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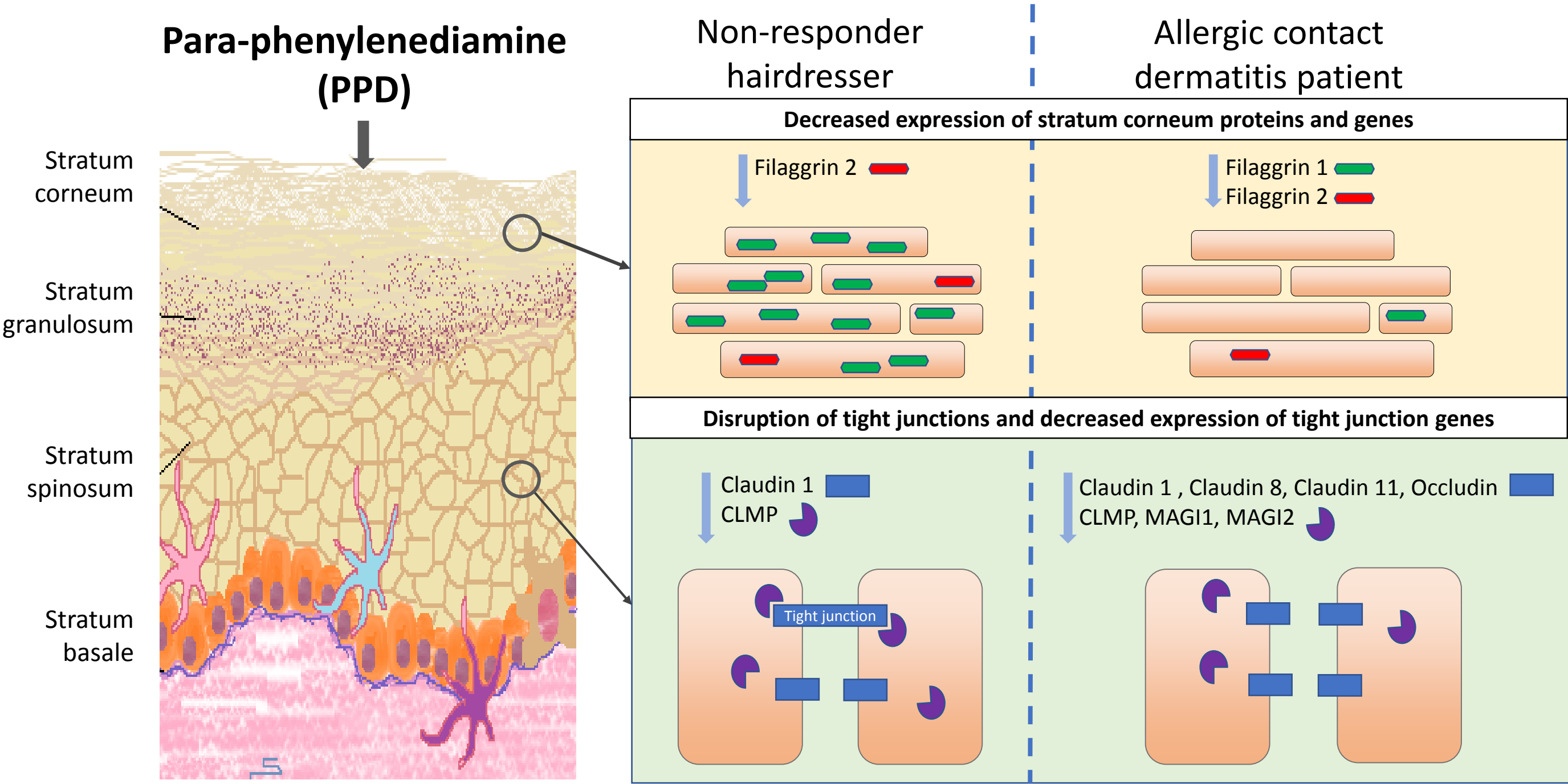
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Conflict of interest:

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Abstract

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

Objective: To investigate effects of PPD exposure on the skin of occupationally exposed individuals with and without clinical symptoms.

Methods: Skin biopsies were collected from 4 mild and 5 severe PPD ACD patients and 7 hairdressers without contact dermatitis 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 analyzed in skin from 4 hairdressers and 1 ACD 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 severe ACD patients after PPD exposure. Claudin-1 (CLDN1), claudin-8 (CLDN8), claudin-11 (CLDN11), CLMP, occludin, MAGI1, and MAGI2 expression were downregulated in severe ACD patients. CLDN1 and CLMP expression were downregulated in non-responding hairdressers and mild ACD patients. Filaggrin-1 (FLG1), filaggrin-2 (FLG2) and loricrin expression were downregulated in ACD individuals. Confocal microscopy images showed down regulation of claudin-1 and filaggrin 1 and 2. In contrast, 3D 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

Better understanding mechanisms of skin barrier damage caused by strong contact allergens can improve treatment and assist in prevention of occupational ACD.

Capsule summary:

Mechanisms of skin barrier defects in para-phenylenediamine allergic contact dermatitis patients and healthy hairdressers after exposure demonstrate significant downregulation of tight junction and stratum corneum proteins.

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, *Stratum 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 two 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 though only 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), which would make most hairdressers sensitized to PPD, but this is not the case. 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). Occupationally exposed individuals might therefore have other mechanisms to protect against PPD exposure induced sensitization and tissue damage. 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, pattern 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 dysregulated TJs and *stratum corneum* (SC) proteins is one of the underlying causes of contact allergic skin diseases (17)

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 ACD 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 of ACD patients but also show the same pattern in non-responding individuals to a lesser extent.

Materials and methods

Study population

The study included two groups: a PPD ACD 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 allergic 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 ACD patients and 11 hairdressers without and ACD 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 PPD ACD patients and 7 biopsies from PPD 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) (25) implementation of Bowtie software (v 1.0.0) (26) 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 analysis between two groups was performed using edgeR Bioconductor package (27). 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 $\log_2$ (fold change) >1 were kept for further analysis. Gene ontology (GO) term enrichment analysis was performed using GOrse Bioconductor package (28) using the Wallenius approximation. Pathway analysis was done using the Enricher platform with the Panther database (29, 30). 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 ACD patient. Upon collection, the biopsies were embedded in TissueTek, frozen in liquid nitrogen and stored at -80°C . The biopsies were cut in $7\text{ }\mu\text{m}$ 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 ACD patients 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 ACD patients with skin lesions with higher degree of severity (graded +2 and +3) (Figure 1B). Gene expression profile of mild ACD individuals (graded +1 or doubtful) clustered more close to vaseline exposed skin from non-responders and PPD ACD patients, as well as PPD-exposed skin from non-responder individuals. The RNA expression in vaseline controls from ACD individuals and non-responder hairdressers did not differ from each other, only one gene out of 21175 sequences showed significant differential expression. 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 upregulated (Figure 2A) and downregulated genes (Figure 2B) and compared between the three groups (upregulated Figure 2C, downregulated Figure 2D). 2636 upregulated genes were found to be unique for the severe ACD group, 53 for the mild ACD group and 22 for the non-responder group, further 376 genes were upregulated in all groups. The shared or unique genes in each group were analyzed for significant pathway associations (Figure 2A). Upregulated pathways in ACD 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 severe ACD group, pathways involved in cell damage and death were also significantly upregulated. The non-responder group shared upregulation of T cells activation and inflammatory cytokine and chemokine signaling. Unique to non-responders was a pathway involved in serotonin degradation. Among the downregulated genes, there were 3382 unique genes in the severe ACD group, 69 genes in the mild ACD group and 17 genes in the non-responders (Figure 2D). The most significantly downregulated gene pathways included cell adhesion, tissue regeneration and cell metabolism (Figure 2B). Association of downregulated 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 ACD skin (Figure 3).

Dysregulation of cell-cell junction genes takes place 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, mild ACD and severe ACD skin groups revealed that 15 out of 32 (47%) TJ genes were significantly downregulated in one or more groups (Figure 3A). All the 15 genes were significantly downregulated in severe ACD individuals, whereas CLDN1 and CXADR-like membrane protein (CLMP) were significantly downregulated in the mild ACD 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 downregulation observed in PPD-exposed ACD 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 gap-junctions, desmosomes and keratins in all groups. The degree of dysregulation correlated with the severity of clinical symptoms and as such, most genes were deregulated in severe ACD reactions (Figure 3D).

PPD causes dysregulation of filaggrin family proteins in ACD skin

In line with downregulated 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 mild ACD and non-responder group, whereas the effect was more pronounced in the severe ACD group, which showed a stronger decrease (Figure 4B). In contrast, involucrin (IVL) was significantly upregulated in the patients with severe ACD reactions but remained constant in mild ACD and the non-responder groups. This pattern was also seen in correlation analyses between differentially expressed TJ and SC genes (Figure E1). Only involucrin expression negatively correlated with other barrier genes in the overall analysis (Figure E1A), all others showed either no correlation or a positive correlation.

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

Changes in barrier protein expression levels and their location in epidermis were visualized by confocal microscopy in skin biopsies from day 4 of patch test with PPD from 4 non-responder hairdressers and 1 severe ACD patient (Figure 5). 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 severe ACD 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 a

complete disruption of the epidermis in the severe ACD individual, causing the disappearance of the net-like structure of both claudin 1 and CLMP.

Exposure to PPD decreased protein expression of FLG1 and FLG2 in both ACD 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 non-responder hairdressers and severe ACD (Figure 5B). FLG2 was also expressed in basal membrane and this expression did not appear to be affected by PPD exposure in non-responder individuals (Figure 5B).

PPD affected expression patterns of claudin-1 and CLMP and decreased the 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 hours 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 skin cultures as a positive control for oxidative stress, and it resulted in a significant decrease in CLDN1 expression. In healthy human skin, 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 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, whereas a decrease in FLG2 expression was observed (Figure 6A-B).

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Discussion

The present study provides an in-depth analysis of the changes in the epithelial barrier components after exposure to PPD, a strong contact allergen often found in dark hair dyes. Hairdressers, who were repeatedly exposed to PPD due to their occupation, but never showed signs of contact dermatitis might tolerate this exposure and we therefore investigated their skin response to PPD exposure. The effect of PPD exposure was compared between 3 groups; non-responder hairdressers, mild and severe ACD individuals. The exact mechanism for sensitization to contact allergens is unknown; one theory is that the patients are sensitized 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 and multiple times. It can be suggested that non-responder individuals have a stable and strong epidermal barrier offering protection and tolerance towards this contact allergen. The analyses of vaseline exposed skins in both groups answered this question. Interestingly, our study did not indicate any differences in skin barrier protein expression in ACD patients and non-responder hairdressers upon vaseline exposure.

Overall, TJ and SC proteins showed similar expression levels in both ACD patients 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 strength and stability between ACD patients and non-responders, but rather a result of immune response, tissue signaling, metabolism or inflammation caused by PPD exposure.

Expression of TJ proteins CLDN1 and CLMP were downregulated in both mild and severe ACD, as well as in non-responders after PPD exposure, suggesting that PPD exposure disturbs the expression levels of these TJ proteins, even when there is no clinical symptoms. The rest of significantly differentially expressed TJ and SC barrier molecules were mainly downregulated upon PPD exposure in the severe ACD group, and to a lesser extent in the

mild ACD group, where inflammation was also present. Confocal microscopy imaging of claudin 1, filaggrin-1 and filaggrin 2 confirms the decrease of these barrier molecules in non-responding individuals. Skin inflammation in AD, contact dermatitis and psoriasis have been reported to down-regulate filaggrin-1, filaggrin-2 and claudin-1 (31, 32). Together this suggests that only CLDN1 and CLMP down-regulation is directly caused by PPD exposure and that the remaining dysregulation of TJ proteins is probably caused by the subsequent inflammation.

PPD is a highly reactive chemical and has a half-life of a few hours on human skin (33). If left in contact with oxygen it polymerizes into its trimer Bandrowski's base, which also has an immune stimulating capacity (34). In healthy skin, PPD is *N*-acetylated to a safe metabolite in the uppermost layers of the skin (35). 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 oxidation of PPD, and these activate innate immune response and recruitment of non-specific lymphocytes and direct them to the contact site (36). Specific epitopes and the type of signals that reach skin-resident antigen-presenting cells are currently unknown. Skin lesions in PPD ACD individuals are persistent and lesions can reoccur in previously exposed sites indicating formation of allergen-specific memory cells in PPD ACD individuals.

Early events in ACD resembles that of irritant contact dermatitis, which involves skin barrier damage or stress resulting in the activation of innate immune cells (37). Most contact allergens, including PPD are known to have irritant properties. In the present study, we attempted to limit the irritant response by using a 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 may suggest that PPD exposure

increases the risk of both irritant contact dermatitis and ACD to other allergens. Damage to the epithelium was evident in the skin of non-responder 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 ACD patients.

Non-responders do not display dysregulation of the epidermal barrier except for CLDN-1 and CLMP on day 4 after PPD exposure. We propose that the higher stability of the epidermal barrier in non-responding individuals upon exposure to PPD is due to differences in immune response and possibly a more efficient conversion of PPD to its safe N-acetylated product. Differences in immune response may be due to a higher threshold for innate immune response activation, resistance to irritant exposure in non-responding individuals or the development of immune tolerance. More research is needed to answer these questions. 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 upregulates its key proteins, strengthening the epidermal barrier. This response might be lost amid the inflammatory environment in individuals with ACD.

In conclusion, we identified the state of skin barrier in terms of tight junction and stratum corneum proteins in individuals with ACD to PPD and in occupationally exposed non-responders. We observed that the majority of TJ and SC proteins were significantly downregulated in the skin, which was significantly reduced in severe ACD patients and to a lesser extent in mild ACD patients. We identified two TJ proteins, CLDN1 and CLMP that are also downregulated in the skin of non-responder individuals upon PPD exposure. This direct barrier damage may be the outcome of the highly sensitizing nature of PPD and emphasizes the causality of occupational exposure.

423

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Table 1. Patient characteristics

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
Mild ACD (4)	F	36.3	? / +1	3/4	4/4	2/4	2/4
Severe ACD (5)	F	45.6	+2/+3	5/5	3/5	3/5	0/5

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 ACD 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 were 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 ACD individuals.

Clinical reactions and corresponding hematoxylin 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= 16 (C). Skin lesions in response to PPD and VAS as control on days 2 and 4 after patch is applied (D).

Figure 2. Pathway analysis of differentially expressed genes after PPD exposure.

Comparison and pathway analysis of significantly upregulated (A) or downregulated (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 $\text{fdr} > 0.015$). Pathway analysis using the EnrichR/Panther databases of up- or downregulated 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 upregulated (C) or downregulated (D) between comparisons.

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 * ($\text{fdr} < 0.015$). Scatter plots for the top 8 most significantly differentially expressed 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 downregulated 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 gap 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 differentially 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 and barrier proteins in non-responder and severe ACD individuals on day 4 after skin patch test with vaseline (VAS) or 1% PPD in VAS. Tight

junction proteins claudin 1(N=4) and CLMP (N=4) (A) and barrier proteins filaggrin 1 (N=1) and filaggrin 2 (N=3) (B).

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% H₂O₂ were used as the negative and positive control, respectively.

Figure E1. 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.

Figure E2. 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) and FLG2 (C)

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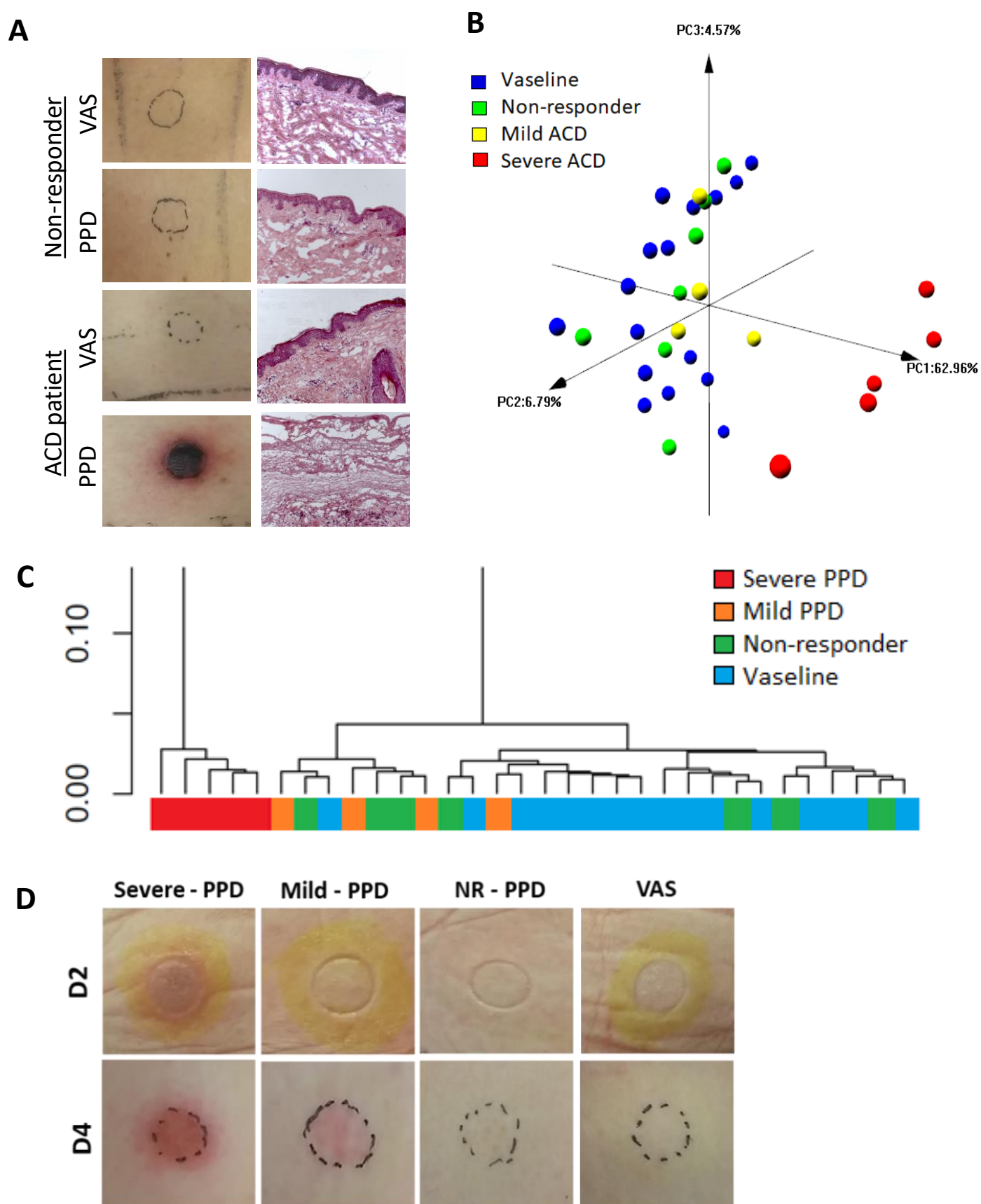


Figure 1 – Steengaard Meisser et Al.

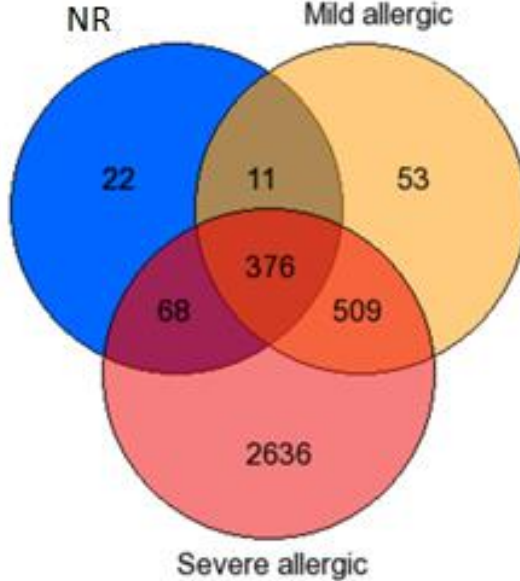
A

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	0.000002599
	T cell activation	13	0.000002384
	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	0.00004866

C



D

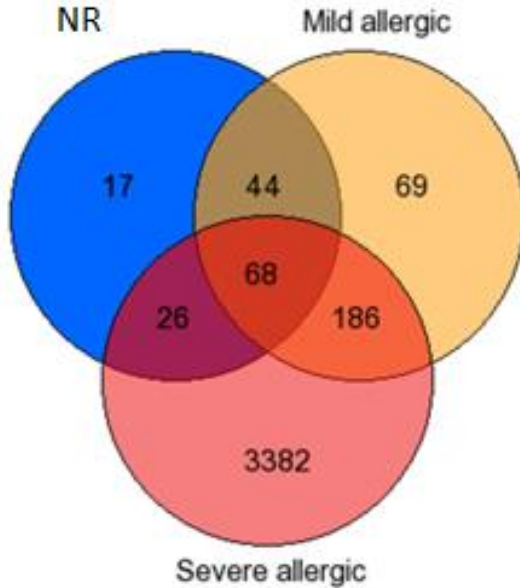
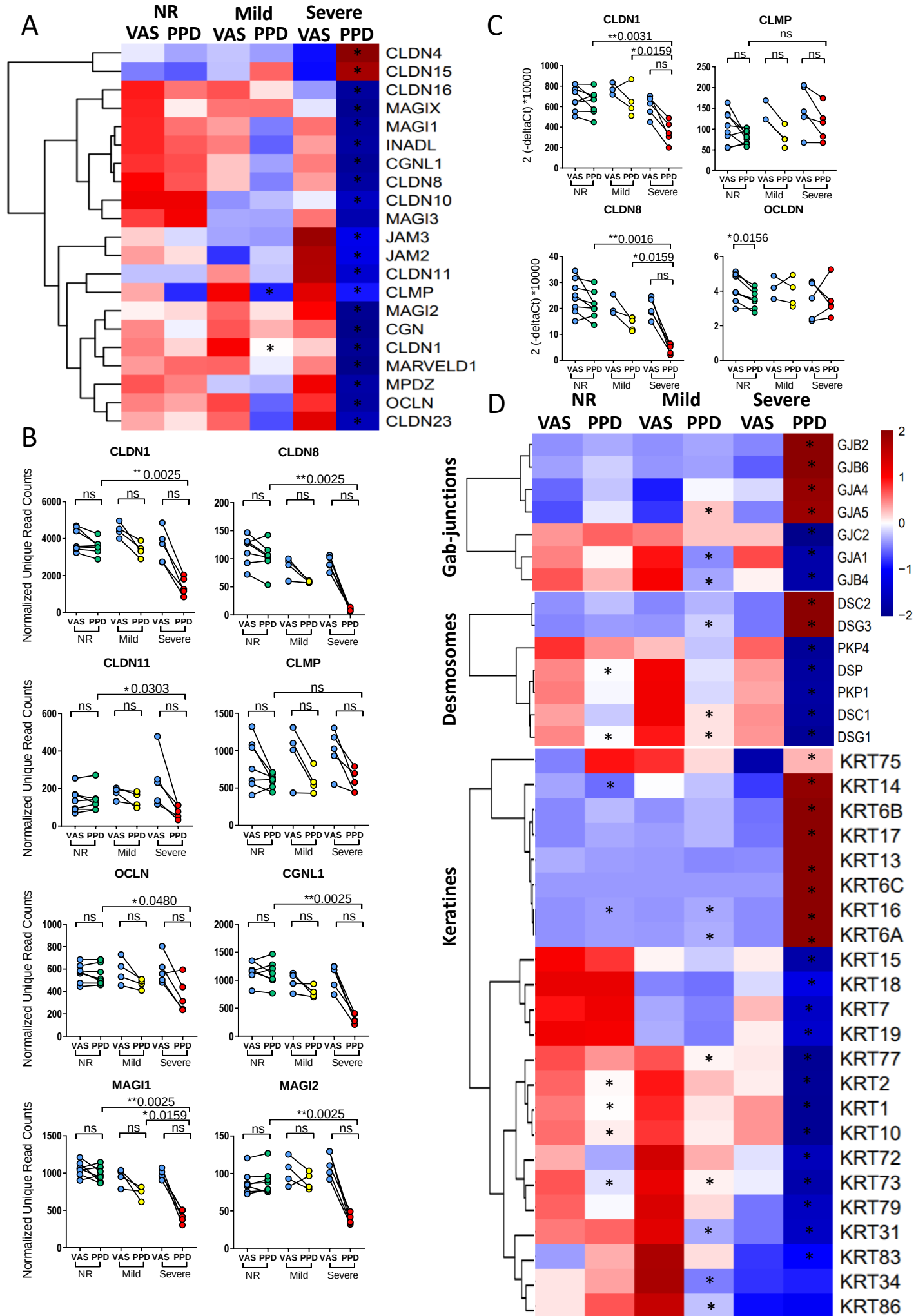


Figure 2– Steengaard Meisser et Al.



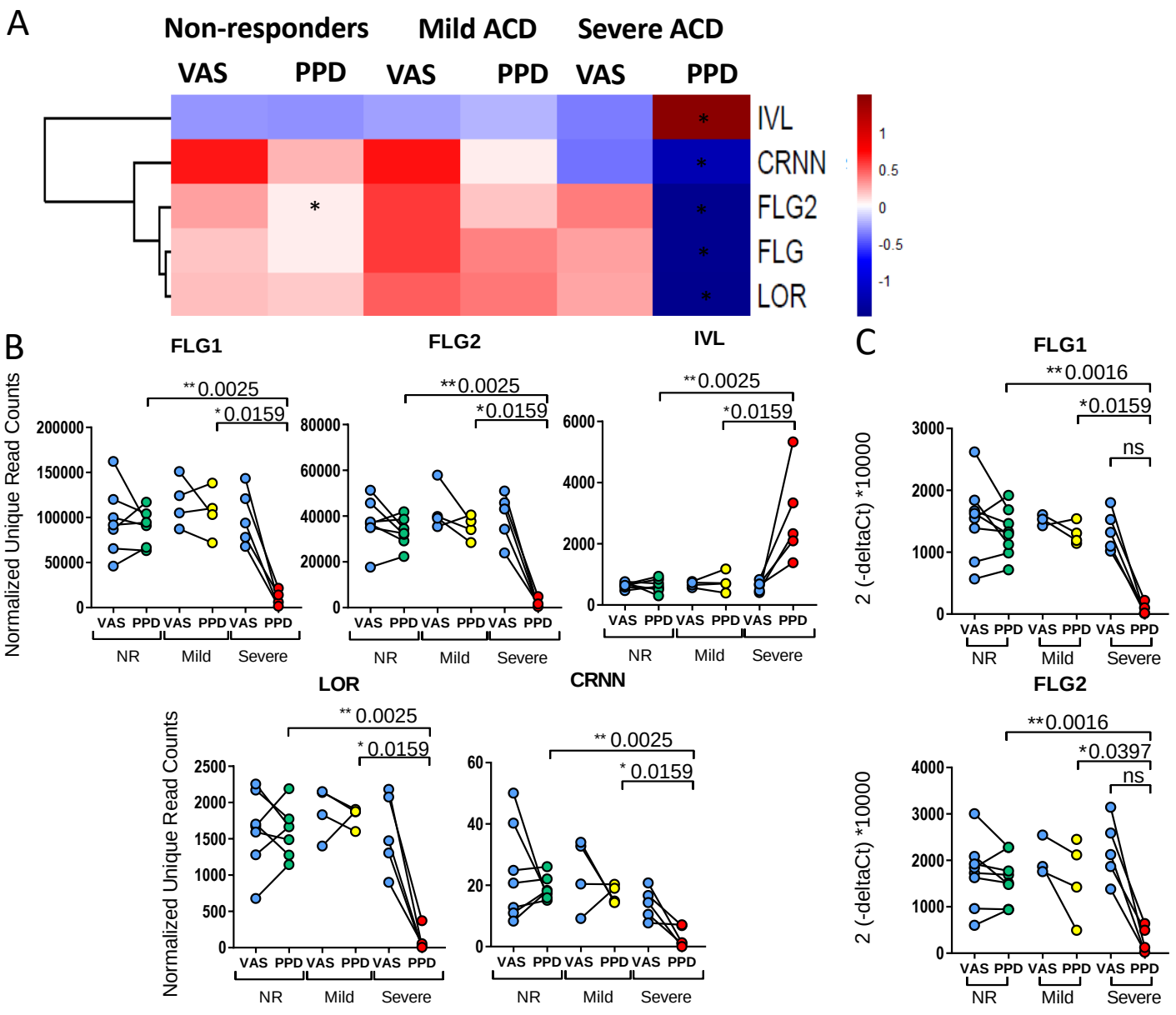


Figure 4– Steengaard Meisser et Al.

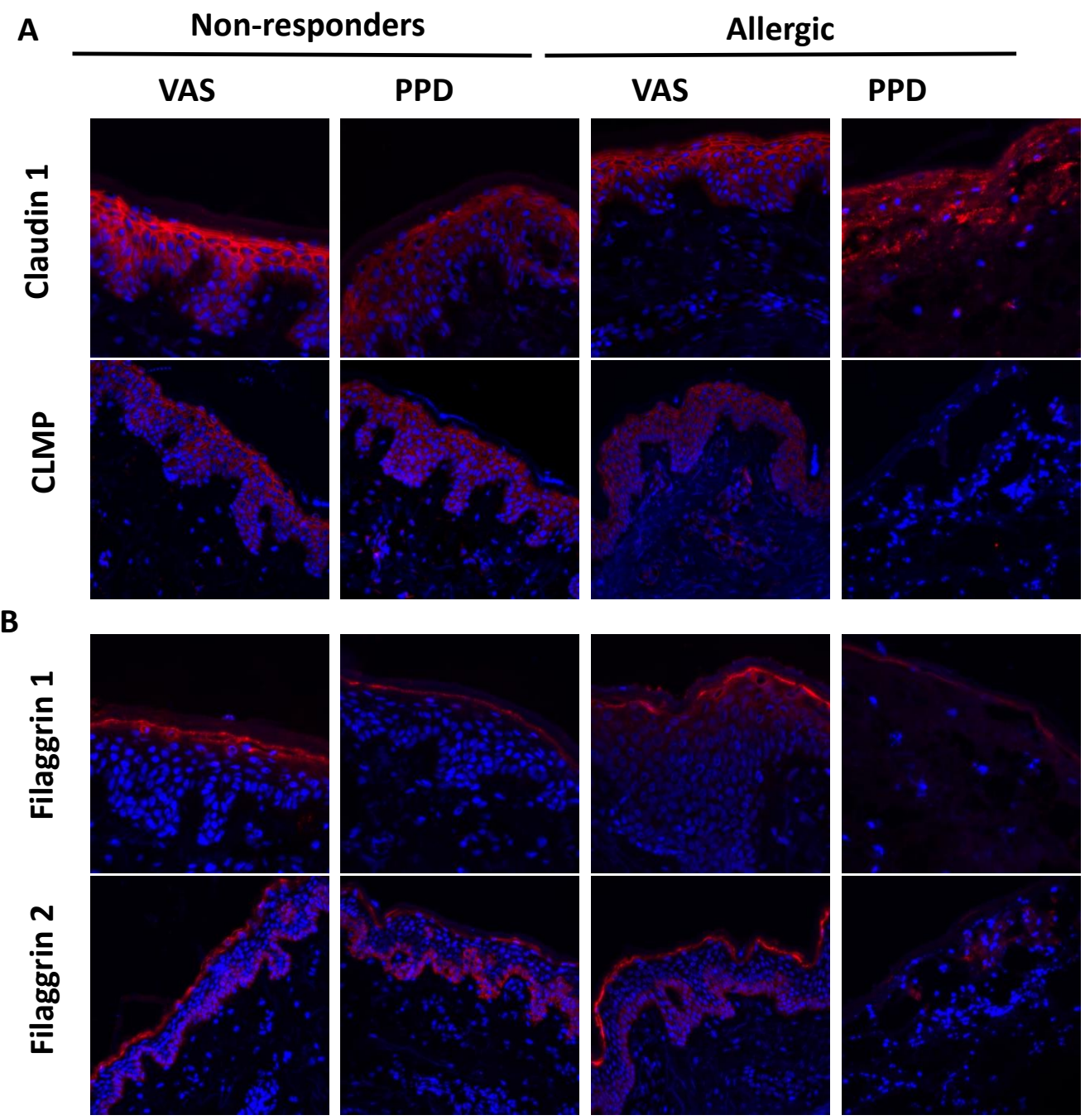


Figure 5 – Steengaard Meisser et Al.

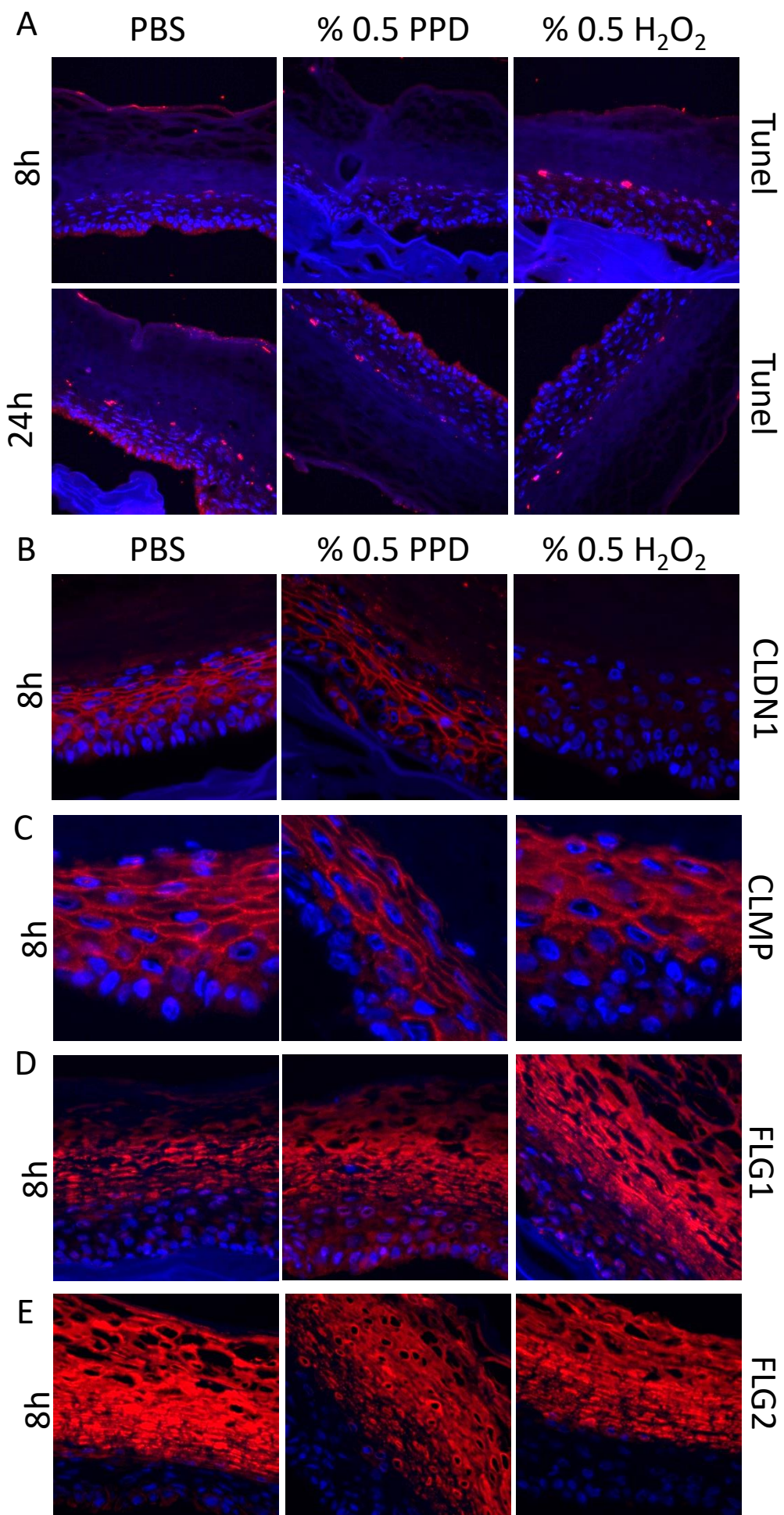


Figure 6 – Steengaard Meisser et Al.

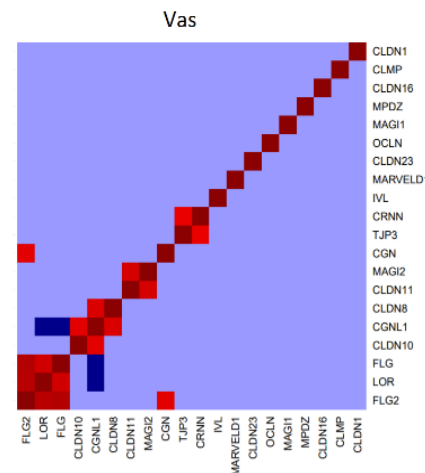
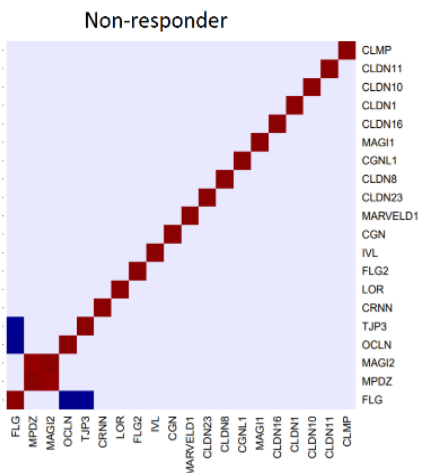
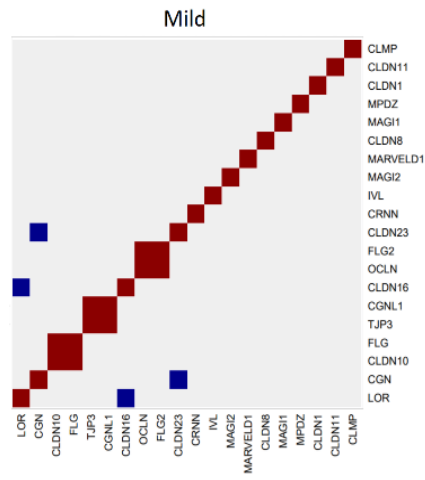
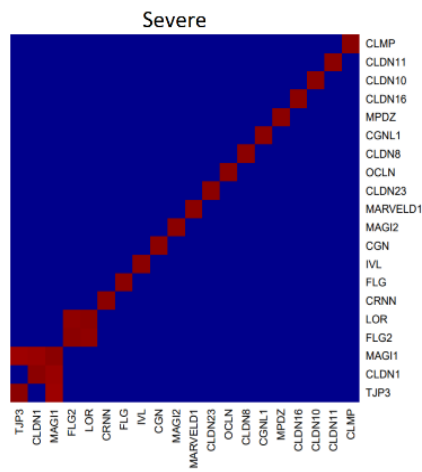
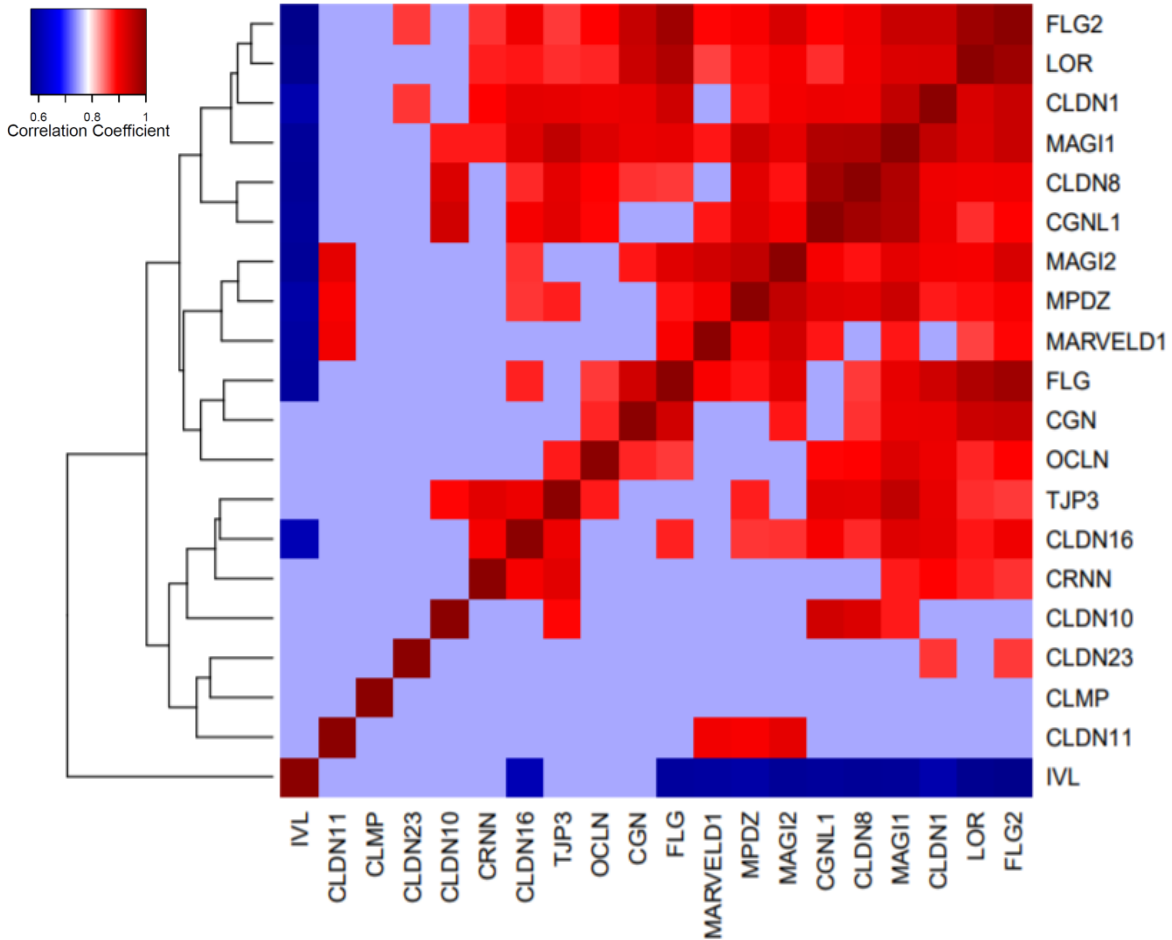


Figure E1 – Steengaard Meisser et Al.

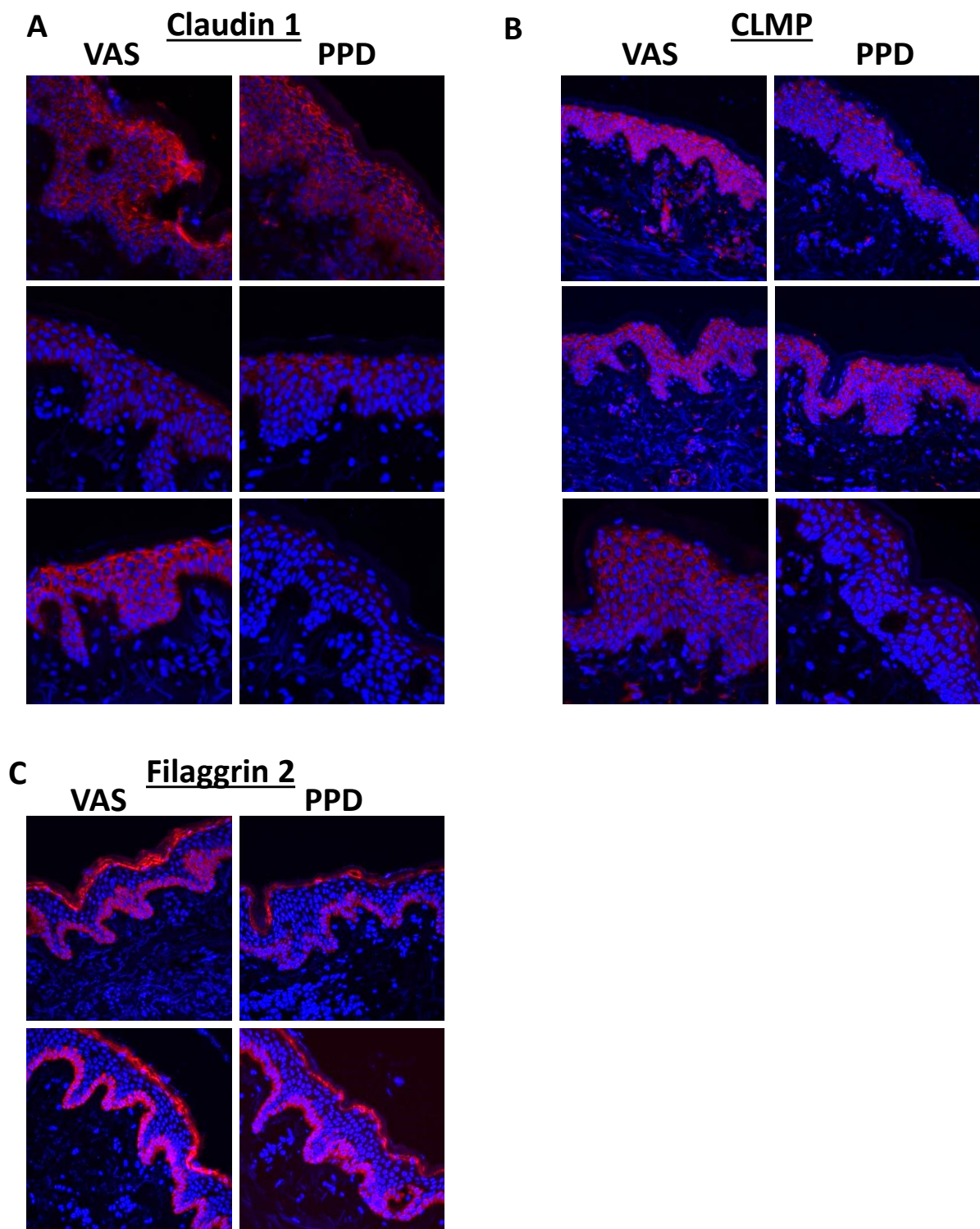


Figure E2 – Steengaard Meisser et Al.