLG:	Filaggrin gene
HSA:	Human serum albumin
HSA:	Immunoglobulin
IL:	Interleukin
IFN:	Interferon
iNKT:	Invariant natural killer T cells
ICD:	Irritant contact dermatitis
JAMs:	Junctional adhesion molecules
MHC:	Major histocompatibility complex
NAT1:	N-acetyltransferase 1 gene
NK cells:	Natural killer cells
NGS:	Next generation sequencing
NOD:	Nucleotide-binding oligomerization domain
PPD:	Para-phenylenediamine
PBMCs:	Peripheral Blood Mononuclear Cells
PD-1:	Programmed cell death protein 1
Treg:	Regulatory T-cell
Th1, 2 or 17:	T helper cells type 1, 2 or 3
TJ:	Tight junctions
Trem:	Tissue resident effector memory T cell
TLR:	Toll-like receptor
PTD:	Toluene-2,5-diamine
VEGF:	Vascular epithelial growth factor
ZO:	Zonula occludens protein

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Summaries

Abstract (English)

Background

Western lifestyle has introduced a wide range of chemicals into our everyday, both in private life and in many occupations. Some chemicals can cause contact allergies and it is estimated that 27% of the European population is sensitized to one or more contact allergens. One of the occupations with the greatest occupational exposure to chemicals is hairdressing. It is well-established that hairdressers for this reason have a high occurrence of both allergic and irritant contact dermatitis. An important cause of allergic contact dermatitis (ACD) is para-phenylenediamine (PPD), a black dye often used in permanent hair dyes. PPD is known to be a very strong sensitizer and can be detected in both blood and urine after the use of PPD containing hair dye. It is puzzling that only some and not all hairdressers develop ACD to PPD given they are exposed daily through their work. PPD is a reactive chemical and it is concerning that many are exposed to PPD, while the long-term effect on skin barrier and skin immunity remains largely unknown.

The overall aim of this thesis was to investigate the effects of PPD exposure on the skin barrier and skin immunology in non-allergic hairdressers in comparison to PPD allergic patients.

Methods

The study is based on data from skin biopsies and blood collected from non-allergic hairdressers and PPD allergic patients. The skin biopsies were collected 4 days after patch testing with 1% PPD in vaseline and vaseline alone as a control. The first sets of biopsies were analyzed for RNA expression by new generation sequencing and the last sets of samples were used for protein visualization by confocal microscopy. Blood samples were taken before the PPD patch test and circulating immune cells were investigated by flow cytometry. Effects of PPD exposure on specific skin barrier proteins was investigated further in 3D human skin cultures (Episkin).

Results

PPD caused deregulation of key proteins in the skin barrier in both PPD allergic patients and non-allergic hairdressers (Manuscript I). In non-allergic hairdressers the tight junction proteins claudin-1 and CLMP were down regulated together with the stratum corneum proteins filaggrin 1 and 2. This was further visible for claudin 1and filaggrin 1 and 2 with confocal microscopy. Keratinocyte cultures showed that PPD down regulates Claudin 1, CLMP and filaggrin 2 but not

filaggrin 1. Allergic individuals had significant more damage to the skin barrier than non-allergic individuals, and the level of barrier damage was directly correlated to severity of the clinical symptoms.

In addition, PPD induced low levels of inflammation in non-allergic hairdressers, despite the absence of clinical symptoms (Manuscript II). The inflammation seen in allergic individuals corresponded well to the clinical severity. Blood analysis of circulating T-cells showed no difference between allergic individuals and non-allergic hairdressers before PPD exposure. The inflammation was seen as an increase in T cells present in the skin biopsies and up-regulation of T-cell activation and effector molecules in both groups. Type 1 effector molecules such as granzyme B, perforin, LTA and LTB were especially increased in allergic patients whereas only LTA and LTB were significant in non-allergic hairdressers. In line with cytotoxic activity PPD induced apoptosis in both groups, though significantly more in the PPD allergic group. Further, in both groups the regulatory co-stimulatory molecule CTLA4 was found up-regulated though no significant increase in other regulatory molecules such as FoxP3 or IL-10 was seen.

Conclusions

Taken together, our results show that PPD causes damage to the skin barrier and induces an immune response in the skin in occupationally exposed individuals. The immune response was predominantly inflammatory, except for the induction of CTLA4, even in the absence of clinical symptoms. This might suggest an induction of low-dose tolerance, but further investigation is needed. Our study shows how PPD distort the immune balance in the skin and thereby highlights the importance of protective measures, especially in risk occupations like hairdressing. The clinical implications could involve an increased risk for other skin diseases over time if the local skin immune balance is permanently distorted.

Resumé (Abstract in Danish)

Baggrund

Den moderne vestlige livsstil har bragt en række kemikalier ind i vores hverdag. Nogle af disse kemikalier kan forårsage allergisk kontakteksem og studier har vist at op til 27% af den europæiske befolkning, er sensibiliseret over for et eller flere kontaktallergener. Et af de fag der er mest i kontakt med allergifremkaldende kemikalier er frisørfaget. Det er veletableret at frisører har en høj forekomst af både allergisk og irritativt kontakteksem. En hyppig årsag til allergisk kontakteksem i frisørfaget er kemikaliet para-phenylenediamine (PPD), som er et mørkt farvestof, der oftest bruges i permanente hårfarver. PPD er et stærkt reaktivt kemikalie med en høj sensibiliseringsevne og efter en normal hårfarvning, kan det spores i både blod og urin. På trods af denne daglige eksponering, er det bemærkelsesværdigt få frisører, som udvikler kontaktallergi over for PPD. Det er foruroligende, at så mange er i direkte kontakt med PPD, da konsekvenserne af denne PPD-eksponering er relativt ukendte for både hudbarrieren og hudens lokale immunforsvar.

Det overordnede formål med denne afhandling var, at undersøge hvordan PPD påvirker hudbarrieren og hudens lokale immunforsvar i henholdsvis ikke-allergiske frisører og PPD-allergiske patienter fra Gentofte hospital, Danmark.

Metoder

Dette studie bygger på hud- og blodprøver fra ikke-allergiske frisører samt PPD-allergiske patienter. Hudbiopsierne blev taget på dag 4 efter lappetest med 1% PPD i vaseline og med vaseline alene som kontrol. Den første del af hudbiopsierne blev brugt til at analysere udtrykket af gener i form af RNA-sekvensering. De resterende hudbiopsier blev brugt til, med confocal mikroskopi, at visualisere udvalgte proteiners forekomst og organisering i huden. Blodprøverne blev taget før påsætning af PPD-lappetest og de cirkulerende T-celler, blev analyseret via flow cytometri. Effekten af PPD på specifikke proteiner i hudbarrieren, blev videre undersøgt i 3D hud-kulturer (Episkin).

Resultater

PPD forårsagede nedregulering af en række vigtige proteiner i hudbarrieren hos både allergiske individer og ikke-allergiske frisører (artikel I). Hos ikke-allergiske frisører var de 2 tight-junction proteiner; claudin-1 og CLMP, nedreguleret samt de 2 stratum corneum-proteiner; filaggrin-1 og filaggrin-2. Med confocal mikroskopi var dette også synligt for claudin-1 samt filaggrin-1 og 2. Analyse af 3D hud-kulturerne viste en nedregulering af claudin-1, CLMP samt filaggrin-2, men ikke filaggrin-1. Hos de allergiske individer var hudbarrieren på celleniveau betydeligt mere

beskadiget, end hos de ikke-allergiske frisører, og skaden stemte overens med deres kliniske symptomer.

PPD forårsagede let inflammation i huden hos de ikke-allergiske frisører på trods af, at de ikke havde kliniske symptomer (artikel II). Hos de allergiske patienter svarede niveauet af inflammation til sværhedsgraden af deres kliniske reaktioner. Derudover viste blodprøverne, at der ikke var nogen forskel mellem allergiske patienter og ikke-allergiske frisører i deres cirkulerende T-celler før PPD-lappetest. Hos begge grupper viste inflammationen sig som infiltrering af T-celler i hudbiopsierne, samt opregulering af gener involveret i T-celle-aktivering og effektor-funktioner. Især gener involveret i et type-1-immunrespons var opreguleret. Dette omfatter granzyme B, perforin, LTA og LTB, hvoraf kun LTA og LTB var signifikante, hos de ikke-allergiske frisører. I direkte forlængelse af dette så vi desuden i begge grupper, at apoptose blev induceret i huden efter PPD-eksponering. Apoptose var betydeligt mere udtalt i den allergiske gruppe end hos de ikke-allergiske frisører. Derudover så vi i begge grupper en opregulering af visse regulatoriske molekyler, deriblandt CTLA4. Dog var der ikke en signifikant opregulering af andre kendte regulatoriske molekyler, såsom FoxP3 og IL-10.

Konklusion

Samlet set fandt vi at PPD forårsager en betydelig skade på hudbarrieren, samt en T-celle medieret immunreaktion i huden hos ikke-allergiske frisører. Med udtagelse af CTLA4 var immunreaktionen overvejende inflammatorisk, på trods af at de ingen kliniske symptomer oplevede. Dette kan være et tegn på en form for tolerance, men det kræver videre forskning at fastslå. Dette studie viser at PPD skubber til immunsystemets balance i huden, selv hos individer uden allergiske reaktioner. Dette fremhæver vigtigheden af gode arbejdsrutiner og beskyttelse, for at mindske eksponeringen, især hos risikofag som frisørfaget. Fremtidig forskning vil kunne afdække om denne påvirkning af hudens immunsystem forøger risikoen for andre hudsygdomme og uønskede reaktioner i huden.

Objectives (aims)

The aim of the thesis was to analyze the mechanisms involved in allergic contact dermatitis to para-phenylenediamine (PPD) and the immune response observed in exposed individuals without allergy.

- How does the skin barrier respond to PPD in allergic and non-allergic individuals?
    - Is there a distinction between direct and indirect damage of tight junctions in the skin barrier after PPD exposure?
    - What is the effect on expression of filaggrin and related proteins in the stratum corneum?
    - What is the extent of the cell damage and induction of apoptosis in the skin after PPD exposure?

- Do occupationally exposed non-allergic individuals have specific immunosuppressive responses to PPD?
    - How do gene expression and immunologically relevant pathways change in the skin after PPD exposure?
    - What does T-cell infiltration in the skin look like after PPD exposure?
    - Are there relevant mechanisms of immune regulation and inflammation in the skin after PPD exposure?

Introduction

Contact dermatitis

Chemicals are widespread. We encounter them in almost every aspect of everyday life in the western world, from the smell of our shampoo to the colors of the clothes we wear. Some chemicals with prolonged or repeated skin contact can cause contact dermatitis. Contact dermatitis is a complex disorder with several clinical phenotypes. Typically, contact dermatitis presents with erythema, infiltration of the skin, possible vesicles in the acute phase, and scaling and fissures in the chronic phase. Etiologically, contact dermatitis can be categorized as either irritant contact dermatitis (ICD) or allergic contact dermatitis (ACD), depending on the trigger. Contact dermatitis is the most frequent occupational disease of the skin in the EU and in many countries it is also the most frequent occupational disease in general, which comes at a substantial cost for society [1, 2].

The professions with a high risk of occupational contact dermatitis include hairdressing, food handling, cleaning and healthcare [3, 4]. Persons with occupational skin diseases often experience loss of work and severe impairment in quality of life [5, 6]. One of the greatest risk factors for irritant contact dermatitis is wet work [7], which is defined as working with wet hands for >2 hours, working wearing waterproof gloves for >2 hours, or more than 20 hand-washes a day [7]. Other risk factors include environmental factors, such as exposure to dry air or extreme temperatures, and the many chemicals and work-related products that may have irritant properties. The first symptoms of ICD are often dry skin followed by inflammation, but without the involvement of an allergen-specific immune response. Treatment for ICD is mainly focused on restoring the skin barrier with emollients. ICD can lead to ACD as the dry and inflamed skin increases the risk of allergen penetration and sensitization [8]. Skin sensitization happens when a chemical is able to activate the adaptive immune system, so that upon re-exposure to a sufficient dose of allergen, ACD is elicited. Clinically, it is not possible to distinguish between irritant and allergic contact dermatitis. The diagnosis of ACD depends on patch testing, an internationally standardized method [9]. Patch testing is performed by applying relevant allergens to the skin using specially designed, ready-made patches or investigator-filled chambers, most commonly to the patient’s upper back. The allergen patches remain on the skin for 2 days; the skin is then inspected for reactions and scored according to severity; the scoring is repeated after 3–4 days and again after 7 days of initial application. The skin reactions can be divided into four categories: no reaction (-), irritant reaction (IR), doubtful reaction (?) or positive reaction

(+1,+2,+3). The severity of the positive reactions is further categorized according to clinical presentation as +1, +2 or +3 reactions where +3 is the strongest reaction. Contact allergy is defined by a positive patch test to an allergen. In cases where the individual is currently exposed to the allergen in a way that can partly or fully explain the dermatitis, the diagnosis of ACD can be made [9].

Epidemiology of contact allergy

Among the general population in Europe, 27% have a contact allergy and 17% are diagnosed with ACD [10]. The highest prevalence is found among women. The most frequent contact allergen is nickel, which causes contact allergy in 22.2% of women and 5.2% of men in the general European population. Other prevalent allergens are fragrances, cobalt, chromium, para-phenylenediamine (PPD)/ 1,4-toluenediamine (PTD) and MI/MCI, which have a prevalence ranging between 0.8 and 5 % in the general adult European population.

Hairdressers

Hairdressing is one of the occupations with the highest incidence of occupational contact dermatitis [4, 11]. Hairdressers are at risk because of their daily contact with allergens and irritants, such as hair dyes, permanent wave solutions, bleach products, shampoos and hair styling products, in combination with excessive wet work [12]. The most common form of contact dermatitis in hairdressers is hand eczema, which has a self-reported 1-year prevalence of 22% in hairdressers in Denmark[5]. Hairdressers stay an average 8.4 years in the trade, including the 4-year training period. Hand eczema and allergy are among the commonest reasons for leaving the trade. Multiple initiatives have sought to prevent or slow the development of hand eczema among hairdressers[13]. In Denmark, additional work routines and personal protective measures were introduced in the hairdressing training establishments in 2011, resulting in some improvement [14]. A similar approach was taken in the Netherlands among healthcare workers, also improving the preventive work routines, but not significantly preventing hand eczema [15]. In Germany, a comprehensive treatment plan for all occupational skin diseases was introduced in 2005 and resulted in better treatment outcome, where more patients were able to return to work and more experienced an overall increase in quality of life [16]. Despite improvements in the treatment of occupational contact dermatitis and in preventive measures, such as work habits and personal protection, hairdressing continues to be a high-risk occupation for contact dermatitis. Even with improved work habits, chemical exposure for hairdressers is still substantial [12]. Hairdressers are not only at risk of skin diseases, but also of airway diseases through their daily work with an array of airborne chemicals, for example persulfates used in bleaching [17]. This

exposure puts hairdressers at risk of other allergic diseases, such as allergic rhinitis and allergic asthma, which can be further exacerbated as a result of exposure to permanent hair dyes [18, 19]. The most common allergens in hairdressers' work-environment are PPD and PTD, which are found in permanent hair dye, glyceryl mono-thioglycolate from permanent wave solution, ammonium persulfates from bleach products, and perfume ingredients added to most of their products. PPD is often highlighted as the most problematic allergen, possibly due to the high frequency of sensitized patients and the severity of reactions. PPD induces a positive patch test in 4.1–11.5 % of all dermatitis patients in Europe, Asia, North America and India [20, 21]. Even when hairdressers wear gloves to protect the skin from exposure, many gloves do not protect against PPD exposure [22]. Not only can PPD be detected systemically in blood and urine from hairdressers working with the chemical, it can also be detected, and to a higher degree, in private users of hair dye [23]. This makes the biological effects of PPD on skin and the immune system relevant for hairdressers as well as every other PPD-exposed person. The main organ that protects the body from PPD exposure from hair dyes and most other sources is the skin.

The skin barrier

The skin is the body's biggest organ and the first line of defense against the environment. The skin not only gives protection against pathogens, along with regulating body temperature and water-loss, it also protects against the chemicals encountered in everyday life. As the modern lifestyle has evolved, our surroundings now include a substantial number of chemicals in both private and occupational settings in the form of cleaning, cosmetic and hygiene products [24]. The complex structure of skin enables it to respond and adapt to the surroundings as part of the immune system [25]. The skin barrier can be looked at as four different entities: the microbiome, the chemical barrier, the physical barrier and the immunological barrier. On the outermost surface, there is the microbiome of commensal bacteria, which helps maintain homeostasis within the skin. Underneath, there is the chemical barrier, which is made up of the ions and small molecules that control the micro-environment, such as the low pH and chemical charge in the upper layer of the skin. The physical barrier is the cell layers and extracellular matrix forming the skin structure and the immunological barrier consisting of both innate and adaptive immune components. The focus of this thesis is on the physical and immunological barrier.

The physical barrier: keratinocytes, junction proteins and all that is in between.

The skin structure is divided in to the epidermis and the dermis (Figure1). The epidermis consists of differentiating keratinocytes that generate the physical skin barrier. Epidermal stem cells on the basal membrane differentiate outwards to keratinocytes and create distinct layers as they flatten, cornify and are shed. Each step of differentiation for the keratinocytes is characterized by expression of different keratins and other barrier molecules. Keratinocytes differentiate from a layer of epidermal stem cells known as *stratum basale* to the layer *stratum spinosum* where the differentiating keratinocytes attach to each other and produce many lipids and other proteins involved in barrier integrity. As they move outwards in the *stratum granulosum*, the keratinocytes cornify, the cell shape flattens and the cells lose their nuclei and release the proteins they have produced. The fully cornified keratinocytes form a dense layer of tightly attached cell envelopes, lipids and peptides, forming the skin surface known as *stratum corneum*. Throughout the epidermis, all cells rely on cell-cell adhesion to properly build the strong structure of the skin.

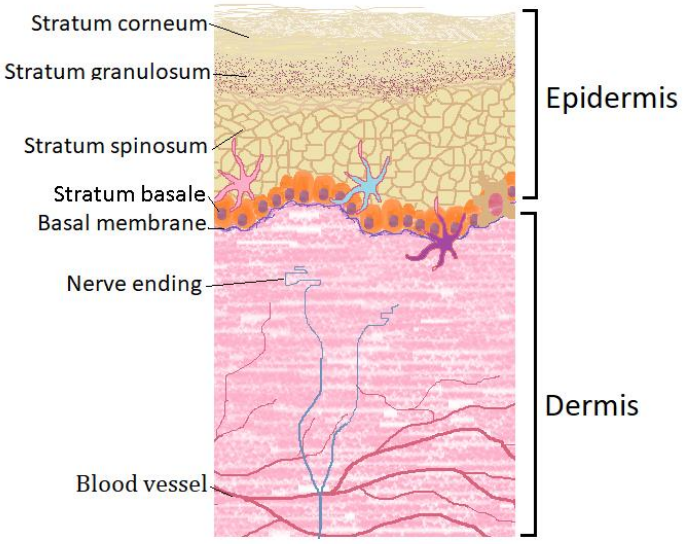

Figure 1, Illustration of human skin structure; Epidermis with the layers of differentiated keratinocytes and Dermis with its connective tissue, blood vessels and nerve endings. (Made in Paint by SSM)

The epidermis starts at the basal membrane—a strong extracellular matrix structure that binds the epidermis to the dermis via a network of collagen fibers and other connective tissue components. The epidermal stem cells are fixed to collagen fibers in the basal membrane via hemidesmosomes, which rely on integrins for adhesion [26]. The epidermal stem cells and the differentiating keratinocytes adhere to each other via a network of junctions. The four main types of cell-cell junctions are tight junctions (TJs), gap junctions, adherens junctions and desmosomes

(Figure 2). The type of junction differs between the different layers in the epidermis, with different junction types also co-locating in different combinations [27].

Cell-Cell Junctions

TJs are complex cell-cell adhesion structures that start to be expressed in the stratum spinosum and are highly expressed in the stratum granulosum [27]. TJs are large protein complexes of transmembrane adhesion molecules and multiple intracellular proteins that support the specific function needed. TJs are organized in tight junction strands on the apical part of the cell surface and function as gates between the cells to control diffusion of smaller molecules according to size and charge [28]. Tight junctions play a particularly important role in preventing water loss from the skin; new research has also revealed a role in cell-signaling, guiding cell behavior and differentiation [29]. TJs transmembrane binding proteins bind to each other between two neighboring cells, pulling the cell membranes together and thereby generating highly specific gates depending on the composition of junctional proteins. The three most common families of transmembrane binding proteins in TJs are claudins, occludins and junctional adhesion molecules (JAMs).

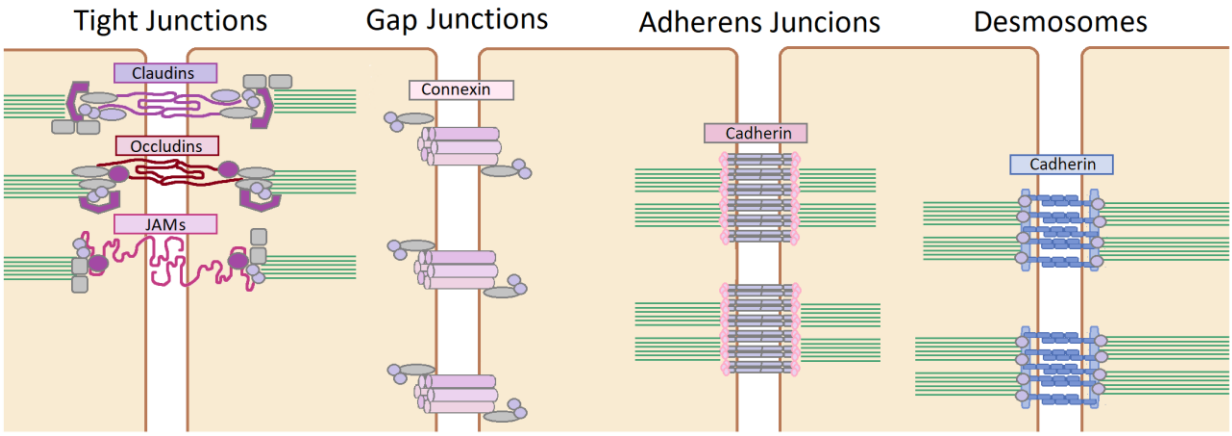

Figure 2, Illustration of junctional complex structures. Tight junctions, Gap junctions, Adherens junctions and Desmosomes. The cells' actin cytoskeleton is shown as green lines. (Made in Paint by SSM)

The largest family of transmembrane TJ binding proteins is the claudins. Humans have 26 identified different claudin proteins, of which claudin 1 is the most abundant in human skin and crucial to proper skin development [30]. Claudin 2, 3, 4 and 7 are also expressed in human skin and are described in different disease settings. The different claudins can interact with each other in the tight junction strands, meaning claudin 1 can pair up not only with claudin 1 from the neighboring cell, but also certain other claudins [31]. In some cases, the different claudins also work as a rescue mechanism for one another, when one type of claudin is decreased, another takes over in its place in an attempt to preserve barrier integrity. The expression pattern of

claudins is therefore often disturbed in skin diseases so that claudins associated with healthy skin are down-regulated and other claudins up-regulated [32, 33].

The second type of tight junction in the skin uses occludin as a transmembrane binding protein. Occludin belongs to a group of transmembrane proteins characterized by a MARVEL domain in the protein structure. Occludin creates TJ complexes very similar to that of claudins but expression of occludin in human skin is generally restricted to the stratum granulosum [27]. The third family of tight junction binding proteins are JAMs. They belong to the thymocyte marker in Xenopus (CTX) family of transmembrane proteins and there are three types: JAM-A, JAM-B and JAM-C. JAMs are expressed in multiple tissues throughout the body. In the skin, mainly JAM-A, and in limited amount also JAM-C, is expressed both in TJs and as independent cell surface receptors. JAM-A and JAM-C are involved in barrier formation and in cell migration, signaling and tissue development [34]. JAM-A regulates the expression of claudin 10 and claudin 15 in epithelial tissue in the gut and affects barrier permeability and tissue inflammation [35]. The different adhesion molecules present in the TJs depend on the tissue requirements, which include the tissues need for structural flexibility, the passage of selected small molecules and particular resistance to certain environmental exposures. Certain other adhesion molecules called tricellulins are prevalent at corner sites where three cells meet and the junction complex requires an additional complex structure [36].

The transmembrane TJ proteins are bound to a dense plate of scaffold and signaling proteins on the intracellular site of the cell membrane [37]. These protein complexes are responsible for binding to the cell cytoskeleton and for the cell signaling properties. Some of the most studied intracellular TJ associated proteins are the family of zonula occludens proteins (ZO-1, ZO-2, ZO-3) also known as tight junction proteins 1–3 (TJ-1, TJ-2 and TJ-3). They are scaffold proteins involved in connecting TJs to the cell cytoskeleton. TJ-1 was one of the first proteins described in the intracellular part of TJs in the skin. It is responsible for the binding of occludin to the cytoskeleton [38]. Another protein associated with TJs is the CAR-like membrane protein (CLMP) from the family of CTX transmembrane proteins. CLMP strengthens and insures stability of TJs and their organization. CLMP is expressed in epithelial tissues, including the skin where it co-locates with TJ-1 and occludin [39]. In humans, little is known about the function of CLMP. It is thought to be involved in epithelial development, partly due to its association with TJs [40], which is supported by the fact that mutations in the CLMP gene cause impaired development of the bowel [41]. Many other proteins are found in the TJ complex, but their function and expression patterns are largely unknown. Therefore, in this study, we looked at all known TJ associated proteins.

Gap junctions are intercellular channels that allow the passage of small molecules between the cytoplasm of two adjacent cells as a form of communication between the two cells. Gap junctions are formed by connexin proteins organized in hemichannels that bind corresponding hemichannels on the adjacent cell to form the gap junction complex [42]. Keratinocytes rely on gap junctions for proper communication throughout the epidermis; mutations in connexin genes are associated with improper skin barrier development [43]. Keratinocytes express different connexins depending on the location in healthy epidermis, or after damage [44, 45].

Adherens junctions are clusters of cadherin proteins linking the cell membrane to the cytoskeleton in adjacent cells. In the skin, they consist mostly of E-cadherin and P-cadherin, which are involved in modulation of both the cytoskeleton and the organization of junction complexes, including tight junctions. Adherens junctions play a crucial role in response to tension and cell differentiation in the epidermis [46].

Desmosomes are junction structures made of cadherins, armadillo proteins and plakophilins, characterized by a desmosomal plaque that binds the transmembrane cadherins on the cytosolic side of the cell membrane [47]. They are widely expressed on the cell surface in all layers of differentiating keratinocytes, foremost providing mechanical strength to the skin. Desmosomes also play a role in cell signaling and cell migration and are mutations associated with developmental problems of both the skin and the heart [48]. Desmosomes are connected to the actin cytoskeleton through flexible intermediate filaments, which together with specialized cadherins, make them more resilient to mechanical pressure than the more rigid adherens junctions [49]. The junctional complexes are all required for proper skin barrier development and many have more than one function as they guide the living cells and assist in signaling between cells, both in healthy and diseased skin. Any changes to the composition of junctional complexes can therefore have a substantial impact on skin barrier function. In this thesis we first looked at the broad composition of junction components and then focused on tight junctions.

Stratum corneum components

Moving outwards in the epidermis, another type of barrier is created by fatty-acids, lipids and proteins which glue the cornified envelopes from keratinocytes together and create the stratum corneum. One of the most studied stratum corneum proteins is filaggrin, which belongs to a family of S100 fused-type proteins. Filaggrin expression starts as pro-filaggrin inside the differentiating keratinocytes in the stratum granulosum where it is stored as aggregates in the cytoplasm. As the keratinocytes move from the stratum granulosum to the stratum corneum, pro-filaggrin is processed and degraded into the biologically active filaggrin protein. Profilaggrin has

a crucial function in Ca+ binding, which contributes to the Ca+ gradient in the epidermis necessary for proper barrier formation [50]. Each profilaggrin molecule is cut into 10 to 12 filaggrin proteins, which vary between individuals [51]. Filaggrin is important for the correct keratin aggregation in the cells and thereby the creation of the structural components of the skin [52]. In the stratum corneum, filaggrin is further degraded into small peptides known as natural moisturizing factors [53]. The production of natural moisturizing factors is crucial for the water binding properties of stratum corneum and thereby healthy, flexible skin. Another important function of natural moisturizing factors is the acidification of the stratum corneum; lowering the pH of the skin contributes to the chemical barrier and inhibits the growth of potential pathogens [54]. Filaggrin is not acting alone in the stratum corneum, other proteins that play a role in barrier integrity include filaggrin-2, loricrin, cornulin, involucrin, repetin, hornerin, trichohyalin and trichohyalin-like protein 1.

The S100 fused-type protein family also includes filaggrin-2, which, like filaggrin, is expressed in keratinocytes in the outer layers of the epidermis and involved in proper formation of the stratum corneum [55], especially the cornification of keratinocytes [56]. Mouse models show a role of filaggrin-2 in barrier function even though the expression pattern of filaggrin-2 in response to skin stimulation differs from that of filaggrin [57]. Fragments of filaggrin-2 also show antimicrobial properties, inhibiting the growth of certain bacteria and thus contributing to host defence [58]. Loricrin is involved in keratinocyte differentiation and is a major structural component of the envelope itself [59]. Loricrin is therefore first expressed in the stratum granulosum where it, among others, interacts with pro-filaggrin[60] and another stratum corneum protein, involucrin, in the assembly of the cornified envelope [61]. Involucrin is often used as a maker of keratinocyte differentiation and the gene is expressed in all stages of differentiation of keratinocytes but its gene regulation and promoter activation differ from early to late stages of differentiation [62].

The major part of the stratum corneum is made up of keratins, which are produced by keratinocytes, as indicated by their name. Keratins are structural proteins known as intermediate filaments and which make up the cytoskeleton of epithelial cells. Keratins are divided in two groups based on their structure: type I and type II keratins [63]. There are 54 identified keratins in the human genome and in multiple associated skin diseases [64]. The skin expresses multiple keratins and the expression changes as the keratinocytes differentiate. Two major components in the epidermis are keratin 1 and 10, which maintain barrier function [65]. Keratin interacts with filaggrin in the differentiated keratinocytes to form the flat cell shape associated with stratum corneum; loss of filaggrin decreases expression of certain keratins, one of which is keratin 10

[66]. Keratin 76 is essential for the correct assembly of tight junctions and thereby for barrier function [67], whereas keratin 16 is specifically expressed in response to skin damage and functions as an activator of the innate immune system [68]. Accordingly, the composition of both keratins and other stratum corneum proteins can function as an indicator of healthy skin, and a shift in keratin expression can be associated with skin stress or damage.

Skin barrier in disease

Disruption of the epithelial barrier is a well-known underlying cause of allergic skin diseases. Disruption not only facilitates allergen penetration but also results in a more inflammation-prone micro-environment due to increased contact between the immune system and the environment. The role of tight junctions in human skin disease is apparent when looking at human genetic polymorphisms that can decrease barrier function. Claudin 1 polymorphisms in humans are associated with increased sensitization to contact allergens [69] and can also cause disease in other organs due to a decreased or collapsed barrier function [70]. One of the most studied diseases involving skin barrier impairment is atopic dermatitis (AD). Polymorphisms in the claudin 1 gene can increase the risk of developing of inflammatory skin diseases, such as AD [71]. Further, individuals with filaggrin-null mutations have an increased risk of AD as well as a greater risk of developing hand eczema due to a decrease in skin barrier integrity[72]. Genetic variations of the filaggrin gene affecting the number of filaggrin repeats in pro-filaggrin have also been shown to cause dry skin if the person has 12 repeats in contrast to 10 or 11 repeats [51]. Studies on the genetic variants in occupations like hairdressing have shown that hairdressers carrying 10 filaggrin repeats showed more damage to cancer related genes in their DNA from blood than the hairdressers with 11 or 12 repeats. But on the other hand, 10 repeats resulted in lower levels of aromatic amines in the blood after occupational exposure than carriers of 11 or 12 filaggrin repeats [73]. In individuals with normal expression of barrier genes, the disruption of tight junctions often goes hand in hand with a decrease in important stratum corneum proteins, such as filaggrin. Down regulation of tight junction proteins, claudin 1 and occludin, and filaggrin is seen in AD lesion skin and the down-regulation correlates with disease severity [74] [75]. Further, down-regulations of claudin 8 and claudin 23 has also been reported in atopic dermatitis, but less is known about their relevance [76] [77]. It is clear that a strong barrier is pivotal in preventing these inflammatory skin diseases, but the maintenance and healing of skin damage might also play an important role in disease severity, especially in the context of contact dermatitis and chemical exposure. Tight junctions are also central in up-holding skin barrier integrity doing the regeneration of human epidermis after barrier damage

[78]. TJ 1 and involucrin co-locate in hyper-proliferating keratinocytes and are thought to create a temporary functional barrier in wounds as they heal. Claudin 1 and occludin have crucial roles in optimal wound healing; accordingly, a decrease in claudin 1 and occludin expression is associated with features of chronic wounds [79]. The effects of claudin 1 and occludin on wound healing are at least partly independent of tight junction formation, resulting more from their roles in keratinocyte migration and signaling. Based on these findings it is clear that barrier components may have important roles in ACD, not only in preventing sensitization but also in eliciting the allergic reaction and proper healing of the skin barrier.

The different junction complexes are highly affected by the immune system, especially the presence of inflammation. Multiple cytokines are known to open the skin barrier, and tight junctions are known to open in response to both the Th2 cytokine IL-4 and the Th1 cytokine IFNγ [80]. Allergic reactions can involve the release of histamine, which suppresses the differentiation of keratinocytes and damages barrier integrity [81]. Inflammation can induce the cytokine Il-33, which can down-regulate claudin 1 expression in keratinocytes [82]. Keratinocytes are not alone in their expression of junction associated proteins, many immune cells also express both cadherins and integrins. In addition, the immune cells in the skin can interact with barrier formation; one mechanism is through the expression of CD44, which interferes with the assembly of tight junctions [83]. Nevertheless, the expression of junction proteins by immune cells might also have other functions. The expression of occludin in epidermal γδT T cells is associated with epidermal stress and the migration to draining lymph nodes [84]. It is clear that a complex crosstalk exists between the functional skin barrier components and the immune system. This crosstalk influences barrier stability and both the development and severity of skin diseases. The crosstalk is also relevant in ACD, and the influence of specific contact allergen exposure is largely unknown.

The immunological barrier: Skin resident cells and signaling factors

In addition to the physical barrier, the skin can also be viewed as an immunological barrier. Healthy skin is home to many immune cells, some reside in the epidermis or the dermis and others are organized in skin-associated lymphoid tissue [85]. But the crosstalk between the barrier and the immune system starts with keratinocytes. Keratinocytes can produce a wide range of signaling molecules to recruit the cell types they need depending on barrier challenges, such as viral or bacterial infection, wounds, DNA damage or other types of cell stress [86]. Human keratinocytes sense barrier changes through a system of receptors, including toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors. Keratinocytes

express TLR 3, 4, 5, and 9, which upon activation result in the production of both proinflammatory cytokines and chemokines [87]. A key feature of keratinocyte activation is the activation of the inflammasome, resulting in production of the active form of IL-1β and Il-18, both of which are inflammatory cytokines important for antigen presentation, macrophages activation and further inflammatory responses [88]. In response to pathogens or barrier damage, keratinocytes are also able to produce antimicrobial peptides (AMPs), which inhibit potential pathogen intrusion and activate innate immune cells [89].

In the human epidermis, there are two types of immune cell: Langerhans cells and allergen-specific resident memory CD8+ T cells (Trms). The first cells to meet to a new antigen are Langerhans cells. They are a specialized type of antigen-presenting cell (APC) which forms a net throughout the epidermis, detecting intruding antigens through a wide range of innate immune receptors. Langerhans cells are involved in initiating the adaptive immune response where they not only present the antigen but also educate and regulate the adaptive immune response [90]. Langerhans cells can differentiate from circulating monocytes, and recent studies suggest that Langerhans cells are involved in skin barrier homeostasis through the induction of specific IL-22 producing Th-cells after CD1a stimulation [91].

The dermis is home to a broader set of innate immune cells, including dermal dendritic cells, macrophages, mast cells and NK cells [92]. Two specialized APCs are found in the dermis: dermal dendritic cells and langerin$^+$ dermal dendritic cells. Both types are effective APCs and dermal dendritic cells are involved in shaping the innate immune response through cell interactions with mast cells [93], which are potent cytokine and chemokine producers in the inflammatory immune responses [94]. Dermal dendritic cells also affect the adaptive immune response by guiding the T-cell response in disease [95]. Macrophages protect the body from pathogens by phagocytosis of the intruder and a massive production of signaling molecules, including various cytokines and chemokines. They can also function as APCs in certain settings [96]. Macrophages support the recruitment of Langerhans cells into the epidermis [97] and the recruitment of monocytes, neutrophils, eosinophils and natural killer (NK) cells from the blood through their cytokine and chemokine production [98]. In damaged skin and wound healing, macrophages are crucial in skin regeneration and clearance of inflammation, as well as in the clearance of allergen in allergic diseases. There are two phenotypes of macrophages in the skin: the classically activated M1 and the alternatively activated M2 phenotype. MI microphages are associated with IFNγ production and other pro-inflammatory cytokines and in some cases the production of toxic components, such as reactive oxygen species, which can contribute to certain skin diseases. In contrast, M2 macrophages are associated with cytokines typical of a Type 2

response, such as IL-4 and IL-13, and have both pro-inflammatory and regulatory abilities depending on the context of the inflammation [99]. The phenotype of macrophages can change over the course of an immune response, depending on the type of damage [86, 100].

Another important source of cytokines in the skin are the innate lymphocyte cells (ILCs), which can also be found in the dermis or are recruited to the dermis during skin inflammation. ILC phenotypes depend on their cytokine profile and they have distinct roles in skin inflammation, wound repair and skin diseases [101, 102]. ILCs with an IL-17 phenotype have been described in contact dermatitis to nickel [103], but more research is needed to clarify their role in the disease. Taken together, all the above-mentioned cells represent the innate immune cells in the skin. They initiate an inflammatory response, but in the case of allergic diseases, an allergen specificity is required, a specificity that is achieved only by the involvement of the adaptive immune response and allergen specific cells.

The adaptive immune system in the skin consists of both circulation T cells patrolling the skin and T cells that have taken up permanent residence in the skin. B cells are less well described in the skin and are generally considered to stay in lymphoid tissue and affect the skin through the production of antibodies. One of the cell subsets that take up permanent residence in the skin is a specialized T-cell subset known as skin resident memory T cells (Trm cells), which are mainly identified by the expression of the surface makers CLA, CD69 and CD103 [104, 105]. These antigen-specific cells appear in at least two different subsets, $CD8^+$ T-cells or $CD4^+$ T-cells [106]. $CD8^+$ Trm cells are primarily located in the epidermis and $CD4^+$ Trm cells in the dermis. Trm cells home to the skin after antigen exposure as part of the immunological memory response. The skin where the antigen encounter occurs has the highest prevalence of antigen-specific cells; nevertheless, antigen-specific Trm cells can also be found throughout the rest of the skin, depending on the antigen [106]. The population of Trm cells in the skin is maintained by homeostasis, especially by the cytokines IL-7 and IL-15 produced by the tissue near hair follicles. As a result Trms cluster near the hair follicles where the cytokine production also ensure proper effector function [107]. Antigen-specific CD8+ Trms sustain a high prevalence in the epidermis even after the corresponding central memory cells have greatly decreased in the blood [106]. Despite a common clonal origin, CD8+ Trms in the epidermis differ in phenotype from central memory CD8+ T cells through a highly controlled expression of effector molecules such as granzyme B and perforin [104]. In contrast, CD4+ Trm cells in the dermis are more closely connected to circulation, and the composition of CD4+ Trms is thereby constantly equilibrating with the CD4+ memory T cells in the blood [108]. Langerhans cells help shape the activation of Trms in the skin, not only in inflammatory responses but also in activating

regulatory Trms and controlling excessive inflammation [109]. Other T cell types might also have important functions in the skin, including invariant T cells [110]. The mucosal barrier have specialized T cell subsets called mucosal-associated invariant T cells (MAIT cells) which are rapid cytokine producers, especially in the tissue [111]. MAIT cells shape both adaptive and innate immune responses at the mucosal surface but their role in the skin or the existence of a skin equivalent subset is less understood even though they have been found in some skin diseases [112, 113].

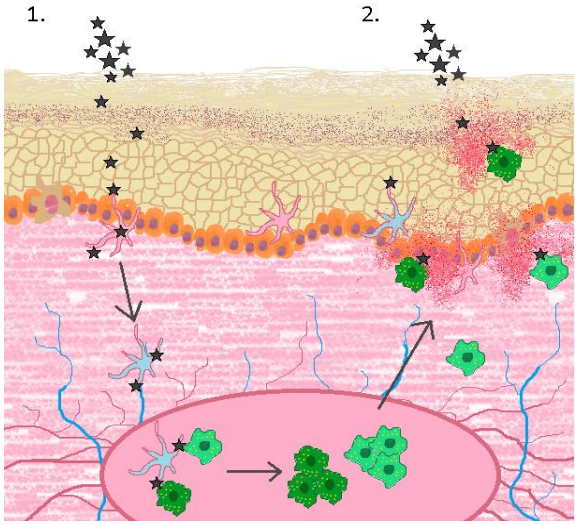

Figure 3. Allergic contact dermatitis, 1. Sensitization to Allergen (stars), allergen is recognised by APCs in the skin which migrate to local lymph nodes and activate allergen specific $CD4^+$ and $CD8^+$ T cells which migrate to the original exposure site in the skin. 2. Elicitation phase, allergen specific T cells recognises and react to allergen encounter and generate allergic inflammation. (Made in paint by SSM)

Both innate and adaptive immune cells are influenced by keratinocytes and changes in the structural composition of the skin. Immune reactions in the skin are highly coordinated through a complex network of signaling pathways giving rise to very specific reactions depending on the overall context. Any damage or defect in either the structural or immunological part of the skin can greatly impact the skin barrier function and cause disease. Exactly how the immune reactions are decided and regulated is largely unknown, but it is a pivotal step in understanding the development of skin diseases, including contact allergy.

The immunology of allergic contact dermatitis

There are at least 4350 chemicals described that can cause ACD [24]. Common for them all is that they need to penetrate the skin and be recognized by and activate the immune system. These chemicals are referred to as haptens, which are small molecules with indirect immune activating abilities as they rely on binding to peptides or proteins for immune recognition. An exception to

this is nickel. Nickel can activate the immune system directly by the binding of TLR4. Accordingly, the mechanism of allergen recognition and immune activation can vary greatly between different contact allergens. ACD is a T-cell mediated delayed type hypersensitivity reaction also referred to as a type IV allergy. ACD is divided in two phases: the sensitization phase and the elicitation phase.

Sensitization

In the sensitization phase the allergen or hapten penetrates the skin and is introduced to the immune system via APCs; this phase is not necessarily clinically visible. The activation of APCs in the skin depends on at least two factors: the recognition of the allergen and a "danger" signal in the form of tissue damage, stress cytokines from keratinocytes or the presence of other innate immune activating factors. A known danger signal in ACD is the activation of the IL-1β-processing inflammasome, which results in the production of pro-inflammatory cytokines [114]. The pro-inflammatory cytokines IL-1β, IL-18 and TNFα are key factors in ACD where they provide the early activation signals to APCs and innate immune cells, stimulate migration of APCs to local lymph nodes and activate local subsets of T cells [115-117]. Keratinocytes can also activate the tissue by the production of vascular epithelial growth factor (VEGF) in response to certain chemical allergens, including PPD, which might further support the migration of immune cells [118].

The recognition of the allergen depends on the formation of allergenic epitopes, which most contact allergens form by binding to peptides or proteins in the skin. The allergen is presented on either MHC class I or MHC class II molecules depending on its chemical nature (Figure 4). Hydrophilic allergens can react with extracellular peptides and proteins that are thereafter engulfed and presented via the exogenous route on MHC class II molecules. Lipophilic allergens can penetrate the cell membrane and thereby also bind to cytoplasmic proteins, which are presented through the endogenous route on MHC class I molecules. The chemical reactivity of a contact allergen also affects its presentation. Some allergens are capable of polymerization or oxidation, which does not necessarily involve specific proteins, and others show highly specific binding patterns. Allergens with strong reactivity can react with multiple proteins and generate multiple allergenic epitopes, making it difficult to isolate allergen-specific cells because multiple epitopes exist and vary from patient to patient [106]. Antigen-presenting cells (APCs) in the skin take up the allergen and migrate to the draining lymph nodes where the allergen is presented to allergen-specific T cells. The recognition of an allergen in ACD occurs foremost through dermal dendritic cells. Although Langerhans cells were originally thought to be the main APC, more

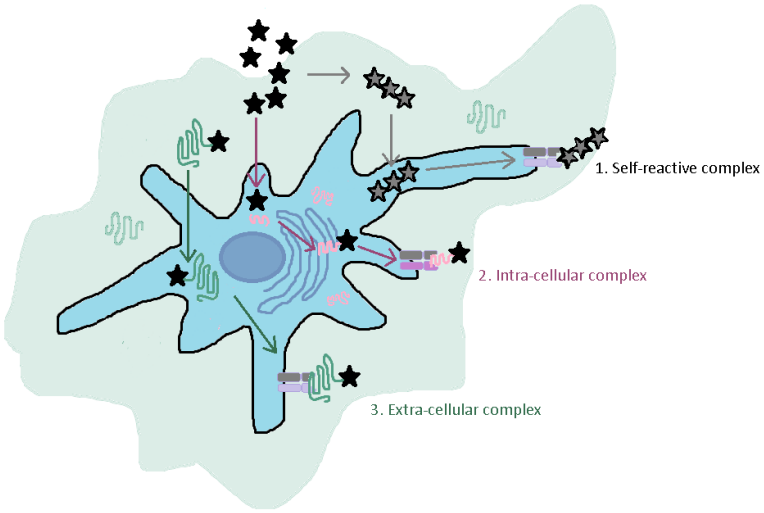

Figure 4. Presentation of contact allergens, allergenic epitopes generated by 3 different routes, self-polymerization and presented via MHC-II (1), reaction with intracellular peptides or proteins and presented via MHC-I (2) or reaction with extracellular peptides or proteins presented via MHC-II (3). (Made in paint by SSM)

recent research has shown that specialized subsets of dermal dendritic cells can alone initiate ACD reactions [95]. The magnitude of the allergic response relies on the number of APCs exposed to the allergen, and this is likely to involve more than one specific dendritic cell type [119]. Further, certain subtypes may also have anti-inflammatory potential, in particular, this has been suggest for Langerhans cells [120]. Upon activation, the APCs migrate to the draining lymph nodes where they locate to the paracortical T-cell area as interdigitating cells, presenting the allergen to naïve T cells. Upon binding to an allergen-specific T cell, the APCs release specific combinations of cytokines. T cells require three signals for proper activation: allergen-specific binding of the T-cell receptor, co-stimulatory signaling through surface receptors and a signal from instructive cytokines. The combination of co-stimulatory molecules and cytokines released by the APC primes the T cells for a certain T-cell response and gives information about which tissue to migrate to [121]. This activation results in a rapid proliferation of allergen-specific T cells which are then released into recirculation ready to attack the allergen-modified cells. The allergen-specific T-cell population forms an allergen-specific memory population, which is generally divided into central memory (Tcm), effector memory cells (Tem) and Trms, as already mentioned. The central memory T cells circulate the lymphoid organs and blood, effector memory cells are primarily found in blood circulation and certain peripheral tissues, and Trms migrate back to the tissue affected initially where they take up permanent residence and play an important role the elicitation of ACD [106].

Elicitation

The second phase of ACD is the elicitation phase. The memory population of allergen-specific T cells is in place and causes an inflammatory response upon allergen encounter. In contrast to the sensitization phase, this phase is widely clinically visible within 2–7 days. The continuant recruitment of cells to the skin contributes to the development and severity of ACD skin lesion. The recruitment is mainly orchestrated by the production of chemokines, most of which are produced by the keratinocytes. The first chemokines expressed in the allergic reaction are those involved in monocyte and macrophage recruitment (CCL2 and CCL22) followed by the T-cell chemoattractants and regulator RANTES and IP-10 (also known as CXCL10), among others [122]. The cytokines involved in ACD shape the allergic reaction from the inflammatory initiation to the control and regulation associated with healing. The IL-1 family cytokines, in particular, play a crucial role in skewing this balance toward inflammation, this includes IL-1β, IL-33 and IL-36 [123]. The combination of cytokines present depends on the allergen and can vary greatly according to the type of allergen involved, including the phenotype of involved T cells [124].

It is common to classify a given immune response and the cell types involved by the cytokines they produce, most importantly the T-cell response. In recent years it has become more evident that most T cells have a certain plasticity and can change cytokine profile in the course of a immune reaction or with prolonged or repeated allergen exposure [125]. Most contact allergens induce a Type 1 response with Th1 cells and IFNγ as the main inflammatory cytokine and sometimes in combination with a Type 3 response with Th17 cells and dominated by IL-17. CD8+ effector T cells drive the allergic Type 1 response with a rapid production of IFNγ and IL-17 [126] and the recruitment of inflammatory monocytic cells to the skin [127]. In response to an allergen, a shift occurs in circulating T-cell phenotypes unique for contact-allergic individuals, where $CD8^+$ T cells increase in number compared with $CD4^+$ T cells [128].

Other inflammatory responses to allergen include a Type 2 response with Th2 cells and the cytokines IL-4, IL-5 and IL-13, also linked to more chronic inflammation and regulation. Type 1 and Type 2 responses are known to inhibit each other, meaning as one response grows in strength the other is inhibited. Many new cytokines are emerging as important in ACD, which means the Type 1/Type 2 classification often over-simplifies the immune responses as each allergen seems to induce its own unique combination of cytokines [124]. Among the other less dominant cytokines involved in ACD are IL-9 and IL-22, which are produced by $CD4^+$ T cells in the skin. IL-9 is present in the allergic reaction, especially in nickel-allergic patients where IL-9 production also inhibits the production of IFNγ by Th1 cells [129]. ACD reactions can be

enhanced by cytokines such as IL-25 – a proinflammatory cytokine that supports Th17 cells by enhancing IL-1β production by dermal dendritic cells [130]. Combinations of certain cytokines may even be able to detect ACD in patients. This has been seen in nickel-allergic individuals where the increase of IL-5 and the simultaneously decrease of IL-8 were characteristic of those with allergy [131]. A particularly unpleasant feature of ACD is itching of the affected skin. Itching is often attributed histamine release of mast cells, but in ACD itching is associated with the cytokine IL-31 produced by $CD4^+$ T cells [132]. The expression of IL-31 is elevated in the skin of ACD lesions and associated with the expression of Th2 cytokines in patients with ACD [133].

The role of $CD8^+$ and $CD4^+$ T cells in ACD is well established, but other T cells also contribute to the pathogeneses. γδT cells have been studied in the DNCB mouse model of ACD where they regulate the immune response by producing IL-12, TNFα, IL-17 and IFNγ, all of which are associated with elicitation of ACD [116]. Although γδT cells have been identified in humans and their levels are elevated in patients with nickel allergy [134], their function in ACD remains to be investigated.

Mechanisms of immune tolerance

Controlling inflammation is a crucial step to avoid unnecessary tissue damage. When a visible reaction is absent after significant exposure to an allergen, tolerance can be suspected. Tolerance is regarded as passive or active; however, clinically, there is no visible difference between the two. Passive tolerance is a lack of reaction because a substance did not penetrate the skin, was not recognized by any cells or did not cause sufficient damage to initiate a response. Active tolerance is a lack of inflammatory reaction because a substance was recognized as not dangerous by regulatory immune cells and induced the production of immune regulatory components inhibiting the formation of inflammation and suppress or regulate other inflammatory T cell subsets, dendritic cells, macrophages and endothelial cells and switch antibody isotypes of B cells [135]. Active tolerance can also be referred to as immunological tolerance and that is the focus of this thesis.

Immunological tolerance in the skin can be achieved in more than one way, the most common method being the induction of regulatory cells. The major player in immunological tolerance is allergen-specific regulatory T cells (Tregs). Tregs express CD25 and FoxP3 and have a regulatory function through the production of regulatory cytokines, such as IL-10, IL-35 and TGFβ, and the expression of the suppressive co-stimulatory molecules CTLA4 and PD-1. Tregs can be found throughout the body, especially in barrier tissue, such as the skin, lung and gut. In

each location the Tregs adapt to the tissue they inhabit, and many sub-types have been described depending on the tissue and regulatory mechanism [136]. The skin homing Tregs express CLA^+ and play a major role in the development and severity of ACD [137]. Induction of Tregs in mouse models of ACD is associated with the development of long-term tolerance to contact-allergen exposure [138] and Tregs decrease both clinical symptoms and the prevalence of inflammatory cells [139]. The regulatory function of Tregs in ACD is foremost attributed to their IL-10 production. IL-10 is a well-studied immune-suppressive cytokine, both in mice and humans, which is known to be involved in inflammatory diseases in humans [140-142]. Il-10 inhibits activation by binding to the IL-10 receptor, which consist of two subunits: IL10Rα and IL10Rβ, expressed on T cells, APCs and most innate immune cells in the skin. Binding of IL-10 inhibits the production and secretion of proinflammatory cytokines from dendritic cells and effector T cells and inhibits the differentiation of Th1 cells while it induces the differentiation of Treg cells [143]. IL-10 also interferes with the dendritic cells' ability to activate inflammatory T cells in ACD [144]. Without IL-10 production, Tregs lose part of their regulatory function and are no longer able to suppress ACD in mouse models [145, 146]. However, despite IL-10 production being an important part of Tregs' regulatory potential, other regulatory mechanisms are also seen, both via other soluble factors and via cell-cell contact. One example is the suppression of ACD mouse-model reactions by Tregs via their production of adenosine, which inhibits the influx of effector T cells from the blood to the skin [146, 147]. Specialized subtypes of Tregs have been described in ACD mouse models based on regulatory mechanisms, surface markers, or cytokine or chemokine receptor expression [148], but the relevance of these subtypes in humans is yet to be determined.

Tregs are not the only cells with regulatory properties in the skin. The tissue itself has regulatory mechanisms in place to protect it from excessive inflammation. An example is the production of IL-10 from keratinocytes and Langerhans cells in response to UV radiation, which is thought to explain the benefits of light therapy in patients with inflammatory skin diseases [149, 150]. UV radiation also affects the Treg population in the ACD mouse model, inhibiting the elicitation phase of ACD [151]. Further, epidermal Langerhans cells play a role in controlling the elicitation phase of ACD as their absence in the skin causes enhanced allergic reactions [152]. This is widely attributed to their interactions with T cells and their ability to produce IL-10 in response to contact allergens [120].

Even though ACD is mediated by cytotoxic T cells and not by the production of antibodies as in other allergic diseases, B cells and mast cells still influence the elicitation phase of ACD. Mast cells can inhibit the proliferation of effector CD8+ T cells in chronic ACD models by removing

T-cell survival cytokines, such as IL-15, from the tissue [153]. B cells are not often described in the skin, but subsets of regulatory B cells are found near inflamed skin and play an important role in skin immune regulation by producing IL-10 and maintaining homeostasis of Tregs [154, 155]. IL-10 producing B cells are thereby able to influence the severity of ACD in mouse models [156], and the production of neutralizing IgG4 antibodies has been implicated in regulation of other allergic skin diseases [157, 158], but human data on the relevance in ACD remains incomplete. Invariant natural killer T cells (iNKT cells) interact with regulatory B cells through their expression of CD1d, where they show a potential regulatory role as iNKT cells capable of suppressing effector CD8+ T-cells in ACD [159]. However, as iNKT cells are also potent producers of inflammatory cytokines, their contribution to ACD in humans is still discussed.

A further level of regulation comes from the cytotoxic CD8+ T cells themselves which can be induced to produce IL-10 as a response to overstimulation. This is also known as exhaustion tolerance and has been widely studied in chronic inflammatory diseases [160]. Although some contact allergens induce IL-10 production in CD8+ T cells, IL-10 alone does not appear to influence the elicitation of ACD [161]. Skin resident memory CD8+ T cells in the epidermis are responsible for most of the damage seen in ACD, but they express an array of inhibitory checkpoint receptors through which the tissue can inhibit their inflammatory response. These checkpoints are also crucial in ACD and blocking of the inhibition leads to enhanced ACD reactions [162].

Repeated exposure to low doses of certain contact allergens can induce tolerance. This was first seen with nickel induced via oral tolerance [163] and later also in other settings. This is referred to as low zone tolerance and relies on crosstalk between Tregs and a special phenotype of dendritic cells with a regulatory phenotype [164]. When looking at occupational exposure to low doses of allergen, this type of tolerance could be relevant, especially for hairdressers, but, to date, data are missing.

Allergic contact dermatitis to PPD

PPD is a highly reactive hydrophilic aromatic amine with a size of 108.1 kDa and registered with CAS no. 106-50-3. PPD is used as a black dye, and although the pure form of PPD is white crystals, it turns black when oxidized. The oxidation reaction is utilized in the dying process where PPD is mixed with color cobblers and an oxidative agent, for example H_2O_2, to generate the permanent black color polymers. The most common source of PPD exposure is from hair dye or black henna tattoos [165, 166], the major risk factors for allergic sensitization to PPD [167]. PPD can also be found in everyday items, such as clothes and rubber, as an ingredient of

different dyes. This can make avoiding PPD difficult for allergic patients [168]. The clinical presentation of PPD allergy depends on the exposure site. Allergic reaction to PPD in hair dye presents as swelling and redness of the scalp, and in some cases also the face and neck, potentially leading to airway obstruction. The reaction can lead to full or partial hair-loss and in extreme cases even reactions to a patch test can take more than one month to disappear [169].

In its pure form, PPD is toxic. Poisoning can happen after ingestion or when it is applied to large areas of the skin. For example, this can occur with black henna tattoos or when coloring the feet and palms, which is popular in some cultures. PPD poisoning is potentially deadly as it can cause neurotoxicity and acute renal and liver failure. After poisoning, PPD can be detected in the blood, urine, stomach and liver of the patients, also post-mortem [170]. Even after hair coloring, small amounts of PPD can be detected in the blood and urine [23, 171]. Notably, some studies find that after limited hair dye exposure, it is primarily the inactivated form of PPD that can be detected in the blood and urine, which could reduce the health risk associated with systemic exposure [172].

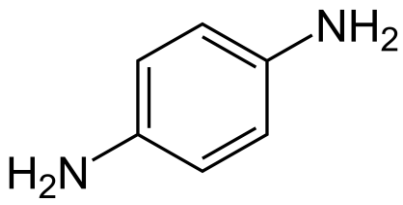

Figure 5. Chemical structure of pare-phenylenediamine (PPD)

The systemic exposure of PPD is foremost a concern for people in regular contact with PPD. The exposure to PPD is the greatest in individuals preparing the hair dye mixture; both the hair dye components and final mixtures are known to induce allergic reactions [173].

PPD's small size and hydrophilic properties make it efficient in penetrating the skin [171]. It penetrates the stratum corneum in less than 30 minutes and has a half-life in the skin of approximately 3 hours [174]. Due to PPD's high reactivity it will auto-oxidize when it comes into contact with oxygen in the air or water molecules in the skin.

Upon PPD exposure, keratinocytes protect the skin from damage by neutralizing PPD via N-acetylation, eliminating the genotoxic potential [175]. Further, monocyte-derived dendritic cells in the dermis can neutralize PPD in the same way [176]. N-acetylation of PPD in the skin is done by the enzyme N-acetyltransferase 1 encoded by the gene NAT1. Polymorphisms in the NAT1 gene that increase enzymatic activity and promote faster acetylation of PPD are to some extent less present in PPD sensitized individuals, but data are overall inconclusive [177].

An allergic response is thought to happen when the neutralizing capacity of keratinocytes is exhausted and the remaining PPD is left to create cell damage. One of the ways PPD damages the cells is by inducing the formation of hydroxyl radicals or reactive oxygen species. These radicals result in oxidative stress and DNA damage in keratinocytes [178]. The damage induces the production and secretion of Il-1β, IL-18 and VEGF from keratinocytes, which activates the surrounding tissue and immune cells [118, 179, 180]. Il-1β and IL-18 are important parts of the inflammasome activation that drives the early phases of ACD. The PPD-induced production of IL-18 in keratinocytes occurs within 3–24 hours of exposure and is regulated by expression of Blimp-1 and NHRP12. Blimp-1 is involved in inducing IL-18 secretion, whereas NLRP12 inhibits IL-18 secretion and thereby inflammasome formation. The damage induced by PPD can also result in the induction of controlled cell death. This can happen as a result of PPD-induced DNA damage or by the induction of certain miRNAs involved in apoptosis and cell-cycle control [181].

Even before PPD penetrates the skin the compound can react with its surroundings. One of the most common oxidation products of PPD is its trimer Bandrowski's base which has been shown to activate T cells in in-vitro[182]. Bandrowski's base can activate T cells from both allergic and non-allergic individuals, whereas PPD more specifically activates only T cells from allergic individuals [183]. Further, only PPD activates dendritic cells, indicating that PPD, rather than Bandrowski's base, forms the epitopes needed for allergic T-cell activation [184]. Accordingly, Bandrowski's base is thought to play only a minor, in any, role in ACD. Among the other oxidation products of PPD, 4-nitroaniline and 4,4'-azodianiline have been identified as potent sensitizers and although they elicit a reaction in the majority of allergic patients tested, their relevance remains debated [185].

After skin penetration, both PPD and its oxidation products can bind irreversibly with specific amino acids in multiple peptides and proteins in the skin, such a cysteine, effectively generating multiple possible allergenic epitopes [186, 187]. One of the more promising allergen epitopes in PPD allergic individuals seems to be the cysteine in human serum albumin (HSA), which is found in many patients, but far from all [188]. Despite multiple efforts, no single epitope has been identified in PPD allergy common for most patients, which becomes more evident when looking at the generated T-cell receptor repertoire in PPD both in cell cultures and from allergic patients [189]. The polyclonal T-cell response gives rise to clones with cross-reactivity to PPD-related substances [190], which further complicates ACD for the affected patients.

Allergen-specific T cells start to infiltrate the skin at 8–24 hours after PPD exposure and have increased in number at 48 hours. The recruitment of immune cells to the site of PPD exposure

depends on the secretions of chemokines. In the stratum corneum, PPD exposure changes the expression of certain chemokines[191], but more data are necessary to truly understand the expression pattern induced by PPD exposure. The infiltrating T cells include classical CD8+ or CD4+ αβT-cells, γδT-cells and Tregs [192]. The phenotype of the T cells involved in the response to PPD is greatly debated because depending on study method and/or model, Th1, Th2 and Th17 responses are detected. The majority of studies today are leaning towards an initial pathogenic Th1/Th17 response driven by IFNγ and IL-17 where the main effector cells are CD8 T cells which with time are then regulated by anti-inflammatory components related to Th2 responses. Immune cells isolated from allergic patients produce significant amounts of IFNγ and low levels of IL-10 after stimulation with PPD. In contrast, cells from healthy individuals showed no response and cells from non-allergic individuals with a history of PPD exposure showed only an increase in IL-10 production [193]. In mice, the allergic reaction to PPD involved both T and B cells and the main cytokines involved included Il-17, IFNγ, IL-10 and to a lesser degree IL-4 [194]. Th2 responses to PPD were found to be very rare in mice after repeated topical application and therefore considered less relevant in PPD ACD [195]. However, other studies, both in vitro studies in human blood and in vivo studies in mice, show the involvement of both T- and B cells with the induction of the Th2 cytokines IL-4 and IL-5 and an important role of IgE and mast cells in the allergic reaction to PPD [196, 197]. Further, γδT cells assist in the allergic response to PPD and, like the αβT-cells, they can produce IFNγ, IL-4 and IL-10 [198]. Taken together, the T-cell response to PPD is more complex than one simple response and crucial information seems to be lacking to make sense of the results at hand.

Like all other inflammatory responses, the allergic response to PPD also needs to be controlled by the body. In the case of repeated exposure to hair dye with PPD, mouse models show the induction of Treg cells and IL-10 production, which decrease reaction severity [199]. In line with the findings in mouse models, allergic patients also show induction of regulatory mechanisms after PPD exposure, both in the form of IL-10 production [193] and the secretion of IL-9 from CD4+ T-cells, which also seems to have an immune regulatory role in ACD [192].

To prevent new, strong sensitizers appearing in consumer products, each new product must undergo testing to assess its sensitizing potential. Previously, this was done in animal models, but today the industry relies on various in vitro assays, not always translating well to real-life settings. As a result, some strong allergens are not noticed before a substantial number of people have developed allergy. It is therefore crucial to understand what makes strong allergens strong, both to develop better treatment and protection for people already affected, and to develop relevant tests for new compounds before they enter the consumer market and potentially cause

an epidemic of ACD in the population. Therefore, this study focuses on understanding the pattern of barrier disruption and immunological changes induced after exposure to PPD in both allergic patients and occupationally exposed hairdressers.

Methods

Study population

This PhD thesis is based on the comparison of two study populations exposed to PPD: a group of occupationally exposed hairdressers without contact allergy and a group of diagnosed PPD allergic individuals. To study the effects of PPD on skin barrier and skin immunity, patients with other diagnosed skin diseases or a history of allergic skin reaction were excluded; these reactions included but were not limited to atopic dermatitis, psoriasis and allergic urticaria. Data on other allergies and skin diseases were checked against the medical files of the PPD-allergic patients, whereas medical data on the non-allergic hairdresser solely depended on their own recollection when asked. All individuals had to be otherwise immune healthy adults between 18 and 60 years of age and not taking any immune modulating medicine. All included participants were women; a table of participants' characteristics can be found in Manuscript I. Study populations and sample collection are summarized in Figure 6.

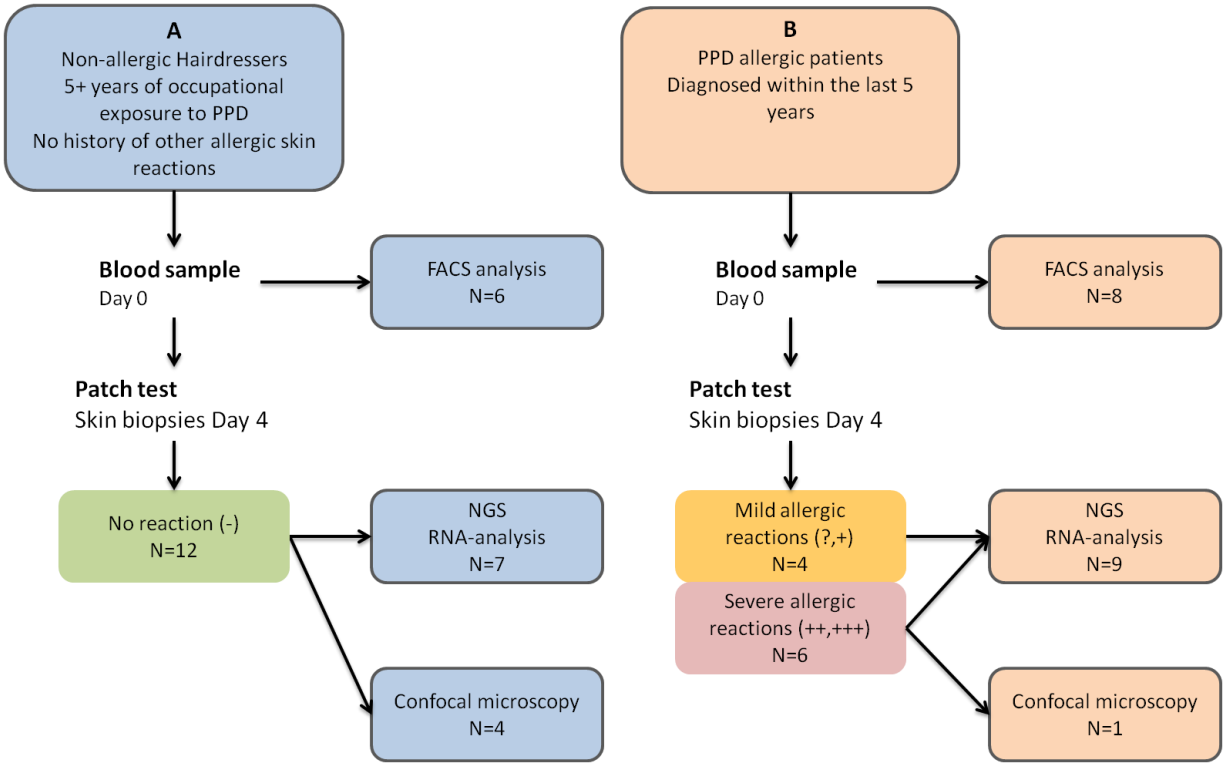

Figure 6. Study population and sampling overview. Samples were collected from two groups to be analyzed by three different techniques: flow cytometry (FACS), next generation sequencing (NGS) RNA analysis and confocal microscopy imaging.

Participants donated 50 ml of blood for PBMC isolation and FACS analysis on Dag 0 before a patch test was applied. Each participant was patch tested with 2 chambers: one with 1% PPD and one vehicle control (Vaseline). The patch tests were left on for 2 days and reactions were read on both Day 2 and Day 4. On Day 4, skin biopsies were collected from both test sites and treated depending on further analysis.

Recruiting a sufficient number of participants was a challenge: most allergic individuals diagnosed at Gentofte hospital had a history of very severe reactions; accordingly, they were understandably reluctant to undergo further exposure to PPD.

Next Generation Sequencing

The RNA expression in the skin following PPD exposure was analyzed by next generation sequencing (NGS). The sequencing was done by the Functional Genomic Center at Zürich University, Switzerland using ribosomal depletion and TruSeq adapters (more details can be found in Manuscript I). The analysis generated expression data of 21,174 RNA sequences with 15,400 expressed genes detected, resulting in the three comparisons outlined in the result section (Figure 8). Differential expressed genes was defined as having a false discovery rate (fdr) below 0.015.

Pathway analysis and further analysis

Pathway analysis based on gene expression is still a developing field and many databases exist with different algorithms calculating the chances of certain pathways being relevant based on known interactions, the number of involved genes and the categorization of said genes. In this study, we used the Enricher platform, which supports Panther, KEGG and many other bioinformatics databases. The different databases vary in how they categorized the genes; it can be based, for example, on biological interactions, cellular processes, chemical structure, or relation to diseases. In this study, we used the Panther and KEGG databases. Panther is short for "protein analysis through evolutionary relationships" and proteins are classified according to four criteria: family and subfamily (structurally related proteins or proteins with the same function), molecular function (e.g. enzymatic function), biological process and pathway. KEGG stands for "Kyoto Encyclopedia of Genes and Genomes" and is a combination of 18 databases that include four categories of data: systems information (including signaling pathways), genomic information, chemical information and health information (including relevance in diseases). The databases contain overlapping data, but the KEGG database is preferred in cases where information on disease-specific pathways is of interest. More information about the databases

can be found here for Panther: http://www.pantherdb.org/about.jsp and for KEGG: https://www.genome.jp/kegg/kegg1a.html.

The visualization of specific pathways and protein interactions among the differentially expressed genes was done using the String and Cytoscape software (more details are in Manuscript II). The String software uses data mining to obtain information from online databases about protein interactions, structural similarities, co-expression and association with biological functions and diseases. In our study, we focused on known protein interactions and biological functions.

Results from the NGS data were confirmed by standard qPCR when possible, and relevant proteins were detected in the skin biopsies using confocal microscopy. Circulating T-cell phenotypes were analyzed by flow cytometry on PBMCs isolated from whole blood.

To further investigate the reaction to PPD in human skin, we used 3D skin cultures from Episkin. Skin cultures are grown from human keratinocytes from mammary skin and are 13 days-old on arrival. The cultures are multilayer air/liquid interface cultures grown on a collagen matrix and comparable to the keratinocytes in the epidermis. As the cultures consist purely of differentiating keratinocytes, only keratinocyte responses can be studied and any contribution of immune cells, innate and adaptive is lacking.

Results and Manuscripts

The NGS analysis resulted in three comparisons as shown in Figure 8. Allergic individuals had numerous differentially expressed genes, both up- and down-regulated. As a control, we compared the Vaseline control group with our two groups and found only one gene to be differentially expressed. This gene was histidine decarboxylase. Therefore, there were no differences in expression of barrier-related genes in the skin before PPD exposure among our groups. The Vaseline control groups from allergic and non-allergic individuals was therefore considered as one group throughout our study.

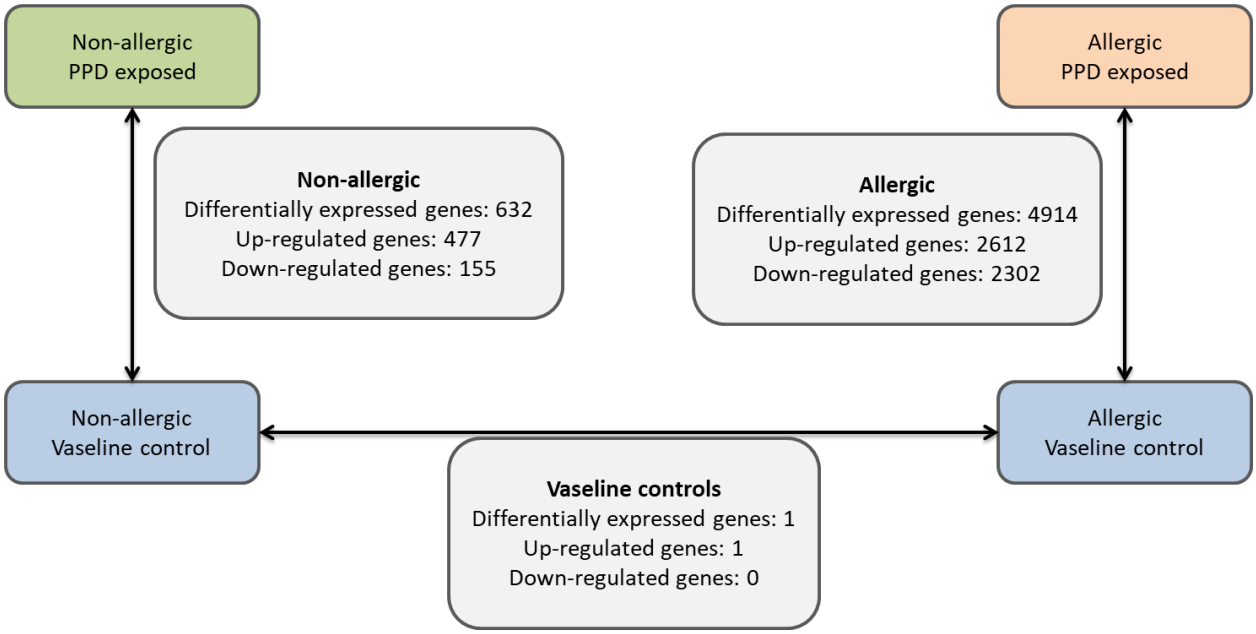

Figure 7. NGS gene expression comparisons among samples. Genes were considered differentially expressed when the false discovery rate (fdr) was below 0.015.

We started by looking into the skin barrier and focused on all barrier-related aspects in Manuscript 1. We then moved to the immune system and focused on the characterization of T-cell reactions in both the allergic and non-allergic group in Manuscript II.

Manuscript I

Skin barrier changes upon exposure to p-phenylenediamine in hair dye exposed individuals with and without development of allergic contact dermatitis

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Conflict of interest:

The authors declare no conflict of interest.

Abstract

Background: *p*-Phenylenediamine (PPD) is a strong contact allergen used in hair dye and known to cause allergic contact dermatitis. Both private and occupational exposure to PPP is frequent, but the effect of PPD exposure in non-allergic occupational exposed individuals is unknown.

Objectives: To investigeate effects of PPD exposure to the skin of occupational expossed individual without clinical symptoms.

Methods: Skin biopsies were collected from 4 mild and 5 severe PPD contact hypersensitive patients and 7 hairdressers without PPD contact allergy on day 4 after patch testing with 1% PPD in Vaseline. RNA-sequencing and transcriptomics analyses were performed and confirmed by qRT-PCR. Protein expression was analysed in skin from 4 hairdressers and 1 contact hypersensitive patient with immunofluorescence staining. Reconstructed human epidermis was used to test the effects of PPD *in vitro*.

Results: RNA-sequencing demonstrated down-regulation of tight junction (TJ) and *stratum corneum* (SC) proteins in skin of severely contact hypersensitive individuals after PPD exposure. Claudin-1 (CLDN1), claudin-8 (CLDN8), claudin-11 (CLDN11), CLMP, occludin, MAGI1, and MAGI2 expression were down-regulated in severely contact hypersensitive individuals. CLDN1 and CLMP expression were down-regulated in non-responding hairdressers. Filaggrin-1(FLG1), Filaggrin-2 (FLG2) and Loricrin expression were down-regulated in contact hypersensitive individuals. Confocal microscopy images showed down regulation of claudin-1 and filaggrin 1 and 2. In contrast, skin cultures showed up-regulation of Filaggrin-1 in response to PPD but down-regulation of Filaggrin-2.

Conclusion: PPD-exposed skin is associated with extensive transcriptomic changes including down-regulation of TJ and SC proteins even in the absence of clinical symptoms.

Clinical implications

Understanding skin barrier damage caused by strong contact allergens can not only improve treatment of allergic contact dermatitis but also assist in prevention of occupational allergic contact dermatitis.

Capsule summary:

Investigation of the skin barrier of healthy hairdressers after PPD-exposure, show specific damage to skin barrier components, overlapping with that seen in ACD patients, despite lack of clinical symptoms.

Abbreviations

ACD: Allergic contact dermatitis
AD: Atopic dermatitis
PPD: *p*-Phenylenediamine
CLDN: Claudin
CLMP: CXADR-like membrane protein
CRNN: Cornulin
FLG: Filaggrin
IVL: Involucrin
LOR: Loricrin
MAGI1: Membrane-Associated Guanylate Kinase Inverted 1
MAGI2: Membrane-Associated Guanylate Kinase Inverted 2
OCLN: Occludin
ROS: Reactive oxygen species
SC: *Stratum corneum*
TJ: Tight junction
VAS: Vaseline

Keywords

Allergic contact dermatitis, *p*-Phenylenediamine, hair dye contact hypersensitivity, tight junctions, S*tratum corneum*, RNA-sequencing.

Introduction

Allergic contact dermatitis (ACD), also referred to as contact hypersensitivity reaction, is mediated by T-cells and the lesion typically appears 2-7 days after exposure to the contact allergen [1]. The presentation and severity of the clinical reactions depends on the exposure site and type of contact allergen, and the diagnosis of contact dermatitis is given after a positive patch test to a clinically relevant contact allergen [2]. ACD reactions to *p*-phenylenediamine (PPD) typically result in eczema and typical symptoms include itchiness, redness and swelling, which can be severe with vesicles at the site of contact allergen exposure. Severe reactions to hair dye can cause swelling of the face and obstruction of the airways, which can even result in hospitalization. Other clinical manifestations include severe scalp eczema and complete or partial hair-loss. Additional stimulus, such as cold and dry weather, abrasive activity in damp conditions, and exposure to chemical or mechanical stress may contribute to the severity of lesions. Together with irritant contact dermatitis, ACD is one of the most abundant occupational diseases in Europe, resulting in a considerable socioeconomic burden due to cost of treatment and loss of productivity [3].

Occupations particularly at risk of developing ACD include hairdressers, who have regular contact with irritants and contact allergens. The hair dye component PPD used in dark permanent hair dyes is a well-known, strong allergen leading to ACD after as little as 2 exposures [1, 4]. Over the past 15 years, the hair dye industry has been attempting to chemically modify or find alternatives to PPD to improve the safety of cosmetic products, thus synthesizing an array of new ingredients with various degree of success [5]. Both hairdressers and consumers still experience a high level of exposure and 0.8% of the general population in Europe is sensitized to PPD [6]. Hair dye is the main source of PPD-exposure, but this contact sensitizing chemical is also an ingredient for temporary black henna tattoos posing a significant sensitization risk in children and young adults [7, 8]. Making individualized hair-dye mixtures or combining different products at home may also increase the exposure to PPD also for personal use [9]. Several studies have demonstrated how

using gloves when handling the dye mixtures and improving working habits can decrease the hairdresser's exposure to PPD [10, 11]. However, some exposure is unavoidable regardless of the health and safety training received by the hairdresser. Contact with PPD might occur when cutting newly dyed hair or by contamination in the workplace [12]. Thus, it is important to gain a better understanding of the effects of PPD exposure on the human body, especially on the skin barrier.

PPD has been shown to sensitize most exposed individuals after only two exposures [13]. Previous results by Uter et al., showed that 20% of the patch tested hairdressers had ACD to PPD based on data collected from the Information Network of Departments of Dermatology (2007-2012) on 824 female hairdressers [14]. PPD is a highly reactive small aromatic compound, and ACD to PPD is thought to be initiated when PPD or its immediate oxidative products, react with proteins in the skin [1]. Among the suggested mechanisms, is the oxidation of the amino acid cysteine in human serum albumin which can then function as an immunogenic epitope [15]. In contrast, acetylation of PPD by *N*-acetyltransferase from keratinocytes deactivates its sensitizing potential [16].

The skin barrier is the first layer of defense against potential pathogens, pollutants and contact allergens. In addition to the physical barrier provided by keratinocytes, the skin barrier contains elements of innate immunity such as anti-microbial peptides, pathogen recognition receptor systems, lipids, ions designed to prevent colonization by pathogens, and protecting the commensal microbiota. The physical barrier of keratinocytes depends on the formation of tight junctions (TJ). TJs seal the epidermal layer at the stratum granulosum against the outer environment. It is now known that disruption of epithelial barrier by deregulated TJs and *stratum corneum* (SC) proteins is one of the underlying causes of contact allergic skin diseases [17]

The TJ family proteins claudin-1 (CLDN-1), CLDN-8 and CLDN-23 are expressed at low levels in the lesional skin of atopic dermatitis (AD) patients. [18, 19] and polymorphisms in the claudin-1 gene is associated with increased sensitization to contact allergens [20]. Filaggrin-1 (FLG) and

filaggrin-2 (FLG-2) are expressed at lower levels in lesional skin of AD patients [21]. FLG mutations in humans lead to a susceptibility to hand eczema, childhood-onset AD and sensitization to contact allergens where the amount of filaggrin in epidermis is correlated with disease severity and quality of life [22-24]. A dysfunctional skin barrier is therefore not only involved in the development but also in the severity of skin diseases.

The full extent of changes in the epidermal barrier in ACD to PPD are currently unknown. In the present study, we report RNA-sequencing on skin biopsies from PPD contact hypersensitive patients and clinically non-responding hairdressers. We demonstrate changes in the level of expression in SC barrier proteins and TJs in PPD-exposed skin, with a multi-level damage to the skin barrier being observed not only in skin from ACD patients but also in non-responding individuals.

Materials and methods

Study population

The study included two groups: a PPD contact hypersensitive patient group (N=10) recruited from the Department of Dermatology and Allergy at Gentofte Hospital, Copenhagen (Denmark) and a group of PPD-exposed hairdressers without ACD referred to as non-responders (N=11). The hypersensitive group was diagnosed with ACD to PPD within the past 5 years. Non-responders were hairdressers with 5 or more years in the field and with no history of ACD, recruited from hairdresser salons and through the hairdresser unions platforms. The participants were adults between 18 and 60 years, further characteristics are listed in table 1. Patients with other inflammatory skin diseases or receiving immunosuppressive medication were excluded. All participants gave informed written consent and the study was conducted in accordance with the Helsinki declaration and approved by local ethics committee HGH-2015-032 (I-Suite nr: 03984).

Patch test

A total of 10 hypersensitive individuals and 11 non-responder hairdressers were patch tested with 20 mg of 1% PPD in vaseline (VAS) and a vehicle VAS control applied in 8 mm Finn chambers on the buttocks, affixed with Scanpore tape with an occlusion time of 48 h. The patch test was scored on days 2 and 4 according to the European Society of Contact Dermatitis (ESCD) criteria (IR, doubtful, 1+, 2+ or 3+) [2]. Punch biopsies (4 mm) were taken on day 4 from both the PPD and vehicle test-site.

RNA sequencing

Nine biopsies from hypersensitive patients and 7 biopsies from non-responders was used for RNA sequencing. The biopsies were placed in RNAlater and frozen in liquid nitrogen before storage at -80 °C. Total RNA was prepared from skin biopsies using the RNeasy Universal Plus Kit (QIAGEN, Hilden, Germany). The quantity and quality of the isolated RNA was determined with Qubit® 1.0 fluorometer (Life Technologies, California, USA) and Bioanalyzer 2100 (Agilent,

Waldbronn, Germany) and samples with RNA integrity number >5.0 were chosen for sequencing. Library preparation for RNA-seq was performed using the TruSeq Stranded mRNA Sample Prep Kit (Illumina, Inc., California, USA).

Total RNA samples (400 ng) were ribosome depleted and then reverse-transcribed into double-stranded cDNA with actinomycin added during first-strand synthesis. The cDNA samples were fragmented, end-repaired and polyadenylated before ligation to the TruSeq adapters. The adapters contain the index for multiplexing. Fragments containing TruSeq adapters on both ends were selectively enriched with PCR. The quality and quantity of the enriched libraries were validated using Qubit® 1.0 Fluorometer and the Bioanalyzer 2100 (Agilent, Waldbronn, Germany). The product is a smear with an average fragment size of approximately 360 bp. The libraries were normalized to 10 nM in Tris-Cl 10 mM, pH 8.5 with 0.1% Tween 20. The TruSeq SR Cluster Kit v4-cBot-HS or TruSeq PE Cluster Kit v4-cBot-HS (Illumina, Inc., California, USA) was used for cluster generation using 8 pM of pooled normalized libraries on the cBOT. Sequencing was performed on the Illumina HiSeq 2500 paired-end at 2x126 bp or single-end 126 bp using the TruSeq SBS Kit v4-HS (Illumina, Inc., California, USA).

Quality control on the raw sequence data was performed with FastQC (v.0.10.0, Babraham Institute, Cambridge, UK) and mapped to the Homo sapiens genome (GRCh38 build) using RSEM (v1.2.12) [PMID: 21816040] implementation of Bowtie software (v 1.0.0) [PMID: 19261174] alignment program with the Ensembl annotation (v 75). Gene and isoform level abundances were quantified as RPKM values. Clustering analyses were performed using the “ward.D2” clustering algorithm implemented in the “hclust” function of R statistics package. Heatmap plots were performed with the function “heatmap.2” implemented in the gplots R package.

Differential expression analyses between two groups were performed using edgeR Bioconductor package [PMID: 19910308]. Genes present in less than 75% of samples in both conditions were excluded. Q-values were calculated using the Benjamini-Hochberg method and genes with a q-

value <0.015 and an absolute value of log2 (fold change)>1 were kept for further analysis. Gene ontology (GO) term enrichment analysis was performed using GOseq Bioconductor package [PMID: 20132535] using the Wallenius approximation. Pathway analysis was done using the Enricher platform with the Panther database [25, 26]. Significant pathways were defined as pathways with an adjusted P-value below 0.01.

Confocal microscopy

Biopsies were collected on day 4 from 4 non-responder hairdressers and 1 PPD hypersensitive patient. Upon collection, the biopsies were embedded in TissueTek, frozen in liquid nitrogen and stored at -80 °C. The biopsies were cut in 7 µm slices, fixed with 4% PFA on glass slides and stained with primary antibodies against claudin-1 (Abcam FLG01), CLMP (Abcam ab171552), filaggrin-1(Abcam ab3137), filaggrin-2 (Abcam ab122011), secondary fluorochrome labeled antibodies, goat anti-rabbit AF546 (Invitrogen A-11010), goat anti-rabbit AF488 (Invitrogen A-11034), and goat anti-mouse AF546 (Invitrogen A-11030). Apoptosis stainings were performed using Click-iT TUNEL Alexa Flour 674 Imaging Assay (Bioscience).

3D Skin cultures

Reconstructed human epidermis grown for 13 days (Episkin, France) were cultured overnight in an air-liquid interface prior to use. Cultures were stimulated with 0.5% PPD in PBS, 0.5% H_2O_2 in PBS or PBS alone. Skin cultures were collected at 8 h and 24 h and imaged by confocal microscopy.

Statistics

Significantly differentially expressed genes were defined as genes with a false discovery rate (FDR) below 0.015. Gene expression between paired samples, PPD-exposed and VAS exposed skin from the same individual were analyzed using the Wilcoxon signed-ranked test and comparison between groups was done using the Mann-Whitney test. Venn diagrams of significantly regulated genes between comparison groups were visualized with the online application “genevenn.sourceforge.net”

Results

Altered gene expression profiles linked to severity of lesional skin

All patch tests from non-responder hairdressers and the histology of tissue sections showed no remarkable changes, whereas hypersensitive individuals showed reactions ranging from mild swelling and redness to severe blister formation (3+ patch test reaction) followed by necrosis (Figure 1A). RNA sequencing, subsequent principal component analysis (PCA) and hierarchical clustering of skin biopsies collected from patch tested individuals showed a unique gene expression profile in PPD-exposed skin in PPD hypersensitive patients with skin lesions with higher degree of severity (graded +2 and +3) (Figure 1B). Gene expression profile of mildly hypersensitive individuals (graded +1 or doubtful) clustered more closely in VAS-exposed skin from non-responders and PPD hypersensitive patients, as well as PPD-exposed skin from non-responder individuals. The RNA expression in vaseline controls from hypersensitive individuals and non-responder hairdressers did not differ from each other, only one gene out of 21175 sequences was significantly differential expressed. Overall, hierarchical clustering of skin biopsies according to the top 2000 genes showed that the transcriptomic signature of PPD-exposed skin depends on the severity of the clinical manifestation. Severe ACD reactions clustered together, whereas samples from mild ACD reactions and non-responder PPD-exposed skin were dispersed (Figure 1C). In addition, inflammatory signs such as erythema, thickening of the skin and inflammatory papules were visible in the skin of severe and mild ACD reactions on day 2, increasing in severity on day 4. Skin characteristics and phenotype did not change with VAS stimulation and in non-responders at day 2 or day 4 (Fig 1D)

Gene expression patterns differ between ACD and non-responder groups

Next, we investigated the enriched pathways for the differentially expressed genes between severe and mild ACD reactions and non-responder skin biopsies. Differentially expressed genes from the three groups were depicted as up-regulated (Figure 2A) and down-regulated genes (Figure 2B) and compared between the three groups (up-regulated Figure 2C, down-regulated Figure 2D). 2636 up-regulated genes were found to be unique for the severely hypersensitive group, 53 for the mildly hypersensitive and 22 for the non-responder group, further 376 genes were up-regulated in all groups. The shared or unique genes in each group were analyzed for significant pathway associations (Figure 2A). Up-regulated pathways in hypersensitive individuals belonged to immune response pathways involved in T-cell recruitment and activation, inflammation, cytokine and chemokine signaling and Toll-like receptor signaling. In the severely hypersensitive group, pathways involved in cell damage and death were also significantly upregulated. The non-responder group shared up-regulation of T cells activation and inflammatory cytokine and chemokine signaling. Unique to non-responders was a pathway involved in serotonin degradation. Among the down-regulated genes, there were 3382 unique genes in the severely hypersensitive group, 69 genes in the mildly hypersensitive group and 17 genes in the non-responders (Figure 2D). The most significantly down-regulated gene pathways included cell adhesion, tissue regeneration and cell metabolism (Figure 2B). Association of down-regulated genes with cellular adhesion and cellular junction pathways, as well as identification of multiple genes with known function on skin barrier in individual gene lists led us to investigate the state of skin barrier and cell adhesion components in PPD hypertensive skin (Figure 3).

Dys-regulation of cell-cell junction genes happens in both non-responders and individuals with ACD

To analyze the expression of the TJ components in the skin, 47 genes with known association of function with the TJ gene family were curated from Gene Ontology (GO), Kyoto Encyclopedia of

Genes and Genomes (KEGG) databases. Out of the 47 genes, 37 were expressed in the skin, taking a mean expression threshold value of >10 unique read counts. Analysis of the TJ gene expression in non-responders, mildly hypersensitive and severely hypersensitive skin groups revealed that 15 out of 32 (47%) TJ genes were significantly down-regulated in one or more groups (Figure 3A). All of the 15 genes were significantly down-regulated in severely hypersensitive individuals, whereas CLDN1 and CXADR-like membrane protein (CLMP) were significantly down-regulated in the mildly hypersensitive group. CLMP and CLDN1 down-regulation did not reach statistical significance in the PPD-exposed non-responder group but showed a similar pattern to the down-regulation observed in PPD-exposed hypersensitive individuals (Figure 3B). Validation of RNA sequencing results with qPCR confirmed the findings for TJ genes CLDN1, CLDN8 and CLMP (Figure 3C). PPD exposure also affected the expression of gab-junctions, desmosomes and keratins i all groups. The degree of deregulation followed the severity of clinical symptoms and as such most genes was deregulated in severe ACD reactions (Figure 3D).

PPD causes deregulation of filaggrin family proteins in the skin of ACD

In line with the down-regulated pathways identified in our differential expression analysis of PPD exposed and unexposed skin groups, we analyzed the mRNA expression of the 9 filaggrin family genes (FLG, FLG2, IVL, CRINN, LOR, RPTN, TCHH, TCHHL1, HRNR) and found 5 (56%) differentially expressed genes in one or more groups after PPD exposure (Figure 4A). Severe ACD reactions to PPD were associated with mRNA down-regulation of 4 filaggrin family members; FLG1, FLG2, loricrin (LOR) and cornulin (CRNN) (Figure 4A). The analyses of expression levels showed a decrease in some individuals in the mildly hypersensitive and non-responder group, whereas the effect was more pronounced in the severely hypersensitive group, which showed a stronger decrease (Figure 4B). In contrast, involucrin (IVL) was significantly up-regulated in the patients with severe ACD reactions but remained constant in the mildly hypersensitive group and

the non-responder group. This pattern was also seen in correlation analyses between differentially expressed TJ and SC genes (Figure E1). Only involucrin was negatively correlated to the other barrier genes in the overall analysis (Figure E1A), all others showed either no correlation or a positive correlation.

ACD patients and non-responder individuals both showed decreased expression of barrier proteins by PPD

Changes in barrier protein expression levels and their location in the epidermis were visualized by confocal microscopy in the skin biopsies on day 4 of patch test with PPD from 4 non-responder hairdressers and 1 severe hypersensitive patient (Figure 5). The TJ proteins claudin 1 and CLMP were localized to the cell surface membrane forming a net-like pattern between the keratinocytes throughout epidermis in VAS controls of both the non-responder hairdressers and the severely hypersensitive individual (Figure 5A). Claudin 1 protein expression decreased after PPD exposure in non-responder individuals but the net-like structure in the epidermis remained intact. PPD exposure had only limited visual effect on CLMP protein expression and the net-like structure in non-responder individuals However, PPD exposure caused complete disruption of the epidermis in severely hypersensitive individuals, causing the net-like structure of both claudin 1 and CLMP to disappear.

Skin exposure to PPD decreased protein expression of FLG1 and FLG2 in both hypersensitive and non-responder individuals. Although an even distribution of both FLG1 and FLG2 proteins was observed in the outer layer of epidermis in the VAS controls of both the non-responder hairdressers and the severely hypersensitive individual (Figure 5B). FLG2 was also expressed in the basal membrane and this expression did not appear to be affected by PPD exposure in the non-responder individuals (Figure 5B).

PPD affected expression patterns of claudin-1 and CLMP and decreased expression of filaggrin-2 in cultures of skin equivalents without inducing apoptosis

Since PPD is highly oxidative, we tested its direct toxicity on healthy skin cultures after 8 and 24 h of exposure. There was no indication of increased apoptosis after PPD exposure, though a general cell death in the basal membrane was observed after 24 h in all cultures. We next investigated the expression of barrier proteins and found slight decrease in CLND1 expression at the cell surface of keratinocytes with no signs of disorganization 8 h after PPD exposure. Hydrogen peroxide was applied to the skin cultures as a positive control for oxidative stress, and it resulted in a significant decrease in CLDN1 expression. In healthy human skin, the CLMP protein is mainly expressed at the cell membrane and, in lower levels, at the cytosol. After exposing skin to PPD for 8 h, we observed a decreased expression but remained localized to the cell surface. In contrast, hydrogen peroxide exposure resulted in dispersion of protein expression at the cell surface, but a more homogenous expression was observed in the cytosol. After PPD or hydrogen peroxide exposure, the FLG1 levels in skin cultures remained unchanged but we observed a decrease in FLG2 expression (Figure 6A-B).

Discussion

The present study provides an in-depth analysis on the changes in the epithelial barrier components after exposure to PPD, a strong contact allergen often found in dark hair dyes. Hairdressers which repeatedly where exposed to PPD through their job but never showed sign of contact dermatitis might tolerate this exposure and we therefore investigated their skin response to PPD exposure. The effect of PPD exposure were compared between 3 groups; non-responder hairdressers, mildly and severely hypersensitive individuals. The exact mechanism for sensitization is unknown; one theory is that the patients are sensitized to contact allergens due to inherent skin barrier defects leading to increased permeability of the skin. Most hairdressers do not develop ACD to PPD despite being exposed to the contact allergen for prolonged periods of time. One explanation could be that they have a stable and strong epidermal barrier offering protection and tolerance towards this contact allergen exposure. However, our study did not indicate any differences in skin barrier protein expression in hypersensitive patients and non-responder hairdressers in the vaseline exposed skin Overall, TJ and SC proteins showed similar expression levels in both mildly, severely hypersensitive individuals and non-responders. In view of these results, we propose that the development of ACD lesions is not driven by inherent differences in skin barrier stability between hypersensitive and non-responder individuals but rather a result of tissue signaling, metabolism or inflammation caused by PPD exposure.

Expression of TJ proteins CLDN1 and CLMP were down-regulated in both mildly and severely hypersensitive individuals, as well as in non-responders after PPD exposure, suggesting that PPD exposure disturb the expression levels of these TJ proteins, even when no clinical symptoms are seen. The rest of the significantly differentially expressed TJ and SC barrier molecules were mainly down-regulated upon PPD exposure in the severely hypersensitive group, and to a lesser extent in the mildly hypersensitive group, where inflammation was also present. Confocal microscopy imaging of claudin 1, filaggrin-1 and filaggrin 2 confirms a decrease of these barrier molecules in

non-responding individuals. Skin inflammation in AD, contact dermatitis and psoriasis have been reported to down-regulate fillagrin-1, filagrin-2 and claudin-1 [27, 28]. Together this suggests that only CLDN1 and CLMP down-regulation is directly caused by PPD exposure and that the remaining deregulation of TJ proteins is probably caused by the subsequent allergic inflammation.

PPD is a highly reactive chemical and has a half-life of a few hours on human skin [29]. If left in contact with oxygen it polymerizes into its trimer Bandrowski's base, which also has immune activating abilities [30]. In healthy skin, PPD is *N*-acetylated to a safe metabolite in the uppermost layers of the skin [31]. When PPD concentrations saturate the *N*-acetyltransferases in the skin, the remaining PPD can oxidize and cleave a wide range of proteins on the cell surface, releasing non-self-cleaved peptides into the tissue environment. Reactive oxygen species (ROS) are formed during the oxidation of PPD, and these activate the innate immune response and the recruitment of non-specific lymphocytes and direct them to the contact site [32]. The specific epitopes and the type of signals that reach skin resident antigen presenting cells are currently unknown. ACD lesions in PPD hypersensitive individuals are persistent and lesions can reoccur in previously exposed sites indicating formation of allergen specific memory cells in PPD hypersensitive individuals.

The early events in ACD resembles that of irritant contact dermatitis and the involve skin barrier damage or stress which results in the activation of innate immune cells [33]. Most contact allergens, including PPD is known to have irritant properties. In our study we attempted to limit the irritant response by using the standard concentration of PPD optimized for detecting ACD without causing clinically visible irritant reactions. The direct damage of the skin barrier caused by PPD in non-responder hairdressers could mean that PPD exposure increase the risk of both irritant contact dermatitis and ACD to other allergens. Damage to the epithelium was evident in the skin of non-respoder hairdressers 4 days after PPD exposure, when the clinical manifestations were fully visible in ACD patients. However, a more extensive analysis at earlier time points is needed to conclude if

there is a difference in immediate response to PPD between non-responders and individuals with ACD.

Non-responders do not display deregulation of the epidermal barrier except for CLDN-1 and CLMP at day 4 after PPD exposure. We propose that the higher stability of the epidermal barrier in non-responding individuals upon exposure to the PPD allergen is due to differences in the immune response and possibly a more efficient conversion of PPD to its safe *N*-acetylated product. Differences the in immune response could involve a higher threshold for innate immune response activation and a resistance to irritant exposure in non-responding individuals or a development of immune tolerance, more research is need to answer that question. The stimulation of healthy keratinocytes in skin cultures led to a higher expression and displayed a more coherent protein localization of major barrier proteins FLG1 and CLDN1. This observation might indicate that a healthy tissue response to PPD exposure up-regulates its key proteins, strengthening the epidermal barrier. This response might be lost amid the inflammatory environment in individuals with ACD.

In summary, we identified the state of skin barrier in terms of tight junction and stratum corneum proteins in individuals with ACD to PPD and in occupational exposed non-responders. We observed that the majority of TJ and SC proteins are significantly down-regulated in the skin and protein expression was significantly reduced in severely hypersensitive patients and to a lesser extent in mildly hypersensitive patients. We identified two TJ proteins, CLDN1 and CLMP that are also down-regulated in the skin of non-responder individuals upon PPD exposure. This direct barrier damage may be the reason for the highly sensitizing nature of PPD and emphasize the problems of occupational exposure.

Acknowledgments

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for her assistance in optimizing immuno fluorescence staining and Anne Marie Topp for her help with recruitment of participants, gathering patients information and patch testing data.

Group (n)	Sex	Age (mean)	PPD reaction score	Other contact allergies	Type 1 allergies	Non-allergic eczema	Occupational PPD exposure
Non-responders (7)	F	32.1	0	0/7	0/7	0/7	7/7
Mildly allergic (4)	F	36.3	? / +1	3/4	4/4	2/4	2/4
Severely allergic (5)	F	45.6	+2/+3	5/5	3/5	3/5	0/5

Table 1. Patient characteristics

Characteristics of the individuals included for NGS analysis. Individuals from the non-responder group did not have diagnosed allergies, atopic dermatitis or contact dermatitis. Occupational exposure was based on the participants work and own report of exposure. All PPD hypersensitive individuals had a history of rash upon PPD exposure either from hair dyes, dark henna tattoos, photographic developer chemicals or other known sources of PPD exposure. Mild reactions was defined as doubtful reactions (?) and positive (+1) and severe reactions was defined as +2 and +3 positive reactions.

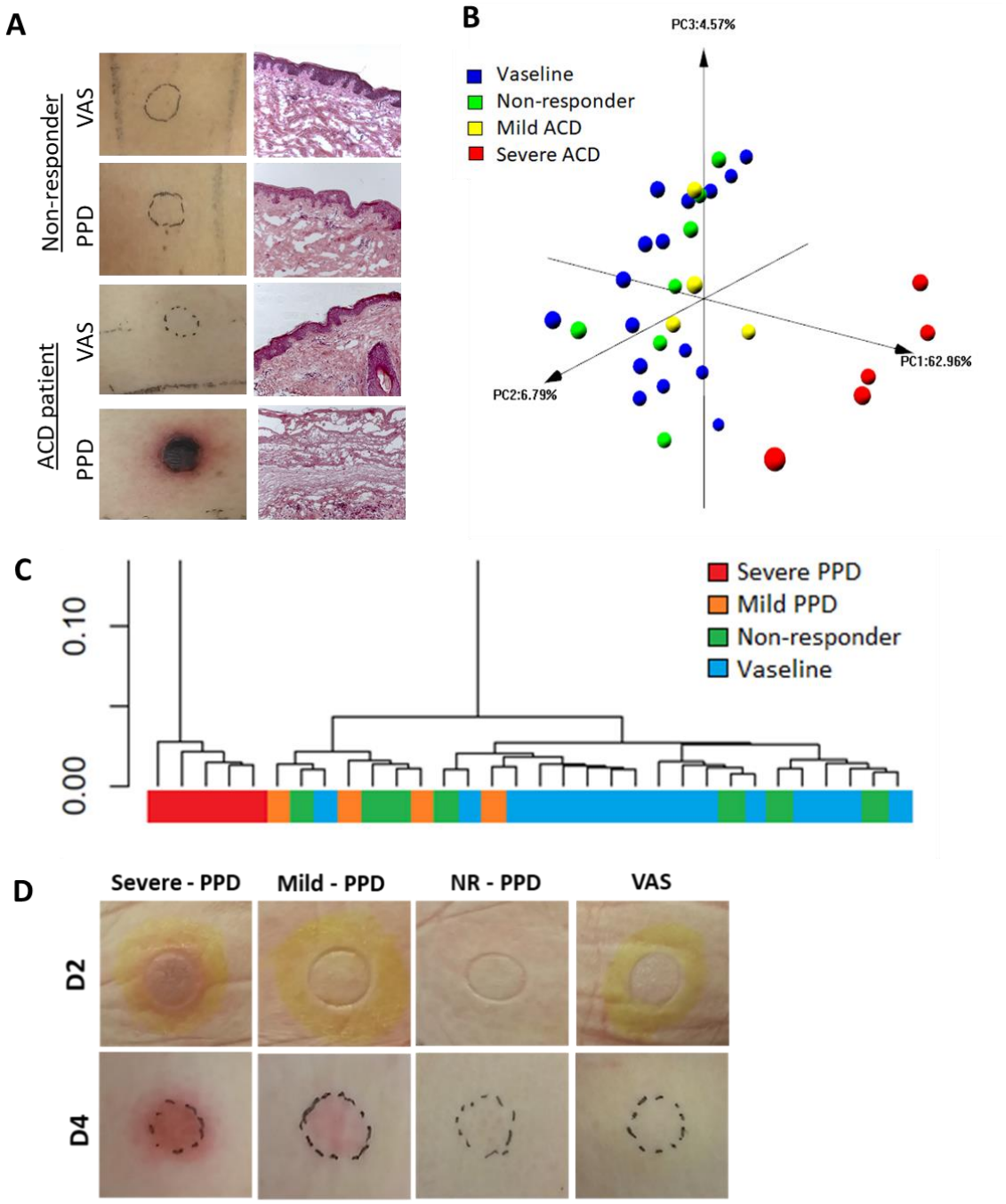


Figure 1. Reactions to PPD patch test in non-responder and hypersensitive individuals. Clinical reactions and corresponding ematoxylin and eosin (H&E) staining of tissues on day 4 after patch testing with 1% PPD in VAS or control (VAS only) (A). 3D PCA plot of sample clustering in top 2000 differential expressed genes demonstrated a distinct clustering of severe lesions (B). Hierarchical clustering of samples in top 2000 differentially expressed genes; severe ACD reactions (red) n=5, mild ACD reactions (yellow) n=4, PPD-exposed skin from non-responder individuals (NR) (green) n=7 and VAS controls (purple) n= 6 (C). Skin lesions in response to PPD and VAS as control on days 2 and 4 after patch is applied (D).

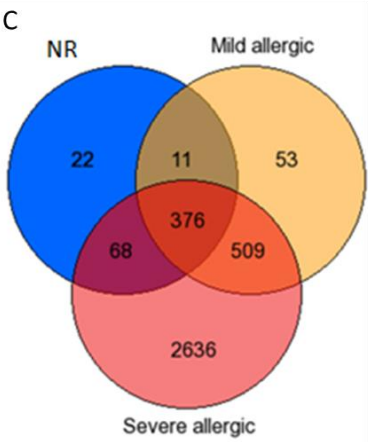
A

Group	Pathway	N	Adjusted P-value
NR	Serotonin degradation	1	0.005488
NR / Mild:	None significant	-	
Mild:	None significant	-	
Mild / Severe	Inflammation mediated by chemokine and cytokine signaling	20	0.00000259
	T cell activation	13	0.00000238 4
	Apoptosis signaling	11	0.001323
Severe:	Apoptosis signaling	39	1.791e-8
	CCKR signaling map ST	46	0.00002091
	Parkinson disease	28	0.00002171
	Interleukin signaling	29	0.00002171
	Integrin signaling	39	0.0009766
	VEGF signaling pathway	17	0.005042
	Inflammation mediated by chemokine and cytokine signaling	41	0.006756
	Toll receptor signaling	16	0.005042
	EGF receptor signaling	27	0.006756
	FAS signaling	12	0.005042
	B cell activation	17	0.006756
Severe /NR	p53 pathway	4	0.002301
All shared	T cell activation	8	0.003221
	Inflammation mediated by chemokine and cytokine signaling	12	0.005764

B

Group	Pathway	N	Adjusted P-value
NR	None significant	-	
NR/ Mild:	Cholesterol biosynthesis.	3	8.096e-6
Mild:	None significant	-	
Mild / Severe	Circadian clock system	3	0.0009422
Severe:	Cadherin signaling	70	1.967e-15
	Wnt signaling (skin tissue regeneration)	98	4.009e-12
Severe /NR	None significant	-	
All shared	Cholesterol biosynthesis	3	0.0000486 6

C



D

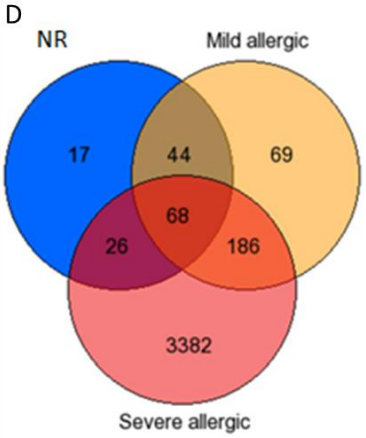


Figure 2. Pathway analysis of differentially expressed genes after PPD exposure. Comparison and pathway analysis of significantly up-regulated (A) or down-regulated (B) genes among severe ACD reactions (red) n=5, mild ACD reactions (yellow) n=4, PPD-exposed skin from non-responder individuals (NR) (blue) n=7 compared to their vaseline controls (significance defined as a fdr > 0.015). Pathway analysis using the EnrichR/Panther databases of up- or down-regulated genes in all groups and in genes overlapping between one or more groups. The number of involved genes in each pathway is indicated by N in the tables. Venn diagrams indicating the

number of unique and shared genes significantly up-regulated (C) or down-regulated (D) between comparisons.

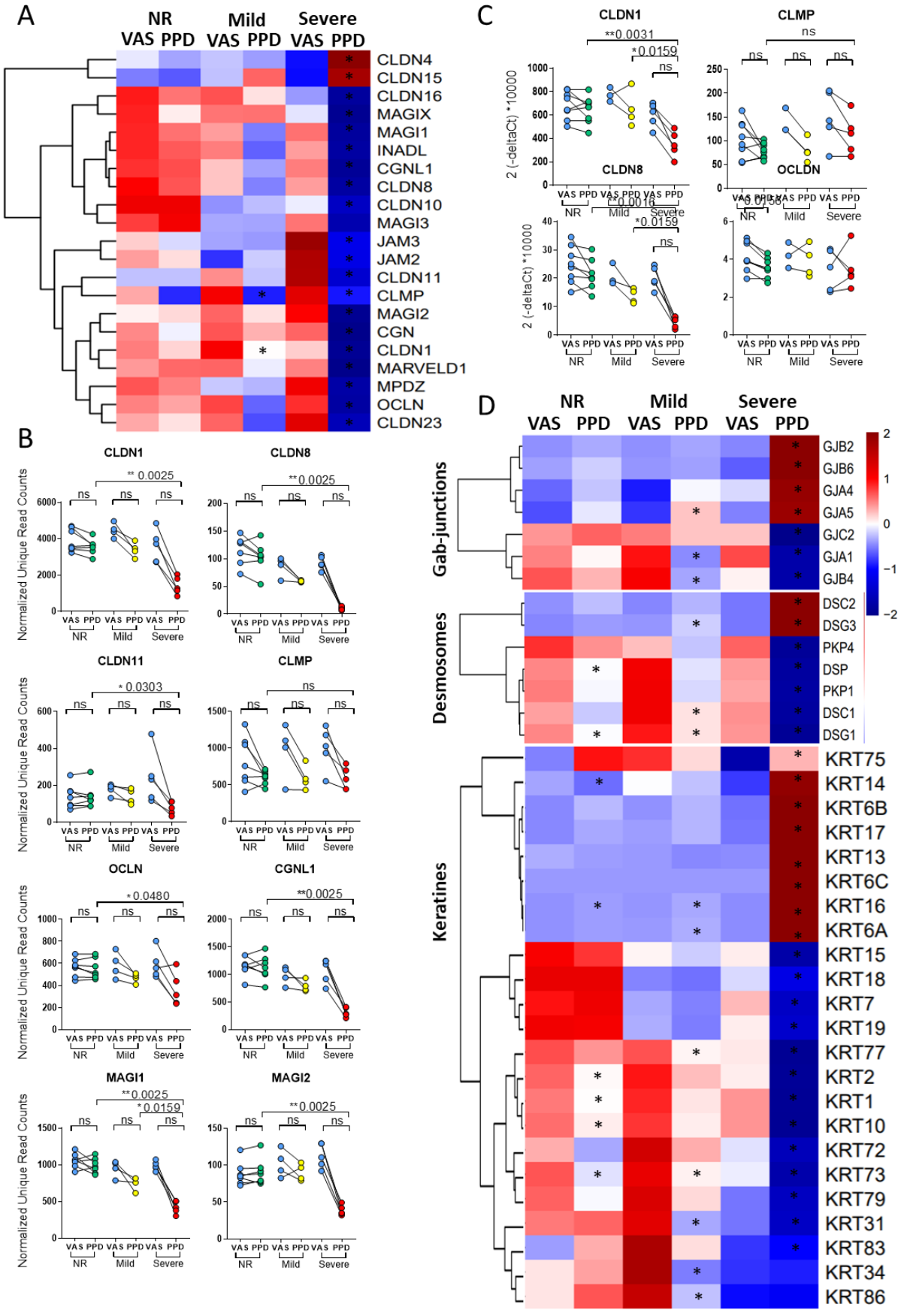


Figure 3. Steengaard Meisser et al.

Figure 3. Significantly regulated TJ proteins in PPD-exposed skin. Heatmap showing the log2 fold change of significantly differentially expressed TJ proteins in PPD exposed skin compared to vaseline controls in one or more groups; non-responder (NR), mild ACD reactions (mild) and severe ACD reactions (severe) (A). significance in the individual groups is indicated by * (fdr < 0.015). Scatter plots for the top 8 most TJ proteins in ACD skin reaction. Statistical comparisons for all subject groups were analyzed using paired or non-paired data (Wilcoxon signed-rank and Mann-Whitney test respectively) (B) qPCR confirmation of several TJ proteins significantly down-regulated in severe ACD skin to PPD (severe reactions N=5, mild reactions N=3/4 (data missing from one vaseline control), NR N=7). mRNA expressions confirmed by PCR were calculated as arbitrary units ($2^{(-\Delta Ct)}$*1000) according to EEF1A expression (C). Heatmap showing the log2 fold change of significantly differentially expressed gab junction, desmosome and keratine genes in PPD-exposed skin compared to vaseline controls in one or more groups; non-responder (NR), mild ACD reactions (mild) and severe ACD reactions (severe), Significance in the individual groups are indicated by * (fdr < 0.015) (D).

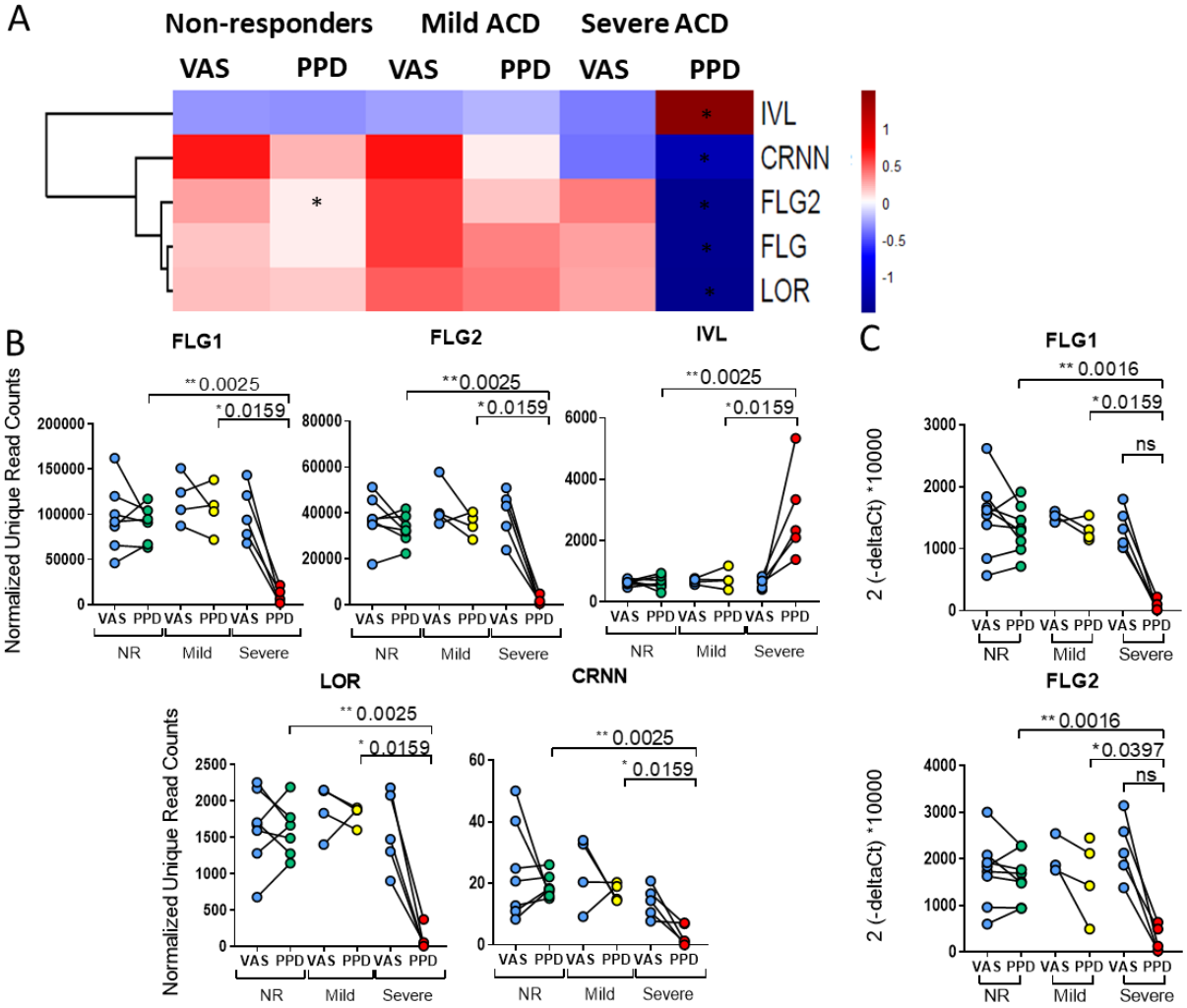


Figure 4. Significantly regulated SC proteins in PPD-exposed skin. Heatmap showing significantly differentailly expressed SC proteins in PPD exposed skin compared to vaseline controls in one or more groups; non-responder (NR), mild ACD reactions (mild) and severe ACD reactions (severe), Significance in the individual groups are indicated by * (fdr < 0.015) (A) significance in the individual groups is indicated by * (fdr < 0.015). (A) Scatter plots for the 5 significantly differential expressed SC molecules in PPD-exposed severe ACD skin reactions, Statistical comparisons between groups, were analyzed using paired or non-paired data (Wilcoxon signed-rank and Mann-Whitney test respectively) (B) qPCR confirmation of FLG1 and FLG2 in severe ACD reactions to PPD (severe reactions N=5, mild reactions N=3/4 (data missing from one vaseline control), NR N=7). Gene expression calculated as arbitrary units ($2^{(-\Delta Ct)}$*1000) according to EEF1A expression (C).

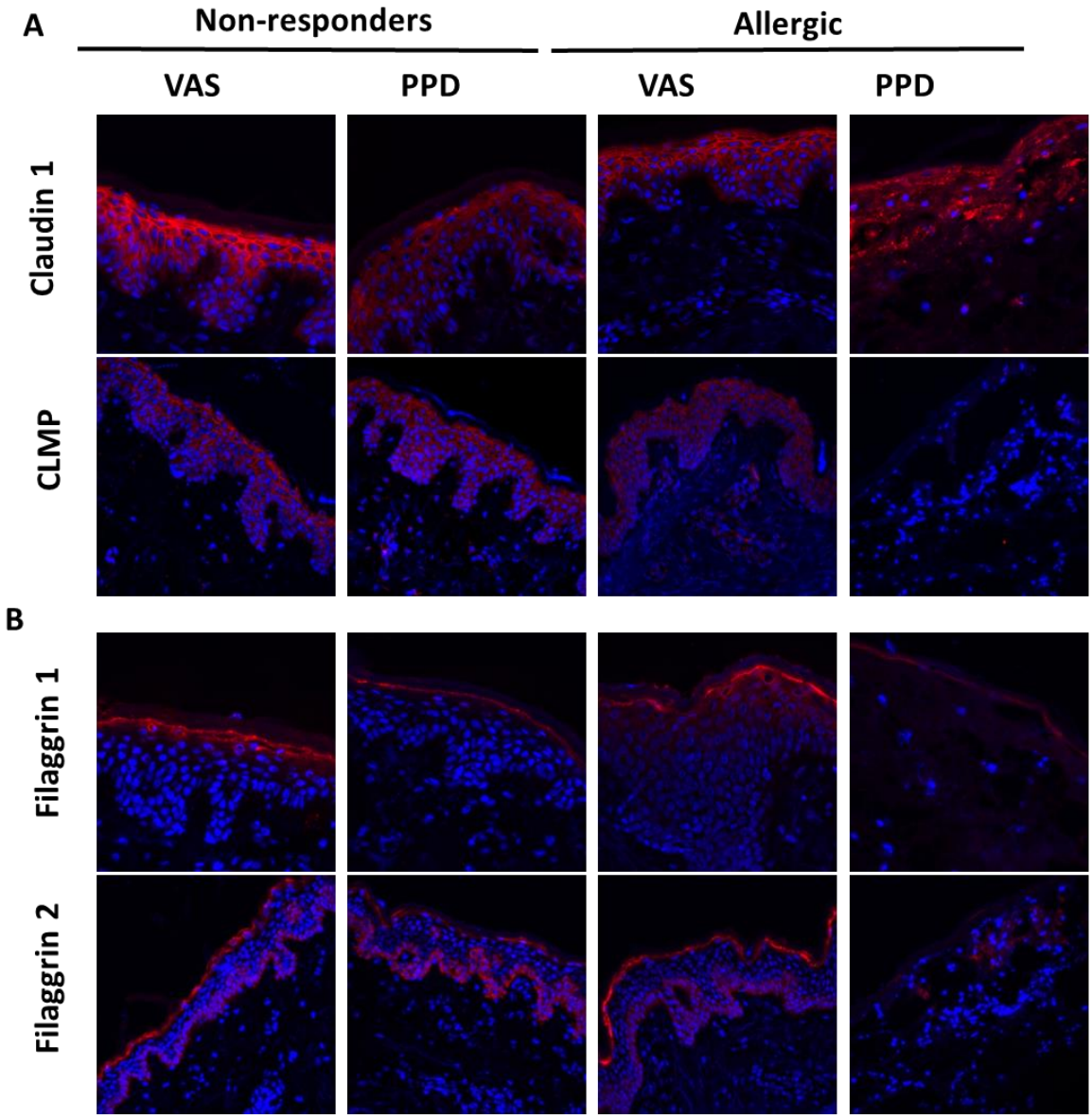


Figure 5. Confocal microscopy images of barrier proteins in human skin biopsies. Immuno fluorescence imaging of TJ proteins CLDN1 and CLMP (A) FLG1 and FLG2 (B) in non-responder (n=4) and severe ACD (n=1) individuals on day 4 after skin patch test with vaseline (VAS) or 1% PPD in VAS.

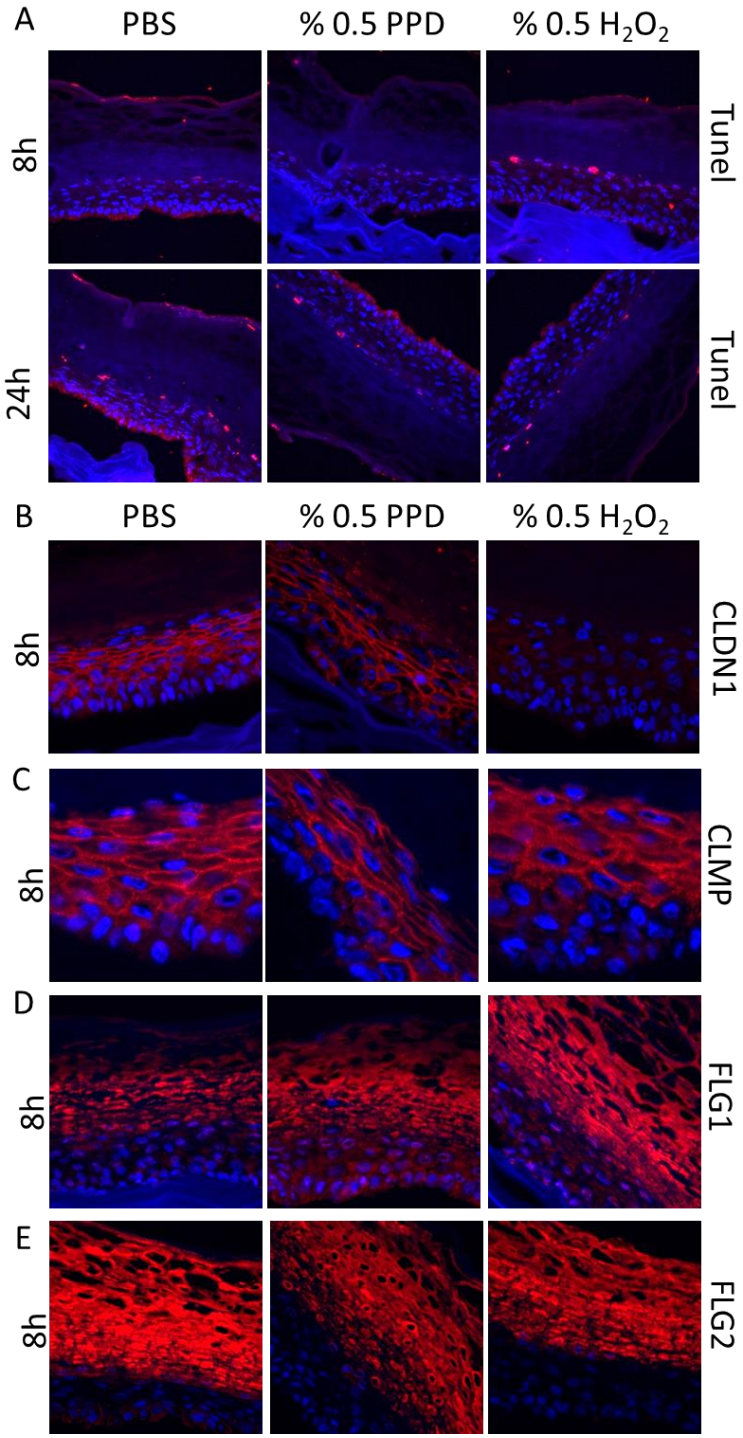
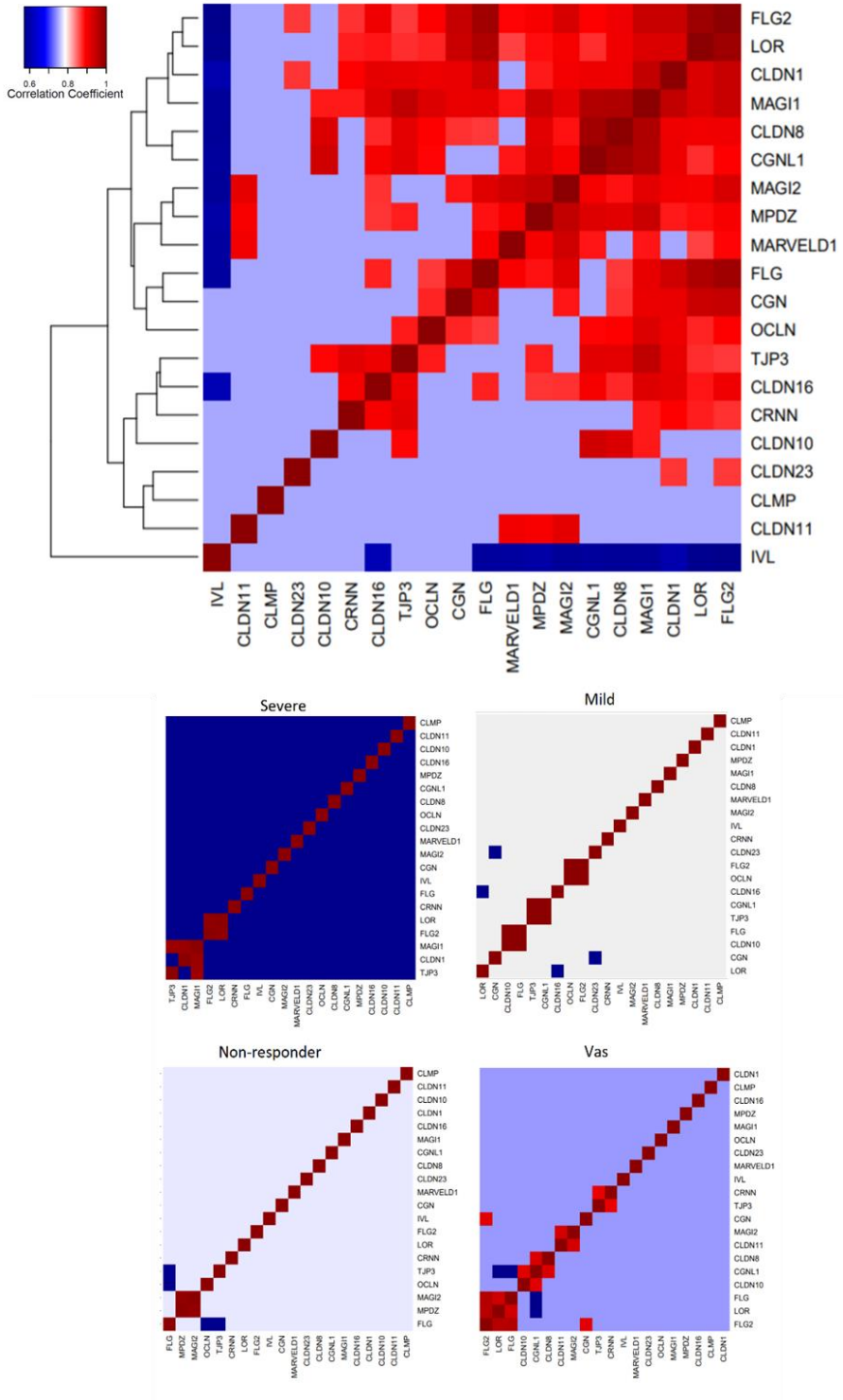
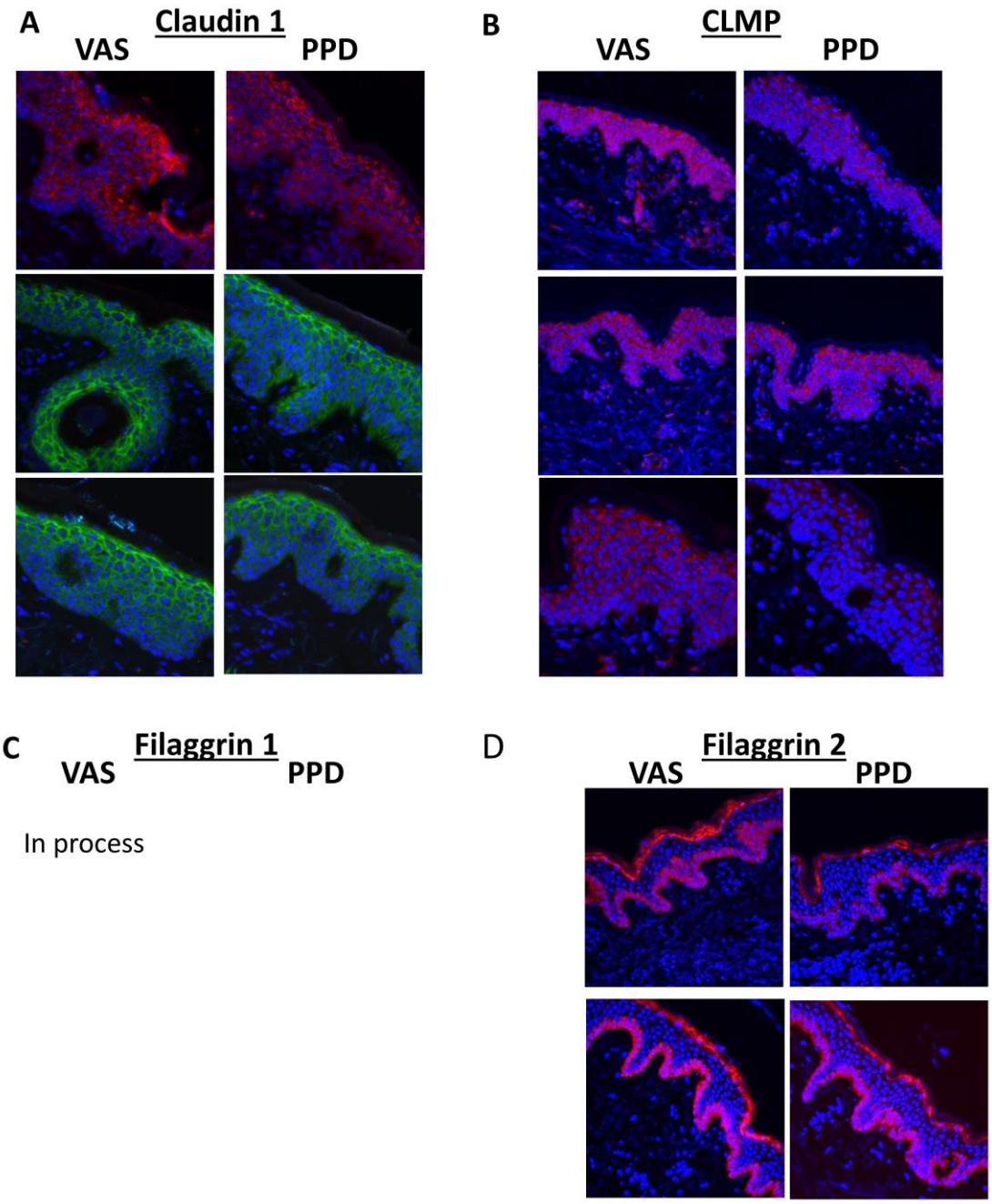


Figure 6. Confocal microscopy images of apoptosis and barrier proteins in skin equivalent cultures, 8 and 24 h after PPD exposure. TUNEL staining showing apoptosis in skin cultures after 8 and 24 hours after contact with PPD (A). Epidermal barrier proteins CLDN1 (B), CLMP (C), FLG1 (D) and FLG2 (E) were stained 8 h after PPD exposure. Vehicle alone (PBS) and 0.5% H_2O_2 were used as the negative and positive control, respectively.



Supp. Figure 1. Correlation of TJ and SC gene expression. Heat map showing Pearson correlation for the differentially expressed TJ and SC genes in PPD-exposed skin, positive correlations (red) and negative correlations (blue), R adjusted for p= 0.01. (A) All samples combined and (B) separate heat maps for the 4 groups, severe ACD reactions, mild ACD reactions, non-responder, and vaseline controls.



Supp. Figure 2. Protein expression of claudin 1, CLMP, filaggrin 1 and filaggrin 2 in non-responder skin after PPD exposure. Additional confocal pictures of the remaining non-responder biopsies. CLDN1 (A), CLMP (B), FLG1 (data not shown) (C) and FLG2 (D) were stained (RED or GREEN) and dapi (Blue).

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Manuscript II

Subclinical up-regulation of immune related genes in skin from non-allergic hairdressers exposed to *p*-Phenylenediamine

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Abstract (244 words, max 250)

Background: *p*-Phenylenediamine (PPD) is a strong contact allergen found in many hair colour products. Interestingly, only few hairdressers regularly exposed to PPD develop allergy to PPD. It is un-clear whether some individuals are true non-responders to PPD or whether there is different interindividual immune responses to PPD.

Objective: To determine and compare the response to PPD in non-allergic individuals regularly exposed to PPD versus the response to PPD in individuals allergic to PPD.

Methods: 11 regularly exposed, non-allergic hairdressers and 10 individuals with known allergy to PPD were patch tested with PPD and vaseline (VAS). Skin biopsies were collected at patch test site after 96 hours and analysed by RNA-sequencing and transcriptomic analyses. Protein expression was analysed by confocal microscopy.

Results: mRNA expression was affected in both non-allergic individuals and individuals allergic to PPD. The most up-regulated pathways in both groups were I) inflammation mediated by chemokine and cytokine signalling II) T cell activation and III) apoptosis. An increased number of genes associated with the 3 pathways were up-regulated in individuals allergic to PPD and the up-regulation of mRNA correlated with patch test reaction severity. Both groups showed signs of T-cell infiltration in the skin and activation of cytotoxic T cells and apoptosis.

Conclusion: We found that exposure to PPD leads to immune activation in both regularly exposed non-allergic and allergic individuals. However, the magnitude and the nature of the immune response elicited by PPD determines whether the patient will experience symptoms of PPD allergy.

Introduction

Contact allergy (CA) is a common condition found in at least 27% of the European adult population [1]. In case an individual with CA is re-exposed to sufficient amounts of the allergen in question allergic contact dermatitis (ACD), an inflammatory skin disease, will elicit. CA is a T cell-mediated immune reaction which can be induced after exposure to contact allergens on the skin. Contact allergens are low molecular weight chemicals that modify self-proteins in a way that they become immunogenic. Following exposure of the skin to a contact allergen in allergic individuals, dendritic cells are activated and migrate to the draining lymph nodes where they present the allergen-modified self-proteins to naïve T cells. This leads to activation of T cells and their further differentiation into effector and memory T cells. A subsequent exposure to the specific allergen results in reactivation of the memory T cells. The activated memory T cells mediate their effector function in the skin at the site of allergen exposure leading to destruction of allergen-modified keratinocytes and the clinical symptoms of ACD [2, 3] . Both $CD4^+$ and $CD8^+$ T cells are involved in the response. Whereas $CD8^+$ T cells mainly play an inflammatory role, $CD4^+$ T cells have both inflammatory and anti-inflammatory roles [4]. In general, Tc1 and Th1 cells are considered the major players in the response via their production of IFN-γ and their killing mechanisms. In addition, both Th2 and Th17 cells can be involved in the response [5]. Finally, different types of regulatory T cells play a role in termination of the inflammatory response and in mediating tolerance to the contact allergen [6].

p-Phenylenediamine (PPD) is one of the most potent contact allergens found in consumer products. PPD is used in oxidative hair dyes but also in a variety of industrial processes. The potency of PPD as a contact allergen is illustrated in a study by Kligman showing that repeated patch testing with 10% PPD using the Maximization Test induced sensitization in 100% of 25 healthy volunteers [7]. The general population is frequently exposed to PPD e.g. 18 % of men and 75% of women in

Denmark have dyed their hair at least once and PPD is found in 16-50 % of oxidative hair dyes on the European marked [8, 9]. It is therefore surprising that only 4.5 % of approximately 3.000 consecutive patients in 12 dermatology clinics had a positive patch test to PPD [10]. The reason for this is unknown, but it could be due to lack of response, subclinical immune activation or to the development of some type of immunological tolerance.

It was previously shown that T cell responses to PPD differed between allergic patients and healthy volunteers [5, 11].Thus, in vitro stimulation with PPD induced T cell proliferation as well as production of Th2 cytokines (IL-4, IL-5, IL-9, IL-13) only in peripheral blood mononuclear cells (PBMCs) isolated from allergic patients and not in PBMC from healthy volunteers [5, 11]. However, the healthy volunteer group in these studies contained both individuals who previously had used hair dyes without any symptoms and individuals who had never used hair dyes, which makes it difficult to conclude on the mechanism behind the lack of response. In the present study, we wanted to investigate the immune response to PPD in detail in allergic and non-allergic individuals regularly exposed to PPD. To do this we recruited allergic patients with mild and severe allergy and a non-allergic group of hairdressers that had be working a minimum of five years as hairdressers without developing PPD allergy. It has been shown that in average hairdressers dye hair 9.3 times a week [12] and even with glove use exposure is seen [13, 14]. We therefore assumed that individuals in our non-allergic group had been heavily exposed to PPD. To investigate the difference in the immune response to PPD in the two groups, all individuals were patch tested with 1% PPD or the vehicle Vaseline (VAS). Skin biopsies were collected 96 hours after exposure and extensive molecular profiling was made. We found a differential gene expression profile of immune related genes in PPD exposed compared to VAS exposed skin in both non-allergic and PPD allergic individuals. The number of up-regulated genes correlated with the severity of the patch test reaction, meaning that the non-allergic individuals had fewest upregulated genes. Thus, we show that in individuals regularly exposed to PPD for many years, with a negative PPD patch test and no

symptoms of PPD allergy, PPD still affects the expression of many genes and signalling pathways although to a lesser extent than in individuals allergic to PPD.

Materials and Methods

Study population

In this study, 11 hairdressers regularly exposed to PPD but with no allergy to PPD and 10 individuals with known allergy to PPD were recruited (for further details on the study population please see Table I in Supplementary Materials and Steengaard Meisser et al, (Manus I). Patients with other inflammatory skin diseases or receiving immunosuppressive medication were excluded. The participants were all females between 24 and 60 years. All participants gave informed written consent and the study was conducted in accordance with the Helsinki declaration and approved by local ethics committee.

PPD patch testing and biopsy collection

All participants were patch tested with 20 mg 1% PPD in vaseline and a VAS control for 48 hours using 8 mm Finn chambers. The patch tests were scored on day 2 and 4 according to criteria in The European Society of Contact Dermatitis' guideline (-, no reaction; ?, doubtful reaction ;1+, 2+ or 3+,weak, strong or extreme positive reaction; IR, irritant reaction) [15]. In some analyses, the group with allergy to PPD was divided in two groups with individuals with mild allergy (patch test score ≤ 1) in one group and individuals with strong allergy (patch test > 1) in the other group based on their highest patch test score. 4 mm punch biopsies were collected on day 4 from both PPD and VAS test sites. The biopsies were either placed in RNA later and frozen in liquid nitrogen before storage at -80°C or embedded in tissue-tek, frozen in liquid nitrogen and stored at -80°C as detailed in Table I in Supplementary Materials.

Gene-expression of immune-related genes

Biopsies were analysed for the expression of immune related genes. The analyses were made on data generated in Steengaard Meisser et al, (Manus I). For detailed description of the methods, please see supplemental materials.

Intracellular staining and flow cytometry

Blood samples were collected from the participants before PPD exposure. The T cell phenotype was determined after PBMC purification by intracellular staining and flow cytometry. For details, please see supplemental materials.

Confocal microscopy

Biopsies were cut in 7 µm slices at -20 degrees Celsius, fixed with 4% paraformaldehyde (PFA) on glass slides and stained at room temperature with primary antibodies against CD3 ((MCA1477T) Bio-Rad in 1:200 dilution and secondary fluorochrome labelled antibodies, goat anti-rabbit AF546 ((A-11010)Invitrogen) in 1:2000 dilution , Apoptosis stainings were done using Click-IT Tunel alexaflour674 imaging assay (Bioscience) according to the protocol provided by the producer. Imaging was done using the Zeiss - LSM 780 System Laser Scanning Microscope and the Zen imaging software. Pictures shown are the maximal intensity picture generated from a 10µm z-stack of 20 pictures.

Statistics

Significant differential expressed genes were defined as genes with a false discovery rate (FDR) below 0.015. Gene expression between paired samples, PPD and VAS exposed skin from the same individual was analyzed using Wilcoxon test and comparison between groups was done using Mann Whitney test. Pathway analysis was done using the Enrichr platform [16, 17] and the Panther database [18]. Significant pathways were defined as pathways with an adjusted P-value below

0.015. Venn diagrams of significantly regulated genes between comparison groups were visualized by the online application “genevenn.sourceforge.net”

Results

PPD-induced expression of immune-related genes in the skin from non-allergic versus allergic individuals

To investigate how PPD affects expression of immune-related genes in the skin from non-allergic individuals and individuals allergic to PPD compared to the expression seen upon exposure to vaseline we analysed the gene expression of immune related genes from data generated in Meisser et al, TJ-manus (Manus-1 in PhD-thesis). Compared to control skin treated with VAS, PPD significantly affected the expression of immune-related genes in both non-allergic and individuals allergic to PPD (Fig. 1A). The differential expression profiles of the top 50 differential expressed immune related genes between allergic and non-allergic individuals (1A) and between mildly and severely allergic individuals (1B) are shown in heatmaps and the top 10 differential expressed genes in each group compared to their VAS controls are labelled in volcano plots (Fig. 1C). Separate clustering of the expression of immune related genes was found between PPD exposed skin (green: non-allergic, orange: mildly allergic, red: severely allergic) and VAS (blue) exposed skin (Fig. 1A and B). The amount of differential expressed genes correlated with the patch test reaction severity as seen in the volcano plots (Fig. 1C). Principal component analysis revealed 3 clusters (Fig. 1D). The PPD exposed skin samples from severely allergic individuals clustered in a clearly distinct cluster. In contrast, two less distinct clusters with one cluster containing all VAS samples, PPD samples from four non-allergic and one mildly allergic and one cluster containing PPD samples from three non-allergic and three mildly allergic individuals. There was a significant overlap between differential expressed immune genes in all three groups, with 116 genes being shared among all groups (Fig 1E). 7 genes were unique to the non-allergic, 10 genes to the mildly allergic individuals and 739 were unique to the severely allergic individuals. RNA analysis of VAS exposed skin from all groups did not differ significantly in gene expression and analysis of circulating immune cells showed no significant difference between non-allergic and allergic individuals before

PPD exposure (Suppl. Fig. 1). This indicated that there was no general difference in the immune status in the skin and blood from allergic versus non-allergic individuals before exposure to PPD. Taken together, exposure to PPD induced immune activation in both non-allergic and PPD allergic individuals even though the non-allergic individuals did not have any clinical symptoms (Steengaard Meisser et al, (Manus I in PhD-thesis).

Up-regulated immunological pathways in PPD exposed skin

Next, we wanted to identify possible immunological pathways induced in skin exposed to PPD in non-allergic, mildly allergic and severely allergic individuals based on differential expressed immune related genes (Table 1). Pathway analysis revealed that the three most significant up-regulated pathways in the skin after PPD exposure in all three groups were i) Inflammation mediated by chemokine and cytokine signalling, ii) T cell activation, and iii) apoptosis. A total of 10, 4 and 26 significant up-regulated pathways were found in PPD exposed skin from non-allergic, mildly allergic and severely allergic individuals, respectively (Suppl. table II). There were no significantly down-regulated pathways found in samples from the non-allergic or in the mildly allergic group. In samples from the severely allergic group 9 immunological pathways were down-regulated, most significantly the angiogenesis pathway (Suppl. table II).

Up-regulated genes related to inflammation mediated by chemokines and cytokines found in PPD exposed skin

To further investigate the specific genes involved in the upregulated pathways, we investigated which chemokines and chemokine receptors differential expressed in PPD compared to VAS exposed skin (Fig. 2). A total of 32 chemokines was significantly differentially expressed in the

skin in one or more of the groups after PPD exposure, though most were found in the severe allergic group(2A). The overlap of significant chemokine and chemokine receptor expression in the 3 groups showed only one gene to be specific for the non-allergic and mild allergic group (Fig. 2B and C). This chemokine was identified as CCL21 and showed increased expression in response to PPD (Fig 2E). All groups shared an up-regulation of 4 chemokine in PPD exposed skin and further 6 chemokines were shared among the mild and severe allergic groups and 21 chemokines was significant only in the severe allergic group (Fig. 2B and suppl. Fig. 2A). The 4 shared chemokine were CXCL10, CCL13, CCL17 and CCL19, which were up-regulated to a higher degree in PPD exposed skin from allergic individuals compared to non-allergic individuals. CXCL10 and CCL17 have also been shown to be up-regulated in nickel-exposed skin from nickel-allergic individuals [19]. Interestingly, the receptor for CCL17, CCR4 was up-regulated in PPD exposed skin from non-allergic, mildly allergic and severely allergic but to a higher degree in PPD exposed skin from allergic individuals compared to that of non-allergic individuals (Fig. 2F). CCR4 has been shown to be expressed on T cells from nickel allergic individuals especially on Th2 cells [19]. In addition, CCR1 was up-regulated in PPD exposed skin from all three groups (Fig. 2F and suppl. Fig. 2). Furthermore, additional chemokine receptors were up-regulated in the allergic groups, 5 shared and 9 unique to the severe allergic group (Fig. 2C and suppl. Fig 2B).

We looked further into the activation of innate immunity and molecules involved in the recruitment of immune cells into the skin and found evidence of innate skin activation after PPD exposure in all groups. The innate skin activation did only result in extensive cell infiltration and noticeable tissue damage in severe allergic individuals (Suppl. Fig 3A), but PPD exposure caused increased expression of compliment component 3 in non-allergic individuals to a higher degree than in allergic individuals (Suppl. Fig 3B). Interestingly, several members of the IL-1 family were among the most differentially expressed genes in PPD exposed skin (Fig. 3 A and B). IL-1β and IL-18 are known to be central for the inflammatory response to contact allergens [20-22]. In contrast, IL-18

binding protein and IL-37 can reduce the inflammation induced by contact allergens [23, 24]. An up-regulation of IL1beta, IL1-receptor and IL18 binding protein and a down-regulation of IL18 was found in PPD exposed skin from severely allergic individuals (Fig. 3C). IL37 was down-regulated in PPD exposed skin from both non-allergic and PPD allergic individuals (Fig. 3C). IL-15, which is known to play a central role in the homeostasis of memory T cells [25] was up-regulated in PPD exposed skin from both non-allergic and PPD allergic individuals (Fig. 3C).

T cell infiltrates skin exposed to PPD in both non-allergic and allergic individuals

As contact allergy is a T cell mediated response and as T cell activation is among the most significant regulated pathways (Table 1), we next examined the expression of T cell related genes further. PPD exposure resulted in an up-regulation of T cell associated genes in both non-allergic and PPD allergic individuals, though most persistent in the severe allergic group (Fig. 4A). A significant up-regulation in the expression of CD3, CD45 and the co-stimulatory molecule ICOS was found after PPD exposure in both non-allergic and PPD allergic individuals (Fig. 4B). In addition, an up-regulation of CD4, CD8 and the co-stimulatory molecule CD28 was found in PPD allergic individuals upon PPD exposure (Fig. 4B). The largest increase in expression of T-cell associated genes was found in the group of severely allergic individuals. To further investigate PPD induced T cell infiltration, biopsies from non-allergic and allergic individuals were stained for $CD3^+$ cells and analysed by confocal microscopy. A slight increase in $CD3^+$ cells in dermis was seen in the non-allergic group whereas the skin from the severely allergic individual showed a massive infiltration of $CD3^+$ cells in both dermis and epidermis (Fig. 4C). Thus, confirming the expression data. Taken together, this shows that even though no clinical response was seen in the non-allergic individuals, PPD exposure induced some T cell recruitment to the skin.

Type 1 T cell associated genes dominate the effector functions found in skin exposed to PPD in both non-allergic and allergic individuals with selective up-regulation of granzyme B and perforin in individuals allergic to PPD

To further investigate T cell responses in the skin following exposure to PPD, we identified the most affected genes related to T cell effector functions. Expression analysis of genes associated with type 1 (Th1 and CTL), type 2 (Th2) and type 3 (Th17 and Tc17) T cell responses showed a predominantly up-regulation of genes involved in type 1 responses in both non-allergic individuals and individuals allergic to PPD (Fig. 5A). Single genes involved in type 2 responses were also up-regulated, but not to the same extent as type 1 genes (Fig. 5A and B). A strong up-regulation of the cytotoxic effector molecules granzyme B and perforin was selectively found in individuals allergic to PPD (Fig 5C). In contrast, lymphotoxin alfa and beta and CTLA4 were up-regulated in both non-allergic and allergic individuals (Fig. 5C). The type 2 associated cytokine IL-13 was only significantly up-regulated in individuals allergic to PPD and strongest in individuals having severe allergic reactions (Fig. 5A-C). The type 3 response did not seem to be induced neither in non-allergic nor allergic individuals (Fig. 5A).

Exposure to PPD induces massive apoptosis in the skin from individuals allergic to PPD but not in non-allergic individuals

A central part of the immune response to contact allergens is the killing of allergen-modified skin cells by allergen-specific T cells. This correlates well with the up-regulation of several genes involved in cytotoxicity described above and with the apoptosis pathway being identified as one of the 3 most significantly up-regulated pathways in the pathway analysis (table 1). To investigate this further, the expression of apoptosis-associated genes identified in the pathway analysis was compared between non-allergic and allergic individuals (Fig. 6A). Individuals allergic to PPD showed a general up-regulation of apoptosis associated genes, most evident in individuals with

severe allergy (Fig. 6A and B), whereas the non-allergic individuals show an up-regulation of only a fraction of the genes (Fig. 6A). Up-regulation of the classical apoptosis receptor FAS was seen in both non-allergic and allergic individuals (Fig. 6C). In contrast, significant up-regulation of the other apoptosis genes like FAS ligand, TNF, BIRC3 and BAX was only seen in allergic individuals (Fig. 6C). To visualize the severity of apoptosis induced in the skin after exposure to PPD, we identified DNA breaks representing the last stages of apoptosis skin sections by Tunel staining (Fig. 6D). The stainings showed apoptosis in single cells in dermis and epidermis after PPD exposure in non-allergic individuals whereas a widespread apoptosis or cell damage throughout all layers of the skin was seen in severely allergic individuals (Fig. 6C).

Discussion

In the present study, we show that exposure of PPD on the skin induces a significant change in the expression of immune-related genes in both non-allergic individuals and individuals allergic to PPD. Furthermore, the number and expression level of affected genes correlated with the level of clinical response induced by the PPD patch test. Interestingly, we found that the three most up-regulated pathways in skin exposed to PPD were identical in the non-allergic individuals and individuals allergic to PPD, namely I) Inflammation mediated by chemokine and cytokine signalling, II) T cell activation, and III) Apoptosis. Thus, we show that in individuals who have been regularly exposed to PPD for many years but with no symptoms of ACD to PPD and a negative PPD patch test, PPD still affects the expression of many genes and signalling pathways although to a lesser extent than in individuals allergic to PPD.

It is well established that exposure to contact allergens on the skin leads to a rapid induction of several inflammatory mediators such as cytokines from the IL-1 family and various chemokines [26, 27]. Skin from PPD allergic individuals showed an up-regulation of several of these including IL-1beta, CCL17 and CXCL10. In non-allergic individuals, exposure to PPD only induced up-regulation of a few chemokines including CCL17 and CXCL10 and none of the classical T cell cytokines. The only significantly differential expressed interleukin in non-allergic individuals was IL-37, which was down-regulated after PPD exposure. A certain threshold of activation is needed for inflammation to be visualized clinically. As we did not see any clinical response in the non-allergic group (Supplemental table I) it could be speculated that PPD only induces a partial activation of the immune response in this group, which is not enough to induce clinical visual reactions.

Both $CD4^+$ and $CD8^+$ T cells are known to play important roles in immune responses to contact allergens [4]. We found that PPD induced T cells infiltration in the skin in both non-allergic and

allergic individuals, however to a much lesser degree in the non-allergic individuals. We found that the T cell response was dominated by a type 1 response, likely mediated by both Th1 and CTL cells. In contrast, other studies have shown that T cell responses induced in individuals allergic to PPD are dominated by type 2 responses as seen by IL-4 and IL-5-producing T cells and that type 1 responses mediated by IFN-γ-producing T cells only seem to play a minor role [5, 11, 28]. This discrepancy probably can be explained by differences in experimental setup and readout. We examined the response to PPD in the skin after *in vivo* exposure to PPD and investigated the response as changes in gene expression, whereas other studies determined the T cell responses by using either PBMC or PPD specific T cell clones that were stimulated *in vitro* followed by cytokine determination by ELISA [5, 11, 28]. However, as we find an up-regulation in the expression of IL13 in skin exposed to PPD in individuals with severe allergy to PPD, our results do not rule out the involvement of type 2 responses in ACD.

An important part of the type 1 response in ACD is the killing of allergen-modified keratinocytes [2, 29, 30] . In accordance, we found an up-regulation of the expression of several mediators involved in T cell cytotoxicity including Granzyme B, Perforin, FAS, FAS ligand, Lymphotoxin A and B in the skin from individuals allergic to PPD after exposure to PPD. In contrast, only some of these, namely FAS, Lymphotoxin A and B were up-regulated by PPD in skin from non-allergic individuals. Thus, suggesting that skin exposure to PPD only induces a partial activation of immune response mediating ACD in non-allergic individuals compared with the response seen in PPD allergic individuals. Another possibility is that PPD exposure induces anti-inflammatory mechanisms to a higher degree in non-allergic compared with PPD allergic individuals. It has previously been shown that regulatory T cells infiltrates the skin upon nickel exposure of non-allergic individuals [31]. However, we did not find differences in gene expression of genes related to regulatory T cells and their function in skin exposed to PPD compared with skin exposed to VAS exposed skin. Nevertheless, we found that CCL21 is up-regulated in PPD exposed skin from non-

allergic individuals but not in skin from PPD allergic individuals. As CCL21 is known to support the development of T cells tolerance [32, 33], this could suggest that more anti-inflammatory mechanisms are induced upon skin exposure to PPD in the skin from those without allergy to PPD compared with those allergic to PPD. Another important step is the regulation of IL-18 which affects the early stages of allergic contact dermatitis [34]. An example of this regulation is IL-18 binding protein which thereby inhibit inflammation and clinical reactions in contact allergy [23]. In our study we only saw a significant down-regulation of IL-37. This might be an important observation as IL-37 has been indicated in multiple regulatory functions in other skin diseases [20, 29, 30], but the role of IL-37 in contact allergy is largely unknown and this should be the focus of future studies.

In this study, we investigated how PPD affects the gene expression in the skin after 96 hours. The relatively late time point gives us the possibility to examine the infiltrating cells at the peak of inflammation, but we cannot see initial differences of the immune response or conclude on the long-term effects. The up-regulated pathways in the severely allergic individuals show many pathways involved in tissue remodelling, wound healing and controlling of inflammation, which is consistent with late stages of inflammation regardless of the inflammatory trigger. Therefore, to get a more complete picture on how PPD affects gene expression in the skin in non-allergic and allergic individuals, further studies including earlier time points are needed.

In conclusion, we find that PPD induces up-regulation of immune-related genes in the skin in both non-allergic individuals and individuals allergic to PPD. However, the level of T cell infiltration and apoptosis in the skin is much higher in PPD allergic individuals. The immune activation in the non-allergic PPD exposed individuals is subclinical which could be due to the activation of anti-inflammatory mechanisms, but further investigations are required. Taken together this could signify that subtle skin changes after daily exposure to PPD as hairdresser could lead to a change in the immune balance and thereby trigger the development of skin diseases.

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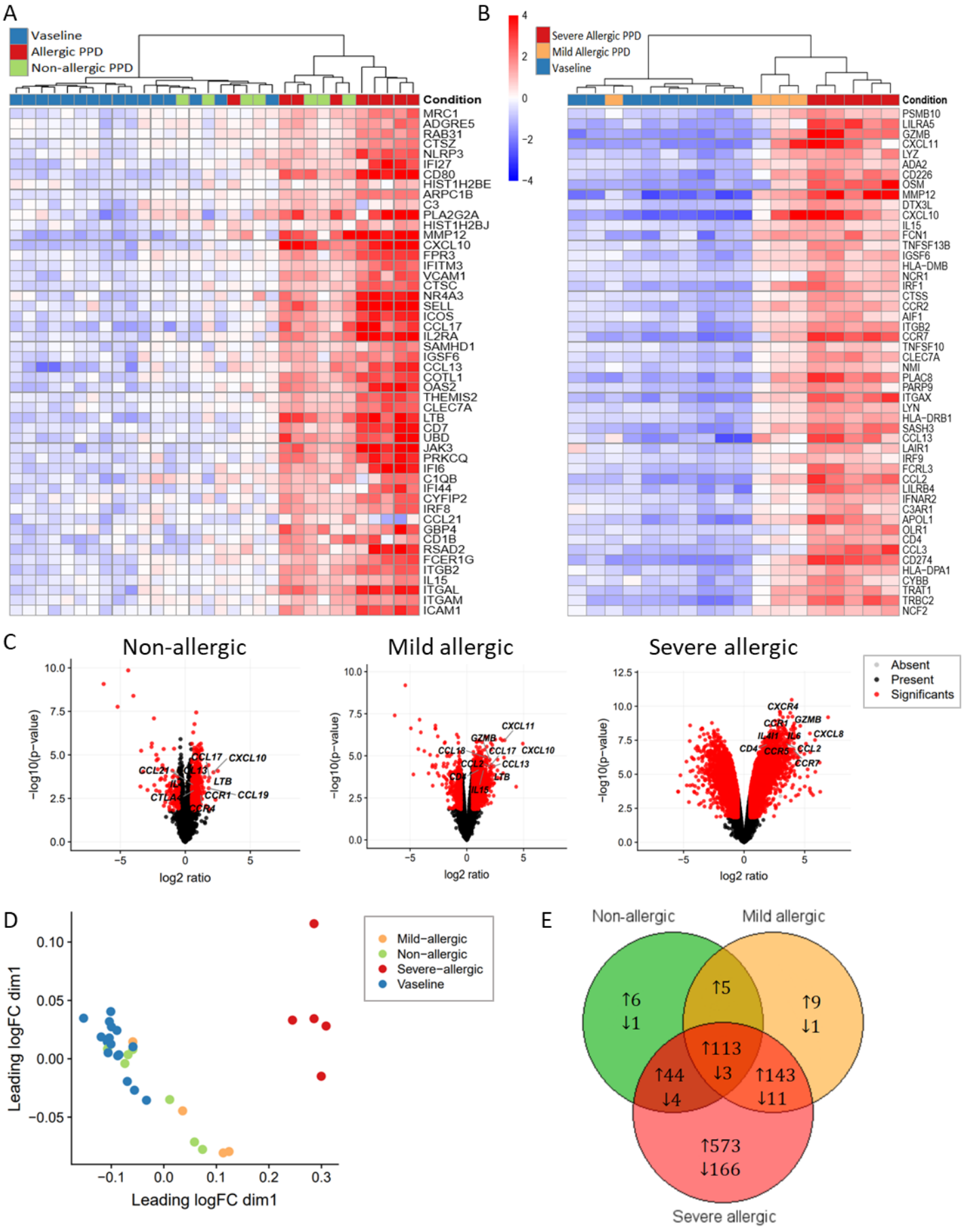


Figure 1 – Steengaard Meisser et al.

Figure 1. Expression of immune-related genes. Top 50 differential expressed immune-related genes in the skin at day 4 of non-allergic (non-allergic PPD) and allergic (allergic PPD) individuals expose for PPD compared to expression seen skin exposed to VAS (A). Top 50 differential expressed immune-related genes in the skin at day 4 between individuals with severe (severe PPD) and mild (mild PPD) allergy to PPD expose for PPD or VAS (B).Volcano plots showing the up- and down regulation of genes in non-allergic, mild allergic and severe allergic patients with a significance 0.015 and the top 10 most significant differential expressed immune-related genes are labelled (C). Multidimensional scaling plot (MDS) showing clustering of samples based on immune-gene expression (D). Venn diagram showing total differential expressed immune gene (false discovery rate < 0.015) in non-allergic individuals (green), mild allergic individuals (orange) and severe allergic individuals (red) after PPD exposure compared to Vaseline exposed skin (E). (Non-allergic n=7, Mild allergic n=4, Severe allergic n=5).

	Non-Allergic Pathways	Adjusted p-value	N	Key genes
up	Inflammation mediated by chemokine and cytokine signaling	2.878e-7	13	CCR1, CCL13, CCL21, CCR4, CXCL10
up	T cell activation	0.000003634	8	CD3d, CD80, NFKB2
up	Apoptosis signaling pathway	0.0003363	7	LTB, DR5, PRKCQ
up	Integrin signalling pathway	0.0005434	8	ITGAM, ITGA4

	Mild Contact Allergic Pathway	Adjusted p-value	N	Key genes
Up	T cell activation	3.724e-11	14	CD86, CD80, CD3E, CD28
Up	Inflammation mediated by chemokine and cytokine signaling	1.658e-8	17	CCR1, CCL13, CCL22, CCL21, CXCL10, CCL8, CCL2, CCR7, CCR5, CCL18, CCR2
Up	Apoptosis signaling pathway	0.00001745	10	GZMB, LTB, TNF
Up	Integrin signalling pathway	0.01390	8	ITGAM, ITGA4, ITGB2, ITGAX

	Severe Allergic Pathways	Adjusted p-value	N	Key genes
Up	Inflammation mediated by chemokine and cytokine signaling	1.464e-27	54	CXCL8, CCR8, CCR7, CCR5, CCR6, CCR4, CCR2
Up	T cell activation	1.936e-25	34	CD86, CD80, CD3G, CD3E, CD3D
Up	Apoptosis signaling pathway	2.760e-24	38	FASLG, TNF, FADD, LTA, BAX, FAS, LTB
Up	B cell activation	1.630e-14	22	LYN, SYK

Down	Angiogenesis	3.630e-7	12	NOTCH2, JAG1, BRAF, PLD1
Down	Ras Pathway	0.00007450	7	MAPK10, BRAF, PDL1
Down	T cell activation	0.00007450	7	VAV3, PPP3CA
Down	Integrin signalling pathway	0.0001667	9	MAPK10, COL1A1, COL3A1, COL1A2

Table 1. Pathway analysis of differentially expressed immunogens (fdr < 0.015). Top 4 pathways for each group is shown based on adjusted P-value. N notes the number of genes involved in the given pathway

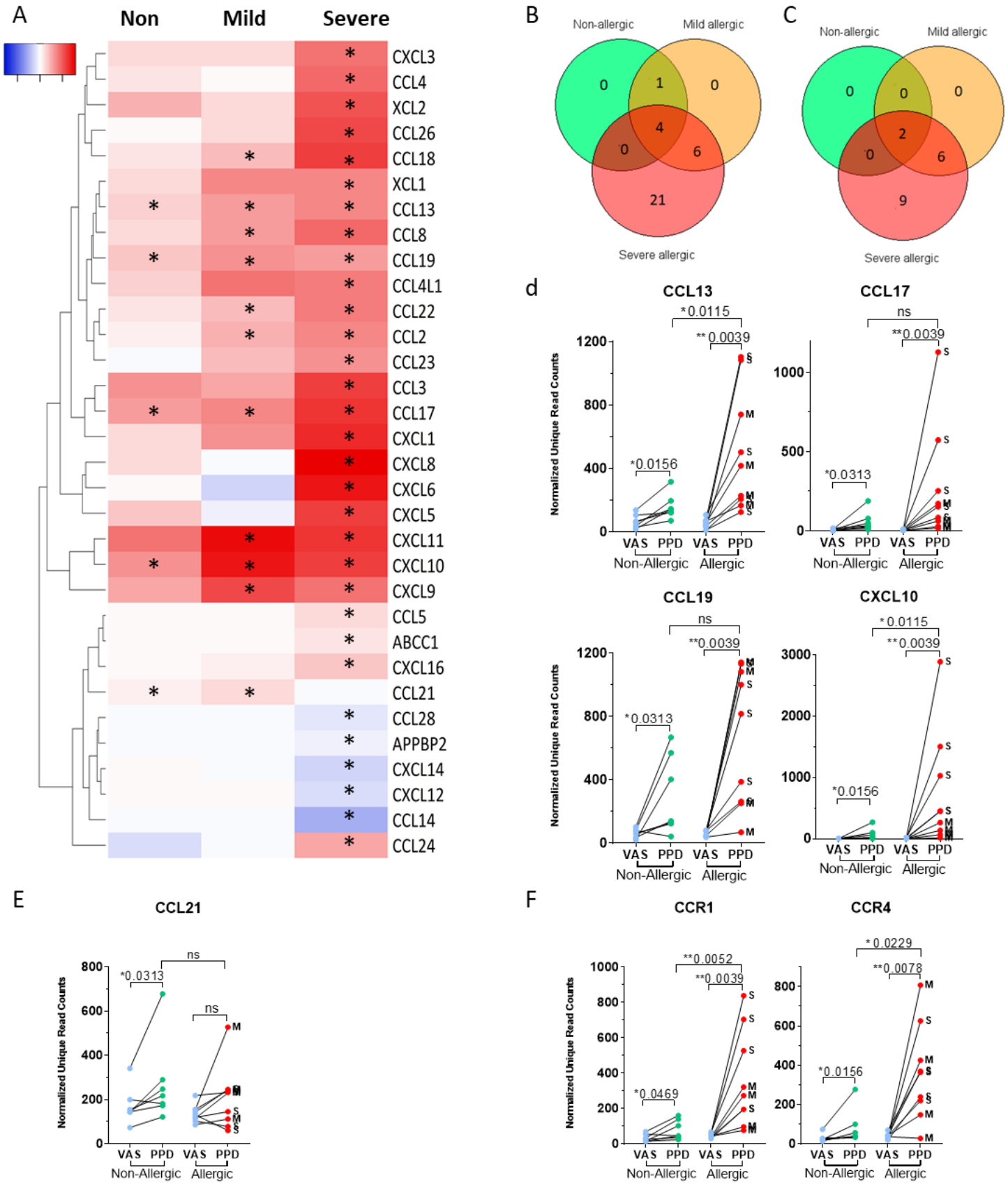


Figure 2 – Steengaard Meisser et al.

Figure 2. Expression of chemokines and chemokine receptors. Heatmap of significant differentially expressed chemokines in one or more of the groups (non-allergic hairdressers (NON), mild allergic individuals (MILD) and severe allergic individuals (SEVERE) compared to their vaseline controls, significance (fdr<0.015) is indicated by *, upregulated (red) and down regulated (blue)(A). Venn diagram showing significant differentially expressed chemokines (B) and chemokine receptors (C) after PPD exposure compared to VAS exposure in non-allergic hairdressers (green), mild allergic (orange) and severe allergic (red) individuals. Significant genes were defined as a false discovery rate < 0.015. Individual plots of the NGS data of (C) chemokine expression in skin samples from non-allergic, (D) chemokines shared among all groups or (E) chemokine receptors shared among all groups. The samples in the allergic group is marked according to reaction severity, mild allergic (m) and severe allergic (s). (Non-allergic N=7, Mild allergic N=4, Severe allergic N=5).

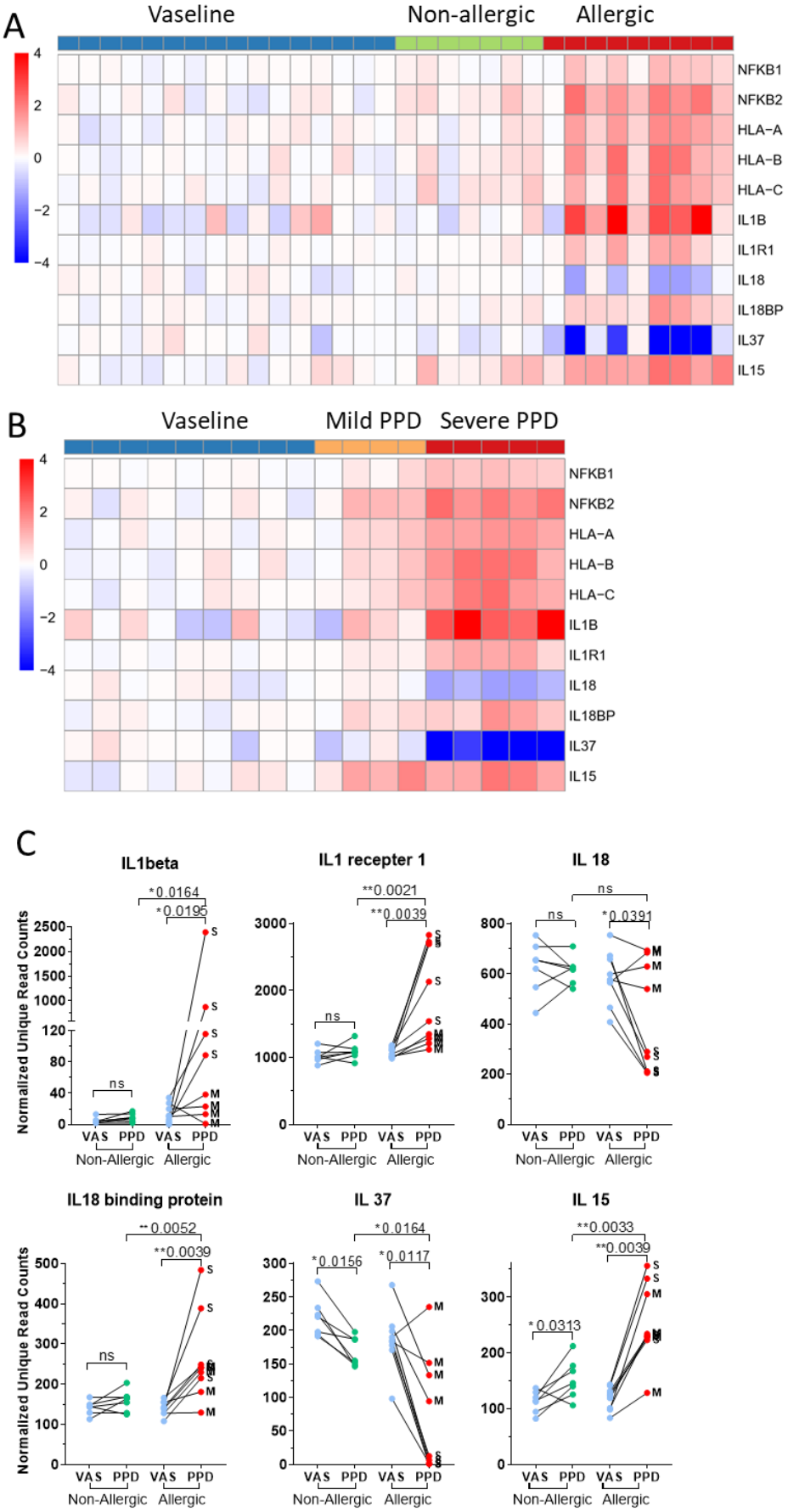


Figure 3 - Steengaard Meisser et al

Figure 3. Innate immune activation after PPD exposure. Heatmap showing genes associated with skin activation by PPD compared to Vas exposed skin from (A) allergic and non-allergic individuals and (B) among mild and severe allergic individuals. (C) Individual plots of the NGS data of differential expressed key genes involved in skin activation. The samples in the allergic group is marked according to reaction severity, mild allergic (m) and severe allergic (s). (Non-allergic N=7, Mild allergic N=4, Severe allergic N=5).

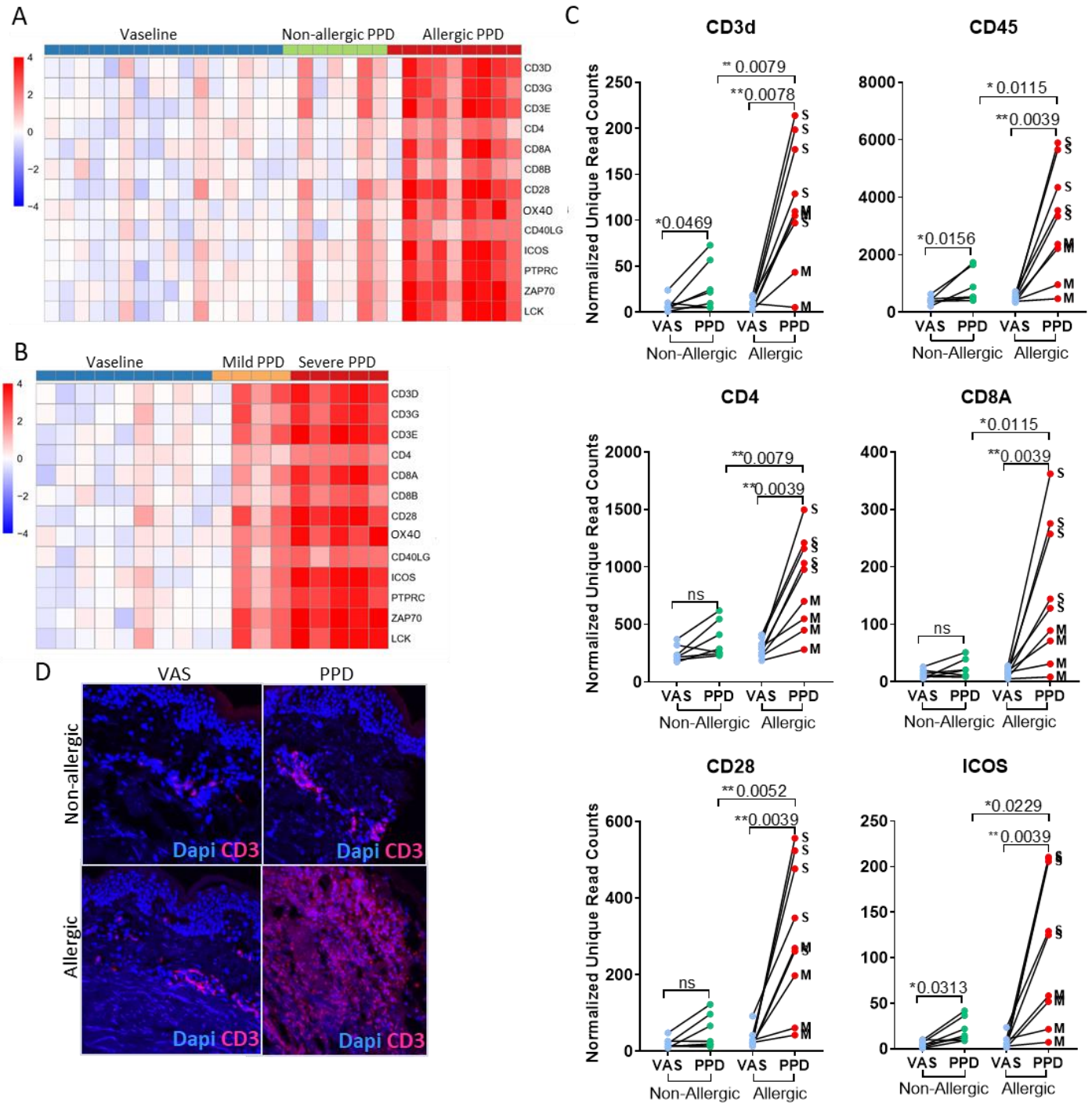


Figure 4 - Steengaard Meisser et al

Figure 4. Skin infiltration of T cells after PPD exposure. Heatmap showing differential expressed gene associated to T cell in PPD compared to Vas exposed skin from (A) allergic vs non-allergic and (B) severe allergic vs mild allergic individuals. (C)Plots of selected genes showing the NGS data of individual samples of each group. The samples in the allergic group is marked according to reaction severity, mild allergic (m) and severe allergic (s). (D) Confocal microscopy staining of CD3 (red) and the cell nucleus (blue) of human skin biopsies on day 4 after PPD or vaseline exposure. (Non-allergic N = 4, Allergic N = 1).

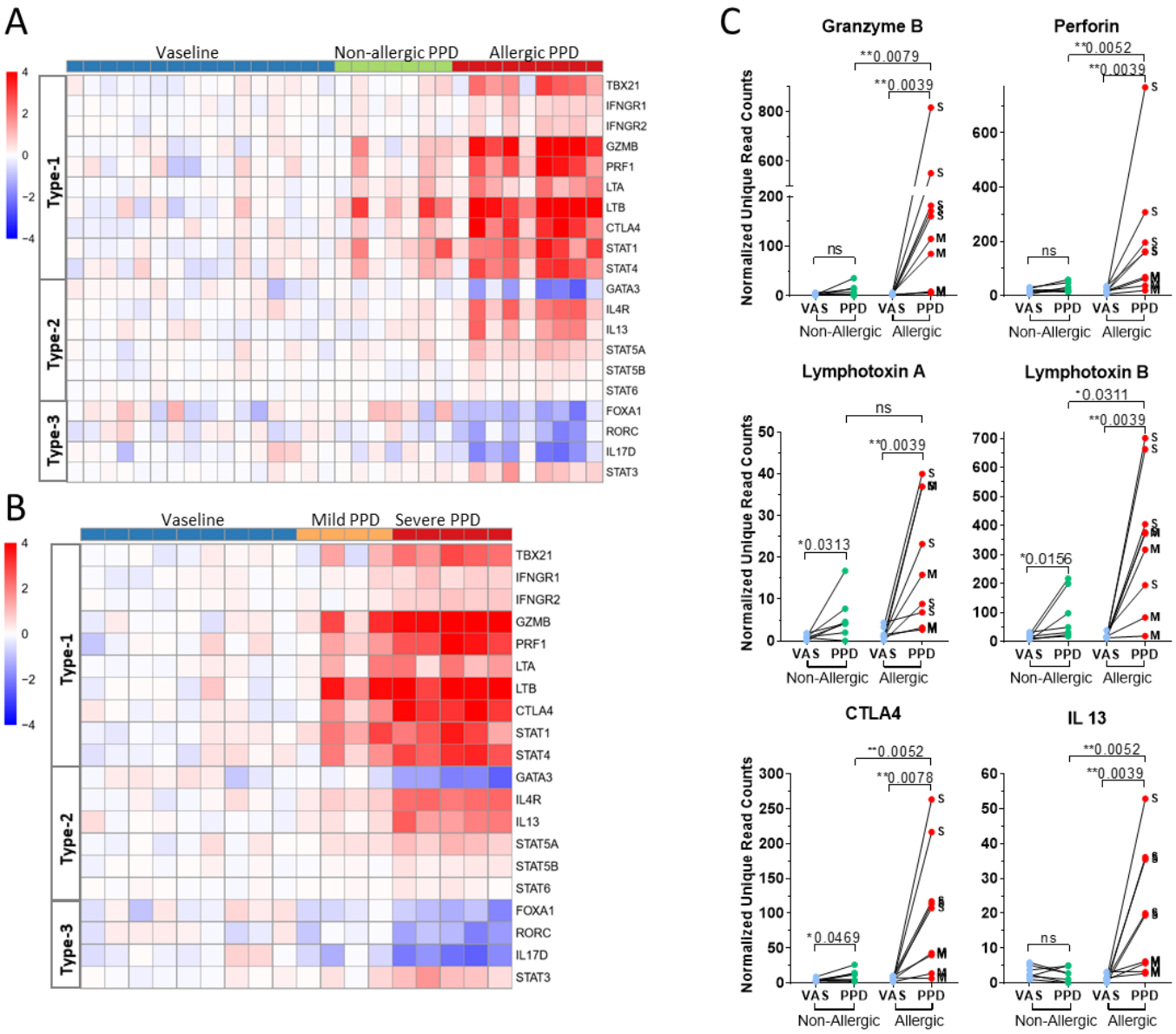


Figure 5- Steengaard Meisser et al

Figure 5. T cell effector functions after PPD exposure. Heatmap showing gene expression associated with T cell effector functions in PPD compared to Vas exposed skin from (A) between allergic and non-allergic individuals and (B) among mild and severe allergic reactions. (C) Individual plots of the NGS data from key T cell effector function genes from the non-allergic and allergic individuals. The samples in the allergic group is marked according to reaction severity, mild allergic (m) and severe allergic (s).

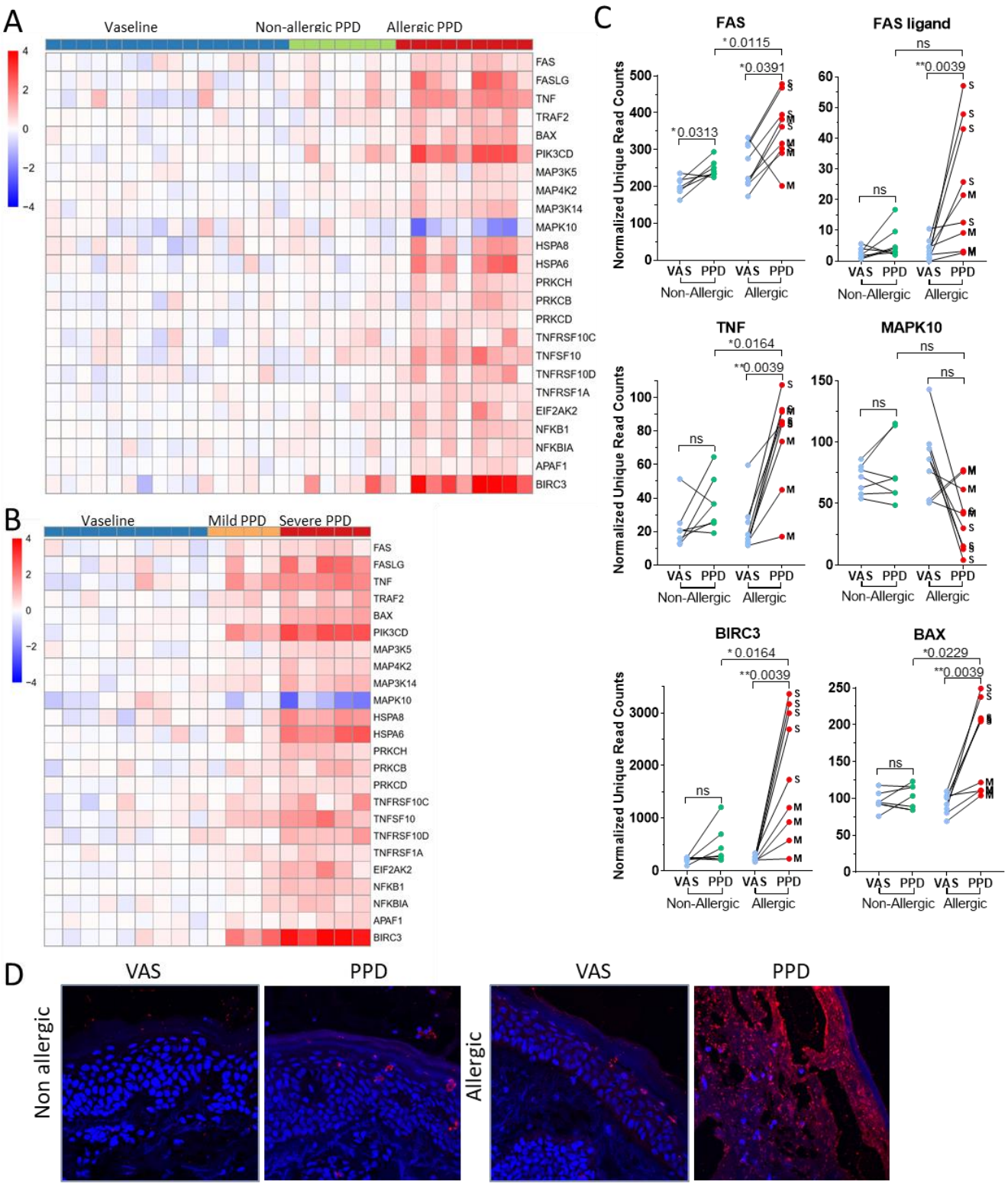


Figure 6- Steengaard Meisser et al

Figure 6. Apoptosis after PPD exposure. Heatmap of apoptosis related differential expressed genes in PPD compared to Vas exposed skin from (A) allergic and non-allergic individuals and (B) severe and mild allergic individuals. (C) Plots of selected genes from the NGS data showing the individual samples of each group. The samples in the allergic group is marked according to reaction severity, mild allergic (m) and severe allergic (s). (D) Confocal microscopy staining of damaged DNA, tunel kit (red) and the cell nucleus (blue) of human skin biopsies on day 4 after PPD or vaseline exposure. (Non-allergic N = 4, Allergic N = 1).

		Sex	Age	Max. Patch test score in study (time of diagnosis)	Exposure to PPD	Analysis Seq (s)/ Histology (h)
Non-allergic		F	29	-	Occupational/Hairdresser	S
		F	35	-	Occupational/Hairdresser	S
		F	26	-	Occupational/Hairdresser	S
		F	28	-	Occupational/Hairdresser	S
		F	50	-	Occupational/Hairdresser	S
		F	27	-	Occupational/Hairdresser	S
		F	34	-	Occupational/Hairdresser	S
		F	25	-	Occupational/Hairdresser	S
		F	42	-	Occupational/Hairdresser	H
		F	39	-	Occupational/Hairdresser	H
		F	42	-	Occupational/Hairdresser	H
		F	45	-	Occupational/Hairdresser	H
Allergic	Mild	F	44	? (+1)	Personal/eyebrow coloring	S
		F	26	? (+2)	Occupational and Personal /hairdresser and hair dye	S
		F	24	? (unknown)	Occupational/hairdresser	S
		F	51	+1 (+1)	Personal / hair dye	S
	Severe	F	56	+2 (+2)	Unknown	S
		F	27	+2 (+2)	Personal/ Textile dye	S
		F	60	+2 (+2)	Unknown	S
		F	28	+3 (+3)	Personal/ Hair dye	S
		F	57	+2 (+3)	Unknown	S
		F	45	+3 (+2)	Personal/ Hair dye and Photography liquids	H

Supplemental table I. Study participants, individual data on each participant. Patch test reactions are shown in our study and in brackets the reaction at the time of diagnosis for the allergic group. Exposure was divided in personal and occupational exposure and activity resulting in PPD exposure.

	Non-Allergic Pathways	Adjusted p-value	N	Key genes
up	Inflammation mediated by chemokine and cytokine signaling	2.878e-7	13	CCR1, CCL13, CCL21, CCR4, CXCL10
up	T cell activation	0.000003634	8	CD3d, CD80, NFKB2
up	Apoptosis signaling pathway	0.0003363	7	LTB, DR5, PRKCQ
up	Integrin signalling pathway	0.0005434	8	ITGAM, ITGA4
up	VEGF signaling pathway	0.007485	4	
up	PDGF signaling pathway	0.01392	5	
up	Ras Pathway	0.01392	4	
up	Interleukin signaling pathway	0.02724	4	
up	Angiogenesis	0.02875	5	
up	B cell activation	0.04557	3	

	Mild Allergic Pathway	Adjusted p-value	N	Key genes
Up	T cell activation	3.724e-11	14	CD86, CD80, CD3E, CD28
Up	Inflammation mediated by chemokine and cytokine signaling	1.658e-8	17	CCR1, CCL13, CCL22, CCL21, CXCL10, CCL8, CCL2, CCR7, CCR5, CCL18, CCR2
Up	Apoptosis signaling pathway	0.00001745	10	GZMB, LTB, TNF
Up	Integrin signalling pathway	0.01390	8	ITGAM, ITGA4, ITGB2, ITGAX

	Severe Allergic Pathways	Adjusted p-value	N	Key genes
Up	Inflammation mediated by chemokine and cytokine signaling	1.464e-27	54	CXCL8, CCR8, CCR7, CCR5, CCR6, CCR4, CCR2
Up	T cell activation	1.936e-25	34	CD86, CD80, CD3G, CD3E, CD3D
Up	Apoptosis signaling pathway	2.760e-24	38	FASLG, TNF, FADD, LTA, BAX, FAS, LTB
Up	B cell activation	1.630e-14	22	LYN, SYK
Up	Interleukin signaling pathway	2.726e-12	24	LI10, IL4R, IL15, IL13, IL1A, IL6, IL23A, IL2A, IL9
Up	Toll receptor signaling pathway	1.285e-11	18	TLR9, TLR8, TLR10, TLR6, TLR2, MYD88
up	Parkinson disease	3.664e-11	22	PSMA7, PSMB10, PLD2
Up	CCKR signaling map ST	1.437e-9	29	ITGB1, SRC, CDC42, CSK
Up	Integrin signalling pathway	1.599e-9	28	ITGB1, ITGAM, ITGB2, ITGAL, ITGAX, ITGB7, ITGA4
Up	Cytoskeletal regulation by Rho GTPase	0.000002315	15	TUBB, ACTB, ACTG1
Up	VEGF signaling pathway	0.00002188	12	NRAS
Up	Ras Pathway	0.00005477	13	STAT1, SHC1, PLD2, NRAS, RAC2, PAK2
Up	PDGF signaling pathway	0.0001878	16	ARHGAP9
Up	Angiogenesis	0.0002922	18	PRKCH, FZD5
Up	Huntington disease	0.0005711	16	ACTR2, APAF1
Up	Axon guidance mediated by netrin	0.001336	7	CDC42, RAC2
Up	EGF receptor signaling pathway	0.001413	14	PRKCH, PRKCB
Up	Ubiquitin proteasome pathway	0.002270	8	UBE2D3, UBE2N
Up	p53 pathway	0.004454	10	APAF1, PIK3CD
Up	FGF signaling pathway	0.005381	12	NRAS, PRKCH
Up	Oxytocin receptor mediated signaling pathway	0.005464	7	VAMP8, PRKCH
Up	Thyrotropin-releasing hormone receptor signaling pathway	0.007068	7	VAMP8
Up	FAS signaling pathway	0.007335	6	FAS, FASLG, CASP8, FADD
Up	Muscarinic acetylcholine receptor 1 and 3 signaling pathway	0.007487	7	VAMP8
Up	JAK/STAT signaling pathway	0.008739	4	SOCS1, STAT1, JAK3
Up	5HT2 type receptor mediated signaling pathway	0.01179	7	VAMP8, SNAP23

	Severe Allergic Pathway	Adjusted P-value	N	Key genes
Down	Angiogenesis	3.630e-7	12	NOTCH2, JAG1, BRAF, PLD1
Down	Ras Pathway	0.00007450	7	MAPK10, BRAF, PDL1
Down	T cell activation	0.00007450	7	VAV3, PPP3CA
Down	Integrin signalling pathway	0.0001667	9	MAPK10, COL1A1, COL3A1, COL1A2
Down	CCKR signaling map ST	0.0002086	9	MAPK10, RPS6, PTK2
Down	VEGF signaling pathway	0.001050	5	
Down	B cell activation	0.009571	4	
Down	EGF receptor signaling pathway	0.01551	5	
Down	Insulin/IGF pathway-protein kinase B signaling cascade	0.001703	4	TSC1

Supplemental table II. Total pathway analysis of differential expressed immunogens after PPD exposure in non-allergic, mildly allergic and severely allergic individuals.

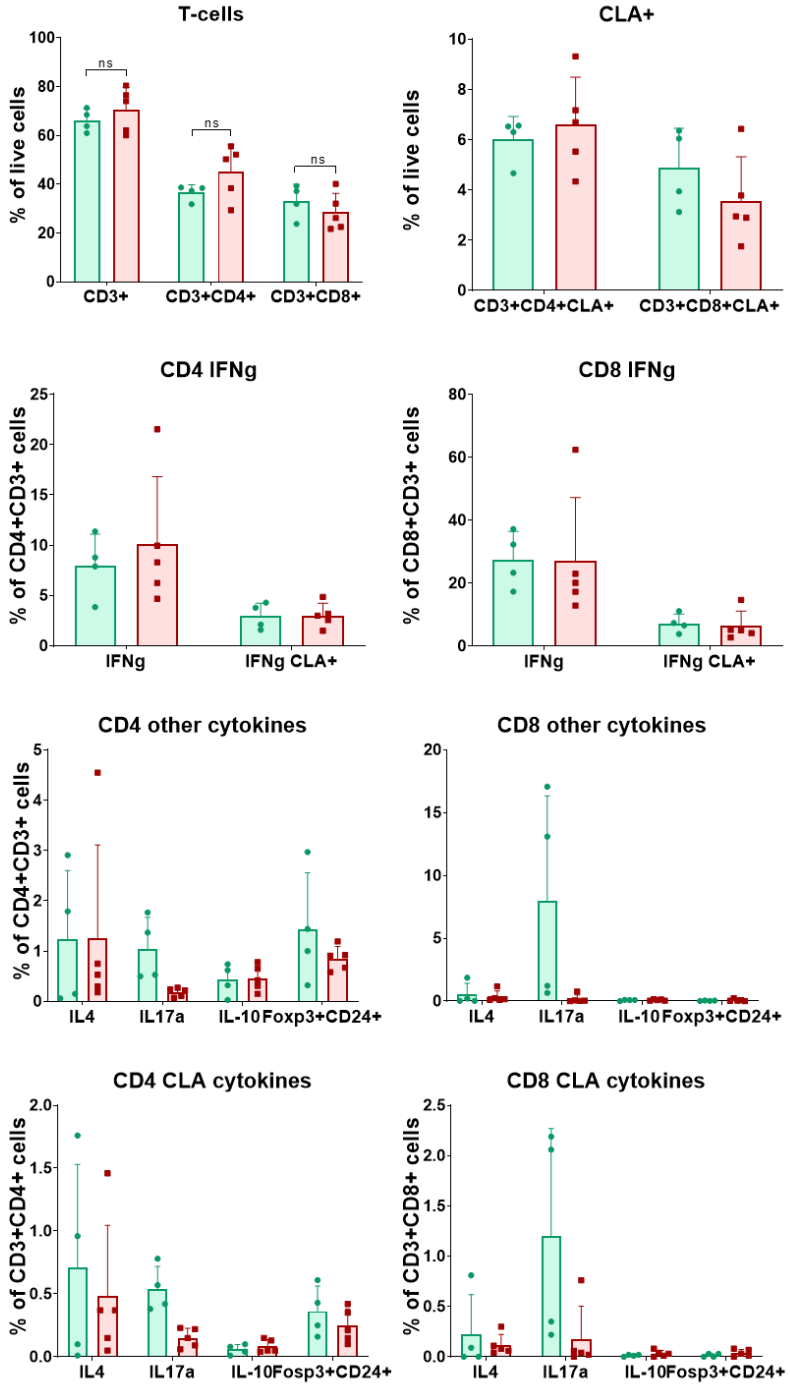
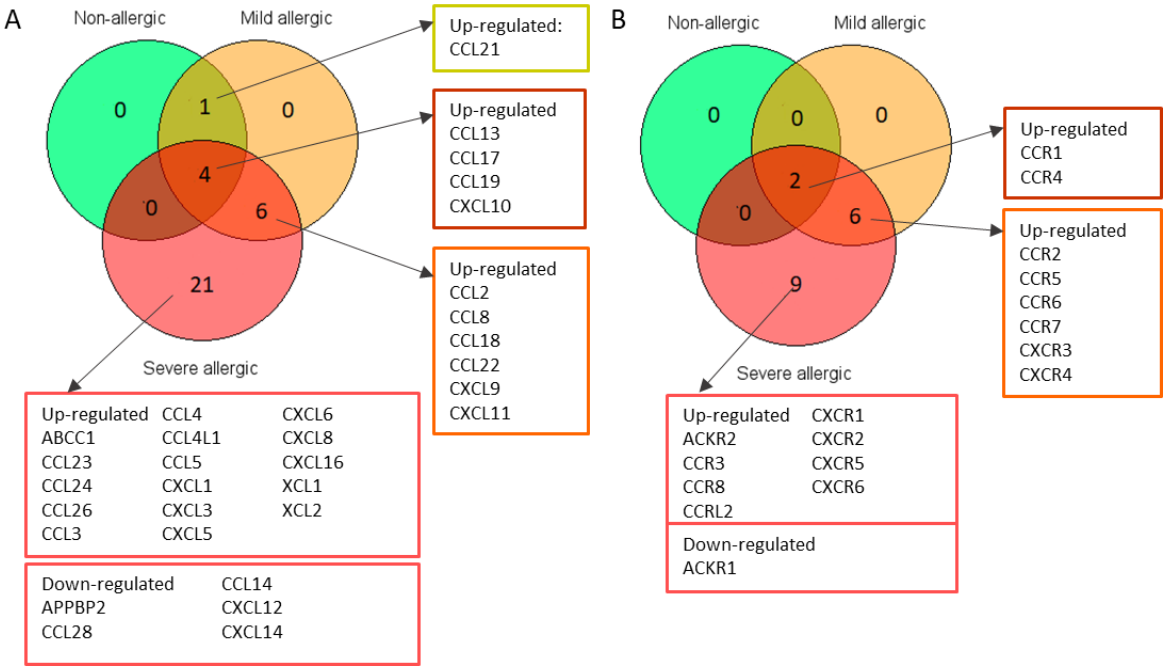
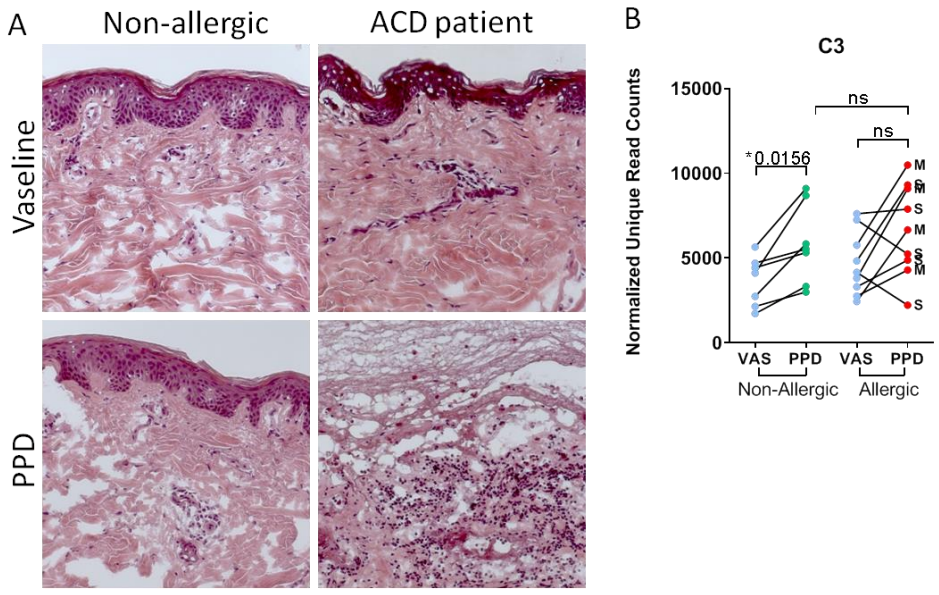


Figure E1, analysis of circulating T cells before PPD exposure

Supplemental Figure 1. Flowcytometry analysis of circulating T-cells in allergic (red) and non-allergic (green) individual before PPD exposure. (allergic N=5, non-allergic N=4).



Supplemental Figure 2. Alternative visualization of chemokines (A) and chemokine receptors(B) and the overlap between the 3 groups; non-allergic individuals (green), mild allergic individuals (orange) and severe allergic individuals (red) after PPD exposure compared to Vaseline exposed skin. Significant genes were defined as a false discovery rate < 0.015. (Non-allergic n=7, Mild allergic n=4, Severe allergic n=5).



Supplemental Figure 3. Hematoxylin and eosin staining of skin biopsies from non-allergic and allergic individuals after exposure to vaseline (upper) and PPD (lower) (A). Activation of compliment and general cell infiltration. RNA NGS data of C3 in non-allergic and allergic individuals (B).

Considerations on Methodology

In the thesis we chose to study PPD based on a number of arguments, from relevance to practical factors. Many other hair dye ingredients with sensitizing properties exist, such as some isothiazolinones and fragrances [200], but it is often difficult to determine if exposure to these ingredients has occurred in individuals if no allergy has developed. In our study it was important to define a group that had not developed allergy after continuous exposure to a known strong allergen. PPD is a well-studied allergen, where exposure among hairdressers is almost certain based on the extensive research of PPD exposure among hairdressers [201, 202]. Research has shown PPD exposure occurs even when gloves are used for certain steps of hair-coloring. None of the hairdressers in this study used gloves when cutting or styling hair after coloring and this alone is known to cause PPD exposure [202]. We were therefore confident that some PPD exposure had occurred in all included hairdressers, though the extent of PPD exposure might vary between individuals. Another reason to use PPD is that PPD allergy in hairdressers still constitutes a significant occupational problem and a deeper understanding of its effects on the skin may be useful in the prevention and treatment of ACD.

This study is based on samples collected from research subjects over a time period of 2 years and the subsequent analysis and supporting experiments. Medical data from the non-allergic hairdressers was based on the individuals own recollection when asked at two separate interviews. As they were not patients, no medical files were available, and we therefore had to rely solely on their own recollection. PPD allergic individuals were included despite having additional allergies. Both type 1 and 4 allergies were frequent as seen in table 1, manuscript I. The information about diagnosed allergies was collected from the patient's medical file at Gentofte Hospital. However, the medical file may not have included information about diagnoses of other diseases from childhood, like atopic dermatitis and outgrown allergies. Together this leads to a possible recollection bias in our medical data in both groups. We tried to minimize the recollection bias by having two separate interviews, one over the phone and one face-to-face. In these interviews all participants were asked about both their medical history and any eczema, allergies, dry skin, itch, red spots or swelling of the skin they might have experienced. Non-allergic hairdressers were only considered if they had a clear negative PPD patch test when tested in this study.

In this study both non-allergic hairdressers and PPD allergic patients were recruited, with recruitment occurring multiple times over the 2 years the study was running. While we could have wished for more participants, the group sizes of 7 and 9 participants for NGS analysis was statistically sound based on the initial power analysis. We aimed for 5 participants in each group for the confocal microscopy staining. Unfortunately, 4 allergic and 1 non-allergic hairdresser were not included, due to never showing up to appointments or cancelling due to fear of strong allergic reactions or logistic difficulties. Our study was therefore not able to truly investigate protein expression and organization via confocal microscopy in PPD allergic individual and as such our results only cover that of non-allergic hairdressers.

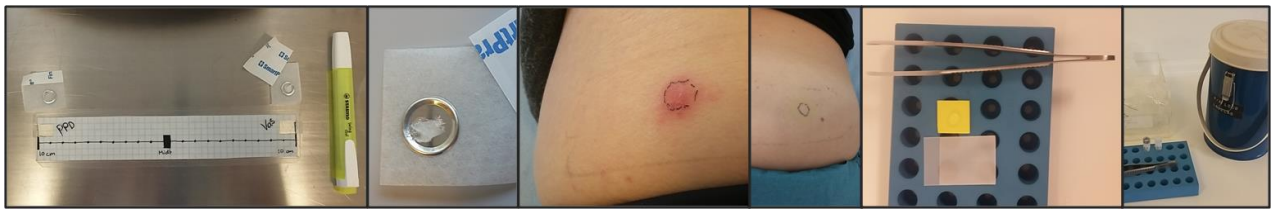


Figure 8. Patch test setup and skin biopsi collection. PPD and Vaseline Patch test was places on upper bttucks/lowerback using a scaffold (1) in finnchanpers (2). Positive reactions (3) and vaseline controls (4) was collected on mooshgummi slides in tissuetek for confocal stanings(5) and in RNAlater for NGS (6).

The patch test and subsequent biopsies were performed on the upper buttocks/lower back of each participant using a scaffold (Figure 8). This skin location was chosen mainly for practical reasons as; chambers are easy to fit and keep in place, there is no sun exposure, the area is big enough to avoid any tattooed areas and any scarring that may occur is easy to hide. On the other hand, this also meant that we studied skin that had not before had significant PPD exposure in any of the groups. It has been suggested that the skin can go through a “hardening” process when repeatedly exposed to an irritant [203]. PPD has both irritant and allergenic properties, and as hairdressers are in contact daily with many irritants the skin on their hands would therefore likely be affected to some degree. Our goal was to determine differences in the skin and immune response to PPD among allergic and non-allergic individuals, and therefore we needed comparable skin locations, like the lower back. Future studies may gain new insight into the effects of long-term repeated exposure of strong contact allergens or skin barrier irritants by looking at skin from the hands of occupational exposed individuals or by developing long lived cell culture-systems. The skin location chosen for this study was adequate for seeing immunological differences among the participants and to test for central immunological memory.

PPD was administered in vaseline in the patch test chamber at a concentration of 1% in accordance with international guidelines [9]. The PPD patch test has been optimized for both sensitivity and specificity over the past decades which is well documented in the literature [204]. Vaseline has minor effects on the skin barrier itself, which is also well studied and therefore a good background for our analysis. Vaseline is a relative thick substance at room temperature which makes it easier to administer on the skin in vivo and PPD is slowly absorbed from the vaseline through the skin. Skin cultures are more fragile due to the small size and the lack of connective tissue, vaseline was therefore not an option to use as vehicle in our in vitro experiments. To introduce as little background disturbance as possible and avoid any additional proteins PPD could react with, we used PBS as a vehicle on our skin cultures. This also allowed us to administer very small volumes so that the skin surface could dry after exposure.

Using NGS RNA sequencing to investigate gene expression in the skin allowed us to look at all genes at the same time, providing us with a good general picture of the whole skin at a certain time point. As a result, it unfortunately becomes impossible to know which cell types are expressing which genes and genes expressed in rare cell types might fall under the limit of detection. The NGS data should therefore never be used alone to draw conclusions on specific cell subsets, but rather be used as a general picture and a basis for further specific investigations. When discussing NGS data we use two parameters; the significance and the log2 fold-change. In our study we decided to define the significance in form of the false discovery rate (fdr) and set the threshold at 0.015. This is stricter than the conventional 0.05 and allowed us a higher certainty of the genes being truly significant, but also increased the risk of ignoring some truly significant genes. The log2 fold-change was used to measure up or down regulation. We used any change detectable in our study, as even small changes can have great effect on the immune cells. Another option would have been to only use changes above 0.5 or 1 which would focus the results on the bigger changes and ignore small and possible irrelevant changes.

The functionality of the skin barrier is often thought of as the permeability of the skin. Multiple methods exist to investigate skin barrier integrity, but all have their own limitations. The least invasive way to test human skin is to measure trans-epidermal water loss (TEWL), but this was not possible in the set-up of our study. We instead tried to investigate the skin barrier integrity in our 3D skin cultures of human skin. This was done by measuring trans-epidermal resistance (TER), but as the skin cultures consisted of 5+ cell-layers the resistance was too high in the intact skin barrier to measure a reliable starting point and we therefore did not include the data in

our manuscripts. Other techniques available either involved killing the cells directly or induced apoptosis over time, thereby ruling out measuring the true RNA expression in the same cultures. Other techniques are being developed and we look forward to including them in future skin research [205, 206].

General discussion

PPD is a well-known potent contact allergen used in permanent hair dyes and in some semi-permanent tattoos. It is one of the largest causes of ACD in hairdressers and PPD exposure contributes to the high prevalence of occupational skin problems in hairdressers. PPD contact allergy also occurs in consumers dying their hair or beard and after receiving black henna tattoos illegally containing PPD. Experimental studies show that healthy individuals may become sensitized after as few as 2 exposures to 10% PPD. [207] It is therefore puzzling that it is only some of the exposed hairdressers or consumers that do develop allergy to PPD.

In this thesis we showed for the first time that exposure to PPD affects central structures in the skin barrier in non-allergic individuals. PPD further activates the immune system causing inflammation both in those allergic to PPD, as expected, but also in the non-allergic group.
We investigated PPD exposed skin of non-allergic hairdressers and patients with diagnosed ACD to PPD. RNA expression was investigated via NGS and protein expression and organisation was investigated via confocal microscopy. Our first theory was that hairdressers may have a stronger physical skin barrier which inhibits the uptake of PPD compared to that of allergic individuals. We looked at the expression of cell-cell junctions and stratum corneum proteins in the epidermis (manuscript I), all of which are key components of a healthy and strong skin barrier. Of all the cell-cell junctions we focused on tight junctions because of their unique properties in the skin and implications in other skin diseases like atopic dermatitis [75, 76]. We identified 2 proteins (claudin1 and CLMP) which were down regulated after PPD exposure in the skin of both non-allergic hairdressers and allergic individuals. A down regulation was also seen in keratinocyte cultures alone and could therefore not be explained by damage caused by inflammatory cells recruited into the tissue after exposure. This was also seen among one of the stratum corneum proteins, filaggrin 2, which was also directly affected by PPD and down regulated after exposure. In contrast, filaggrin 1 was only down regulated in the skin biopsies and not in the keratinocyte cultures. We could therefore conclude that filaggrin1 was not directly affected by PPD exposure but indirectly by the induction of low levels of inflammation, which involved other cell types in addition to the keratinocytes. Other studies have shown that filaggrin expression is negatively correlated with the amount of inflammation in the skin in other skin diseases, which supports our results. Allergic individuals did not surprisingly suffer a more pronounced disruption of the skin barrier which included many more genes. This barrier disruption was directly correlated with the severity of the clinical reactions and thereby also skin inflammation.

The second theory of this thesis was that some hairdressers develop a form of tolerance to PPD over time. This has to some extent been observed in mice [199] and we therefore investigated immune cells and signaling components in the PPD exposed skin (manuscript II). We saw that PPD induced the up-regulation of multiple immune genes, with the top 3 pathways induced in all participants involving inflammatory cytokine and chemokine signaling, T-cell activation and apoptosis. It was thereby clear that PPD caused some degree of inflammation also in non-allergic hairdressers. PPD exposure lead to infiltration of T-cells in the skin of both non-allergic hairdressers and the allergic individuals. The phenotype of the infiltrating T-cells suggested a primarily type 1 response with primarily LTA and LTB up-regulated in non-allergic hairdressers and not other effector molecules associated with cytotoxicity. This was in line with the induction of apoptosis also seen in the pathway analysis. Apoptosis was seen in both dermis and epidermis after PPD exposure, though it was not enough to cause clinical symptoms in the non-allergic hairdressers. The allergic individuals did not surprisingly show a significantly higher level of inflammation than the non-allergic hairdressers. The allergic inflammation also resulted in extensive tissue damage, apoptosis and the up-regulation of wound healing components.

A big question in this study was the direct damage caused by PPD. PPD is toxic to human cells and induces ROS production in keratinocytes which can damage the cells enough to induce apoptosis [180]. But that on its own is not enough to cause an allergic reaction. PPD will also have to create an epitope for the immune system to recognize. PPD is a very reactive small molecule and no single PPD binding T cell receptor clone has been identified [182, 188, 189]. It is therefore reasonable to assume that more than one epitope is responsible for the allergic reaction. One known reaction of PPD is with cysteine residues in HSA [188] but PPD can also react with cysteine residues in other proteins. The epidermis is a relative cysteine rich tissue with cysteine making up 2.55% of the amino acid present in the proteins in the upper layers of the epidermis [208] compared to the 1.38% of the average human protein [209]. Cysteine residues are also found in higher percentages in small proteins (below 150 residues) compared to bigger proteins, 2% vs. 1% respectively [210] and many of the proteins involved in tight junction formations are small proteins, including claudin 1. The proteins affected by PPD exposure in our study all contained multiple cysteine residues, though further research is needed to know if PPD reacts directly with cysteine residues in these proteins.

In contrast to the direct damage caused by PPD, the indirect damage is most likely caused by the induction of inflammation. Both tight junction and filaggrin family protein expression are

affected by inflammation, especially filaggrin 1 and loricrin expression that decreases in the presence of inflammatory cytokines [211]. Interestingly, filaggrin 1 deficiency alone does not alter the expression of tight junctions [212], whereas defective tight junction formation results in a decrease in filaggrin 1 monomers and a defective stratum corneum [213]. Damage to claudin 1 could therefore also contribute to the deregulation of the stratum corneum. Keratinocytes can activate inflammation in the skin via IL1β production but IL1β caused up-regulation of claudin 1 in keratinocyte cultures, and thus seemed to strengthen the skin barrier [214]. This may work as a defense mechanism, but interestingly PPD managed to induce a decrease in claudin 1 anyway.

Our data showed an up-regulation of certain regulatory molecules in the skin of both allergic participants and non-allergic hairdressers. Most significant was CTLA4 and CXCL10 as shown in manuscript II, but also other important genes showed signs of up-regulation, though not significant. These included FoxP3 and PD-L1. These could still be relevant even if we did not reach significance in this study, as we only had a small group of participants, and as such these molecules should not be ruled out but studied further. We could not identify the phenotype of any specific cells or examine regulatory potential of individual T cell subsets in our data. We could therefore not identify a specific mechanism of tolerance, but our data suggests that some level of regulation takes place after PPD exposure.

Our NGS analysis of the vaseline control samples showed that only one gene out of more than 20.000 differ between the two groups before PPD exposure. The gene was histidine decarboxylase (HDC) which is responsible for the production of histamine and a major player in IgE mediated allergic disease. The PPD allergic group did suffer from multiple IgE mediated allergies in addition to their contact allergy, but no one reported IgE mediated allergic episodes during our study period, this being neither skin or respiratory symptoms. Research in IgE mediated allergies do implicate the skin [215], but a clear link to contact allergy is yet to be found. The relevance of HDC expression in the skin of contact allergic patients was out of the scope of this thesis and therefore not further investigated, but it should be included in future research.

The data generated in this thesis contributes to the understanding of the effects of exposure to PPD on the skin. Other investigations have shown that PPD exposure may occur from contamination of surfaces in the hairdressing saloon such as on trolleys, tables and hairbrushes [202]. PPD exposure on hands may occur accidently, if no gloves are used or when gloves are

not used correctly [216]. Our investigation shows that PPD may not only sensitize but also cause barrier disruption and inflammation in non-allergic individuals. These effects may potentially promote development of contact dermatitis, even if no sensitization occurs. It stresses the need for proper skin protection among hairdressers and the need of finding less reactive alternatives to PPD in hair dying products. Even though private consumers can protect their hands and suffer less frequent PPD exposure, they are likely facing the same challenges as hairdressers in using gloves correctly. Even after correct glove use, some contact with scalp, facial and neck skin is unavoidable and thereby poses a risk of sensitization and skin damage. Exposure can therefore only truly be avoided by not using PPD containing products.

Over the past 15 years the cosmetic industry has tried to modify or find alternatives to PPD to make cosmetic products less sensitizing, thus generating an array of new components with various success [203, 204]. The most used alternative to PPD is 1,4-toluenediamine (PTD). Unfortunately, PTD is only vaguely better than PPD [205] and as the search continues, many new aromatic amine-based dyes have been tested with various potential [206, 207]. Because many of these compounds are related to PPD, cross-elicitation might occur in PPD sensitized individuals. Cross reactivity has great clinical relevance as the patients can encounter cross reactive components from many unsuspected places, not only hair dye. Cross reactivity is partly due to the generation of multiple T-cell receptor clones with a broad cross reactivity to similar structural compounds [186]. The severity of cross-elicitation reactions correlates to the severity of patients allergic reactions to PPD [208]. The most promising alternative to PPD today is 2-methoxymethyl-p-phenylenediamine (mePPD), but some cross elicitation is still an issue [204, 209, 210].

The indication of tolerance induction may be a stepping stone to find a cure of severe allergy to PPD if low-dose exposure can induce an allergen specific immune tolerance. Whereas it might be a risk factor of other skin diseases if the PPD exposure causes an unspecific decrease in responsiveness to damage signals in the skin. These questions are therefore important for future research.

Conclusions

This thesis focused on the effects of PPD exposure in the skin barrier and the local immune system, especially that of occupational exposed individuals without allergic symptoms. It contributes to the field with the following main findings:

- PPD causes damage to the skin barrier in occupational exposed individuals, even in the absence of clinical symptoms.

- PPD exposure down-regulates the expression of tight junction proteins claudin 1 and CLMP.

- PPD exposure down-regulates the expression of stratum corneum proteins filaggrin 2 directly and indirectly filaggrin 1.

- PPD exposure induces low degrees of inflammation in the skin of non-allergic hairdressers, predominantly a type 1 response.

- PPD exposure induces T-cell infiltration and activation in the skin of both allergic patients and non-allergic hairdressers.

- PPD exposure up-regulates expression of type 1 T cell effector molecules in the skin of both allergic patients and non-allergic hairdressers.

- PPD exposure induces apoptosis of cells in the epidermis and dermis in both allergic patients and non-allergic hairdressers.

- Occupational exposure to PPD might induce low-dose tolerance with the involvement of CTLA4.

Perspectives for further research

This thesis provides information on the damages and disruption caused by PPD exposure to the skin in non-allergic individuals. It is the first step to investigate development of tolerance to PPD, if such a tolerance truly exists. The question is which mechanisms of tolerance could be responsible for the lack of allergic reactions to PPD seen in hairdressers? This could be further investigated in a mouse model of low dose tolerance, which has already been established. Because of the significant differences between the skin immune system in mouse and human, human skin models are of particular interest. This study only focuses on the effect of PPD in occupationally exposed individuals, as such further research should include non-exposed individuals as a comparison, although it is important to note that because most contact allergens are present in many different products it might not be possible to identify a truly non-exposed group of people, but rather a low-exposed group. In the scenario where a mechanism of tolerance is identified, it might be possible to induce a similar tolerance in allergic patients. This will pave the way for creating immunotherapy for contact allergy and ACD. One of the major concerns of inducing tolerance is to induce tolerance of cancer cells, thereby increasing the risk of skin cancer. Future research should therefore aim to identify an allergen specific tolerance, which will involve the identification of key epitopes in PPD allergy. Other studies have tried to identify PPD specific T-cells clones without success and because of the many possible targets of PPD it is not likely to find one, but rather a group of dominant clones. A key element in the investigation of PPD allergic epitopes would be to look at TCR clones in the skin from a large number of PPD allergic individuals.

It should be investigated if the effects on the skin barrier, in the case of no sensitization occurring, can lead to irritant contact dermatitis or if the absence of clinical symptoms are a sign of "hardening" of the skin to tolerate repeated irritant exposure. It is therefore also important to investigate the kinetics of the skin barrier damage to determine if this damage can become permanent if exposures are repeated over time. Many of these questions will require new model systems and methods to study living cells in skin tissue over time.

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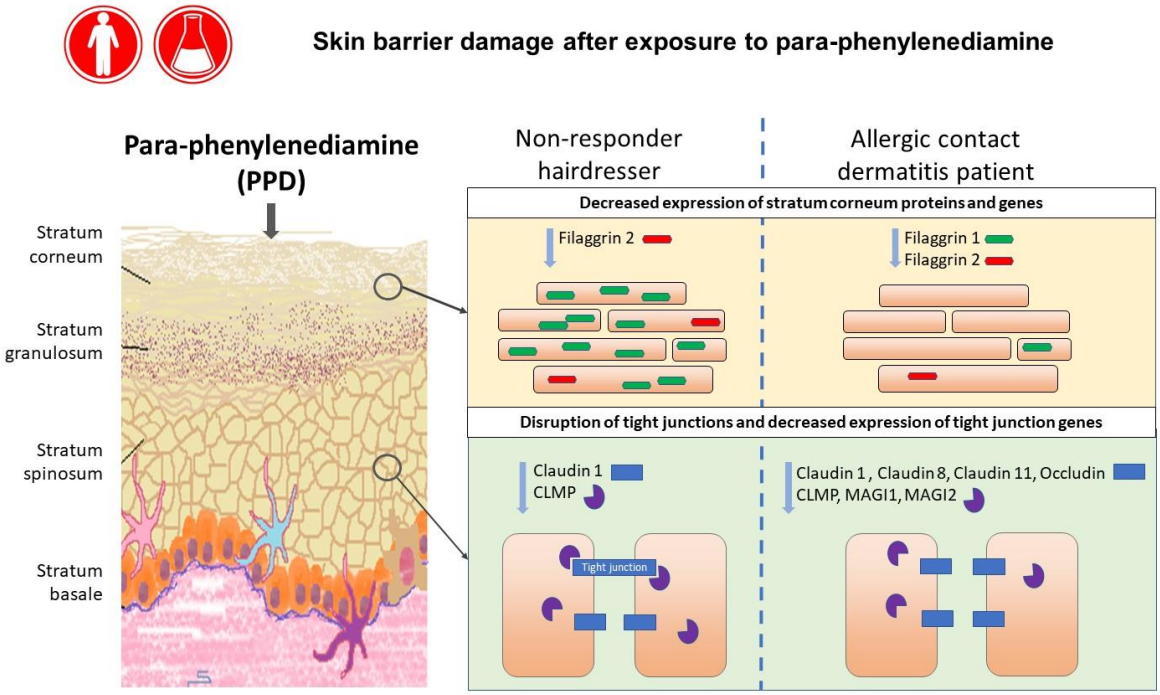
Revision Sheet

Manuscript I

The publication status of manuscript I has changed since the submission of this thesis. The manuscript has been accepted and published in Journal of Allergy and Clinical Immunology (JACI). DOI: https://doi.org/10.1016/j.jaci.2019.11.023

The title was changed to: Skin barrier damage after exposure to para-phenylenediamine and a graphical abstract was added (shown below).



Public defence

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