**Fakultät für Medizin**

**Klinik und Poliklinik für Dermatologie und Allergologie am Biederstein**

# Novel diagnostic tools and markers for inflammatory skin diseases

**Dr. med. Natalie Verena Garzorz-Stark**

Vollständiger Abdruck der von der Fakultät für Medizin der Technischen Universität München zur Erlangung des akademischen Grades eines

**Doctor of Philosophy (Ph.D.)**

genehmigten Dissertation.

**Vorsitzender:** Univ.-Prof. Dr. Arthur Konnerth

**Betreuer:** Univ.-Prof. Dr. Kilian G. Eyerich, Ph.D.

**Prüfer der Dissertation:**

1. Univ.-Prof. Dr. Carsten Schmidt-Weber

2. Univ.-Prof. Dr. Dr. Johannes Ring

Die Dissertation wurde am 01.03.2016 bei der Fakultät für Medizin der Technischen Universität München eingereicht und durch die Fakultät für Medizin am 19.04.2016 angenommen.

# Table of Contents

# Summary

The inflammatory skin diseases psoriasis and eczema are highly prevalent and relevant diseases implying a devastating quality of life for affected individuals on the one hand and enormous socio-economic costs on the other hand. Many novel effective therapies have been developed in the past decades for both diseases, yet eczema and psoriasis are still underdiagnosed and undertreated diseases. Due to their complexity and heterogeneity not sufficiently acknowledged in most studies, the pathogenesis of psoriasis and eczema is still not fully understood and new models are needed to achieve a substantial scientific break-through. In my thesis, I studied three models to decipher disease-specific signatures.

First, I demonstrated that patients suffering from alopecia areata, a T cell-mediated autoimmune disease of the hair follicle, are an ideal model to study disease-specific pathways of inflammatory skin diseases, as these patients are prone to develop various T cell-driven inflammatory skin diseases. Depending on specific environmental triggers, cutaneous inflammation is driven towards disease-specific patterns of inflammatory skin diseases resembling the ones found in single affected patients.

With my second model, the human imiquimod model, I showed that imiquimod cream induced skin reaction in humans leads to a homogenous reaction independent of the genetic background. Imiquimod is a TLR7 agonist that is commonly used to induce psoriasis in murine models. In humans, I demonstrated that the reaction mimicked characteristics of acute contact dermatitis but also reflected important pathways of psoriasis such as the IL-23 pathway. Therefore, the imiquimod model has the potential to be an alternative to murine models for explicit (interventional) questions to be studied in patients.

A cohort of patients affected by psoriasis and eczema simultaneously represents the third model. By intraindividually comparing disease signatures of eczema and psoriasis, background noise from interindividual variability was limited and disease-specific mechanisms could be distinguished from general cutaneous inflammation. Using this approach genes and signaling pathways regulated in common and exclusively for each disease were identified. Since psoriasis and eczema phenotypes often overlap, accurate diagnostics is

impossible in special cases, not to speak about predicting the clinical outcome of an individual patient. Therefore, I aimed to build a disease classifier based on this data set which was superior over current gold standard methods. I established a molecular classifier on the level of immunohistochemistry and real-time PCR based on the two genes *NOS2* and *CCL27* that diagnosed psoriasis and eczema with high sensitivity and specificity in patients suffering from classical forms and subtypes of psoriasis and eczema. Moreover, this disease classifier gave a clear prediction for therapeutic response in indistinct patients that was in line with the subsequent clinical course.

This thesis presents a new scientific approach using specific models that give new insights into the pathogenesis of the heterogeneous diseases of psoriasis and eczema. Integrating these models with future approaches will lead to precise diagnostics on molecular level and therefore enable therapeutic regimen tailored for individual patients.

# List of Abbreviations

| | |
|---|---|
| AA | Alopecia areata |
| ACD | Acute contact dermatitis |
| AD | Atopic dermatitis |
| ad | Adde (fill up to) |
| AE | Atopic eczema |
| BMI | Body mass index |
| CCL27, *CCL27* | Chemokine (C-C motif) ligand 27 |
| CCR (e.g. CCR5) | C-C chemokine receptor ( e.g. C-C chemokine receptor type 5) |
| CD | Cluster of differentiation |
| CLA | Cutaneous lymphocyte-associated antigen |
| *CLEC4G* | C-type lectin family member 4 |
| CTLA4 | Cytotoxic T-lymphocyte-associated protein 4 |
| CXCL (e.g. CXCL17) | Chemokine (C-X-C motif) ligand (e.g. Chemokine (C-X-C motif) ligand 17) |
| CXCR (e.g. CXCR4) | C-X-C chemokine receptor (e.g. C-X-C chemokine receptor type 4) |
| DALY | Disability-Adjusted Life Years |
| DAPI | 4',6-diamidino-2-phenylindole |
| DC | Dendritic cell |
| $dH_2O$ | Distilled water |
| DNA | Deoxyribonucleic acid |
| EDTA | Ethylenediaminetetraacetic acid |
| EGF | Epidermal growth factor |
| ELISA | Enzyme Linked Immunosorbent Assay |
| FACS | Fluorescence-activated cell sorting |
| *GDA* | Guanine deaminase |
| GM-CSF | Granulocyte-macrophage colony-stimulating factor |
| GWAS | Genome-wide association study |
| $H_20$ | Water/aqua |
| HE stain | Hematoxylin and eosin stain |
| IDEC | Inflammatory dendritic epidermal cell-like dendritic cells |
| IFN (e.g. IFN-γ) | Interferon (z.B. Interferon γ) |
| Ig (e.g. IgG) | Immunoglobulin (e.g. Immunoglobulin G) |
| IL- (e.g. IL-37) | Interleukin (e.g. Interleukin 37) |
| iNOS | Inducible nitric oxide synthase |
| IRF-1 | Interferon regulatory factor 1 |
| ISD | Inflammatory skin disease |
| JAK | Janus kinase |
| *KLK13* | Kallikrein-related peptidase 13 |
| LC | Langerhans cell |
| LDL | Low-density lipoprotein |
| LL-37 | Cathelicidin-related antimicrobial peptide |
| LTF | Lactotransferrin |
| MC | Molecular classifier |
| n | Number of samples |
| NE | Neutrophil elastase |

| | |
|---|---|
| nE | Naturally occurring eczema |
| NFAT | Nuclear factor of activated T cells |
| NF-κB | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| NKG2D | Natural-killer group 2, member D |
| noPL | No pseudolymphoma-like dermatitis (Imiquimod study) |
| *NOS2* | Inducible nitric oxide synthase |
| *NPTX1* | Neuronal Pentraxin 1 |
| PASI | Psoriasis Area Severity Index |
| PBS | Phosphate buffered saline |
| pDCs | Plasmacytoid dendritic cells |
| PDE4 | cAMP-specific 3',5'-cyclic phosphodiesterase 4 |
| PL | Pseudolymphoma-like dermatitis (Imiquimod study) |
| *PLA2G4D* | Phospholipase A2, group IVG |
| Pso | Psoriasis |
| *RHCG* | RH family, C glycoprotein |
| RIN | RNA integrity number |
| RNA | Ribonucleic acid |
| RT | Room temperature |
| S. *aureus* | Staphylococcus aureus |
| RT-PCR | Real-time polymerase chain reaction |
| SCORAD | Scoring of Atopic Dermatitis |
| *SELE* | E-selectin |
| SEM | Standard error of the mean |
| *SOST* | Sclerostin |
| STAT | Signal transducer and activator of transcription |
| Std | Standard deviation |
| SVM | Standard vector machine |
| Tc | Cytotoxic T cell |
| *TCN1* | Transcobalamin1 |
| TGF-β | Transforming growth factor beta |
| *TGM1* | Transglutaminase 1 |
| Th | T helper cell |
| TIP - DC | TNF/iNOS-producing dendritic cell |
| TLR (e.g. TLR7) | Toll-like receptor (e.g. Toll-like receptor 7) |
| *TMPRSS11D* | Serine transmembrane protease 11D |
| TNF-α | Tumor necrosis factor alpha |
| Treg | Regulatory T cell |
| TSLP | Thymic stromal lymphopoietin |
| U | Unit |
| USD | US dollar |
| VEGF | Vascular endothelial growth factor |
| VLA-1 | Very Late Antigen-1 |
| Vs | versus |
| YLD | Years Lost due to Disability/ Years Lived with Disability |
| YLL | Years of Life Lost |

# List of Tables

# List of Figures

# 1 Introduction

## 1.1 The global burden of psoriasis and eczema

Skin diseases are amongst the most prevalent human illnesses across all cultures and ages. According to the global atlas of disease burden of the World Health organization, skin conditions are the fourth leading cause (Table 1) of nonfatal burden in 2010 expressed in the measure of "Years Lived with Disability" (YLD) (Hay *et al.*, 2014) which – in contrast to commonly used mortality measures – records the burden of living with a disability.

Table 1: Years lost due to disability (YLD) ranks when considering skin conditions collectively
Table modified from Hay et al., 2014.

| **Cause** | **Global YLD** | **YLD rank** |
|---|---|---|
| Low back pain | 80,666,896 | 1 |
| Major depressive disorder | 63,239,334 | 2 |
| Iron-deficiency anemia | 42,505,250 | 3 |
| ***Skin conditions*** | ***33,717,725*** | ***4*** |
| Neck pain | 32,650,797 | 5 |
| Chronic obstructive pulmonary disease | 29,420,262 | 6 |
| Other musculoskeletal disorders | 28,247,230 | 7 |
| Anxiety disorder | 26,847,326 | 8 |
| Migraine | 22,362,507 | 9 |
| Diabetes mellitus | 20,791,397 | 10 |

Together with infectious diseases, the inflammatory skin diseases psoriasis and eczema (= dermatitis) are the most important conditions among the recorded skin diseases resulting in high numbers of YLD with eczema as leading cause across all ages (Figure 1). According to a systematic analysis of Murray et al. who investigated the "Disability-Adjusted Life Years" (DALYs) for 291 diseases and injuries in 21 regions between 1990 and 2010, the DALYs for eczema showed an increase of 29.1% and the DALYs for psoriasis even increased by 42.8% within these 20 years. DALYs not only take into account

the YLD, but also the "Years of Life Lost" (YLL) due to dying prematurely (Murray *et al.*, 2012).

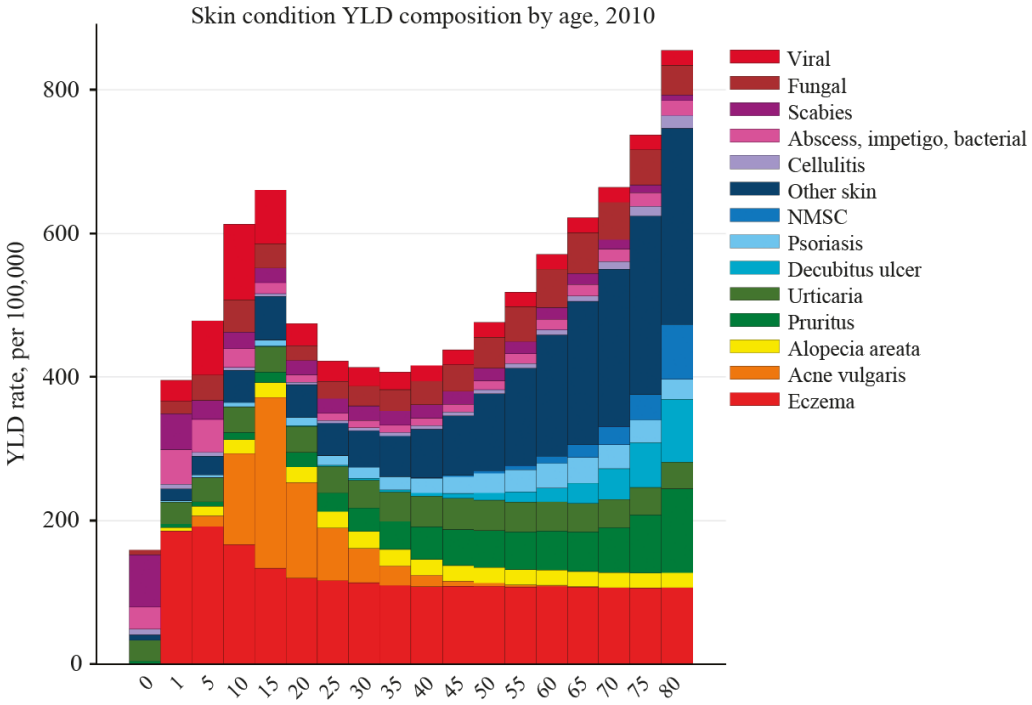


Figure 1: Skin condition Years Lost due to Disability (YLD) composition by age, 2010
YLD rate per 100,000. NMSC = non-melanoma skin cancer. Figure modified from Hay el al., 2014.

The significant contribution of psoriasis and eczema to the YLD rate is based on the high prevalence rates and early onset (< 30 years) of these diseases but also on the high average disability rates reducing quality of life. It is estimated that worldwide 2% of the population suffers from psoriasis (Christophers, 2001). The prevalence of atopic eczema[1] has more than doubled in the past three decades in industrialized countries, now affecting 2 to 10% of adults and up to 30% of children (Bieber, 2008). However, increasing incidence is also detected in developing countries indicating that atopic eczema has become a global challenge (Deckers *et al.*, 2012).

The quality of life is severely reduced in psoriasis and eczema patients. Patients with psoriasis reported a reduction of physical and mental functioning comparable to that seen for example in cancer, arthritis and heart disease (Augustin *et al.*, 2008; Rapp *et al.*, 1999).

[1] Atopic dermatitis/eczema is the most common type of dermatitis/eczema fulfilling the Hanifin and Rajka criteria as listed in Table 3

Generalized atopic dermatitis scored higher on the Children's Life Quality Index (measure for the impairment of quality of life) than any other disease including diabetes and epilepsy apart from cerebral palsy (Beattie and Lewis-Jones, 2006). On the one hand, the impact of psoriasis and eczema on the quality of life is based on the skin manifestations themselves which are stigmatizing (Hrehorow *et al.*, 2012; Roosta *et al.*, 2010) and impairing daily life due to itch, sleep disturbances and fatigue symptoms (Bieber, 2008; Gowda *et al.*, 2010; Silverberg *et al.*, 2015; Szepietowski and Reich, 2015). On the other hand, a main contributor to reduced YLD lies in the variety of comorbidities that are associated with psoriasis and eczema.

Atopic eczema has been shown to precede allergic asthma and hay fever, a concept called "Atopic march" (Spergel, 2010). 50% of all patients with atopic eczema develop asthma and/or allergic rhinoconjunctivitis within their first year of life and probably 85% below 5 years of age (Nutten, 2015). Moreover, eczema is a strong risk factor for IgE-mediated food allergy in infants: children with eczema were shown to be 11 times more likely to develop peanut allergy and almost six times more likely to develop egg allergy by 12 months than children without eczema (Martin *et al.*, 2015). Besides, a positive association of atopic eczema with attention-deficit hyperactivity disorder (ADHD) was reported (Deckert *et al.*, 2014). Suspected positive associations with other inflammatory diseases such as diabetes mellitus, multiple sclerosis could not yet be conclusively proven, but there is evidence for atopic eczema as risk factor for the development of rheumatoid arthritis and inflammatory bowel diseases (Schmitt *et al.*, 2015). On molecular level, increasing evidence for atopic eczema as systemic disease emerges: Ewald et al. recently reported for the first time an association between the genomic fingerprinting of atopic eczema with the atherosclerosis signaling pathway including genes associated with vascular inflammation such as *SELE* encoding for Selection E and IL-37. *SELE* in turn has been independently associated with coronary heart disease and carotid artery atherosclerosis and its expression in the vascular endothelium of the dermis of atopic eczema patients could be shown (Ewald *et al.*, 2015).

In contrast to eczema where evidence for systemic involvement is still to be gained, psoriasis is now rather regarded as systemic disease with skin manifestation than as an isolated skin disease. In line with this, 78% of psoriasis patients have at least one comorbidity (www.derma-atlas.de). 20% of psoriasis patients are suffering from disabling arthritis, and compared to the normal population, prevalence rates for cardiovascular risk factors

such as arterial hypertension (64% of psoriasis patients), diabetes, adiposity (56% of psoriasis patients) and lipometabolic disorders (Gottlieb and Dann, 2009; Mehta *et al.*, 2012; Prey *et al.*, 2010; Qureshi *et al.*, 2009), (www.derma-atlas.de), are significantly increased. Moreover, severely affected psoriasis patients were shown to have an increased risk for myocardial infarcts and strokes (Armstrong *et al.*, 2013). Both body mass index (BMI) and psoriasis risk are significantly positively associated (Nelson *et al.*, 2015; Wolk *et al.*, 2009), a finding that is supported by laboratory examinations which could show elevated serum lipids and oxidated low-density lipoproteins (LDLs) contributing to the elevated risk for atherosclerosis in psoriasis patients (Villanova *et al.*, 2013).

Apart from physical impairment, psychological comorbidities are relevant in psoriasis and eczema patients. A recent cross-sectional multicenter study in 13 European countries revealed that psoriasis and eczema are associated with significantly elevated prevalence rates of depression and anxiety. Patients with psoriasis showed a significant association with suicidal thoughts and more than 60% of both psoriasis and eczema patients reported suicidal ideation because of their skin (Dalgard *et al.*, 2015). Not only the patients, but also their families are deeply affected: Parents of children suffering from atopic eczema have reported high stress levels associated with their children's disease and it could be found that taking care of a child with severe atopic eczema was more stressful than caring for a child with diabetes type I (Carroll *et al.*, 2005).

Beyond individual and social burden, both psoriasis and (atopic) eczema have a substantial impact on direct and indirect health costs worldwide. According to a study of Verboom et al., the direct health care costs of atopic eczema go up to 2,559 USD (US dollar) per patient and year (Verboom *et al.*, 2002). Apart from direct health costs, indirect costs such as loss of productivity due to sick leaves are of high relevance. For chronic hand eczema, a common manifestation of eczema, these factors have been well studied. It could be found that societal costs for occupational hand eczema amounted on average 8,799 Euro per patient and year in Germany with indirect costs making up 70% of total costs (Diepgen *et al.*, 2013). This is a high number for the health system considering the fact that occupational contact dermatitis, mostly displaying as chronic hand eczema, alone makes up about 30% of all occupational diseases (Diepgen, 2003).

Also psoriasis has a strong socio-economic impact. In the US alone, the total burden of psoriasis was estimated 112 billion dollar per year with more than 30% of this amount

due to productivity losses. Besides, patients with psoriasis would pay a lifetime cost of 11,498 USD for relief of physical symptoms and emotional health (Brezinski *et al.*, 2015; Vanderpuye-Orgle *et al.*, 2015).

## 1.2 Psoriasis and eczema: undertreated and underdiagnosed widespread diseases

Though psoriasis and eczema are widespread diseases, they are broadly undertreated. Summarized by Blauvelt et al. "…up to 49%, 36%, and 30% of patients with mild, moderate, and severe psoriasis, respectively, reported receiving no treatment, and 20% – 30% of patients with moderate or severe psoriasis reported treatment with topical medication alone. Additionally, dissatisfaction with treatment is reported by more than half of patients…" (Blauvelt *et al.*, 2015). According to a population-based survey of Lebwohl et al., only 10% of severely affected psoriasis patients receive adequate treatment with classical oral anti-psoriatic treatments and/or biologicals (Lebwohl *et al.*, 2014). Besides, psoriasis associated comorbidities such as arthritis and cardiovascular risk factors are not only underdiagnosed but also undertreated (Ahlehoff *et al.*, 2012; Haroon *et al.*, 2013; Kimball *et al.*, 2012). Also for eczema, the situation is parlous: According to Hanifin et al., 31.6 million people of the US population met the empirical symptom criteria for eczema defined by itching/scratching and red/inflamed rash or excessive dryness/scaling. Besides, 17.8 million met the empirical criteria for atopic eczema defined by the same criteria as eczema but additionally included skinfold location, early onset, symptoms lasting, or a physician diagnosis of asthma/allergic rhinitis/hay fever. However, most cases were not diagnosed by a physician indicating the underdiagnosis but also the undertreatment of this condition (Hanifin *et al.*, 2007).

Whereas the situation on the side of patient care seems devastating, research focusing on novel therapeutics has been characterized by continuous success: For both diseases and in particular for psoriasis, many specific therapies targeting cytokines and other mediators of well-characterized signaling pathways have been developed and approved during the last decade (Table 2).

Table 2: Psoriasis drugs in clinical use (left) and under investigation in clinical trials (right)

Selection of novel drugs according to clinicalTrials.gov and https://services.psoriasis.org/drug-pipeline/index.php. Topical treatments are not included.

| Approved psoriasis drug | Mechanism of action | Psoriasis drug in phase II/III | Mechanism of action |
|---|---|---|---|
| Conventional immunosuppressive drugs | | Ixekizumab | Humanized monoclonal anti-IL-17A antibody (Griffiths *et al.*, 2015) |
| Methotrexate | Acts as a folic acid antagonist, antiproliferative, immunomodulatory (Nast *et al.*, 2012) | Brodalumab | Human monoclonal anti-IL-17 receptor A monoclonal antibody (Lebwohl *et al.*, 2015) |
| Cyclosporine | Inhibits the activity of the calcium-calmodulin-calcineurin complex and thus nuclear factor of activated T-cells (NFAT) dependent production of proinflammatory cytokines (Nast *et al.*, 2012) | HD203 | Biosimilar of etanercept (Yi *et al.*, 2012) |
| Fumaric acid | Inhibition of proinflammatory cytokines via NF-κB inhibition and inhibition of maturation of dendritic cells (Atwan *et al.*, 2015; Nast *et al.*, 2012) | Janus kinase (JAK) inhibitors (Tofacitinib etc.) | Blockage of proinflammatory signaling cascade via JAK/STAT pathway (Hsu and Armstrong, 2014) |
| Retinoids | Antiproliferative and immunomodulatory effects (Nast *et al.*, 2012) | Guselkumab and BI 655066; Tildrakizumab | Fully human monoclonal anti-IL23p19 antibody (Gordon *et al.*, 2015; Krueger *et al.*, 2015a); Humanized monoclonal anti-IL23p19 antibody (Papp *et al.*, 2015c) |
| Biologicals and others | | Tregalizumab (BT-061) | Humanized anti-CD4-specific monoclonal antibody (Helling *et al.*, 2015) |
| Etanercept | Fusion protein functioning as TNF-α receptor antagonist (Leonardi *et al.*, 2003) | ABP 501, GP 2017 | Biosimilars of Adalimumab |
| Adalimumab | Fully human monoclonal anti-TNF-α antibody (Gordon *et al.*, 2006) | Certolizumab pegol | PEGylated IgG fragment (Fab, Fc-free) anti-TNF-α antibody (Reich *et al.*, 2012) |
| Infliximab | Chimeric mouse-human monoclonal anti-TNF-α antibody (Reich *et al.*, 2005) | Namilumab | Neutralizing human IgG1 anti-GM-CSF monoclonal antibody |

| | | | |
|---|---|---|---|
| CT-P13 (Remsima®, Inflectra®) | Biosimilars of Infliximab (McKeage, 2014) | IMO-3100 | Synthetic DNA-based antagonist of TLR7, TLR8 and TLR9 (Suarez-Farinas *et al.*, 2013a) |
| Ustekinumab | Fully human monoclonal anti-IL-12p40 antibody (Griffiths *et al.*, 2010) | Abatacept | Fully human fusion protein of the extracellular domain of CTLA-4 and Fc portion of human IgG1 (Mease *et al.*, 2011) |
| Secukinumab | Fully human monoclonal anti-IL-17A antibody (Langley *et al.*, 2014) | Ponesimod | Oral modulator of sphingosine 1-phosphate receptor 1 (Vaclavkova *et al.*, 2014) |
| Apremilast | PDE4 inhibitor (Papp *et al.*, 2015b) | SRT2104 | Activator of Sirtuin (Krueger *et al.*, 2015b) |

These specific therapies have proven superior effects over broadly acting immunosuppressive therapies with limited side effects (Schmitt *et al.*, 2014). The proinflammatory cytokine tumor necrosis factor α (TNF-α) as well as IL-12 and IL-23 have been shown to play a major role within the pathogenesis of psoriasis and thus, blocking either these cytokines or their receptors have been proven highly successful for therapy (Gottlieb *et al.*, 2005; Griffiths *et al.*, 2010; Nestle *et al.*, 2009). Only in 2015, two novel therapies namely secukinumab, an anti-IL-17A antibody (Langley *et al.*, 2014), and apremilast, a PDE4 inhibitor that regulates inflammation on intracellular level (Papp *et al.*, 2015b), have been approved for psoriasis treatment in the European Union. For atopic eczema, there are currently therapies under investigation in clinical trials, but no specific therapy has been approved, yet. Dupilumab, the most advanced agent, is an anti- IL-4 and anti-IL-13 receptor antagonist targeting two major cytokines in atopic eczema and has shown promising results in a phase II b trial (Thaci *et al.*, 2015). Currently it is under investigation in a phase III trial for the treatment of atopic eczema.

Regarding the rich therapy pipeline, the situation of undertreatment seems inapprehensible. One reason for the dichotomy of high disease burden and insufficient patient care may for sure partly lie in the perception that psoriasis and eczema are – as per se they are not life threatening diseases – rather cosmetic and trivial diseases that do not require (systemic) therapy (Blauvelt *et al.*, 2015; Carroll *et al.*, 2005).

The major reasons for the unsatisfying situation, however, lies in

a) The lack of biomarkers for individual patients and

b) The lack of precise diagnostic tools due to the complexity and heterogeneity of both diseases

*The lack of biomarkers*

Exemplified for dupilumab and secukinumab, the most specific therapies for both diseases, respectively, the insufficient situation for biomarkers to predict therapeutic outcome becomes clear. Not in all patients suffering from psoriasis and eczema the two therapeutic agents show satisfying efficacy: 20% of patients under anti-IL-17 antibody secukinumab do not show an improvement of skin lesions of at least 75% (Langley *et al.*, 2014) and only around 70% of eczema patients are dupilumab responders (Beck *et al.*, 2014). Due to the lack of biomarkers, the question of who will benefit from the therapy or not, cannot be answered beforehand and thus, one needs to follow the trial-and-error principle.

*The lack of precise diagnostic tools due to the complexity and heterogeneity of both diseases*

Equally important and closely connected to the lack of biomarkers is the fact that both psoriasis and eczema are "underdiagnosed" diseases: Both diseases are not only complex and incompletely understood, but also carry like umbrellas broadly various heterogeneous phenotypes under one term which hamper accurate diagnostics.

For both psoriasis and eczema, genome wide association studies (GWAS) have been performed unraveling a variety of susceptibility loci for the respective disease. Up to date 31 risk loci have been reported for atopic eczema. Apart from the well described filaggrin null mutation resulting in epidermal barrier deficiency (Irvine *et al.*, 2011) these variants include candidate genes playing important roles for the regulation of innate host defense and T cell function (Ellinghaus *et al.*, 2013; Genetics *et al.*, 2015). For psoriasis the list is even longer with 41 independent genome-wide significant susceptibility loci that comprise candidate genes regulating T cell function, innate host defense including interferon-mediated antiviral responses as well as IL-17 signaling in human keratinocytes (Tsoi *et al.*, 2015; Tsoi *et al.*, 2012). This data set has substantially contributed to our understanding of the underlying factors dysregulated in psoriasis and eczema and may help to define

the baseline risk for individual patients in the future, which in turn may be connected to specific therapy outcome. However, though big cohorts of many thousands of patients have been deeply genotyped and many risk loci have been found, the individual risk of patients cannot be predicted. For psoriasis, even a combination of 10 risk loci rather that individual single nucleotide polymorphism (SNP) only account for 11.6% of the genetic variance in psoriasis and thus only a small fraction of psoriasis heritability can be captured by the known common risk variants (Chen *et al.*, 2011). A recent study showed an improved risk predictive model; however, as it was performed in the Han Chinese population with generally low psoriasis prevalence, the model may be of limited use in the general population (Yin *et al.*, 2015). The situation is similar for eczema: It is estimated that based on 11 susceptibility loci in eczema only 14.4% of the heritability for atopic dermatitis can be explained (Ellinghaus *et al.*, 2013).

The best characterized gene, *FLG* encoding for filaggrin, is altered in only 30% of atopic eczema patients and approximately 8% of the general population carries loss-of-function mutations without being affected (Eyerich and Novak, 2013). This shows that not only genetics underlying the diseases is not yet fully understood, but also that already on genetic level there is a high heterogeneity, which is in particular reflected on clinical, histopathological and molecular level. Thus, according to Barker the "…time has come to move away from lumping all clinical variants together and to develop a new taxonomy based on a combination of clinical and genetic features."(Barker, 2014).

## 1.3 The heterogeneity of psoriasis and eczema

### 1.3.1 Genetic and immunopathologic concepts of psoriasis and eczema

The variety of clinical and histological phenotypes seen in psoriasis and eczema is a result from a multitude of disease promoting factors based on genetic background (s. above) and environmental influences interacting in complex manner.

For psoriasis the most common model of disease evolution proposes in a simplified manner the sequence of consecutive and parallel events (Boehncke and Schon, 2015; Nestle *et al.*, 2009): Based on a genetic predisposition for dysregulation of immune responses and keratinocyte differentiation, an initial trigger such as mechanical trauma or microbial

products induces local cell damage. This in turn leads to the activation of plasmacytoid dendritic cells (pDCs) in the skin by complexes of self-DNA and anti-microbial peptides. Upon their activation, large amounts of interferon α (IFN-α) are released which – via activation and subsequent migration of dermal dendritic cells to the draining lymph nodes – promote the differentiation of naïve T cells into Th17 and Th1 cells. Together with Th2 and Tc cells all expressing the chemokine receptors C-C chemokine receptor type 6 (CCR6), CCR4 and C-X-C chemokine receptor type 3 (CXCR3) Th1 and Th17 cells migrate to the dermis along chemokine gradients. For disease maintenance, T cells are possibly kept in the skin by putative autoantigens and secrete further cytokines such as IL-17A, IL-22 and TNF-α which activate keratinocytes, induce their proliferation, trigger the production of antimicrobial peptides (e.g. LL-37, β-defensins) as well as the release of chemokines such as chemokine (C-X-C motif) ligand 8 (CXCL8) recruiting neutrophils to the lesion site. The inflammatory milieu reinforced and maintained by many feedback loops also includes T cell migration into the epidermis via interaction between collagen IV and the very late activation antigen 1 (VLA-1), activation of TNF-α and iNOS (TIP) producing dendritic cells (Tip-DCs) and perivascular immune cell clusters stimulated by macrophages. Besides, tissue reorganization via fibroblast activation and the release of growth factors such as vascular endothelial growth factor (VEGF), epidermal growth factor (EGF) and transforming growth factor β (TGF-β) contributing to hyperplasia and angiogenesis with its characteristic vascular architecture are main hallmarks of psoriatic inflammation (Figure 2).

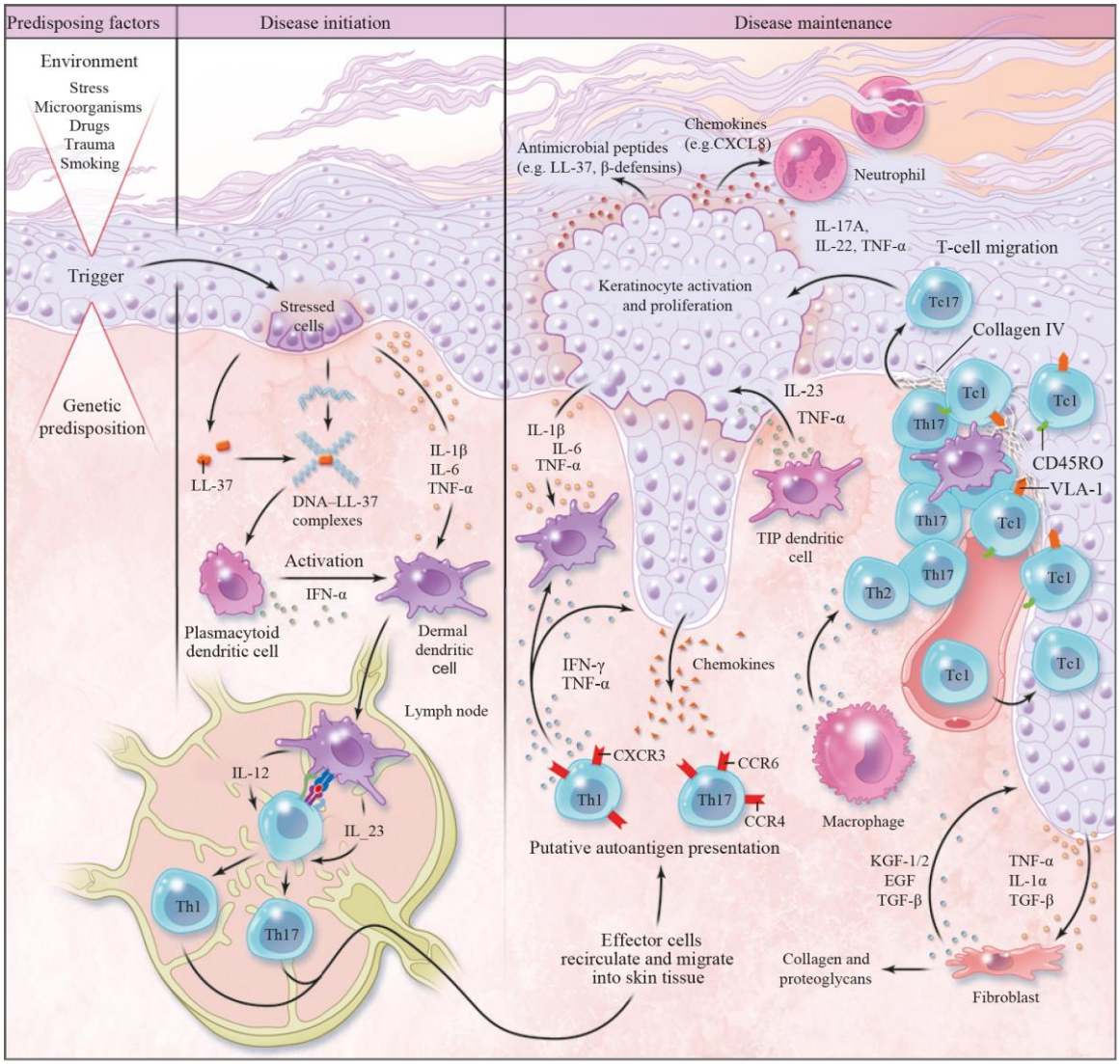


Figure 2: Proposed model of the immune pathogenesis of psoriasis.

Based on genetic predisposition and environmental triggers, dendritic cells (DCs) are activated via complexes of self-DNA and antimicrobial peptides (e.g. LL-37). DCs migrate to the local lymph nodes and activate the differentiation of naïve T cells into Th1 and Th17 cells. During disease maintenance, T cells (Tc1, Th17, Th2, and Th1) migrate to the dermis and further to the epidermis where they are kept by putative antigens. A large amount of cytokines (IL-1β, TNF-α, IL-17 etc.) is released by T cells, keratinocytes, dendritic cells, macrophages and other immune cells that lead to a complex intercellular interaction resulting in keratinocyte activation and hyperproliferation, angiogenesis, neutrophilic microabscesses and other hallmarks of psoriatic skin. Figure modified from Nestle et al., 2009.

Also for eczema, there is a complex interaction of epidermal barrier dysfunction and altered immune system, of genetic background and environmental triggers (Bieber, 2008; Guttman-Yassky *et al.*, 2011b; Weidinger and Novak, 2015). Already in clinically unaffected skin, there is notable cytokine release by keratinocytes, reduced expression of epidermal barrier proteins such as filaggrin and the cornified envelope proteins involucrin and loricrin. Defects of the epidermal barrier facilitate the penetration of epicutaneous antigens and allergens that encounter Langerhans cells and eventually lead to B cell and

T cell priming in the lymph nodes and subclinical inflammation with increased numbers of Th2 and Th22 cells and their signature cytokines (IL-4, IL-5, IL-13, IL-22) already at this stage. These cytokines create a tissue environment that prevents effective induction of antimicrobial peptides, which in turn favors abundance of *Staphylococcus aureus* also at this preclinical stage (Nomura *et al.*, 2003b). The initial mechanisms triggering acute flares are not yet clarified. However, this phase is characterized by increasing amounts of *Staphylococcus aureus* and *Staphylococcus aureus* derived proteases and toxins that increase barrier dysfunction on the one hand (Kong *et al.*, 2012), but also induce skin-homing receptor cutaneous lymphocyte-associated antigen (CLA) on T cells and thus inflammation on the other hand (Bieber, 2008). Besides, *Staphylococcus aureus* can cause IgE-mediated sensitization as well as direct induction of mast cell degranulation (Nakamura *et al.*, 2013; Weidinger and Novak, 2015). T cell recruitment to the skin is further amplified by keratinocyte derived chemokines attracting T cells. Th2 cells in turn induce keratinocytes to produce thymic stromal lymphopoietin (TSLP) which activates Th2 cells in a positive feedback loop via activation of dendritic cells, thus reinforcing the Th2 dominated milieu (Guttman-Yassky *et al.*, 2011b). The epidermal barrier defect facilitates penetration of allergens and antigens through the skin, which are taken up by dendritic cells bearing specific IgE bound to FcεRI. This results in further amplification of T cell activation, inflammation and initial responses of Th1 cells. Together with keratinocytes they release neuropeptides, kinins and cytokines such as IL-31 inducing pruritus (Weidinger and Novak, 2015). This in turn leads to the sequence of scratching, tissue damage, and release of (self)-antigens possibly mimicking microbial structures (to which the patient is already sensitized), eventually inducing IgE autoantibodies that perpetuate the inflammation (Bieber, 2008; Weidinger and Novak, 2015). In chronic conditions hyperplasia indicating progression of epidermal barrier defect, skin remodeling and ongoing neuroinflammation dominate the picture (Weidinger and Novak, 2015) (Figure 3).

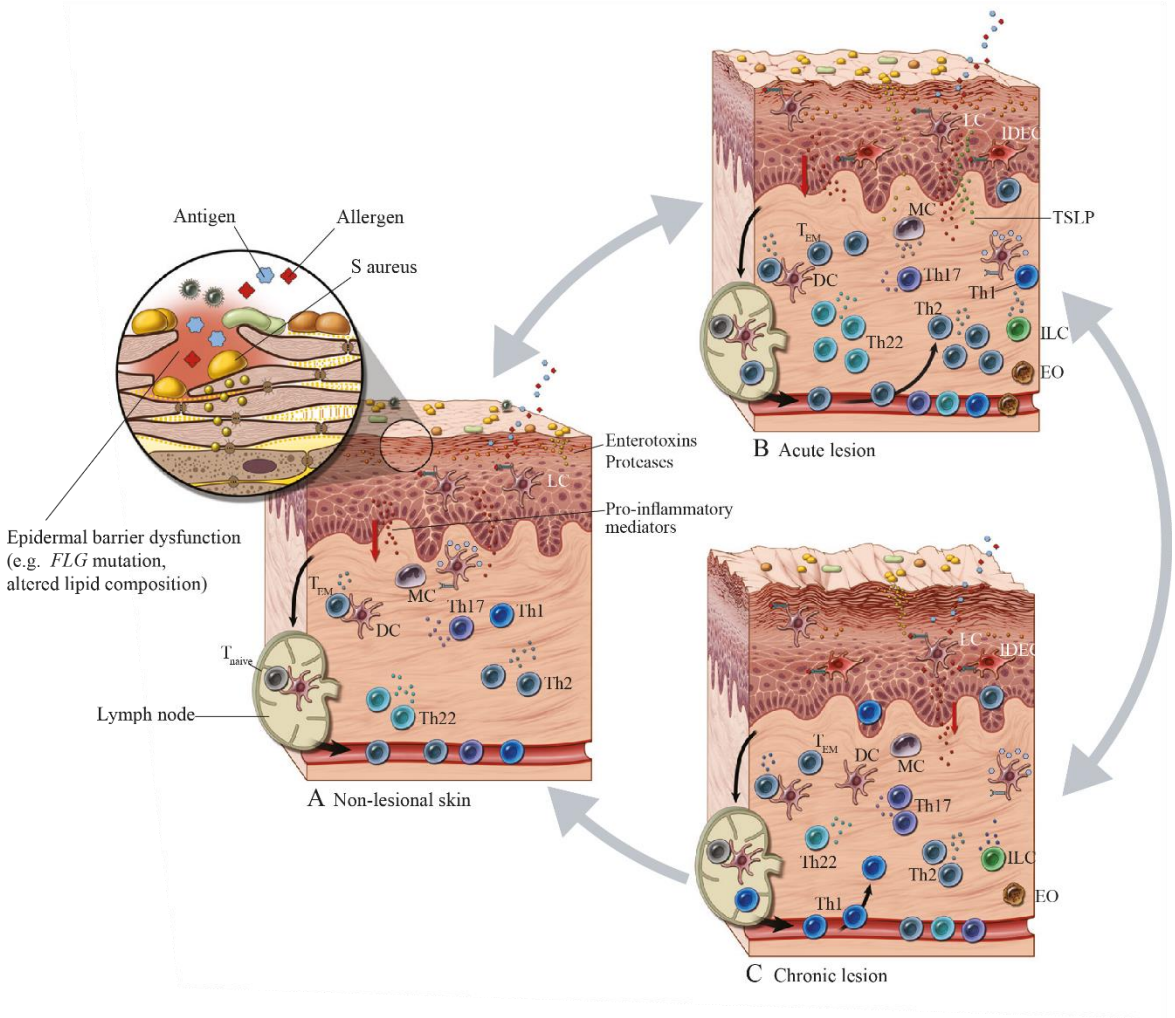


Figure 3: Main inflammatory processes driving the evolvement of acute and chronic lesions in atopic eczema

(A) In non-lesional skin of patients, altered epidermal barrier leads to the production of proinflammatory cytokines from keratinocytes. It also facilitates uptake of antigens and allergens encountering Langerhans cells (LC) and inflammatory dendritic epidermal cells (IDEC). They in turn induce activation of local effector memory T cells ($T_{EM}$) but also prime naïve T cells and B cells in the lymph node. Th2 and Th22 dominated infiltration leads to increased release of IL-4, IL-13 and IL-22 downregulating antimicrobial peptides which favor abundance of *Staphylococcus aureus*. (B) The transition to acute lesion is characterized by mutually amplifying processes of epidermal barrier disruption, increased colonization with *Staphylococcus aureus* and skin inflammation dominated by Th2, Th22 and Th2-cytokine-producing type 2 innate lymphoid cells (ILC) and initial induction of Th1 and Th17 responses. Cytokines of Th2 cells and dendritic cells increase the numbers of eosinophils (EO) and mast cells (MC). (C) In chronic lesions inflammation and barrier defects are further reinforced now also favoring Th1 and Th17 responses leading to epidermal hyperplasia, cutaneous remodeling and neuroinflammation. Modified from Weidinger et al., 2015

At first glance, psoriasis and eczema seem to show mutually antagonistic regulation of important checkpoints of the immune system. There is e.g. a reduced antimicrobial axis in eczema compared to increased production of antimicrobial peptides in psoriasis. Also eczema is dominated by Th2 cells in circulation and infiltrate, whereas psoriasis clearly shows polarization towards Th1/Th17 cells (Guttman-Yassky *et al.*, 2011a). Though the list of opposites could be continued, overlaps – in particular due to the heterogeneity of eczema – are high. Guttman-Yassky et al. e.g. could show that when stratifying eczema in intrinsic forms (not accompanied by high serum IgE) and extrinsic variants (accompanied by high serum IgE), intrinsic but not extrinsic eczema is more significantly correlated with Th17 and Th1 derived cytokines hinting at a difference of Th17 and Th22 activation between intrinsic and extrinsic forms. Regarding T cell response, one might argue that intrinsic eczema is closer to psoriasis than to extrinsic eczema.

### 1.3.2 Clinical variety of psoriasis and eczema

Psoriasis vulgaris, the most common presentation of psoriasis, is characterized by sharply demarcated plaques covered with silvery-white scales that can be preferably found on extensor sites of the body such as elbows and knees, but also in retroauricular and umbilical regions (Figure 4 B). Nails are affected in 15-50% of the patients including nail pitting, oil spots and onycholysis (Oji and Luger, 2015). For the evaluation of disease severity and monitoring of therapy outcome many different scoring systems are used such as the Physician static global assessment (PSGA) or the Overall lesion assessment (OLA) (Feldman and Krueger, 2005). One of the most common one, however, is the Psoriasis Area and Severity Index (PASI) based on the clinical criteria erythema, infiltration, desquamation and affected body surface area (Oji and Luger, 2015). Moderate-to-severe psoriasis is generally defined by PASI > 10 (Mrowietz *et al.*, 2011) and affects up to one third of all psoriasis patients (Boehncke *et al.*, 2011), whereas the majority of patients suffers from mild psoriasis (PASI $\leq$ 10) . The clinical diagnosis of psoriasis is usually straightforward but the disease is heterogeneous and can also manifest as e.g. inverse psoriasis affecting flexural folds (Figure 4 A) or acutely occurring guttate psoriasis with a rather exanthematic appearance (Figure 4 C). However, it can also manifest as palmoplantar psoriasis on hands and feet and as scalp psoriasis affecting the hairy scalp. A less common variant is psoriatic erythroderma affecting the whole skin. In these cases,

the differentiation of psoriasis from eczema is often challenging, leading to misdiagnosis of patients (Figure 4 D-H).

Atopic eczema is an even more heterogeneous condition which is not least mirrored in the dissent of its classification and definition (Brenninkmeijer *et al.*, 2008). The Hanifin and Rajka criteria for diagnosing atopic eczema are widely accepted including the main hallmarks of the disease, namely pruritus, chronicity of the condition and allergic background as major criteria (Table 3). The associated atopy stigmata such as the Dennie-Morgan lines and hyperlinearity of the skin can be found within the minor criteria.

Table 3: Hanifin and Rajka criteria for atopic eczema

At least three major and three minor criteria have to be fulfilled for the diagnosis of eczema. (Diagnostic features of atopic dermatitis. Hanifin JM, Rajka G. Acta Derm Venereol Suppl (Stockh) 1980; 92:44–7).

| | |
|---|---|
| Major criteria | Pruritus |
| | Typical morphology of dermatitis affecting flexural surfaces (adults) or face and extensor surfaces (children) |
| | Chronic-relapsing course of the disease |
| | History of atopic diseases in family history or patient's history |
| Minor criteria | Facial hallmarks: facial pallor or erythema, pityriasis alba, Dennie-Morgan lines (infraorbital folds), infraorbital darkening, cheilitis, recurrent conjunctivitis, anterior neck folds |
| | Triggers: Environmental and emotional factors, food allergy/intolerances, skin irritants (wool, solvents) |
| | Complications: Susceptibility to skin infections, predisposition to keratoconus and anterior subcapsular cataracts |
| | Other: Early onset of disease, sebostasis, ichthyosis, hyperlinearity of palms, keratosis follicularis, susceptibility to hand and foot dermatitis, nipple eczema, white dermographism, perifollicular accentuation, Typ I sensitization, elevated IgE- levels, itch upon sweating |

Like for psoriasis, there is a variety of scoring systems for the disease severity of atopic eczema. In this context, The Scoring of Atopic Dermatitis (SCORAD) has been found to be among the most valid instruments for evaluation of disease severity. Comparable to psoriasis the amount of affected body surface area is evaluated as well as clinical parameters such as redness, skin thickening and dryness. Besides the assessment of objective criteria, subjective symptoms such as pruritus and sleeplessness are also included (Schmitt *et al.*, 2013). Mild atopic eczema is defined by SCORAD < 25, moderate atopic

eczema by SCORAD < 50 and severe atopic eczema by SCORAD > 50 (Oranje *et al.*, 2007; Schmitt *et al.*, 2013).

In contrast to psoriasis, lesions are – apart from early childhood manifestations – preferentially found in flexural folds and in contrast to the thick plaques in psoriasis, efflorescences mainly comprise erythematous macules, papules or papulovesicles as well as crusts and erosions due to scratching in acute status (Figure 4 J). Poorly demarcated plaques and patches as well as lichenifications dominate the picture of chronic lesions (Bieber, 2008; Weidinger and Novak, 2015). Like psoriasis, atopic eczema displays various clinical phenotypes: For example, when affecting the scalp, atopic eczema is often difficult to distinguish from seborrheic dermatitis and nummular atopic eczema may not be distinguished from non-atopic nummular-microbial eczema. Nevertheless, there is not only high overlap between atopic and non-atopic manifestations of eczema. Sometimes it is even difficult to distinguish eczema from psoriasis: scalp eczema may display similar phenotypes as scalp psoriasis and palmoplantar manifestations as well as erythrodermatic variants of psoriasis and eczema are often indistinguishable (Figure 4 D-H). Especially when displaying eczematous variants, psoriasis patients may complain of itching, a symptom typically related to eczema. And in contrast, in particular chronic plaque-like psoriasiform lesions of eczema may not go along with itch (Guttman-Yassky *et al.*, 2011a). Here, further diagnostics and careful documentation of medical history may facilitate diagnostic procedure: In contrast to psoriasis, colonization of the skin with *Staphylococcus aureus* can be found in more than 90% of the patients with atopic eczema and during acute flare its proportion within the skin commensals rises (Bieber, 2008; Kong *et al.*, 2012). In addition, information about the course of the diseases including comorbidities as well as determination of general and specific IgE levels help in differential diagnosis. Atopic eczema but not psoriasis is commonly associated with elevated levels of IgE and atopic disorders such as asthma, allergic rhinoconjunctivitis and food allergies (Karimkhani *et al.*, 2015; Mansouri and Guttman-Yassky, 2015).

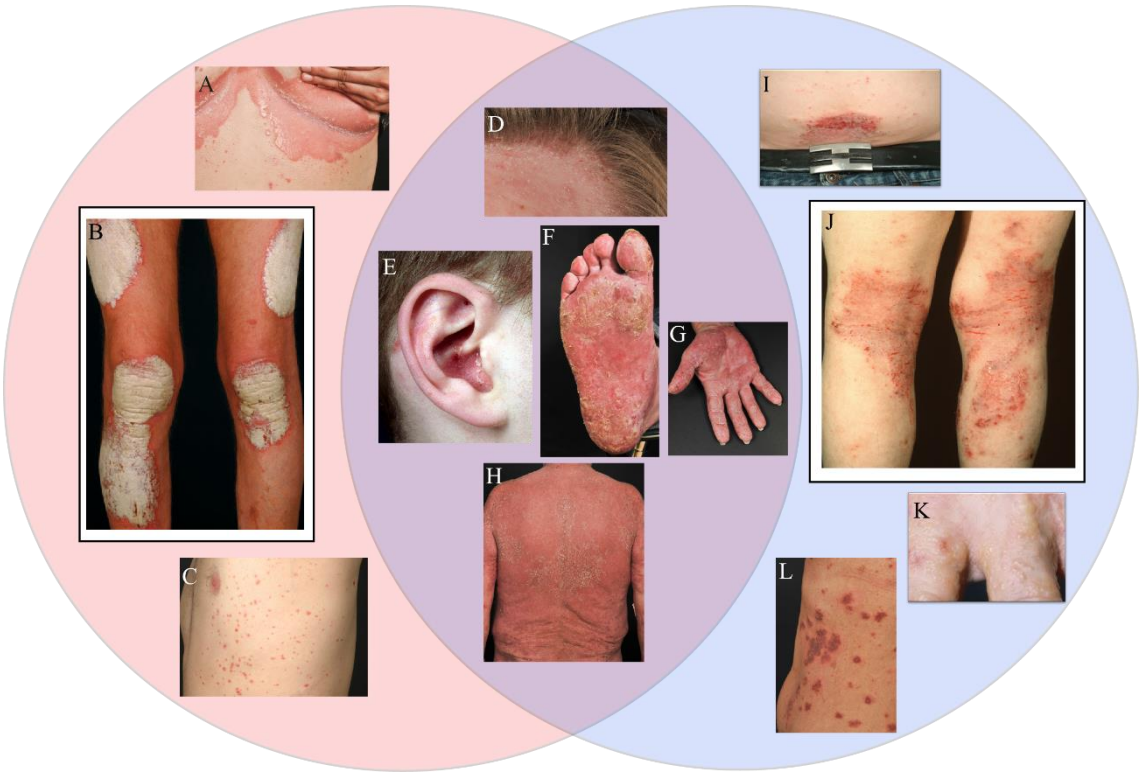


Figure 4: The variety of clinical phenotypes of psoriasis and eczema showing broad overlap for particular phenotypes

Selection of different subtypes of psoriasis (encircled in red) and eczema (encircled in blue) indicating problems in differential diagnostics for overlapping phenotypes of both diseases (violet area). Inverse psoriasis (A), plaque psoriasis (B), guttate psoriasis (C), scalp psoriasis or seborrheic scalp eczema? (D), ear canal psoriasis or eczema? (E), plantar psoriasis or foot eczema? (F), palmar psoriasis or hand eczema? (G) psoriatic or eczematous erythroderma? (H), allergic contact eczema (I), atopic eczema (J), dyshidrotic hand eczema (K) and nummular eczema (L).

### 1.3.3 Histopathological variety of psoriasis and eczema

In suspect clinical cases, histopathological analysis is performed to buttress or clarify the diagnosis. Eczema usually shows spongiosis, serum crust, increased numbers of eosinophils and mast cells, whereas psoriasis presents with elongated epidermal ridges, infiltrates of neutrophils and thinning of suprapapillary plates. However, some features such as acanthosis, hyperparakeratosis, agranulosis and vasodilation are shared by both diseases and thus impede diagnostics (Figure 5). For example, difficulties occur when distinguishing seborrheic scalp dermatitis from scalp psoriasis as seborrheic dermatitis displays psoriasiform hyperplasia and neutrophil infiltration. In particular, the differential diagnosis between palmoplantar psoriasis and hand or foot eczema is challenging due to the anatomic properties of the palms and plantae (Aydin *et al.*, 2008; Garzorz and Eyerich, 2015; Yoon *et al.*, 2013).

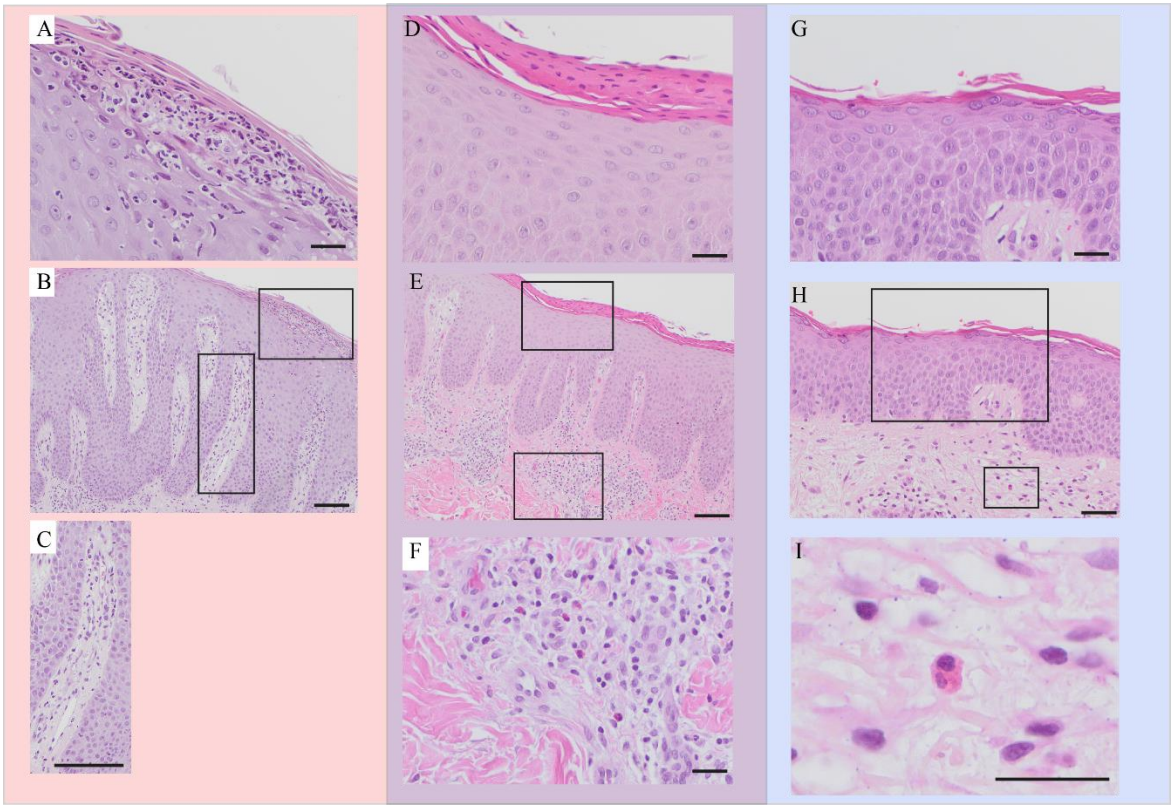


Figure 5: Common and shared histopathological characteristics of psoriasis and eczema

Psoriasis (A-C) is characterized by elongated epidermal ridges and hyperkeratosis. Parakeratosis, hypogranulosis and intraepidermal neutrophil abscesses (A) as well as dilated, elongated capillaries (C) are further hallmarks. Eczema (G-I) in contrast typically presents with irregular acanthosis, but also with spongiosis and often normal granular layer (G) as well as with increased numbers of eosinophils (I). However, phenotypes often overlap (D-F), showing psoriasis-like features such as psoriasiform epidermal architecture (E) and parakeratosis (D), but also eczema-like features such as spongiosis (D) and eosinophilic infiltrate (F). (Scale bars in B, C and E: 100 µm, in H: 50 µm and in A, D, F, G and I: 20 µm).

## 1.4 Approaches to unravel the pathogenesis of psoriasis and eczema

### 1.4.1 Intraindividual comparison of psoriasis and eczema

The complex interplay of genes and environment has hampered the understanding of the pathogenesis of psoriasis and eczema. The concordance rate for atopic eczema among dizygotic and monozygotic twins is only 15% and 77%, respectively (Bieber, 2008), similar to psoriasis with concordance rates of 20% and 73% in dizygotic and monozygotic twins, respectively (Barker, 2014). This data shows that heritability is indeed a major factor; however, it becomes more and more clear that the role of environmental factors is at least of the same relevance.

To overcome differences of underlying genetic background and individual environmental exposure, Eyerich et al. studied a rare cohort of patients simultaneously suffering from psoriasis and eczema. These patients are an ideal model to compare disease pathogenesis as different skin diseases can be investigated within the same patient, at the same time and organ. Choosing this approach it could be shown that distinct T cell subsets infiltrate eczema lesions and psoriasis plaques, with a Th2 dominance in atopic eczema and a Th1 and Th17 dominance in psoriasis. This was also reflected in the cytokines produced by the respective T cell lines, with high amount of IL-4 as a Th2 cytokine and interferon γ (IFN-γ) and IL-17 as Th1 and Th17 related cytokines (Eyerich *et al.*, 2011). The parallel occurrence of antagonistic inflammatory reactions indicates that distinct local antigen triggers define the course of the disease rather than intrinsic epithelial alterations.

### 1.4.2 Patients suffering from alopecia areata and coexistent inflammatory skin diseases

The concept that specific triggers rather induce the clinical phenotype than genetic background is not only strengthened by patients suffering from both psoriasis and eczema, but generally by patients suffering from (auto)-immune conditions of the skin accompanied by eczema, psoriasis or other inflammatory skin diseases. In this context, alopecia areata (AA) seems to be an interesting model: The T cell mediated autoimmune disease AA is the most frequent cause of patchy hair loss, with a life-time risk of 1.7-3.8% in the general population (Gilhar *et al.*, 2012). Recently, the key pathogenic event in AA has been demonstrated for the $NKG2D^+$ $CD8^+$ cytotoxic T cells infiltrating the upper part of the hair bulb without affecting the regenerative stem cells (Xing et al., 2014). AA bases on genetic predisposition as demonstrated in early epidemiologic studies (McDonagh and Tazi-Ahnini, 2002; Rodriguez et al., 2010) and genome-wide association studies (Petukhova et al., 2010). However, environmental triggers such as stress, infections and hormone imbalances are important players within the disease pathogenesis (Gilhar et al., 2007; McElwee et al., 2013; Wasserman et al., 2007). Interestingly, patients suffering from AA have been demonstrated to be prone to also develop other inflammatory and autoimmune conditions of the skin such as eczema (Goh et al., 2006). As such these patients represent a further interesting subgroup which might again enable intraindividual comparison of disease patterns while subtracting genetic background signals but also to

dissect the interplay of genes and environmental factors in the pathogenesis of inflammatory skin diseases.

### 1.4.3 Animal models of inflammatory skin diseases

Both psoriasis and eczema are complex and heterogeneous diseases. Therefore it does not seem surprising that animal models that fully represent the complex phenotype in humans have not yet been developed (Schon, 2008). There is not one defined animal model for inflammatory skin diseases, but rather several models reflecting a limited cut-out of the complex human reality which result in a psoriasis or eczematous like skin inflammation, whether the underlying mechanism is fully understood or not. Exemplified for psoriasis five major approaches to mimic features of psoriasis have been followed in rodent models (Gudjonsson *et al.*, 2007; Schon, 2008): Spontaneous mutations which result in a more or less psoriasis-like phenotype (e.g. $Scd1^{ab}$/$Scd1^{ab}$, $Sharpin^{cpdm}$/$sharpin^{cpdm}$, and Ttc7fsn/$Ttc7^{fsn}$). However, on molecular and cellular level these models seem to develop independently of T cells, which are main drivers of inflammation in humans and they do not respond to anti-psoriatic therapies. This approach is contrasted by models rather focusing on the immunology of disease, e.g. by using adoptive transfer of immune cells such as $CD4^+$ T cells. Very common are also transgenic mice in which e.g. suspected targets (e.g. TGF-α, IL-6) are overexpressed in keratinocytes. Probably closest to human disease are xenotransplantation models. Here, e.g. non-lesional human psoriatic skin is grafted onto AGR mice that lack B and T cells and IFN-γ receptors (Boyman *et al.*, 2004). However, one of the most "simple" models to date is the imiquimod model. Here, a cream containing imiquimod, a TLR7 ligand, is applied daily on the back of BALB/c mice and after six days erythema, scaling and thickness of the skin appear resembling plaque psoriasis. Also on cellular and molecular level, main features of psoriasis are reflected such as accumulation of neutrophils and T cells as well as expression of IL-23 and IL-17, key cytokines in the pathogenesis of psoriasis (van der Fits *et al.*, 2009). However, once application of imiquimod cream is stopped, skin lesions resolve indicating that the chronicity of psoriasis is not reflected by this model, not to speak about missing comorbidities such as arthritis which are seen in subsets of human psoriasis patients (Flutter and Nestle, 2013). A transcriptomic profiling study comparing five of the most common psoriasis mouse models with human psoriasis revealed that gene expression patterns were similar,

in particular for the domain of epidermis (Swindell *et al.*, 2011). However, when it came to immunology the divergence to human psoriasis was higher and differences between the mouse models were more variable making us aware that studies in animal models have to be evaluated carefully, especially in terms of molecular mechanisms and targets and when translating results to the human system.

## 1.5 Aim of the thesis

As outlined in the previous sections both psoriasis and eczema are complex and heterogeneous diseases. Though a broad palette of different therapies has been established, therapy response cannot be predicted as reliable biomarkers are missing and diagnostic tools are limited to descriptive clinical and histomorphological evaluation. In addition, murine models used for both diseases are limited in validity, as both diseases are not solely based on clear alterations of genetic background or other well-characterized deviations in molecular pathways that could be mimicked by animal models. Instead, the categories of "psoriasis" and "eczema" comprise various different phenotypes and stratification of patients according to genetic and molecular markers is still in the fledgling stages. In order to establish more homogeneous and thus less complex subgroups to better understand the pathogenesis but also to develop precise diagnostics on molecular level, novel approaches need to be implemented. To reduce the difficulties of complexity and heterogeneity of both diseases typically confusing advances in the field, I chose three different research approaches and models to answer my research questions (questions highlighted in boxes):

### 1.5.1 The model of alopecia areata (AA)

As outlined above, patients suffering from alopecia areata (AA) and coexisting skin diseases serve as an interesting model to elucidate disease-specific signatures independently from genetic background and eventually, unravel the complex interplay between genetics and environmental influences. These patients are genetically predisposed to develop inflammatory reactions of the skin at other locations than the scalp. However, before investigating these patients on molecular level, it is mandatory to not only validate their increased susceptibility to develop inflammatory skin diseases, but also to confirm that clin-

ical and histological phenotypes of inflammatory skin diseases in these patients are unequivocal and indistinguishable from the ones seen in single affected patients. Therefore, the aim of this project was to perform an intraindividual comparison of clinical, histological and T cell phenotype of AA and inflammatory skin diseases.

1. Are patients suffering from AA prone to develop inflammatory skin diseases at other sites of the body than the scalp?
2. Is the clinical and histological phenotype of inflammatory skin diseases and AA different or equal in patients suffering from AA and inflammatory skin diseases alone or concomitantly?
3. Is the model of AA useful to study inflammatory skin diseases?

### 1.5.2 The imiquimod model in humans

In Europe, imiquimod containing cream (Aldara® 5% cream) is approved for external treatment of genital warts, superficial basal cell carcinomas and actinic keratoses (http://www.ema.europa.eu/docs/de_DE/document_library/EPAR_-_Product_Information/human/000179/WC500023122.pdf). Observational studies in humans reported that patients with psoriasis but also one case without history of psoriasis who have been treated with imiquimod cream for actinic keratosis or basal cell carcinomas showed exacerbation of psoriasis or even newly induced psoriasis (Patel *et al.*, 2011). This phenomenon has been translated into a psoriasis mouse model (van der Fits *et al.*, 2009) in which mice daily treated with imiquimod cream develop a psoriasis-like dermatitis after approximately six days (s. 1.4.3 Animal models of inflammatory skin diseases). On the one hand, mouse models such as the imiquimod model do have their limitations reflecting the complex phenotype of psoriasis in humans. On the other hand, adequate alternatives for research are limited, as there are no proper human skin models or other methods to induce psoriasis in humans. The situation is similar for eczema. Here, however, at least self-limited eczema such as acute contact dermatitis and irritant dermatitis can be induced in humans directly by local application of the allergen or irritant (Lee and Maibach, 1995; Martin, 2012; Quaranta *et al.*, 2014a).

The aim of this thesis was to study the application of imiquimod in humans to validate previous observations in humans that imiquimod cream induces or exacerbates psoriasis,

but also to elucidate which kind of inflammation imiquimod cream would induce in humans. The goal was to determine which aspects of psoriasis and/or eczema would be reflected by the imiquimod model and if – as imiquimod may result in a standardized and thus reproducible robust iatrogenic induced skin inflammation – at least some of the complex pathways of psoriasis and eczema could be studied and altered within this model in humans. As such, the imquimod model in humans could be a valid alternative for using mouse models.

1. What kind of inflammation does imiquimod cream induce in healthy volunteers but also in patients suffering from psoriasis and eczema?
2. What features of psoriasis and eczema are reflected by the imiquimod-induced inflammation?
3. Is the human model of imiquimod-induced inflammation useful to study psoriasis and/or eczema?

### 1.5.3 The model of patients suffering from psoriasis and eczema concomitantly

Investigating patients suffering from both psoriasis and eczema simultaneously, Eyerich et al. showed a mutual antagonism of T cells within the distinct lesion sites of the skin (Eyerich *et al.*, 2011). To unravel the distinct disease-specific signatures, these double affected patients were sought to be studied on the level of gene expression. Big cohort studies analyzing transcriptional profiles of psoriasis and eczema patients are limited by various, non-considered individual differences in genetic background and environmental factors (Guttman-Yassky *et al.*, 2009; Nomura *et al.*, 2003a; Wenzel *et al.*, 2008). With our approach, this "background noise" of genes that are unspecifically involved in skin inflammation could be eliminated by computational analysis while specific disease characterizing genes and pathways would become clear. Thus, complexity of diseases due to confounding interindividual differences could be reduced.

In a first step, I aimed to validate the assumption that psoriasis and in particular eczema are broadly heterogeneous diseases. In patients suffering from plaque psoriasis and a variant of eczema (atopic eczema, nummular eczema, dyshidrotic eczema and allergic contact dermatitis to nickel) concomitantly, the question if the clinical and histological variety of eczema would be also reflected on molecular level was to be answered.

Second, I aimed to clarify if – within the list of potentially differentially regulated genes and pathways between psoriasis and eczema – there is a possibility to create a disease classifier based on the expression of single genes that would be able to distinguish eczema independent of its subtype from psoriasis in clear, but also unclear cases.

1. Proof of concept: Is eczema a heterogeneous disease?
2. Is there a disease-specific signature of psoriasis and eczema (all subtypes) on molecular level?
3. Is there a possibility to establish a disease classifier for psoriasis and eczema independent of the subtype based on single genes that would support routine diagnostic procedure for clinically and histologically unclear cases?

# 2 Material and Methods

## 2.1 Material

| Material for T cell experiments, FACS and ELISA | |
|---|---|
| Reagents | |
| BD Cytofix/Cytoperm Kit | BD Biosciences (San Jose, USA) |
| α-CD3 | BD Biosciences (San Jose, USA) |
| α-CD28 | BD Biosciences (San Jose, USA) |
| IL-2 Proleukin; stock 10000Units/ml | Novartis (Basel, Switzerland) |
| DMEM/F12 | Invitrogen (Karlsruhe) 11320-074 |
| DMSO | Merck (Darmstadt) 317275 |
| DPBS w/o $Ca^{2+}Mg^{2+}$ | Invitrogen (Karlsruhe) |
| EDTA, 0.5 mM | Invitrogen (Karlsruhe) |
| Glutamine | Invitrogen (Karlsruhe) |
| GolgiPlug (with Monensin) | BD Biosciences (San Jose, USA) |
| GolgiStop (with Brefeldin A) | BD Biosciences (San Jose, USA) |
| Cytofix/Cytoperm | BD Biosciences (San Jose, USA) |
| human AB-serum | Sigma-Aldrich (St. Louis, USA) |
| Ionomycin | Sigma-Aldrich (St. Louis, USA) |
| PMA | Sigma-Aldrich (St. Louis, USA) |
| Sodium-pyruvate | Invitrogen (Karlsruhe) 11360 |
| Non-essential amino acids | Invitrogen (Karlsruhe) 11140 |
| Pen/Strep | Invitrogen (Karlsruhe) 15140 |
| Sodium azide solution | Carl Roth GmbH + Co. KG (Karlsruhe) |
| RPMI | Invitrogen (Karlsruhe) 21874 |
| Sodium chloride (NaCl) | Carl Roth GmbH + Co. KG (Karlsruhe) |
| KCl | Merck KGaA (Darmstadt) |
| KOH | Carl Roth GmbH + Co. KG (Karlsruhe) |
| $Na_2HPO_4$ | Merck KGaA (Darmstadt) |
| $KH_2PO_4$ | Merck KGaA (Darmstadt) |
| Citric acid monohydrate | Merck KGaA (Darmstadt) |
| Fetal calf serum (FCS) | Perbio Science (Vastra Frolunda, Sweden) |
| DMSO | AppliChem (Darmstadt) |
| $H_20_2$ | Sigma-Aldrich (St. Louis, USA) |

| | |
|---|---|
| 2 N $H_2S0_4$ = ELISA stop solution | Merck KGaA (Darmstadt) |
| Citrate buffer | Citric acid monohydrate 8,41g<br>$dH_20$ 180 ml<br>mix to dissolve and adjust pH to 3.95, fill up to 200 ml with $dH_20$ |
| Tetramethylbenzidine (TMB) | Sigma-Aldrich (St. Louis, USA) |
| TMB stock, produced 1 day before use | TBM 24 ml<br>Ethanol 500 µl<br>DMSO 500 µl |
| Substrate buffer | citrate buffer 5500 µl<br>$H_20_2$ 2,55 µl<br>TMB 55 µl |
| Tween 20 Detergent | Merck Millipore , Merck KGaA (Darmstadt) |
| Bovine serum albumin (BSA) | Sigma-Aldrich (St. Louis, USA) |
| T cell proliferation medium (10% human serum) | RPMI 450 ml<br>human serum 50 ml<br>glutamine 5 ml<br>Pen/Strep 5 ml<br>non-essential amino acids 5.6 ml<br>sodium pyruvate 5.6 ml |
| Freezing medium | DMEM 60%<br>FCS 40%<br>DMSO10% |
| FACS buffer | DPBS w/o $Ca^{2+}Mg^{2+}$<br>FCS 5%,<br>sodium azide 0.02% |
| ELISA wash buffer stock (20x dPBS) | $Na_2HPO_4$ 46.4 g<br>$KH_2PO_4$ 8 g<br>NaCl 320 g<br>KCl 8g<br>$dH_20$ 1900 ml<br>mix to dissolve and adjust pH to 7.0, fill up to 2000 ml with $dH_20$ |
| ELISA wash buffer | 0.05% Tween 20 Detergent in dPBS |
| ELISA Assay diluent (BD-ELISA) | dPBS with 10% FCS |
| ELISA Assay diluent (R&D-ELISA) | dPBS with 0.1% BSA and 0.05% Tween 20 Detergent |
| FACS antibodies | |
| mouse anti-IFN-γ FITC (clone B27) | BD Biosciences (San Jose, USA) |
| mouse anti-IL-17A PE (clone SCPL1362) | BD Biosciences (San Jose, USA) |
| mouse anti-IL-22 AF647 | BD Biosciences (San Jose, USA) |
| mouse anti-TNF-α AF700 (clone Mab11) | BD Biosciences (San Jose, USA) |
| mouse anti-IL-4 PeCy7 (clone 8D4-8) | BD Biosciences (San Jose, USA) |
| ELISA | |
| Human IL-4 ELISA set (555194) | BD Biosciences (San Jose, USA) |

| | |
|---|---|
| Human TNF-α DuoSet ELISA (DY210) | R&D Systems (Minneapolis, USA) |
| Human IL-17 DuoSet ELISA (DY317) | R&D Systems (Minneapolis, USA) |
| Human IL-22 DuoSet ELISA (DY782) | R&D Systems (Minneapolis, USA) |
| Human INF-γ DuoSet ELISA (DY317) | R&D Systems (Minneapolis, USA) |
| Tools and materials | |
| 24-well non-tissue culture plates (for T cells) | BD Biosciences (San Jose, USA) 351147 |
| 96-well non-tissue culture plates (for T cells) | BD Biosciences (San Jose, USA) 351172 |
| 96-well tissue culture plate round bottom | Sarstedt (Nümbrecht) 83.1837.500 |
| Corning® 96-well flat bottom high bind microplates (ELISA) | Sigma-Aldrich (St. Louis, USA) |
| 384- well plates | Thermo Scientific (Massachusetts, USA) |
| Microtubes, 2 ml, PP | Sarstedt (Nümbrecht) 72.694.006 |
| CryoPure Tube, 1.8 ml | Sarstedt (Nümbrecht) 72.379 |
| Pipette 1, 5, 10, 25 ml | Greiner bio-one (Frickenhausen) 760180 |
| Pipette 10 ml | Greiner bio-one (Frickenhausen) 607107 |
| Pipette 5 ml | Greiner bio-one (Frickenhausen) 606180 |
| Pipette 1 ml | Greiner bio-one (Frickenhausen) 604181 |
| Syringe 50 ml | B. Braun Melsungen AG (Melsungen) 87288107 |
| Microtube 1.5 ml, SafeSeal | Sarstedt (Nümbrecht) 72.706.400 |
| Falcon, 15 ml | BD Biosciences (San Jose, USA) 352096 |
| Cellstar Tubes, 50 ml | Greiner bio-one (Frickenhausen) 2279261 |
| Cluster Tube | Thermo Scientific (Massachusetts, USA) |
| Filtropur, V25, 0.2 µm | Sarstedt (Nümbrecht) |
| Falcon, 15 ml | BD Biosciences (San Jose, USA) |
| Cellstar Tubes, 50 ml | Greiner bio-one (Frickenhausen) |
| 1.3 Technical devices | |
| FACS LSRFortessa | BD Biosciences (San Jose, USA) |
| FlowJo Software | FlowJo (Ashland, USA) |
| Microscope Axiovert25 | Carl Zeiss microscopy (Jena) |
| Incubator HeraCell | Heraeus (Hanau) |
| Sterile bench HeraSafe | Heraeus (Hanau) |
| Centrifuge Megafuge 1.0R | Heraeus (Hanau) |
| HydroSpeed ELISA Plate washer | TECAN (Männedorf, Switzerland) |

| | |
|---|---|
| Epoch Microplate Spectrophotometer | BioTek (Winooski, USA) |
| | |
| Material for immunohistology experiments | |
| 2.1 Reagents | |
| Citric acid monohydrate | Merck KGaA (Darmstadt) |
| Sodium hydroxide solution (NaOH) 1N | Merck KGaA (Darmstadt) |
| Hydrochloric acid (HCl) 5N | Merck KGaA (Darmstadt) |
| KCl | Merck KGaA (Darmstadt) |
| $Na_2HPO_4$ | Merck KGaA (Darmstadt) |
| $KH_2PO_4$ | Merck KGaA (Darmstadt) |
| Trizma base | Sigma-Aldrich (St.Louis, USA) |
| EDTA | Merck KGaA (Darmstadt) |
| Sodium chloride (NaCl) | Carl Roth GmbH + Co. KG (Karlsruhe) |
| Tween 20 Detergent | Merck Millipore , Merck KGaA (Darmstadt) |
| Roticlear® | Carl Roth GmbH + Co. KG (Karlsruhe) |
| Citrate buffer | Citric acid monohydrate 4.2 g<br>add to 2 l with $dH_20$<br>adjust pH to 6.0 with NaOH |
| EDTA buffer | 0.37 g EDTA<br>$dH_20$ 1000 ml<br>Mix to dissolve<br>Adjust pH to 8.0 using 1 N NaOH |
| Tris Buffer 10x | Trizma Base 60.5 g<br>add to 700 ml with $dH_20$<br>adjust the pH to 7.6 with HCl<br>add NaCl 90 g<br>add to 1000 ml with $dH_20$ |
| PBS 10x | $Na_2HPO_4$ 14.4 g<br>$KH_2PO_4$ 2.4 g<br>NaCl 80 g<br>KCl 2g<br>$dH_20$ 1000 ml<br>mix to dissolve and adjust pH to 7.4 |
| Bond ™ Wash Solution 10x | Leica Biosystems (Wetzlar) |
| Bond ™ Epitope Retrieval Solution 1 and 2 | Leica Biosystems (Wetzlar) |
| Bond ™ Dewax Solution | Leica Biosystems (Wetzlar) |
| Bond ™ Polymer Refine Red Detection Kit | Leica Biosystems (Wetzlar) |
| Sudan Black B | Sigma-Aldrich (St. Louis, USA) |
| Hydrogen peroxide ($H_2O_2$) 30% solution | Sigma-Aldrich (St. Louis, USA) |
| DAPI (4′,6-Diamidin-2-phenylindol) | Sigma-Aldrich (St. Louis, USA) |
| Vectashield Antifade Mounting Medium | Vector Laboratories (Burlingame, USA) |
| Eukitt® quick-hardening mounting medium | Sigma-Aldrich (St. Louis, USA) |

| | |
|---|---|
| PERTEX ® rapid drying medium | Clinic pharmacy |
| Ethanol, isopropanol (denatured) | Clinic pharmacy |
| 2.2 Antibodies and sera | |
| Normal goat serum | Life Technologies (Carlsbad, USA) |
| Normal donkey serum | Life Technologies (Carlsbad, USA) |
| Bond primary antibody diluent AR 9352 | Leica Biosystems (Wetzlar) |
| Alexa Fluor® 488 goat anti-rabbit IgG | Life Technologies (Carlsbad, USA) |
| NorthernLights ™ 557 donkey anti-mouse IgG | R&D Systems (Minneapolis, USA) |
| Monoclonal mouse anti-CCL27/CTACK antibody (Clone 124302) | R&D Systems (Minneapolis, USA) |
| Monoclonal rabbit anti-iNOS antibody (clone K13-A) | Novus Biologicals (Littleton, USA) |
| Rabbit IgG isotype control | Novus Biologicals (Littleton, USA) |
| Mouse IgG2A isotype control | R&D Systems (Minneapolis, USA) |
| Rabbit anti-CD4 (clone SP35) ready-to-use | Zytomed Systems GmbH (Berlin) |
| Mouse anti-CD8 (clone SP16) ready-to-use | Zytomed Systems GmbH (Berlin) |
| Rabbit anti-goat IgG AR 9352 | Leica Biosystems (Wetzlar) |
| Rabbit anti-Ki67 ready-to-use | Menarini Diagnostics (Florence, Italy) |
| Polyclonal goat anti-IL-17 antibody | R&D Systems (Minneapolis, USA) |
| Polyclonal rabbit anti-neutrophil elastase antibody | Abcam (Cambridge, United Kingdom) |
| Monoclonal rabbit anti-TLR7 antibody | Abcam (Cambridge, United Kingdom) |
| Monoclonal mouse anti-CD303 (BDCA-2) antibody | Dendritics (Lyon, France) |
| 2.3 Tools and materials | |
| Rotilab disposable weighing trays | Carl Roth GmbH + Co. KG (Karlsruhe) |
| Stirring bars and metal spoons | Diverse manufacturers |
| Syringe driven filter unit Millex-HV PVDF 0,45mm | Merck Millipore , Merck KGaA (Darmstadt) |
| Omnifix 20 ml syringe | B. Braun Melsungen AG |
| StainTray slide staining system | Sigma-Aldrich (St. Louis, USA) |
| Staining dishes | Diverse manufacturers |
| PAP PEN MaxTag™ hydrophobic barrier pen | Rockland antibodies & assays (Gilbertsville, USA) |
| Microscope slides Superfrost Plus 25x75 mm | Gerhard Menzel Glasbearbeitungswerk GmbH & Co. KG (Braunschweig) |
| Microscope cover slips 24x60 mm | Gerhard Menzel Glasbearbeitungswerk GmbH & Co. KG (Braunschweig) |
| Nail polish colorless | P2 cosmetics (Karlsruhe) |
| 2.4 Technical devices | |
| inoLab pH 7110 (pH meter) | WTW Wissenschaftlich-Technische Werkstätten GmbH (Weilheim) |

| | |
|---|---|
| Kern 770 (precision balance) | Kern & Sohn GmbH (Balingen) |
| Pressure cooker “Perfect” (4,5 l) | Württembergische Metallwarenfabrik (Geislingen) |
| IX73 Inverted fluorescence microscope equipped with 4x/0.13, 20x/0.45 and 40x/0.60 objectives and a XM10 monochrome cooled CCD camera with cellSens Software | Olympus (Tokio, Japan) |
| BX45 widefield microscope equipped with 10x/0.25, 20x/0.40 and 40x/0.65 objectives , an SC30 digital camera and cellSens Software | Olympus (Tokio, Japan) |
| Universal Oven (for deparaffinization) | Memmert **(**Schwabach) |
| BOND-MAX Autostainer system | Leica Biosystems (Wetzlar) |
| Microtome RM 2255 | Leica Biosystems (Wetzlar) |
| | |
| RNA experiments | |
| 3.1 Reagents | |
| RNaseZap® Solution | Life Technologies (Carlsbad, USA) |
| UltraPure DEPC-treated water | Life Technologies (Carlsbad, USA) |
| Chloroform ≥ 99.8% | Sigma-Aldrich (St. Louis, USA) |
| Ethanol absolute | Merck KGaA (Darmstadt) |
| 2-Propanol ≥ 99.5% | Carl Roth GmbH + Co. KG (Karlsruhe) |
| miRNeasy Mini Kit | Qiagen (Venlo, Netherlands) |
| RNase-Free DNase Set | Qiagen (Venlo, Netherlands) |
| RNAlater RNA Stabilization Reagent | Qiagen (Venlo, Netherlands) |
| QIAzol Lysis Reagent | Qiagen (Venlo, Netherlands) |
| Paxgene™ Tissue Container | PreAnalytiX (Hombrechtikon, Switzerland) |
| PAXgene™ Tissue miRNA Kit | PreAnalytiX (Hombrechtikon, Switzerland) |
| Agilent RNA 6000 Nano Reagents Part I | Agilent Technologies (Santa Clara, USA) |
| Agilent RNA 6000 Nano Ladder | Agilent Technologies (Santa Clara, USA) |
| FastStart Universal SYBR Green Master (Rox) | F. Hoffmann-La Roche AG (Basel, Switzerland) |
| High capacity cDNA reverse transcription kit | Life Technologies (Carlsbad, USA) |
| Agilent Low Input Quick Amp Labeling Kit (one color) | Agilent Technologies (Santa Clara, USA) |
| Agilent One-Color RNA Spike-In Kit | Agilent Technologies (Santa Clara, USA) |
| Agilent Gene Expression Hybridization Kit | Agilent Technologies (Santa Clara, USA) |
| Agilent Gene Expression Wash Buffer 1 and 2 plus 10% Triton X-102 | Agilent Technologies (Santa Clara, USA) |
| 3.2 Tools and materials | |
| Eppendorf research plus pipettes 10, 100, 200 and 1000 | Eppendorf AG, Hamburg (Deutschland) |

| | |
|---|---|
| Filter Tips (10 µl, 20 µl, 200 µl, 1000 µl) | Starlab (Blakelands, United Kingdom) |
| Stainless Steel Beads 5mm | Qiagen (Venlo, Netherlands) |
| Cellstar Tubes, 50 ml | Greiner bio-one (Frickenhausen) |
| Eppendorf® Safe-Lock microcentrifuge tubes: 0,5 ml, 1,5 ml and 2 ml | Eppendorf AG (Hamburg) |
| SurePrint G3 Human Gene Expression v3 8x60K Microarray Kit | Agilent Technologies (Santa Clara, USA) |
| Agilent Hybridization Gasket Slides plus SureHyb Microarray Hybridization Chamber and Agilent Slide holder | Agilent Technologies (Santa Clara, USA) |
| Caso vacuum bags | Caso Germany (Arnsberg) |
| RNA Nano Chips | Agilent Technologies (Santa Clara, USA) |
| 3.3 Technical devices | |
| Agilent 2100 Bioanalyzer plus 2100 Expert Software pre-installed, chip priming station and vortexer | Agilent Technologies (Santa Clara, USA) |
| Nanodrop ND1000 UV-vis Spectrophotometer | Peqlab (Wilmington, USA) |
| Vortex Genie | Bender + Hobein AG (Zurich, Switzerland) |
| Eppendorf Thermomixer 5437 | Eppendorf AG, Hamburg (Deutschland) |
| Eppendorf Centrifuge 5417R | Eppendorf AG, Hamburg (Deutschland) |
| TissueLyser II plus TissueLyser Adapter Set 2x24 | Qiagen (Venlo, Netherlands) |
| SensoQuest labcycler | SensoQuest GmbH (Göttingen) |
| Techne TC-412 Thermal Cycler | Bibby Scientific Limited (Staffordshire, United Kingdom) |
| ViiA ™ 7 Real-Time PCR System plus 384- well block and software | Life Technologies (Carlsbad, USA) |
| Heraeus Biofuge Pico | Heraeus (Hanau) |
| VTX-3000L Mixer Uzusio | LMS Consult GmbH & Co. KG (Brigachtal) |
| Agilent G2545A Hybridization Oven | Agilent Technologies (Santa Clara, USA) |
| Agilent SureScan Microarray Scanner Bundle plus Feature Extraction software | Agilent Technologies (Santa Clara, USA) |
| Heidolph MR3001 magnetic stirring hotplate | Heidolph Instruments GmBH & Co. (Schwabach) |
| IKA magnetic stirring plate IKAMAG REO | IKA-Werke GmbH & CO (Staufen) |
| GTH 175/MO digital thermometer | Greisinger electronic GmbH (Regenstauf) |
| Caso VC vacuumizer | Caso Germany (Arnsberg) |
| | |
| Acquisition of skin biopsies | |
| 4.1 Reagents | |
| Xylonest 1% with Adrenalin 1:200 000, 50 ml | AstraZeneca (London, United Kingdom) |

| | |
|---|---|
| Octeniderm Antiseptic solution | Schülke & Mayr GmbH (Norderstedt) |
| Paraformalin | Fischar Otto GmbH & Co. KG (Saarbrücken) |
| Aldara® 5% cream | Meda Pharma (Solna, Sweden) |
| 4.2 Tools and materials | |
| FeatherDisposable Scalpel No.11, Nr. 280711, Feather | Feather Safety Razor Co. (Osaka, Japan) |
| Finn chambers on scanpor, large for epicutaneous testing | Hermal (Reinbek) |
| Dafilon 4/0 met.1,5 sutures, Braun REF 0936235 | B. Braun Melsungen AG (Melsungen) |
| Sterican single-use needle 20G and 30G | B. Braun Melsungen AG (Melsungen) |
| 2 ml syringe | B. Braun Melsungen AG (Melsungen) |
| Biopsy Punch 6mm | Stiefel Laboratorium GmbH (Offenbach am Main) |
| Sterile Surgical Set (including scissors, forceps) | Common surgical tools, mixed manufacturers |
| Raucodrape 90x75 cm surgical drape | Lohmann & Rauscher GmbH & Co. KG (Neuwied) |

## 2.2 Methods

### 2.2.1 Patient cohorts

All patients were admitted to the Department of Dermatology and Allergy of the Technische Universität München and were diagnosed based on clinical presentation and histopathological examination if necessary. For all studies, patients gave their written consent to participate in the respective study and the study was approved by the local ethic committee (project number 2773/10). All studies followed the declaration of Helsinki.

#### 2.2.1.1 Alopecia areata cohort

The cohort of patients suffering from alopecia areata (AA) comprised 112 AA patients who were diagnosed based on clinical presentation and on trichogram in unclear cases. Patients were investigated for their clinical phenotype, and interviewed by questionnaires regarding onset of the disease and family history. Statistics concerning the study population are part of the results section (s. 3.1.1 The prevalence of alopecia areata (AA) and coexisting inflammatory skin diseases). From five of the 23 patients also suffering from an inflammatory skin disease, skin specimens (6 mm punch biopsies) could be obtained

from lesional skin at sites of AA and the corresponding inflammatory skin disease, respectively. Furthermore, an independent cohort of single affected patients (n = 42) suffering from AA (n = 9), lichen planus (n = 10), psoriasis (n = 10) or atopic eczema (n = 13) alone was built to validate results from immunohistochemistry experiments of the cohort of AA patients with coexisting inflammatory skin disease.

2.2.1.2 Imiquimod study: cohort and study design

Fourteen volunteers participated in the imiquimod study. Mean age of patients was 47.57 ± 1.55 years and 21.4% of patients were male. Three patients suffered from atopic eczema, four patients had a background of psoriasis, one patient suffered from both psoriasis and atopic eczema simultaneously, and six participants were healthy volunteers. Patients treated with immune-efficient medication prior to material sampling were excluded from the study (wash-out phase six weeks for systemic, two weeks for local treatment). Over a period of 30 days, Aldara® 5% cream containing imiquimod was applied every three days on healthy skin at three spots on the back occlusively (covered by chambers for epicutaneous testing). At day 4, 14 and 28 a 6 mm punch biopsy was taken from the imiquimod cream treated areas (at each time point one of the spots treated with imiquimod cream, respectively) as well as one punch biopsy from healthy skin at day 4. From one eczema patient participating in the study, also one punch biopsy could be obtained from eczematous skin and one patient with psoriasis participating in the study also donated one biopsy from psoriatic skin.

For histopathological and immunohistochemical analysis, an independent cohort of psoriasis and eczema hematoxylin and eosin stained (HE)-samples (psoriasis, n = 11; eczema, n = 13) was established.

For analysis of gene expression, Aldara® 5% cream treated skin samples were compared to a group of psoriasis (n = 24 samples), naturally occurring eczema (n = 14 samples) and acute contact dermatitis (ACD, n = 10 samples). In detail, from13 patients suffering from psoriasis and naturally occurring eczema simultaneously, one punch biopsy was obtained from affected psoriatic and eczematous skin, respectively. From 10 patients suffering from psoriasis and ACD to nickel concomitantly, one punch biopsy from psoriatic skin and skin affected by ACD, respectively, was obtained. The two remaining biopsies for psoriasis and eczema were obtained from participants of the study (s. above).

#### 2.2.1.3 Cohort for the disease classifier

##### *2.2.1.3.1 Cohort of patients for the investigation of the heterogeneity of eczema, the disease-specific signatures of psoriasis and eczema and the disease-classifier*

Twenty-four patients suffering from coexisting plaque-type psoriasis and naturally occurring eczema (atopic eczema, n = 6; nummular or dyshidrotic eczema, n = 7) as well as patients with plaque-type psoriasis and type IV sensitizations to nickel (ACD, n = 11; induced eczema) were in included with age ranging from 18 to 60 years. Patients receiving immune-deficient medication before sampling (wash-out phase six weeks for systemic and two weeks for local treatment) were excluded from the study. Severity scores were defined using the SCORAD (for atopic eczema) and PASI (for plaque-type psoriasis) system. Mean age of patients was 45 ± 11 years, 33% of patients were male, all were Caucasians, 40% were smokers and mean BMI was 22.4 ± 4.8. Skin biopsies (6mm) were obtained from one eczema lesion, one psoriasis plaque and non-lesional skin of all patients.

##### *2.2.1.3.2 Validation cohort for the disease classifier*

The 15 most significantly differentially regulated genes between psoriasis and eczema resulting from the transcriptome analysis of the cohort from section *2.2.1.3.1* were validated in a cohort comprising 53 patients with psoriasis (n = 25.41% male patients, mean age 47 ± 13 years) or eczema (n = 28.38% male patients, mean age 35 ± 15 years). Nineteen patients (nine with psoriasis and 10 with eczema) were included in a subcohort to train the classifier and the remaining patients were included in a subcohort to test the classifier. Skin biopsies (6mm) were obtained from eczematous or psoriatic lesions as well as from non-lesional skin of all patients.

##### *2.2.1.3.3 Follow-up cohort to validate the classifier in a larger patient collective, in subtypes and unclear cases*

To validate the classifier in a larger collective of typical cases of psoriasis and eczema a cohort comprising 88 patients with plaque psoriasis or eczema (psoriasis, n = 45; eczema, n = 43) was built. Skin biopsies (6mm) were obtained from eczematous or psoriatic lesions as well as from non-lesional skin of all patients.

Four patients within this first cohort (n = 1 for psoriasis, n = 3 for eczema) contributed two biopsies of lesional skin, respectively (n = 92 samples). Within this cohort of 88 patients and 92 samples, no autologous healthy skin could be obtained from two eczema patients and one psoriasis patient (in total 89 samples with corresponding autologous skin). The cohort of subtypes of psoriasis and eczema consisted of 31 patients with clinical variants and subtypes of psoriasis and eczema, respectively: nummular eczema (n = 8), palmoplantar and scalp variants (n = 6), erythroderma (n = 2), guttate psoriasis (n = 6), inverse psoriasis (n = 3) and patients with coexisting psoriasis and eczema (n = 6). Five patients showing discrepancies between clinical and histological picture and five patients showing both clinical and histological unclear phenotypes build up the cohort of unclear cases to be tested by the classifier. Mean age of all patients was 47 ± 18.3 years, 57.6% of patients were male, 36.3% were smokers and mean BMI was 27.6 ± 6.3. For all cohorts, patients treated with immune-efficient medication prior to material sampling were excluded from the study if not indicated otherwise (wash-out phase six weeks for systemic, two weeks for local treatment). Moreover, all patients were deeply analyzed for anamnestic, clinical, histological and laboratory criteria (s. section 2.2.2). Severity scores were obtained using the SCORAD and the PASI system, respectively.

### 2.2.2 Psoriasis and eczema score

To evaluate HE stained samples of Aldara® 5% cream treated skin for criteria of psoriasis and eczema, samples were scored according to the psoriasis and eczema score (Table 4). Each criterion was evaluated separately: Absence of a characteristic was evaluated with 0 point, mild presence of the characteristic with 1 point and full presence of the characteristic with 2 points. Thus, a maximum of 16 points could be achieved for the psoriasis score and a maximum of 12 points for the eczema score.

Table 4: Criteria of psoriasis and eczema score

| **Psoriasis score** | **Eczema score** |
|---|---|
| Parakeratosis | Orthokeratosis |
| Neutrophils in epidermis | Retained stratum granulosum |
| Regular acanthosis | Irregular Acanthosis |
| Papillary pattern of the dermis | Spongiosis |
| Suprapapillary thinning | Exocytosis of Eosinophils |
| Dilation of blood vessels | Serum Crust |
| Neutrophils in dermis | |
| Hypogranulosis | |

### 2.2.3 Sample acquisition

Patients' skin was locally anesthetized (Xylonest 1% with Adrenalin 1:200 000) and the surgical area of interest was disinfected with Octeniderm antiseptic solution. 6 mm skin punch biopsies from skin lesions and clinically non-involved skin were taken of all patients (Imiquimod study and classifier study). For the project of alopecia areata and co-existing inflammatory skin disease, 5 mm punch biopsies were taken from patients with AA and eczema (n = 2), AA and psoriasis (n = 1), AA and lichen planus (n = 1), and AA and eczema plus psoriasis (n = 1) from lesional skin at sites of AA and corresponding inflammatory skin disease, respectively. Before obtaining biopsies, patients gave their written informed consent. Biopsies were divided into two (for some experiments into three) equal parts with a scalpel: One part was sent for routine histology to confirm the diagnosis, the other part was stored in PaxGene Tissue Container or RNAlater RNA Stabilization Reagent until isolation of total RNA. If the biopsy was cut into three parts, the third part was used for isolation of T cells.

### 2.2.4 Immunohistochemistry

Skin samples were fixed in 10% formalin and embedded in paraffin. 4 µm sections were cut and dewaxed for 25 min at 65°C. Stainings were performed by an automated BOND system according to the manufacturer's instructions: After rehydration and antigen retrieval in a pH 6 citrate buffer based epitope retrieval solution (Leica), sections were incubated with monoclonal antibodies against CD4 (rabbit anti-CD4, Zytomed Systems)

and CD8 (mouse anti-CD8, Zytomed Systems) as well as with rabbit anti-67 (Menarini Diagnostics), goat anti-IL-17 (R&D Systems), rabbit anti-neutrophil elastase (Abcam), rabbit anti-TLR7 (Abcam) and mouse anti-CD303 (Dendritics) antibodies. Secondary polymeric alkaline phosphatase (AP)-linked anti-rabbit antibody and anti-mouse antibody were applied and the complex was visualized by the substrate chromogen Fast Red. For goat anti-IL-17 a goat bridge (Polyclonal goat anti-IL-17 antibody, R&D Systems) was applied before application of the secondary antibody. Eventually, slides were counterstained with haematoxylin. As a negative control, primary antibodies were omitted or replaced with an irrelevant isotype-matched monoclonal antibody. Positive cells of each slide were counted in two visual fields per condition by two independent investigators in a blinded manner.

### 2.2.5 Immunofluorescence

Before immunofluorescence staining, paraffin mounted slides were dewaxed at 65 °C for 25 min. Then, sections were rehydrated in consecutive washes with Roticlear (two changes à 10 min), followed by isopropanol (two changes à 5 min), 96% and 70% ethanol (one change à 5 min, respectively) and $dH_2O$ (one change à 5 min). Antigen retrieval was performed in a pressure cooker with boiling citrate buffer (approx. 96°C) for 7 minutes followed by a washing step with Tris buffer and a blocking step with peroxidase 3% for 15 minutes sections at room temperature (RT). Before applying the antibody mix (anti-iNOS antibody, Novus Biologicals, 1: 250, and anti-CCl27 antibody, 1:20, R&D Systems) for 1 h at RT and then overnight, sections were washed with Tris buffer and blocked with 10% normal goat serum and 10% normal donkey serum for 1 h. After overnight incubation, slides were rinsed with Tris buffer and incubated with secondary antibody mix (488 goat-anti rabbit antibody, Life Technologies, 1:500 and 557 donkey anti-mouse antibody, R&D Systems, 1:500) in the dark for 1 h at RT. After rinsing with Tris buffer, sections were incubated in 0.1% Sudan Black B, diluted in 70% ethanol followed by a washing step with 0.02% Tween 20 diluted in PBS and several changes of $dH_2O$. Before mounting the sections in Vectashield Mounting Medium, incubation with DAPI for two minutes was performed. Then, images in the blue (DAPI), red (CCL27) and green (iNOS) channel of an Olympus IX73 inverted fluorescence microscope were taken and analyzed as described below (s. section 2.2.15.2).

### 2.2.6 T cell isolation from skin biopsies

T cells were isolated from freshly taken skin biopsies. Samples were placed in 24-well plates – pre- coated with α-CD3 (0.75 µg/ml α-CD3) for 1 h - containing T cell culture medium, 0.75 µg/ml α-CD28 and 60 U/ml IL-2. Fresh medium containing 60 U/ml IL-2 was replaced three times a week and T cells emigrated from tissue samples were expanded and harvested for flow cytometry analysis. T cells were incubated at 37°C, 5% $CO_2$.

### 2.2.7 Enzyme Linked Immunosorbent Assay (ELISA) and T cell stimulation

24-well plates were coated with α-CD3 (0.75 µg/ml α-CD3 in dPBS) for 2 h at 37°C. T cells isolated from tissue (s. section 2.2.6) were thawed and placed in the 24-well plates containing T cell culture medium (RPMI medium supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 1% nonessential amino acids, 1% penicillin/streptomycin, 10% human AB serum) and 0.75 µg/ml α-CD28. After 24 h, T cells were removed from α-CD3 coated 24-well plates and transferred to non-coated 24-well plates. T cell culture medium substituted with IL-2 to a final concentration of 60 U/ml IL-2 was changed every two to three days. After two weeks T cells (150.000 T cells/ per well) were transferred to a 96-well plate and cells of each sample were either stimulated with T cell medium plus 0.75 µg/ml α-CD3 (2 h prior) and 0.75 µg/ml α-CD28 or remained unstimulated with T cell medium only. After 48 h of incubation, cell-free supernatant was harvested and stored at -80°C until further analysis by ELISA. T cells were incubated at 37°C, 5% $CO_2$.

To measure cytokines in T cell supernatants, classical sandwich ELISA were used. Briefly, a 96-well plate was coated with a capture antibody overnight (Plate preparation). After multiple washing steps and a blocking step, supernatants were added (Assay Procedure). Then the detection antibody was added and detection was visualized by an enzyme conjugate. All ELISA assays were performed using commercially available kits following the manufacturer's instructions.

### 2.2.8 FACS analysis

Intracellular cytokine staining of isolated T cells was performed using a kit (BD Biosciences) according to the manufacturer's instructions. Briefly, T cells were stimulated with 10 ng/ml PMA and 1 µg/ml Ionomycin for 6 h in the presence of GolgiStop containing

Monensin. After 2 h, GolgiPlug containing Brefeldin A was added. Cells were fixed and permeabilized with Cytofix/Cytoperm. Afterwards, incubation with primary antibodies (Table 5) for intracellular markers was performed. Cells were analysed using a LSR Fortessa (BD Biosciences) and data were illustrated by FlowJo software.

Table 5: Staining panel for intracellular markers

| Intracellular marker | | |
|---|---|---|
| Antibody | Fluorochrome | Dilution |
| mouse anti-IFN-γ | FITC | 1:1000 |
| mouse anti-IL-4 | PeCy7 | 1:50 |
| mouse anti-IL-17 | PE | 1:50 |
| mouse anti-TNF-α | AF700 | 1:1000 |

### 2.2.9 Isolation of total RNA

Total RNA was isolated with the PAXgene™ Tissue RNA Kit according to the manufacturer's protocol. The RNA yield and quality was determined with a Nanodrop ND1000 UV-vis Spectrophotomer. Moreover, the RNA integrity numbers (RIN) were measured using the 2100 Bioanalyzer (Agilent) according to the manufacturer's protocol (Agilent RNA 6000 Nano Kit). In the course of experiments, however, the miRNeasy Mini Kit was used due to higher RIN and RNA yields gained with this method. Briefly, the biopsies placed in 700 µl of QIAzol Lysis Reagent were cut into smaller pieces before homogenization using the TissueLyser. Further steps were performed as indicated by the manufacturer's instructions including a DNase digestion step before RWT buffer washing steps.

### 2.2.10 cDNA synthesis and Real-time PCR (RT-PCR)

For amplification of genes of interest, first cDNA was synthesized from 500 ng total RNA and transcribed using the High Capacity cDNA Reverse Transcript Amplification Kit (Applied Biosystems) according to the manufacturer's protocol.

Primers amplifying genes of interest were designed using the publicly accessible Primer3 software (http://frodo.wi.mit.edu/primer3/). A list of used primers is shown in Table 6.

Real time PCR reactions were performed in 384-well plates using the Fast Start SYBR Green Master mix (Roche Applied Science) and fluorescence development was monitored using the ViiA7 Real Time PCR machine (Applied Biosystems). The expression of transcripts was normalized to expression of 18S ribosomal RNA as housekeeping gene. Data were expressed as fold change, relative to non-lesional skin as calibrator. Relative quantification was determined according to the formula: $(RQ) = 2^{-\Delta\Delta Ct}$.

Table 6: Primer sequences used for the validation of the molecular disease classifier (MC)

| Target | Sequences |
|---|---|
| *SOST* | 5'- AGCTGGAGAACAACAAGACC-3`<br>5'- TCACGTAGCGGGTGAAGTGCA-3` |
| *PLA2G4D* | 5'- ACACCAGTCATCCTGTGTGG-3`<br>5'- CCGTGACTGAGTCCTCATCA-3` |
| *IL36G* | 5'- CATGCAAGTATCCAGAGGCTC-3`<br>5'- GGCCATACAGATCCATGATC-3` |
| *NOS2* | 5'- GTTCTCAAGGCACAGGTCTC-3`<br>5'- GCA GGT CAC TTA TGT CAC TTA TC-3` |
| *KLK13* | 5'- CTGACAACATGTTGTGTGCC-3`<br>5'- CAGTGTTCTGTTACAGACCAG-3` |
| *GDA* | 5'- CCAGAACATCGACTTTGCAGA-3`<br>5'- CAAGCTGTGGTTGTTCCATTC-3` |
| *IL36A* | 5'- CGAGGAAGGACCGTATGTCT-3`<br>5'- TGAGTCCATTCAGGCCCAGGT-3` |
| *TGM1* | 5'- TCGAAGGCTCTGGGTTACAGA-3`<br>5'- ACGACTGGCGCAGTGTCACT-3` |
| *NPTX1* | 5'- ACGAGCTGGTCCTCATTGAGT-3`<br>5'- GATGTGGTGCCACTTGCCATC-3` |
| *CCL27* | 5'- AGGTCATCCAGGTGGAACTGC-3`<br>5'- TCAAACCACTGTGACAGGCTG-3` |
| *CLEC4G* | 5'- GAAGCAGACGGCGGCGCTGGGT-3`<br>5'- TCTCCTGCTCCATCAGCTTC-3` |
| *IL-13* | 5'- GAGCTCATTGAGGAGCTGGTCA-3`<br>5'- CATGCCAGCTGTCAGGTTGAT-3` |
| *TCN1* | 5'- GTCAACCACTTCACTCCTG-3`<br>5'-AGGACAGCCATTGCACCAGTA-3` |

| | |
|---|---|
| *TMPRSS11D* | 5'- AGAATCCTTGGAGGCACTGA-3`<br>5'-GCAGTAGAGCCAGGTGGAAT-3` |
| *RHCG* | 5'- CAGCTGCTCATCATGACTTTCTTCC-3`<br>5'-CTGTCTCTCCTTGCTCTGCTCTAGGT-3` |
| *18S* | 5'-GTAACCCGTTGAACCCCATT-3`<br>5'-CCATCCAATCGGTAGTAGCG-3` |

### 2.2.11 Whole-genome expression arrays

Full human *in situ* transcriptomics from skin biopsies were obtained using the human gene expression microarray kits from Agilent. Briefly, 50 ng RNA of each sample was first mixed with Agilent One-Color Spike-In Mix (positive controls derived from Adenovirus transcripts for monitoring microarray procedure) and then transcribed into cDNA using the AffinityScript RNase Block Mix from the Agilent Low Input Amp Labeling Kit according to the manufacturer's instructions. Samples were then amplified and labeled using the T7 RNA Polymerase from the same kit, resulting in Cy3 labeled anti-sense cRNA. Eventually, samples were hybridized (Agilent Gene Expression Hybridization Kit) to a SurePrint G3 Human GE 8X60K BeadChip (Agilent Technologies). After washing the hybridized arrays, fluorescence signals were detected by reading the arrays in the microarray scanner system iScan (Agilent Technology). The Agilent Feature Extraction software was used to extract fluorescence signals and unprocessed expression data.

### 2.2.12 Analysis of whole-genome expression arrays

#### 2.2.12.1 Imiquimod study

For statistical analysis of microarray data, R software (http://www.r-project.org) and the limma package from Bioconductor (http://www.bioconductor.org) were used for reading the arrays. Then QualityMetrics for quality control of arrays from Bioconductor was applied. Data was background-corrected using the "normexp" method (also from limma package) and then normalized between the arrays by the "quantile" method (from limma package). Control probes and low expressed probes with normalized signal intensities less than 10% of the 95th percentile of the negative controls of each array were filtered out. Within-array replicates for each probe were averaged by using avereps (from limma package). Then "blastn"

(https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch) was used to map the 60-basepairs-long nucleotide sequences from the array (= probes) to the UCSC release of the human transcriptome available as RefSeqIDs. Only 100% accurate mappings were considered and saved. Out of all probes, 13,430 did not match with 100% accuracy to a position of the human transcriptome. Probes mapping to two different RefSeq IDs were checked if they corresponded to the same gene (via Gene Symbol). The genes corresponding to the RefSeqID were received via the AnnotationDbi (from Bioconductor) that used the org.Hs.eg.db annotation Package. Eventually, 2,229 probes had to be taken out from analysis because the mapped to several genes. In total, 26,664 probes mapped with 100% accuracy to a unique gene. The surrogate variable analysis (sva) from Bioconductor was used to estimate artifacts from microarray data. One surrogate variable for the data set was calculated and we included this variable as a covariate in our regression model. Using the "lme4" package of the R software (https://cran.r-project.org/web/packages/lme4/index.html), a linear mixed-effects (LMM) model was fitted to the data with the REML (restricted maximum likelihood) criterion by using one model per probe. The different patients were included as random effects (= random intercepts). Thus, the gene expression level for each patient was adjusted individually. The estimated coefficients from these models are comparable to fold changes. P values for the coefficients were calculated using the mixed function from the "afex" package of the R software (https://cran.r-project.org/web/packages/afex/index.html) which applies the Kenward-Roger approximation for the degrees of freedom. P values were adjusted for multiple testing by Bonferroni correction. Genes were defined as significant when the adjusted p value was below 0.05 and top hits were defined when the fold change was > 2.5. Analysis was performed by Linda Krause.

#### 2.2.12.2 Heterogeneity of eczema, Molecular signature of psoriasis and eczema

For the analysis of microarrays in this study, the first steps of processing were as described for the arrays of the imiquimod study. However, after averaging within-array replicates, the $\log_2$fold change of the paired samples was computed for each disease sample against the corresponding non-involved skin. A linear fixed-effects model was fit for each individual gene to estimate expression differences between the compared groups of samples. Empirical Bayes approach was used to moderate the SEs of the normalized $\log_2$fold

changes. Finally, two-sided moderated paired t-statistics and log-odds of differential expression (B statistics) and raw and adjusted p values (false discovery rate) controlled by Benjamini-Hochberg were computed to identify genes that were differentially expressed between the different disease groups. Genes with an absolute $log_2$fold change > 2 and a corrected p value < 0.05 were defined as significantly differentially expressed hits. Analysis was performed by Bettina Knapp.

### 2.2.13. Gene enrichment analysis

For the imiquimod study, the "mgsa" package was used based on a Bayesian modeling approach for enrichment analysis (Bauer *et al.*, 2010) . The analysis was performed for the genes significantly regulated in a) naturally occurring eczema (nE), b) acute contact dermatitis (ACD) and c) psoriasis. Then, all significantly regulated genes from the PL cohort (= cohort comprising those samples showing a pseudolymphoma-like inflammation induced by imiquimod cream) were tested for their presence in the GO terms of nE, ACD and psoriasis using Fisher's exact test. P values of Fisher's exact test were adjusted for multiple testing with the Bonferroni correction. The number of tests for which we adjusted was the number of tested GO terms (n = 13,786). GO Terms with a p value < 0.05 were called significantly enriched in the PL cohort. Analysis was performed by Linda Krause.

For the enrichment analysis concerning the study of the heterogeneity of eczema and the molecular signature of psoriasis and eczema the “topGO” package from Bioconductor using the “weight01” method was used on the hit genes that were a) significantly differentially regulated in naturally occurring eczema and b) the top hit genes that were significantly differentially regulated in acute contact dermatitis. For the molecular signature of psoriasis and eczema, the same method was used on a) the hit genes significantly differentially regulated in psoriasis but not in eczema, b) the hit genes significantly differentially regulated in eczema but not in psoriasis and c) the hit genes significantly regulated in both psoriasis and eczema. Analysis was performed by Dr. Bettina Knapp.

### 2.2.14 Network analysis

For the imiquimod study, shared GO pathways between nE and PL, ACD and PL, and psoriasis and PL with significant adjusted p values < 0.05 were further analyzed using the ConsensusPathDB database (http://consensuspathdb.org/). Here, using the "Induced network modules analysis" all genes from the resulting GO pathways (= seed genes) were further analyzed to disclose functional interactions between gene sets. By including genes that are not in the list of "seed genes" but connect two or more seed genes with each other ("intermediate genes) and are likely to be associated with the phenotype (though they may not be regulated on mRNA level and thus do not appear among the seed genes) functional networks can be made visible. Intermediate genes are ranked according to the significance of association with the seed genes given their overall connectivity in the background network. This is quantified by a z-score calculated for each intermediate node with the binomial proportions test. The z-score can be controlled resulting in less or more compact networks. For the genes resulting from the GO term "immune response", a z-score of 20 was chosen and for the genes resulting from the GO term "negative regulation of cytokine production", a z-score of 3 was chosen. Only gene regulatory interactions were shown and non-connected seed nodes were not depicted.

### 2.2.15 Classifier buildup

#### 2.2.15.1 Molecular classifier (MC) on the level of RT-PCR

For the initial classifier the R software and the R package "e1071" (http://CRAN.R-project.org/package=e1071) using standard vector machines (STM) was applied to 19 of the 53 patients of the validation cohort (s. section 2.2.1.3.2) serving as a training cohort. For normal distribution of data, the RT-PCR values of the 15 genes were transformed using the logarithm to the base 10. Normality was tested for using the Shapiro-Wilk normality test ($p = 0.05$) and differential expression was tested using a two-sample, two-sided Welch's t test followed by a Bonferroni p value correction. The two most significantly differentially downregulated (*CCL27*, adjusted p value = $5.31 \times 10^{-4}$) or upregulated (*NOS2*, adjusted p value = $1.53 \times 10^{-6}$) genes were selected for the final classifier. The scaled and log-transformed data of the two genes was used as training set for a C-classification using linear kernel function with C = 1. To train the classifier, a 10-fold cross-validation was used. The classifier was eventually tested on the data of the remaining 34

patients by predicting the disease class and computing probability predictions based on the trained model. Analysis was performed by Dr. Bettina Knapp.

To refine probability predictions, calculations were modified in the follow-up cohort by building a molecular classifier using logistic regression. The RT-PCR values were transformed using the logarithm to the base of 10. The MC predicted disease state (psoriasis or eczema) directly from transformed RT-PCR values of *NOS2* and *CCL27*. The model was calculated using the generalized linear model function in R, with the family binomial and the logit link function. The model was trained on 88 clear patients and tested with patients of unclear disease state. To infer the robustness of the model a 10-fold cross validation (CV) was performed. The CV result for sensitivity was 98 ± 8.4% and for specificity 100 ± 0%. Analysis was performed by Linda Krause.

2.2.15.2 Molecular classifier (MC) on the level of immunofluorescence

An image-processing program was built using Python 2.7 software (http://www.python.org) exerting multistep image analysis. First, the three fluorescent channels of all images were separated and subjected to a thresholding operation removing all pixels with values > 5 fold of mean fluorescent intensity. Then images were analyzed for three parameters: the difference of mean expression in red and green fluorescent channel (1), 2D Fourier transformation (2) and convolution (3). For (1) the red and green channel, respectively, were divided into areas of 10x10 pixels and characterized by calculating the means (m) and standard deviations (std) of all segments' fluorescence intensities. Segments were removed from further analyses when std/m > 2x of std/mean of the segment with the smallest std/mean. The difference of the sum of all means of the red channel ($\sum m_{red}$) and the sum of all means of the green channel ($\sum_{green}$) was calculated ($\sum m_{red} - \sum m_{green}$) and the resulting values were normalized on a scale ranging from 0 to 1. For (2), the Fast Fourier Transformation (FFT) of Python software was applied on the red and blue channel to return the spacial frequencies of the images, thus quantifying the observation of nuclear distribution of CCL27 in eczema (characterized by a large amount of high frequencies) and cytoplasmic distribution of CCL27 in psoriasis (characterized by a large amount of low frequencies). The 2D Fourier signals were translated into a 1D array by summing up all line segments around the maximum center. The frequency content of the red and the blue channel was compared calculating the ratio of the two arrays. In the case of eczema,

where nuclear stain (blue channel) and CCL27 stain (red channel) were distributed similarly, the frequency distributions converged and resulted in ratios close to 1. The opposite situation was given for psoriasis. For (3), the congruency of the blue and red channel was analyzed using the convolution function in Python program. Here, a sharp peak was obtained when high congruency occurred, whereas a smeared out maximum was detected in the case of low congruency. The three values for each image were plotted as spheres in a 3D graph and interconnected for better visualization of the two different diagnostic groups of psoriasis and eczema. Analysis was performed by Dr. Sebastian Stark.

### 2.2.15 Statistical analysis

#### 2.2.15.1 Alopecia areata study

Differences regarding T cellular infiltrate in AA versus eczema, psoriasis, or lichen planus, respectively, were tested for statistical significance using Mann-Whitney-test. If applicable, results were given as mean values ± standard error of the mean (SEM).

#### 2.2.15.2 Imiquimod study

For the analysis of the ELISA experiment, values were first log10 transformed, whereas for the analysis of the psoriasis and eczema score, the non-transformed values of all samples were used. For the immunohistochemical markers, the mean of the four values (resulting from two blinded observers and two visual fields) of each sample was used. For the CD4/CD8 ratio, the mean (CD4)/mean (CD8) was calculated. Then the Kruskal-Wallis rank sum test was applied over the four groups (PL, noPL, AE, Pso). When the Kruskal Test showed a p value $< 0.05$ a post-hoc test was performed by pairwise comparisons using Dunn's-test for multiple comparisons of independent samples from the PMCMR (Pairwise Multiple Comparison of Mean Ranks) package or the R software (http://CRAN.R-project.org/web/packages/PMCMR). Analysis was performed by Linda Krause.

#### 2.2.15.3 Classifier study

To correlate expression of *NOS2* and *CCL27* with clinical and histological features, feature data type appropriate statistical tests were used. Statistical significance for categorical features with two levels was determined using a Welch two-sample t-test, and for those with more than two levels the analysis of variance (ANOVA) was applied. Significance for features on the interval scale was determined using Pearson's product moment

correlation coefficient. The term “association” refers to categorical features, whereas the term “correlation” refers to features on the interval scale. Only associations with a controlled false discovery rate of less than 10% were selected. All listed p values were adjusted using the Benjamini-Hochberg procedure unless indicated otherwise. Significance levels were chosen as follows: * = $p < 0.05$, ** = $p < 0.01$ *** = $p < 0.001$. Results are given as mean ± SEM unless indicated otherwise. Analysis was performed by Linda Krause.

# 3 Results

## 3.1 Part 1: Alopecia areata – a platform to study gene-environment interaction in the pathogenesis of chronic inflammatory skin diseases

The results presented in this section were published in the article: "Dissecting susceptibility from exogenous triggers: The model of alopecia areata and associated inflammatory skin diseases" (Garzorz N, Alsisi M, Todorova A, *et al.* (2015). *Journal of the European Academy of Dermatology and Venereology : JEADV*. 29, 2429-2435).

### 3.1.1 The prevalence of alopecia areata (AA) and coexisting inflammatory skin diseases

To verify that patients with AA have a genetic background that predisposes to various chronic inflammatory skin diseases depending on individual susceptibility and environmental triggers, we first validated previous reports about higher rates of inflammatory skin diseases among AA patients on epidemiological level. In our study, 112 patients with AA were included amongst whom 23 suffered from at least one coexisting inflammatory skin disease (ISD). Most frequent was the co-occurrence of AA and atopic eczema (17 patients), followed by AA and vitiligo (eight patients), AA and psoriasis (six patients), and AA and lichen planus (one patient), (Table 7). More female than male patients were affected (62.5% versus 37.5%, respectively) and within the group of AA+ISD (Alopecia plus coexisting inflammatory skin disease) the percentage of female patients was even higher as compared to the AA alone group (Table 8). Age at onset of AA was normally distributed, ranging from 2 years of age until 70 years of age (Figure 6 A). In the AA+ISD group, AA occurred at earlier age compared to the AA alone group (31 years versus 36 years, respectively) (Table 8). The major difference between the two groups was the higher rate of the most severe phenotype of AA, AA universalis, in the AA+ISD group (47.8% vs 35.9%), (Figure 6 B). In accordance with this, there was a higher occurrence of nail affection (39.1% vs 15.7%) and therapy discontinuation (60.9% vs 25.8%) in the AA+ISD group compared to the AA alone group hinting at more severe courses of disease in the AA+ISD group (Table 8).

Table 7: Prevalence of coexisting inflammatory skin diseases (ISD) in the study cohort of alopecia areata (AA) patients. Modified from Garzorz et al., 2015.

| AA and coexistent ISD | Cases (n) | % |
|---|---|---|
| AA | 112 | 100 |
| AA + Atopic eczema | 17 | 15.2 |
| AA + Vitiligo | 9 | 7.1 |
| AA + Psoriasis | 6 | 5.4 |
| AA + Lichen planus | 1 | 0.6 |

Table 8: Clinical characteristics of AA patients in the study cohort.

Twenty-three out of 112 study patients suffered from coexistent ISD. Females were more frequently affected by AA than males (62.5% vs. 37.5%) and the percentage of affected women was even higher in the AA+ISD group. Modified from Garzorz et al., 2015.

| Clinical characteristics | AA+ISD (n = 23) | AA only (n = 89) |
|---|---|---|
| Gender | | |
| Male n (%) | 7 (30.4) | 35 (39.3) |
| Female n (%) | 16 (69.6) | 54 (60.7) |
| Ratio | 0.4 | 0.6 |
| Age at time of screening in years, mean value (range) | 43 (23-65) | 46 (12-77) |
| Age of AA onset in years, mean value (range) | 31 (7-65) | 36 (2-70) |
| Nail affection n (%) | 9 (39.1) | 14 (15.7) |
| Positive family history of AA n (%) | 4 (17.4) | 15 (16.9) |
| Therapy discontinuation n (%) | 14 (60.9) | 23 (25.8) |

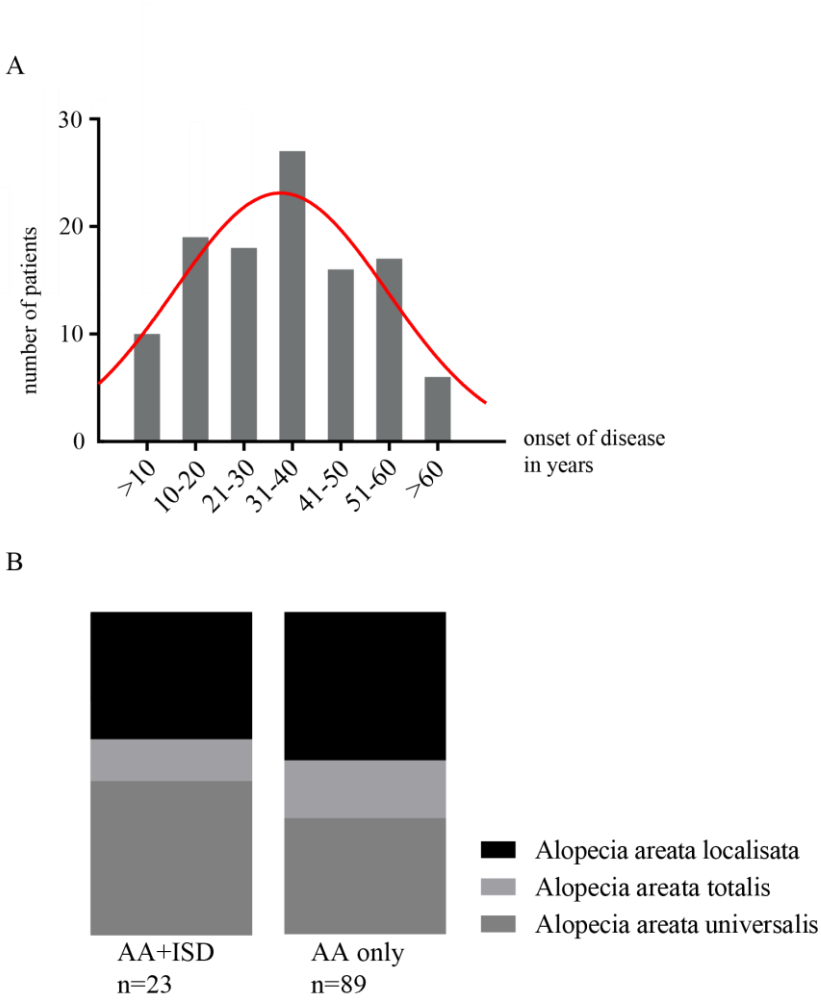


Figure 6: Clinical characteristics of AA patients in the study cohort

Within the study cohort (n = 112 patients), age at onset of AA was distributed normally (A). Compared to the AA alone group, the prevalence of the most severe phenotype of AA, AA universalis, was higher in the AA + ISD group (47.5% vs. 35.9%). Modified from Garzorz et al., 2015.

### 3.1.2 Typical morphology of coexisting AA and inflammatory skin diseases

To elucidate the potential of AA and coexistent ISD as a model to study the interplay of environmental and genetic factors of chronic inflammatory skin diseases we sought to analyse clinical and histological phenotypes of AA and ISD. It could be demonstrated that the clinical picture of atopic eczema (AE), psoriasis or lichen planus were classical and in-distinguishable from the picture seen in patients not suffering from AA (Figure 7 A-F). To clarify if AA patients develop typical inflammatory skin diseases or whether the AA background may have altered the phenotype, biopsies were obtained from five of the 23 AA patients (two with AE, one with psoriasis, one with lichen planus, and one with

both AE and psoriasis). According to the clinical phenotype, all specimens showed disease-specific features according to the diagnosis on the level of histopathology. A dense lymphocytic infiltrate around the hair follicle (known as the "swarm of bees" pattern) as well as lymphocytes within the follicular epithelium, classical hallmarks of active AA, could be observed. At more chronic lesions, miniaturized hair follicles were observed (Figure 7 a and b). In contrast, AE biopsies displayed the typical morphology of spongiosis and acanthosis accompanied by lymphocytic and eosinophilic infiltrate (Figure 7 e). In psoriasis, acanthosis, tortured and dilated capillaries as well as classical neutrophilic microabscesses and lymphocytic infiltrates were observed (Figure 7 d). Lichen planus showed the classical well-described hallmark of interface dermatitis composed of a dense lymphocytic infiltrate at the basal membrane zone (Figure 7 c).

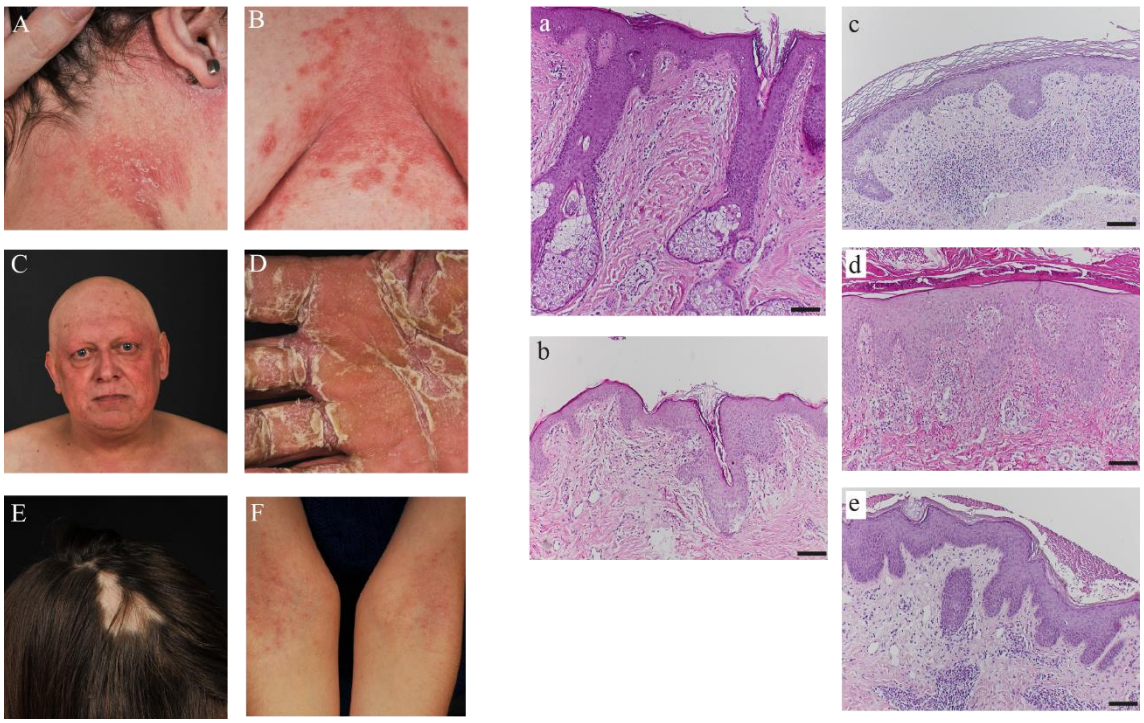


Figure 7: Clinical (A-F) and histopathological (a-e) phenotypes of AA and coexisting ISD.

Clinical phenotypes of an AA patient with AA localisata (A) and eczema (A) as well as psoriasis (B), of an AA patient with AA universalis (C) and eczema (D) and of an AA patient with AA localisata (E) and eczema (F). Histology showed classical signs of active AA (a), chronic AA (b), lichen planus (c), psoriasis (d) and eczema (e), details see text. (Scale bars in a-e: 100 µm). Modified from Garzorz et al., 2015.

### 3.1.3 Distinct ratio of cytotoxic and helper T cells in AA and inflammatory skin diseases

To verify the hypothesis that specific environmental triggers elicit distinct cutaneous inflammation, we investigated the inflammatory T cell pattern of ISD in AA patients and compared it with the inflammatory T cell pattern of ISD in single affected patients by performing immunohistochemistry for $CD4^+$ and $CD8^+$ T cells (n = 5; Figure 8). An independent cohort (IC) of single affected patients with AA (n = 9), lichen planus (n = 10), psoriasis (n = 10) and AE (n = 13) was established and co-analyzed to compare disease-specific T cell infiltrates independent of the AA background. All AA biopsies share a similar percentage (p value = 0.39) of $CD8^+$ T cells compared to $CD4^+$ T cells (CD4/CD8 ratio 1.45, n = 5 vs CD4/CD8 ratio 1.3 ± 0.2, n = 9 in IC) with lichen planus (CD4/CD8 ratio 1.3, n=1 vs CD4/CD8 ratio 1.39 ± 0.2, n = 10 in IC). In contrast to the AA lesions, AE in both cohorts (CD4/CD8 ratio 4.7, n = 3 vs CD4/CD8 ratio 4.1 ± 1.8 n = 13 in IC) was dominated by CD4+ T helper cells rather than cytotoxic CD8+ T cells (p value < 0.0001). In the case of psoriasis, the ratio was lower in the IC cohort (CD4/CD8 ratio 1.39 ± 0.4, n = 10) than in the cohort of double affected patients (CD4/CD8 ratio 2.7, n = 2).

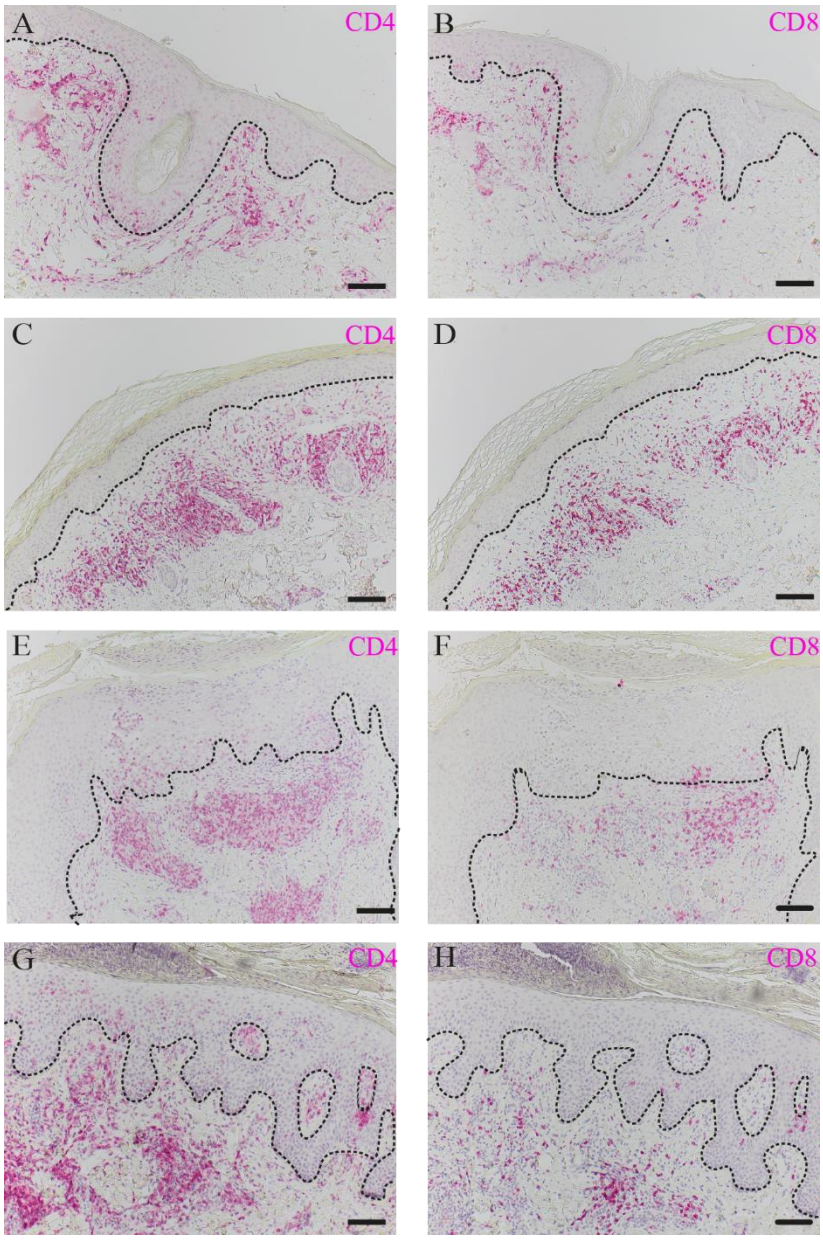


Figure 8: Infiltration of $CD4^+$ T helper cells and $CD8^+$ cytotoxic T cells in AA and ISD

AA (A and B) and lichen planus (C and D) showed a lower CD4/CD8 ratio than eczema (E and F) and psoriasis (G and H) which were dominated by $CD4^+$ T cells. (Scale bars: 100 µm). Modified from Garzorz et al., 2015.

### 3.1.4 Promiscuous T cell infiltrate in AA

Next we sought to characterize the type of T cellular immune response in lesional skin of AA and coexisting ISD. After isolation of T cells from punch biopsies of lesional skin, intra-cellular cytokine production was investigated using flow cytometry. Although we found a cytotoxicity-dominated T cell infiltrate with a high percentage of lesional CD8+ T cells in AA (Figure 8 B), AA lesions were found to be infiltrated by a broad variety of

cytokine-producing cells (Figure 9 A and B). The majority of infiltrating T cells produced the Th1 cytokines IFN-γ (52.3%) and TNF-α (45.6%) but also a high number of IL-17 (4.5%) and IL-4 (16.5%) producing T cells were found.

Intraindividual comparison of AA with inflammatory skin diseases revealed distinct T cellular infiltrates. The relative amount of IL-4+ T cells was higher in AE lesions (17.6%) and in AA (16.5%) compared to psoriasis (11.0%) and lichen planus (11.9%; Figure 9). Indeed, the highest relative percentage of IL-17 producing T cells was observed in AA (4.5%) and psoriasis (2.6%) versus AE (0.45%) and lichen planus (0.7%). Lichen planus was clearly dominated by IFN-γ+ and TNF-α+ T cells (62%; Figure 9 E and F). Though interindividual differences were found to be high, a unique pattern for AA regarding the cytokine production of lesional T cells in all patients with AA was found, independent of the coexisting inflammatory skin disease.

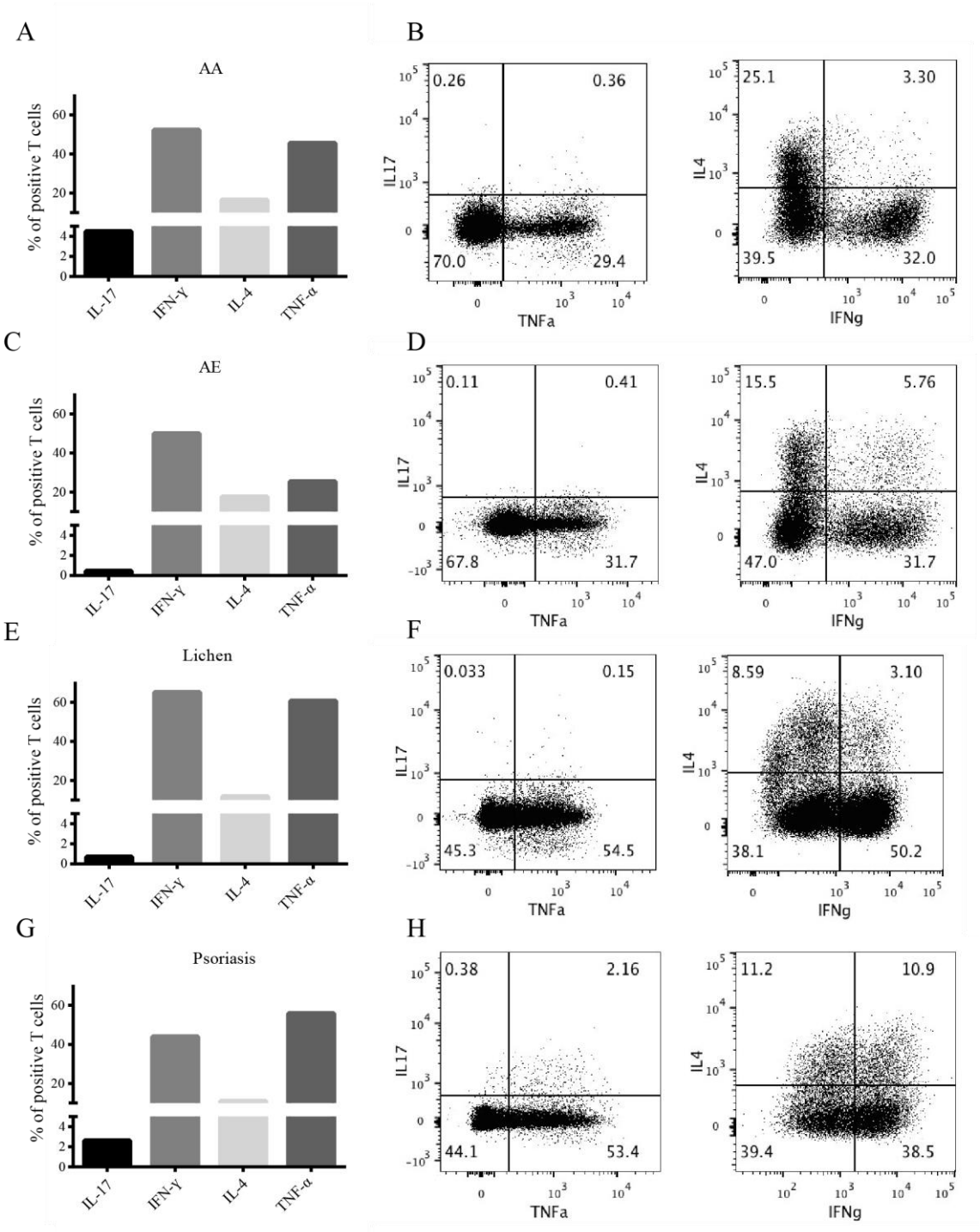


Figure 9: Cytokine staining of T cells isolated from patients with AA and coexistent ISD (n=5).

AA (A and B) and AE (C and D) showed higher amounts of IL-4 producing T cells compared to lichen planus (E and F) dominated by INF-$\gamma^+$ and TNF-$\alpha^+$ T cells (E and F) and psoriasis (G and H) which showed the highest amounts of IL-17 producing T cells together with AA. Modified from Garzorz et al., 2015.

## 3.2 Part 2: The human imiquimod model: A model for psoriasis in humans?

In parallel to investigating AA as a model to study gene-environment interaction in the pathogenesis of chronic inflammatory skin diseases, we investigated standardized and easy-to-use human models to mimic inflammatory skin diseases. In particular, imiquimod cream as outlined in section 1.4.3 has evolved as a popular mouse model for psoriasis or more precisely, for psoriasiform dermatitis. The goal of this project was to determine which aspects of psoriasis or eczema would be reflected in the human imiquimod model.

### 3.2.1 Application of imiquimod cream in humans induces a local, self-limited eczematous skin reaction

Upon application of topical imiquimod cream (Aldara® 5% cream) repeatedly every three days over a period of 30 days, 11 out of 14 participants developed an eczematous-like reaction peaking at day 4 (3/11) or at day 14 (4/11) or day 28 (4/11), (Figure 10).

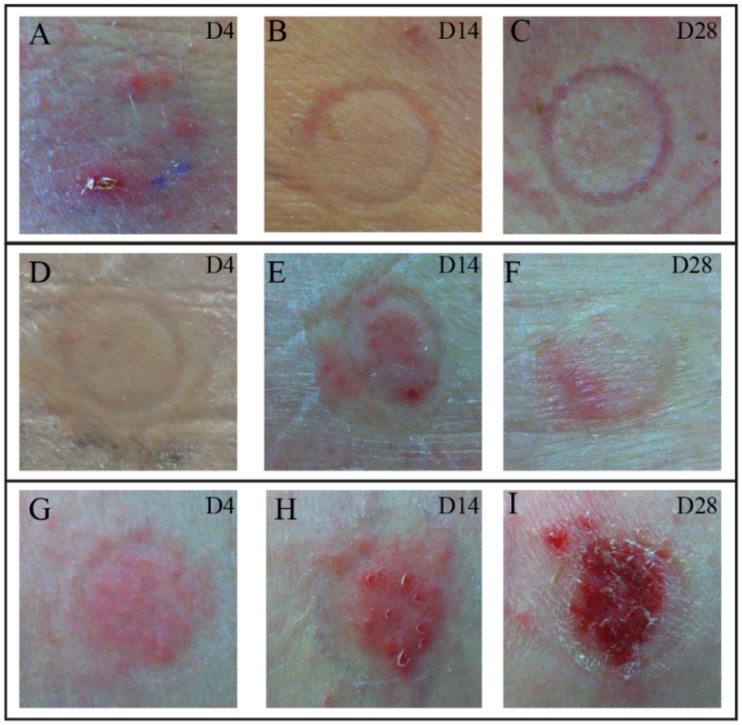


Figure 10: Clinical phenotypes of eczematous-like skin reactions caused by topical application of Aldara® 5% cream over the timecourse of 30 days.

Three out of 11 participants of the study showed the clinically strongest inflammatory reaction at day 4 (A-C), 4/11 patients were reaching the peak of inflammation at day 14 (D-F) and 4/11 patients at day 28 (G-I). Pictures show one representative patient of each group.

Three out of 14 prticipants only showed slight local irritation in the treated area. All lesions cleared after cessation of application of imiquimod cream indicating self-limitation of inflammation after withdrawal of local stimulus. The reactions were characterized by non-scaling red plaques, papules or maculae, in some cases with central erosion, and differences were not observed regarding the different skin disease backgrounds of volunteers (Table 9).

Table 9: Characteristics of participants of the imiquimod study and their patterns of reaction to imiquimod cream

Skin lesions were evaluated like atopy patch tests on a scale from 0 (no reaction) to slight (+), medium (++) and strong (+++). In red color, the peak of inflammation of each participant over the treatment period is indicated. Participants were either healthy or suffering from eczema and/or psoriasis.

| Sex | Age | Background | ID of volunteer | D4 | D14 | D28 |
|---|---|---|---|---|---|---|
| m | 35 | Atopic eczema | KE | + | ++ | +++ |
| f | 61 | Healthy | AE | ++ | + | + |
| f | 42 | Psoriasis | GS | +++ | ++ | + |
| f | 29 | Healthy | HK | + | ++ | +++ |
| f | 45 | Atopic eczema | KA | + | +++ | ++ |
| m | 33 | Psoriasis | KD | + | +++ | ++/+++ |
| m | 46 | Healthy | KT | 0 | | |
| f | 40 | Psoriasis and atopic eczema | MPG | + | + | ++ |
| f | 51 | Psoriasis | OC | ++ | +++ | + |
| f | 53 | Healthy | RU | ++ | + | + |
| f | 59 | Psoriasis | SS | 0 | | |
| f | 70 | Atopic eczema | TI | 0 | | |
| f | 47 | Healthy | UH | +/++ | + | ++ |
| f | 55 | Healthy | YH | + | ++ | + |

### 3.2.2 Skin reactions induced by imiquimod cream rather show characteristics of eczema than hallmarks of psoriasis on the level of histopathology

In a next step, skin reactions were investigated on the level of histopathology analyzing HE-stained tissue sections by two blinded histopathologists. The diagnosis of dermatitis in all samples from the 11 out of 14 participants who showed clinical signs of inflammation upon imiquimod cream was made. Hallmarks of psoriasis such as microabscess, parakeratosis and hypogranulosis were absent, instead spongiosis and other characteristics of eczema were found. In addition, the three patients clinically evaluated as non-responders, showed slight signs of eczema. Interestingly, the both for psoriasis and eczema atypical finding of a pseudolymphoma-like lymphocytic infiltrate characterized by "bottom heavy" infiltrates, deeply penetrating into dermal tissue was made in 10 out of the 11 participants who showed marked clinical signs of inflammation. Five out of eleven patients not only showed pseudolymphoma-like infiltrate at the peak of inflammation but also at preceding and subsequent timepoints (Figure 11 B- E). To facilitate following analyses, all samples of the imiquimod cream treated group were either assigned to the group of "pseudolymphoma-like reaction" (PL) or to the group of "not showing pseudolymphoma-like characteristics" (noPL).

To validate the clinical phenotype of eczema further, the cumulative histopathological psoriasis and eczema score was determined for each sample by two blinded observers. An independent cohort of psoriasis and eczema HE-samples (psoriasis, n = 11; eczema, n = 13) was established to compare the results from the imiquimod cream treated group to the histopathological psoriasis and eczema scores of classical psoriasis and eczema phenotypes. Whereas in the independent cohort of clear psoriasis samples a mean psoriasis score of 11.14 ± 0.79 could be reached, the group of eczema samples from the independent cohort of clear eczema– as expected – only reached a mean psoriasis score of 3.92 ± 0.77 as compared to the PL (2,81 ± 0.68) and noPL (1.05 ± 0.31) group. Accordingly, determination of the eczema score led to high mean scores within the group of eczema (6.25 ± 0.65), PL (6.09 ± 0.43) and noPL (5.95 ± 0.37) and a significantly lower mean score for psoriasis (1.86 ± 0.35), (Figure 11 F and G)

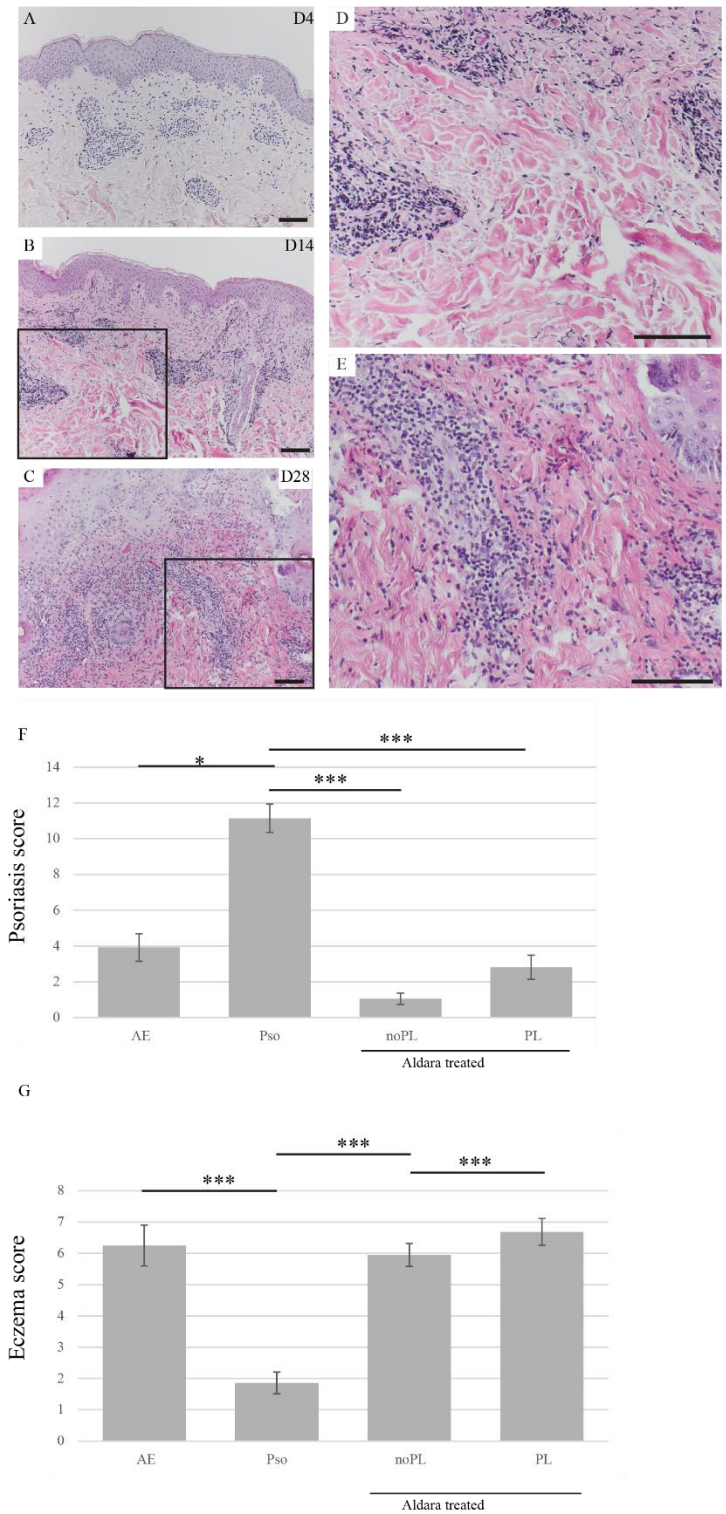


Figure 11: Histopathological diagnosis of "pseudolymphoma-like" dermatitis of skin treated with imiquimod cream.

In (A-E) patient KE is exemplarily presented. Whereas at day 4 only an eczematous reaction was diagnosed, the patient showed deep lymphocytic infiltrates already at day 14 and at day 28 (clinical peak of inflammation). Infiltrates in (B) and (C) are shown at higher magnification in (D) and (E), respectively. Applying the histopathological psoriasis (F) and eczema score (G) on imiquimod cream treated skin as well as on classical phenotypes of psoriasis and eczema shows clear similarity between scores of PL, noPL and eczema (AE) samples and not with psoriasis. (Scale bars in A-E: 100 µm).

### 3.2.3 Imiquimod cream induces cellular infiltration comparable to psoriasis rather than eczema

On histopathological level both PL and noPL phenotypes clearly resembled eczema with pseudolymphoma-like characteristics. As both psoriasis and eczema scores mainly focus on the architecture of skin lesions (e.g. acanthosis, parakeratosis, vascular structure) and apart from neutrophils and eosinophils do not consider cellular characteristics, we performed immunohistochemistry for $CD4^+$ and $CD8^+$ T cells as well as for dendritic cells and neutrophils. We found a clear trend for a lower CD4/CD8 ratio in psoriasis (1.44 ± 0.34) and PL (1.06 ± 0.12) as compared to eczema (3.67 ± 1.61) and noPL (7.4 ± 2.9) (Figure 12 A-E). Also for neutrophils, the number was higher in psoriasis (18.56 ± 4.34) and PL (12.2 ± 6.5) than in noPL (0.875 ± 0.5) and eczema (5.6 ±2.53) (Figure 12 I and J). A marked difference was seen for dendritic cells: Here, the number of dendritic cells in PL and noPL (PL: 18.01 ± 3.68; noPL: 8.31 ± 3.92) was higher than in psoriasis (psoriasis: 5.23 ± 2.12) and even more prominent than in eczema (2.88 ± 1.62) hinting at a prominent role for dendritic cells in imiquimod cream treated skin (Figure 12 F-H). This is mirrored in a higher expression of TLR7 in both PL (22.63 ± 5.85) and noPL (9.05 ± 5.86) as compared to psoriasis (0.54 ± 0.5) and eczema (2.76 ± 2.67) indicating recruitment of TLR7 positive dendritic cells upon application of the TLR7 agonist imiquimod (Figure 13).

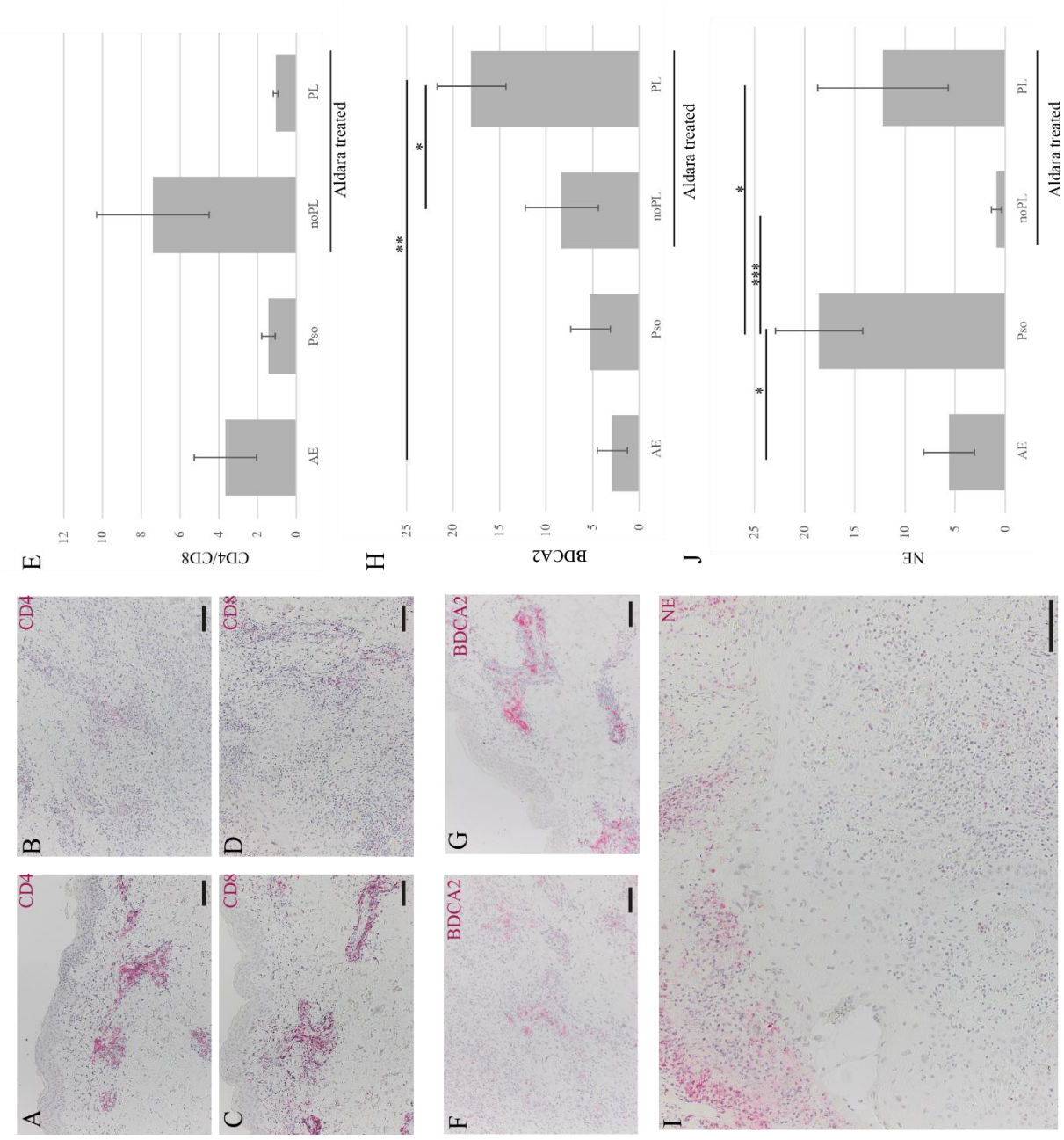


Figure 12: Cellular infiltrate in imiquimod treated skin compared to psoriasis and eczema.

Infiltrate of T helper cells (A and B), cytotoxic T cells (C and D) and dendritic cells (F and G) illustrated for two examples of PL. Neutrophils are visualized by staining for neutrophil elastase (NE) and exemplarily shown for PL (I). Both the proportion of $CD4^+$ and $CD8^+$ cells (E) as well as infiltrates of dendritic cells (H) and neutrophils (J) seen in PL rather resemble the situation seen in psoriasis.

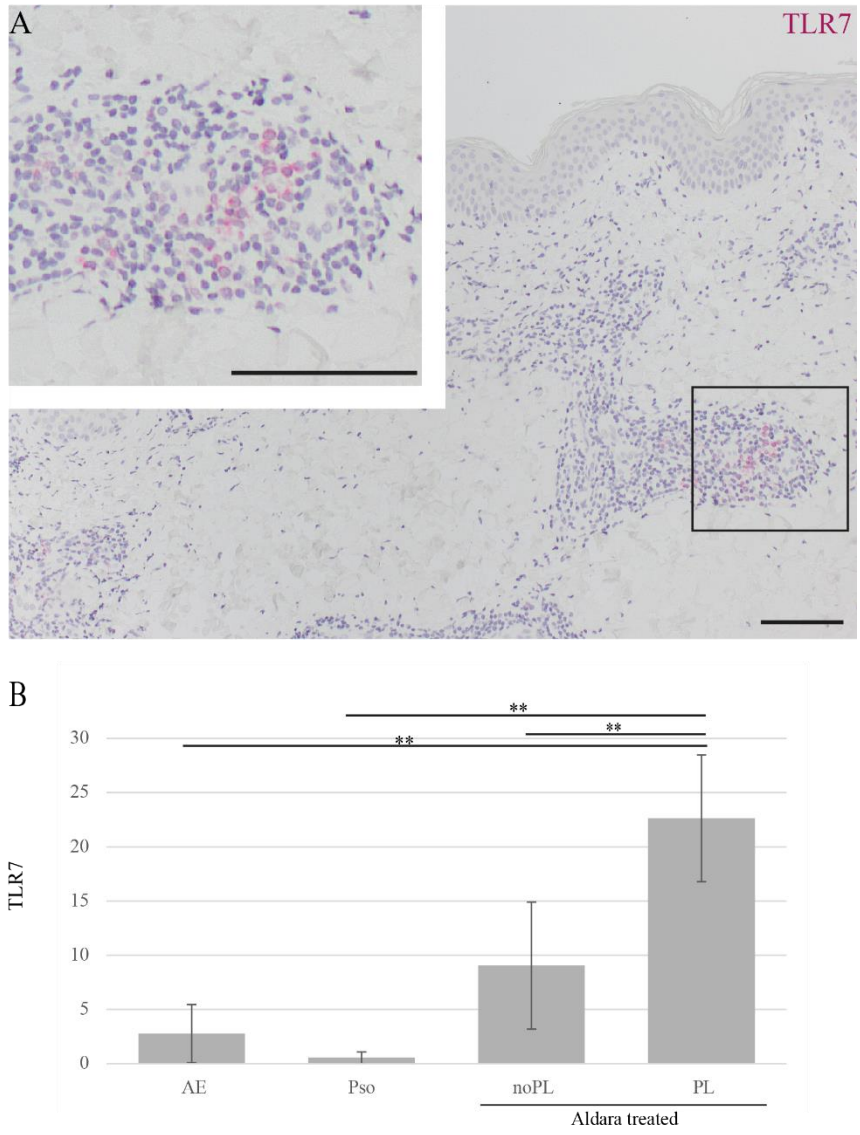


Figure 13: TLR7 expression in imiquimod cream treated skin

Staining for TLR7 shows marked positive expression in cellular infiltrate (A). TLR7 expression is higher in PL and no PL compared to psoriasis and eczema (B).

### 3.2.4 *In vivo* T cell responses in imiquimod cream treated skin is distinct from psoriasis and eczema

For deeper characterization of the T cellular immune response, T cells from punch biopsies of lesional imiquimod treated skin were isolated, stimulated and cytokines of T cell supernatant was measured by ELISA. For comparison to cytokine profiles of psoriasis and eczema an independent cohort of T cell supernatants from eczema samples (n = 9) and psoriasis samples (n = 10) was established. Despite the comparable CD4/CD8 ratio seen in PL and Psoriasis on immunohistochemical level, cytokine production differed

markedly between PL and psoriasis. Here, both PL and noPL showed a trend for higher amounts of IFN-γ and less amounts of TNF-α and IL-4 as compared to psoriasis and eczema (Figure 14).

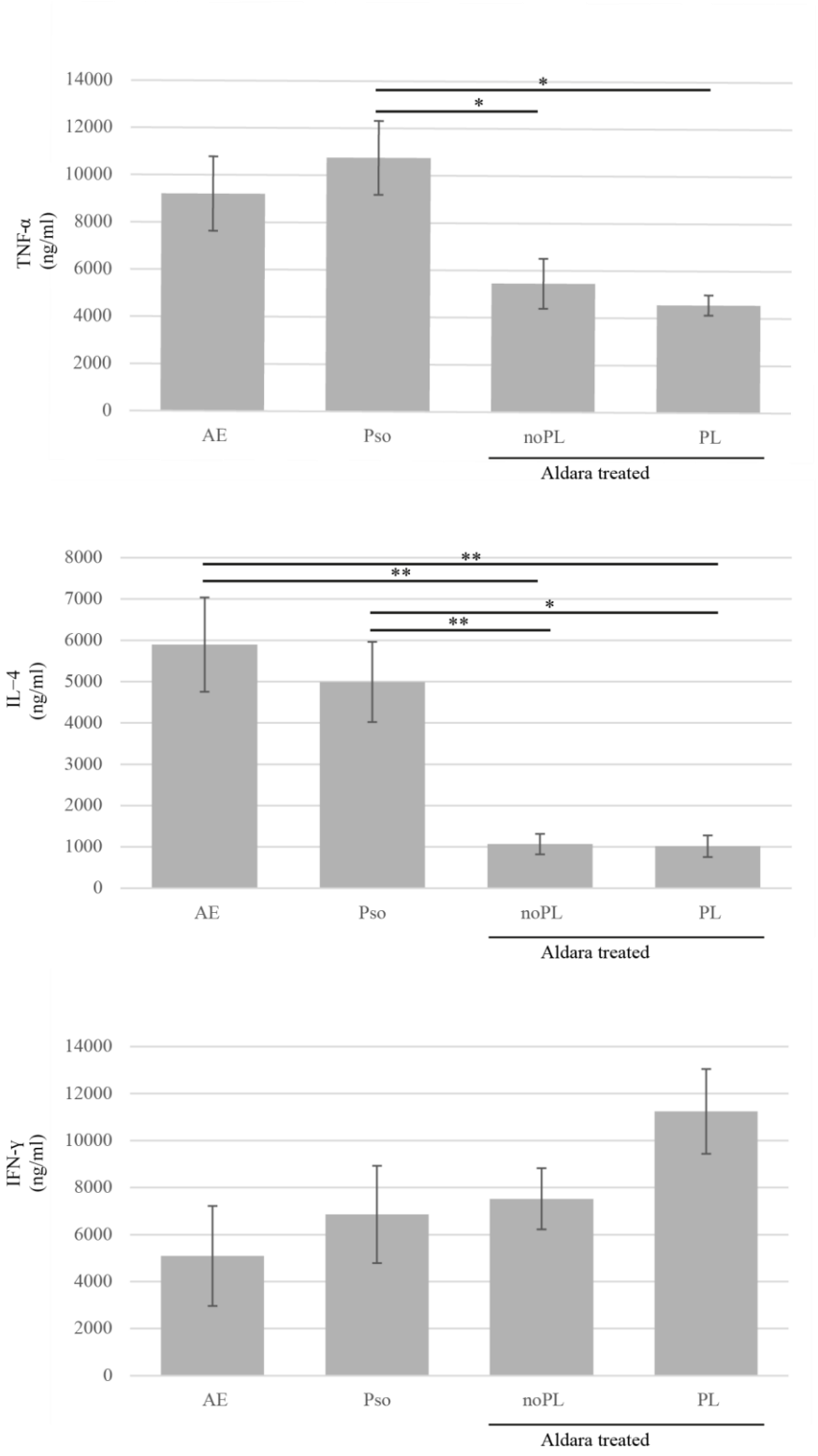


Figure 14: Cytokine profile of T cells isolated from imiquimod treated skin compared to psoriasis and eczema.

### 3.2.5 Molecular signature of PL and noPL compared to psoriasis, eczema and acute contact dermatitis

Comparison of imiquimod cream treated skin with psoriasis and eczema on histological, immunohistochemical and cellular level led to mixed results with similarities to eczema on the level of tissue architecture and features comparable with psoriasis concerning cellular infiltrate. In summary, however, it remains unclear which pathological processes and molecular pathways of psoriasis and/or eczema are reflected by the imiquimod model in humans. We thus performed whole-genome expression arrays of both PL and noPL samples and compared them to a group of psoriasis (n = 24) and eczema (n = 24). As eczema has shown to present with a rather heterogeneous picture (s. section 3.3.1) we included both naturally occurring eczema (n = 14) and acute contact dermatitis (ACD) to nickel (n = 10) into our disease comparison.

We found that when looking at all genes (significantly and non-significantly) regulated between PL and the other three inflammatory skin conditions, ACD showed the highest correlation with PL (correlation coefficient: 0.78) (Figure 15 A), whereas both psoriasis and eczema only reached a correlation of 0.56 (eczema) (Figure 15 C) and 0.57 (psoriasis) (Figure 15 E). Looking at all significantly regulated genes, 65% of the significantly down- or upregulated genes in ACD are also regulated in PL and the percentage is even higher when only looking at the top hits (fold induction > 2.5) of ACD also regulated in PL (80.2%) (Figure 15 B). For eczema only 37.1% of significantly regulated genes are also regulated in PL (Figure 15 D) but again, only considering the top hits the number is higher with 64.3% of top hits in eczema also regulated in PL (Figure 15 D). The lowest number of genes and top hit genes regulated also in PL is seen for psoriasis with 31.2% for all significantly regulated genes and 41.8% for all top hits (Figure 15 F).

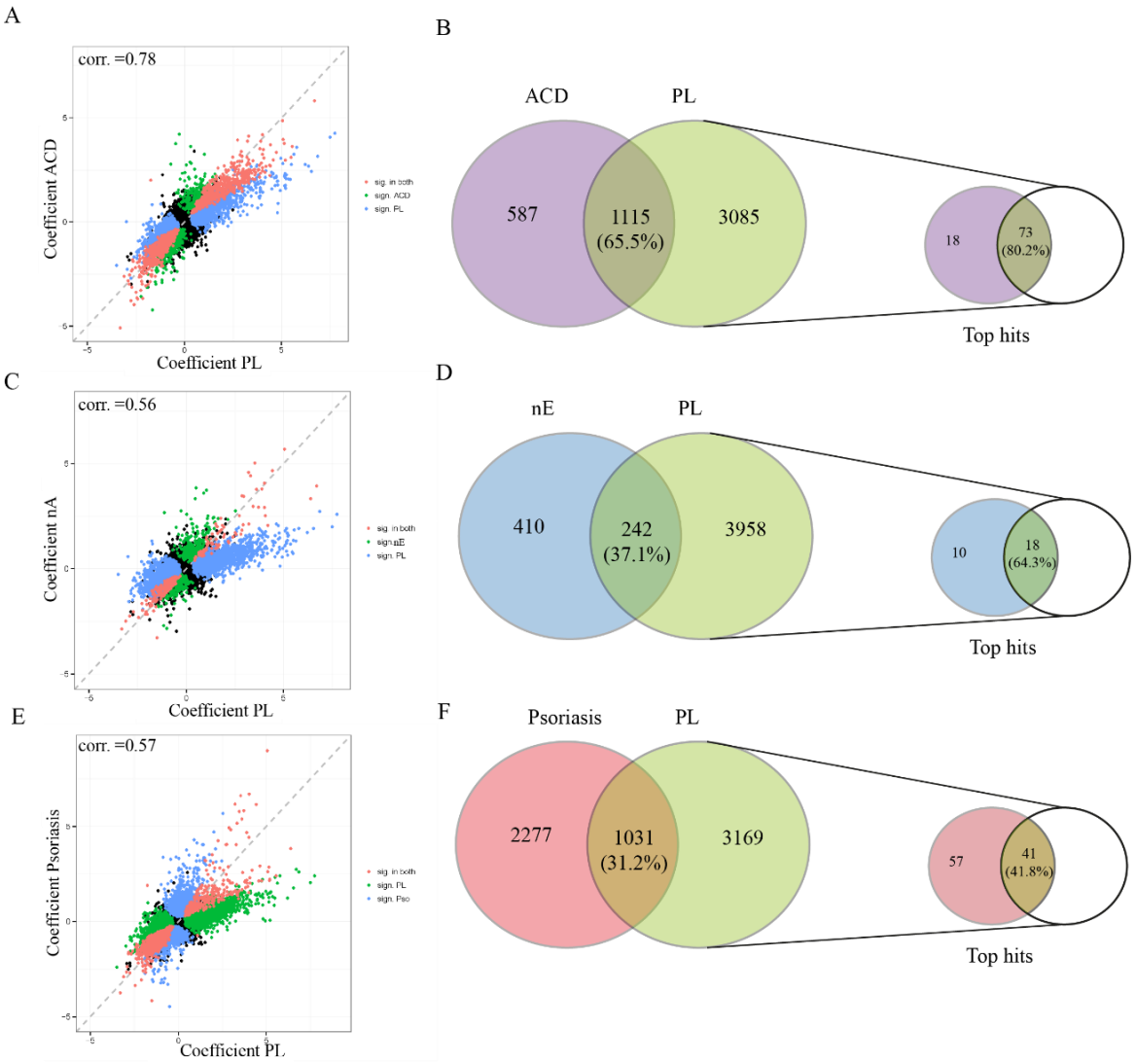


Figure 15: Comparison of imiquimod cream treated skin on molecular level with naturally occurring eczema (nE), ACD and psoriasis.

Comparison of imiquimod cream treated skin resulting in pseudolymphoma-like dermatitis (PL) with ACD (A and B), nE (C and D) and psoriasis (E and F). Looking at the entirety of significantly and non-significantly regulated genes, PL showed the highest correlation with ACD (A), followed by psoriasis (E) and eczema (C). A high proportion of genes significantly up- or downregulated in ACD is also significantly regulated in PL (65.5%) and looking at the top hits (fold induction > 2.5), the percentage of genes is even higher (80.2% of top hits in ACD are also regulated in PL), (B). In contrast, only approximately 30% of genes significantly regulated in psoriasis and eczema are also regulated in PL (D and F).

Interestingly, the correlation between noPL and PL is highest with a correlation coefficient of 0.91 indicating that in both conditions most genes are regulated in the same direction; however, the level of fold induction seems to make the difference between PL and noPL. Accordingly, most of the significantly regulated genes in noPL are also regulated in PL (95.9%) (Figure 16).

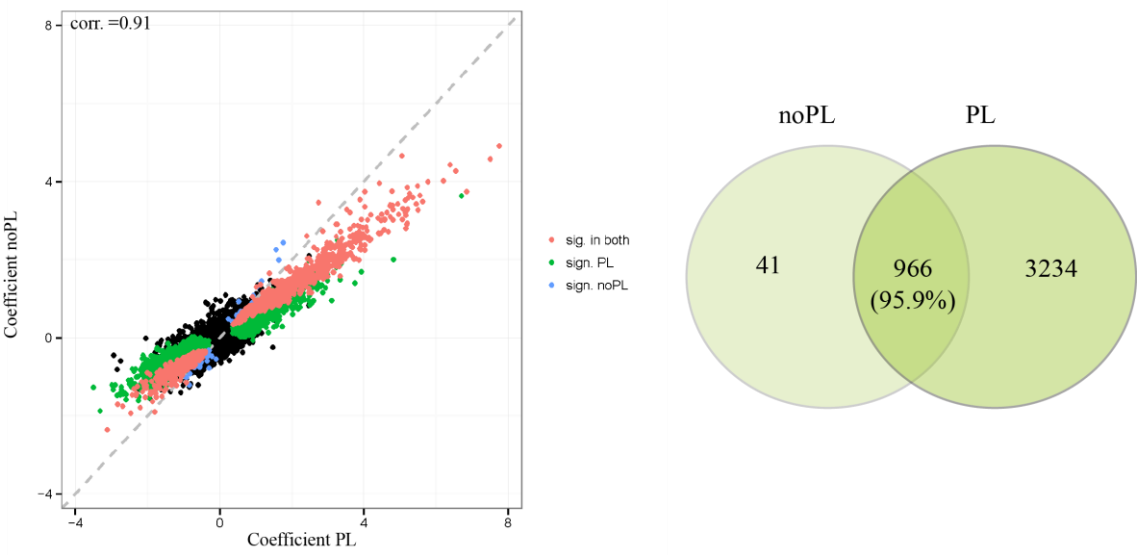


Figure 16: Correlation of regulated genes in PL and noPL

A high overall correlation of significantly and non-significantly regulated genes between PL and noPL is observed (corr. coeff.: 0.91). Looking at the percentage of significantly regulated genes only, the percentage of overlapping genes is high (95.9%) indicating that only the level of induction makes the difference between PL and noPL.

We next looked at the top hit genes (log fold induction > 2.5) for ACD, psoriasis and naturally occurring eczema (nE) and categorized them into the three groups “epidermis”, “immune system” and “metabolism”. We compared the expression of these top hit genes to the respective expression in PL. Concerning the epidermis we found that in particular genes of the S100 family, exerting antimicrobial functions (*S1007A, S100A7, S100A8* and *S100A9*) were significantly upregulated in all four conditions indicating general regulatory mechanism of the epidermis during inflammation (Figure 17). Concerning the immune system, we found the highest number of genes commonly regulated when comparing ACD to PL. Only four top hit genes exclusively up- or downregulated in ACD were not regulated in PL hinting at broadly common mechanisms of immunoregulation of PL and ACD. The lowest number of top hit genes and thus only little overlap to regulated genes in PL was found in the heterogeneous group of nE. As both psoriasis and nE are heterogeneous conditions as compared to ACD, which is triggered in uniform mode by nickel (s. section 3.3.1), we suspected to have overlooked commonly regulated mechanism of nE and psoriasis with PL, respectively, when only considering top hit genes with a log fold induction of > 2.5.

We therefore performed gene enrichment analysis to determine the most active Gene Ontology (GO) annotations for psoriasis, ACD and nE (Figure 18). Then all significantly regulated genes in PL were tested for their presence in these GO terms. The highest percentage of genes commonly regulated in PL and ACD/psoriasis/nE within the respective GO term was found for the Go term "immune response" (15.0% concordance, shared between ACD and PL), followed by "negative regulation of cytokine production" (12.0% concordance, shared between psoriasis and PL) and "cellular modified amino acid metabolic process" (10.8% concordance, shared by psoriasis and PL).

The GO terms "immune response" and "negative regulation of cytokine production" were shown to be statistically significant and the commonly regulated genes between PL and psoriasis or PL and ACD within these two terms were further investigated by the ConsensusPathDB database. Here, within a list of defined seed genes (here: commonly regulated genes in PL and psoriasis or PL and ACD from the relevant GO terms) gene-gene interactions and functional relationships and thus pathways are made visible by interconnecting these seed genes with "intermediate genes" that are likely to be associated with the phenotype. I found that both PL and ACD were characterized by activation of the NF-κB / interferon regulatory factor 1 (IRF-1) signaling pathway leading to the induction of apoptosis (*CASP8* = caspase 8). Along with the induction of apoptosis, in both ACD and PL IL-2/IL-2R signaling seems to play a major role by inducing granzyme B (*GZMB*) that is crucial for the induction of target cell apoptosis by cytotoxic T cells (Figure 19). Investigating commonly regulated genes of psoriasis and PL of the GO term "negative regulation of cytokine production" with ConsensusPathDB, we found regulation of IL-23, a key cytokine for psoriasis, mostly initiated by activation of NF-κB and IL-1β (Figure 20) in both settings.

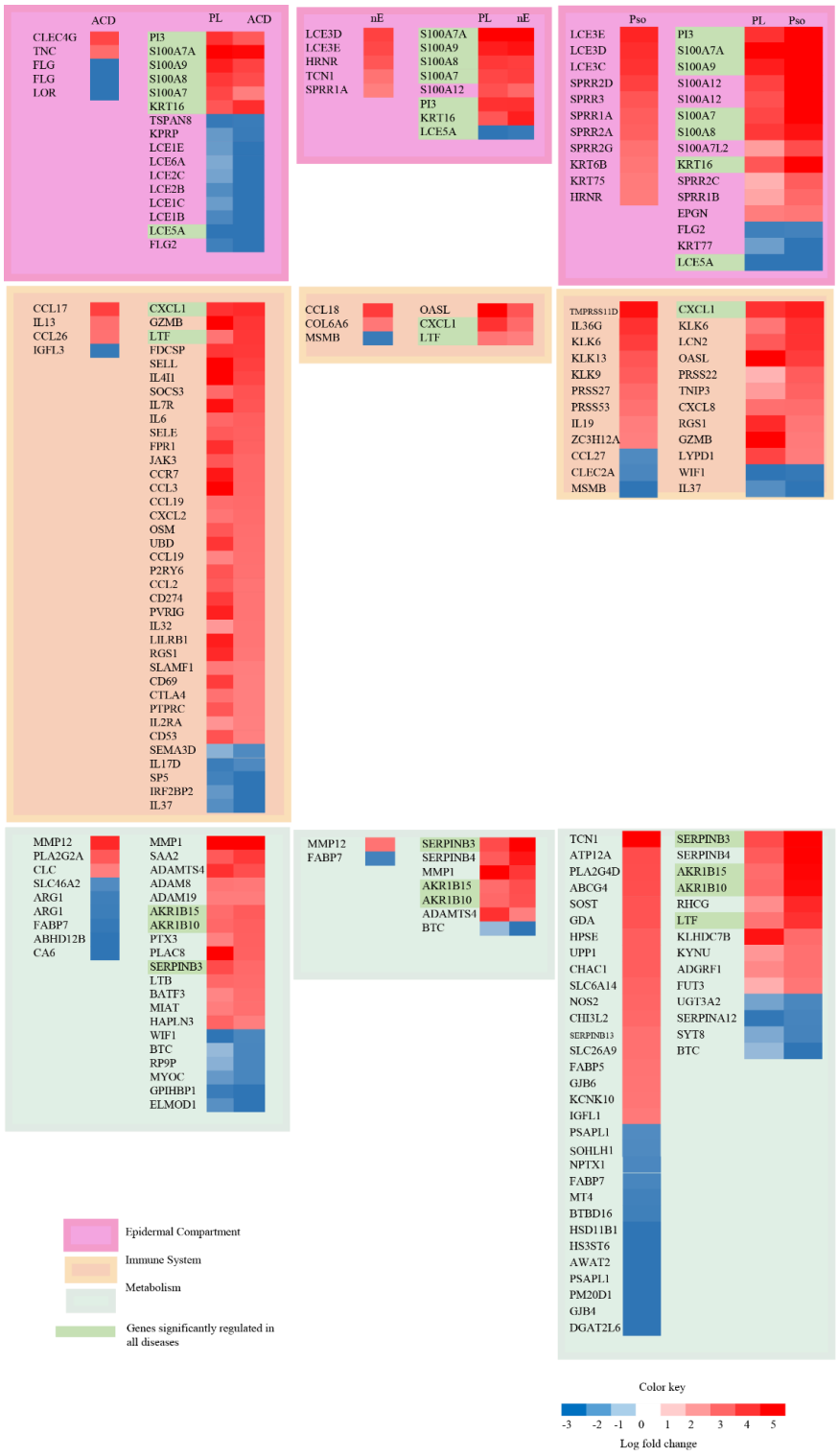


Figure 17: Top regulated genes in psoriasis (Pso) and/or PL, naturally occurring eczema (nE) and/or PL and acute contact dermatitis (ACD) and/or PL

Heat map of all top regulated genes significantly up- or downregulated in ACD and/or PL (left side), nE and/or PL (middle) and psoriasis and/or PL (right). Genes exclusively regulated in ACD/nE/Pso are depicted on the left side of each disease comparison, while genes regulated commonly in ACD/nE/Pso and PL are listed on the right side of each disease comparison. The histogram shows the color code for log fold induction. For top hit genes, only genes with a log fold change > 2.5 for ACD/nE/Pso were chosen and compared to PL. Genes regulated > 2.5 log fold in ACD, nE, Psoriasis (Pso) and PL were highlighted in green.

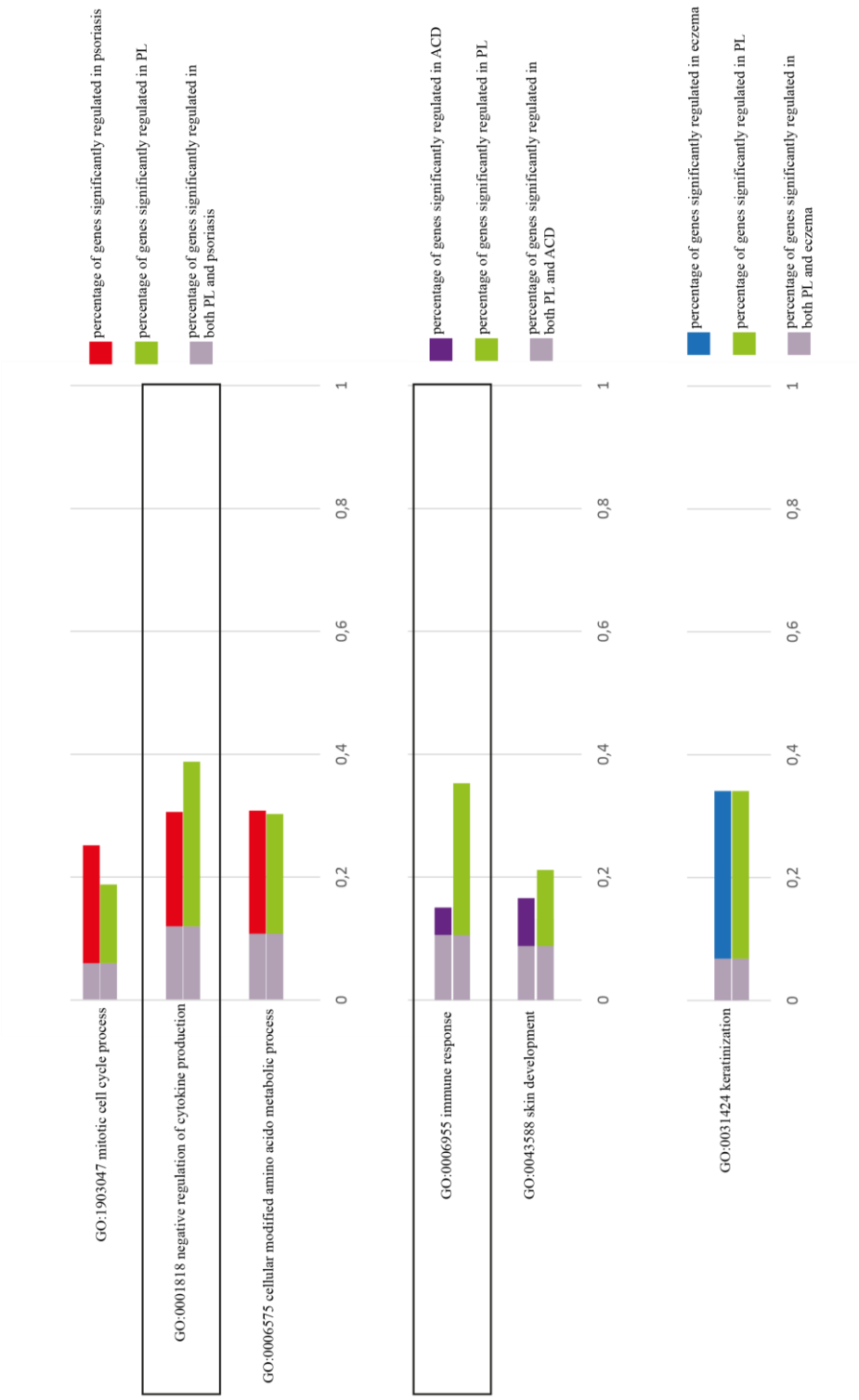


Figure 18: Signaling pathway analysis of psoriasis, ACD and naturally occurring eczema (nE) compared to PL

For psoriasis, ACD and naturally occurring eczema (nE) the GO terms with the highest estimate (lowest false positive discovery) were chosen and the percentage of genes active in the respective term are depicted in the graph (green: PL, red: psoriasis, violet: ACD and blue: eczema). All significantly regulated genes in PL were tested for the GO terms found in ACD, psoriasis and eczema, respectively, and the percentage of genes found in PL regulated in the respective GO term is depicted in green. The percentage of genes commonly regulated in ACD/eczema/ psoriasis and PL is shown in grey color. Highlighted in black boxes are GO terms with adjusted p values < 0.05.

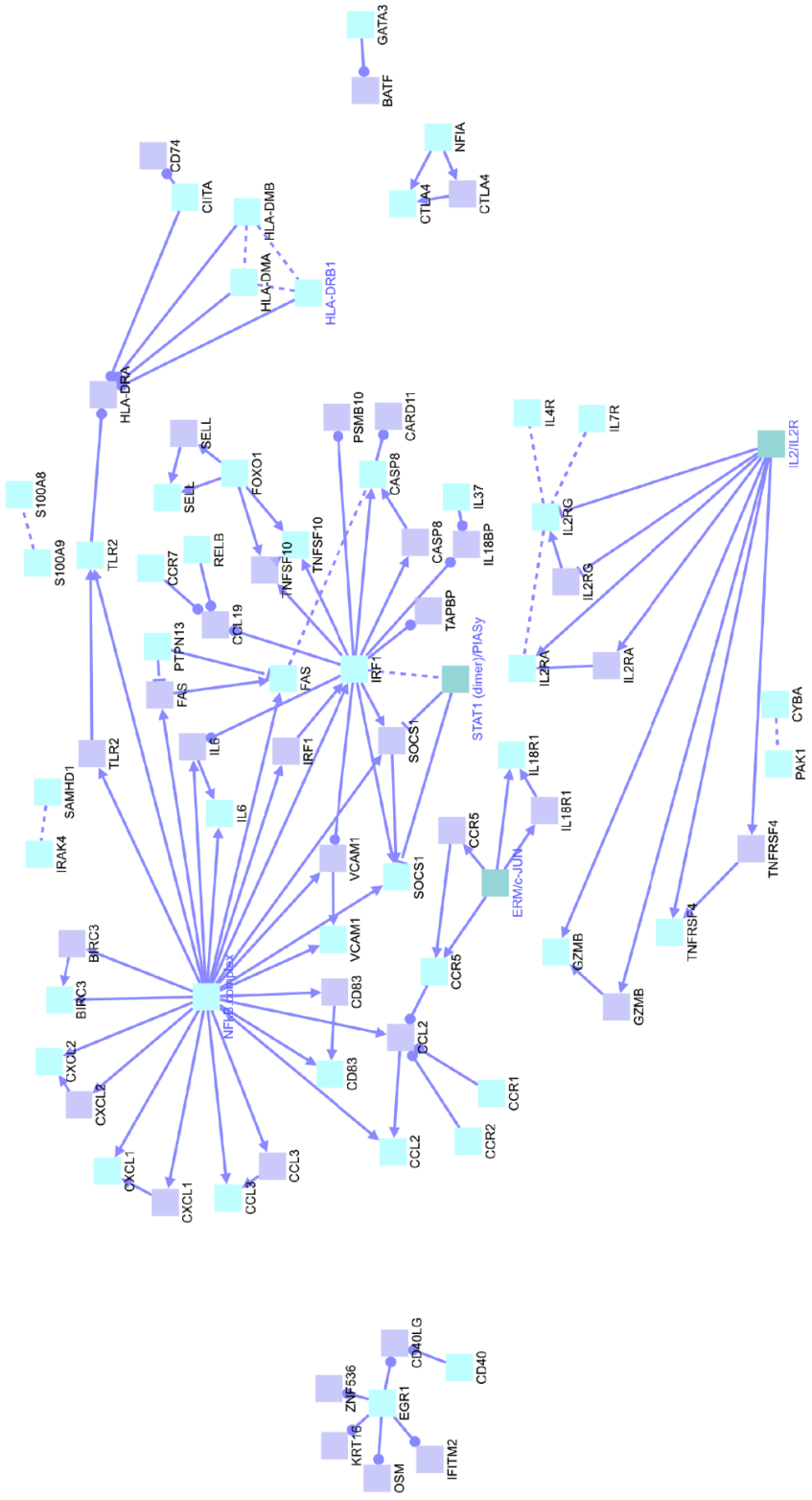


Figure 19: Activation of the NF-κB /IRF-1 signaling pathway shared by both ACD and PL

Induced network modules analysis by ConsensusPathDB reveals activation of the NF-κB /IRF-1 signaling pathway in both ACD and PL. Genes are depicted in violet color, proteins in light blue color and protein complexes in turquoise color. Black node labels denote seed nodes and purple node labels denote intermediate nodes. Arrow shaped edges indicate “activation”, circle shaped edges indication “interaction” and edges shaped as vertical lines indicate “inhibition”.

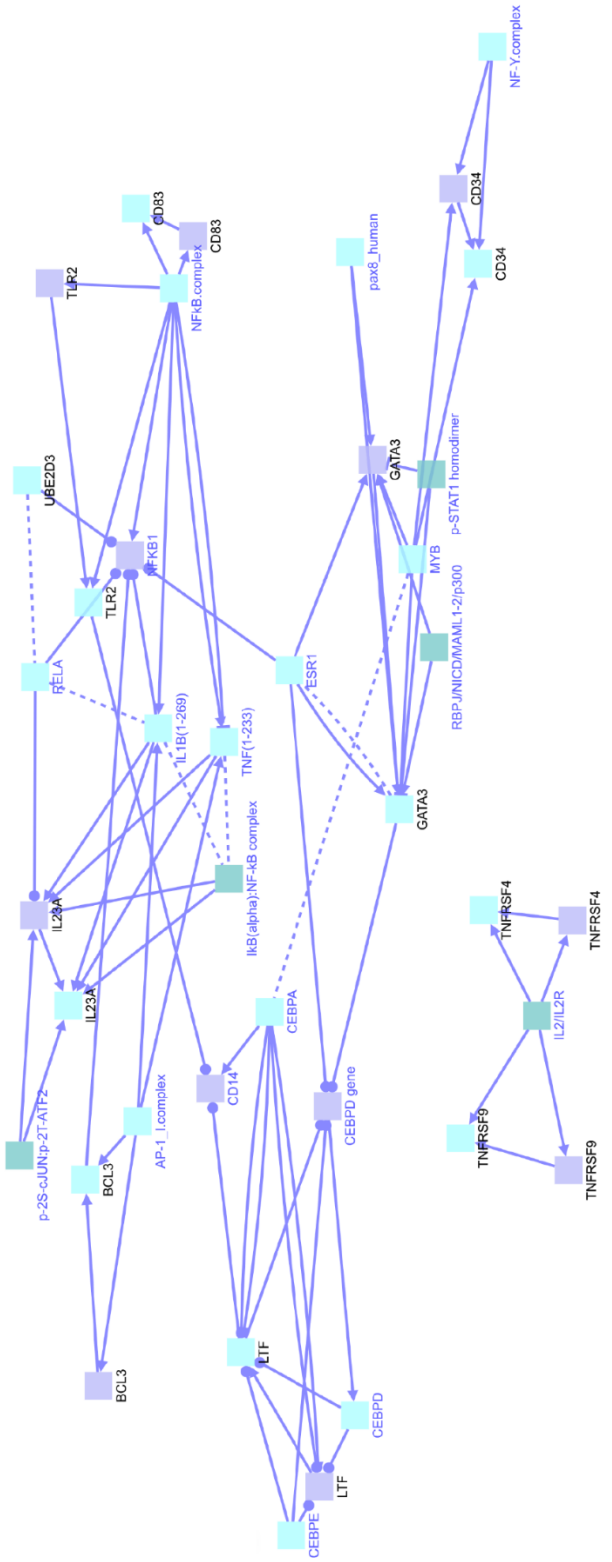


Figure 20: Activation of IL-23 signaling pathway shared by both Psoriasis and PL

Induced network modules analysis by ConsensusPathDB reveals activation of IL-23 signaling in both psoriasis and PL. Genes are depicted in violet color, proteins in light blue color and protein complexes in turquoise color. Black node labels denote seed nodes and purple node labels denote intermediate nodes. Arrow shaped edges indicate “activation”, circle shaped edges “interaction” and edges shaped as vertical lines indicate “inhibition”.

## 3.3 Part 3: A novel molecular disease classifier for psoriasis and eczema

While in the imiquimod study, we focused on a standardized homogeneous skin reaction and analyzed similarities and differences to psoriasis and eczema we now investigated a group of patients suffering concomitantly from both psoriasis and eczema. Although this group is rather heterogeneous – as different types of eczema for example were included – these patients represent an ideal model to decipher disease-specific inflammatory responses. By intraindividual disease comparison, complexity can be reduced, as common inflammatory mechanisms can be reduced and noise from interindividual variability can be subtracted.

The results presented in this section were published in the article: "Intraindividual genome expression analysis reveals a specific molecular signature of psoriasis and eczema" (Quaranta M, Knapp B, Garzorz N, *et al.* (2014). *Science translational medicine* 6:244ra90.). The following section is based on this article as well as on the manuscript entitled: "A novel molecular classifier for psoriasis and eczema" submitted to the *Journal of Experimental Dermatology* in January 2016 by Garzorz N, Krause L, Lauffer F *et al.* The results presented in section 3.3.1 and 3.3.2 have been developed together with Bettina Knapp and Maria Quaranta, whereas my primary focus has been the development of the disease classifier (section 3.3.3).

### 3.3.1 The heterogeneity of eczema: A proof of concept

To verify the clinical observation of a broad heterogeneity within the disease category of eczema (s. section 1.3.2 and 1.3.3), we first investigated if the heterogeneity of different eczema subtypes would also be present on molecular level. As a proof of concept, this analysis marks an important pre-analysis for the disease classifier developed in the following.

We performed whole-genome expression arrays from lesional and autologous non-lesional skin. Biopsies were taken in the group of patients being affected by both psoriasis and an eczema variant – a model with its described advantages of data generation (see section 1.4.1 Intraindividual comparison of psoriasis and eczema). In total, the cohort comprised 24 patients with psoriasis and coexisting atopic eczema (n = 6), psoriasis and

coexisting nummular or dyshidrotic eczema (n = 7) or psoriasis and coexisting allergic contact dermatitis to nickel (n = 11). In a first attempt, variability was shown to be too high to decipher eczema subtype specific gene expression patterns – not the least due to small numbers in each group - when trying to cluster each entity separately (data not shown). Therefore, a subtype analysis of naturally occurring (atopic eczema, nummular and dyshidrotic eczema) vs induced eczema (allergic contact dermatitis, ACD) was performed.

It could be shown that variability in the ACD group was smaller as compared to naturally occurring eczema. Compared to autologous non-involved skin, 172 genes were regulated exclusively in ACD (90 upregulated, 82 downregulated), but not in naturally occurring eczema. In contrast, only 28 genes were exclusively regulated in naturally occurring eczema (22 upregulated, 6 downregulated). Thirty-three genes were regulated in common (27 upregulated, 6 downregulated, Figure 21).

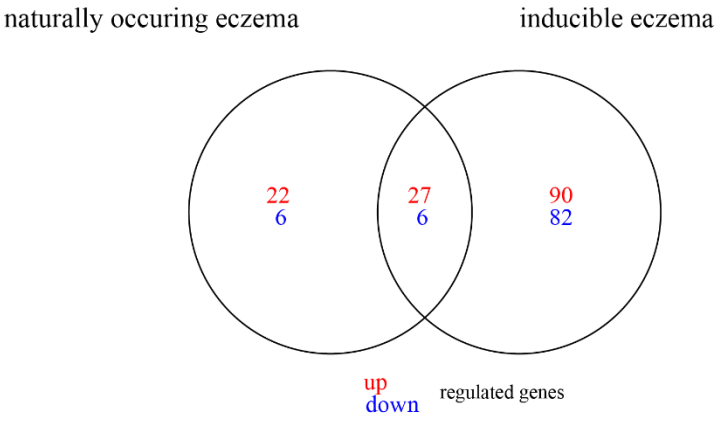


Figure 21: Comparison of gene expression between eczema subtypes

Number of genes significantly up- or downregulated compared to autologous non-involved skin in patients suffering from psoriasis and a variant of eczema simultaneously (n = 24), as illustrated using Venn diagrams and Volcano plots. Modified from Quaranta et al., 2014.

Looking at the genes in more detail, they were assigned to the three categories of “Epithelium”, “Immune system” and “Metabolism”. Interestingly, it could be found that the epithelial antimicrobial response was similar in both naturally occurring eczema and ACD: Members of the defensin family (e.g. *DEFB4*) as well as of the S100 proteins (*S100A7, S100A7A*) were significantly upregulated in both naturally occurring eczema

and ACD. However, a mutually antagonistic picture was observed for the late differentiation markers: Members of the LCE family (e.g. *LCE3A*, *LCE3C*) were upregulated in naturally occurring eczema, but in contrast strongly downregulated in ACD only. Extracellular matrix proteins such as *HAS3* and *EPSTI1* as well as numerous cell-cell adhesion molecules such as *ICAM-1* were exclusively upregulated in ACD (Figure 22).

Looking at the immune system a prominent acute immune response was detected in ACD rather than in naturally occurring eczema. Inflammasome associated genes such as those encoding for IL-1β, AIM2 (absent in melanoma 2) and IFIT3 (Interferon-induced protein with tetratricopeptide repeats 3) as well as a variety of genes encoding for chemokines including the neutrophil attracting chemokine CXCL8 or the Th1 associated chemokines CXCL9, CXCL10, and CXCL11 were exclusively upregulated in ACD skin (Figure 22).

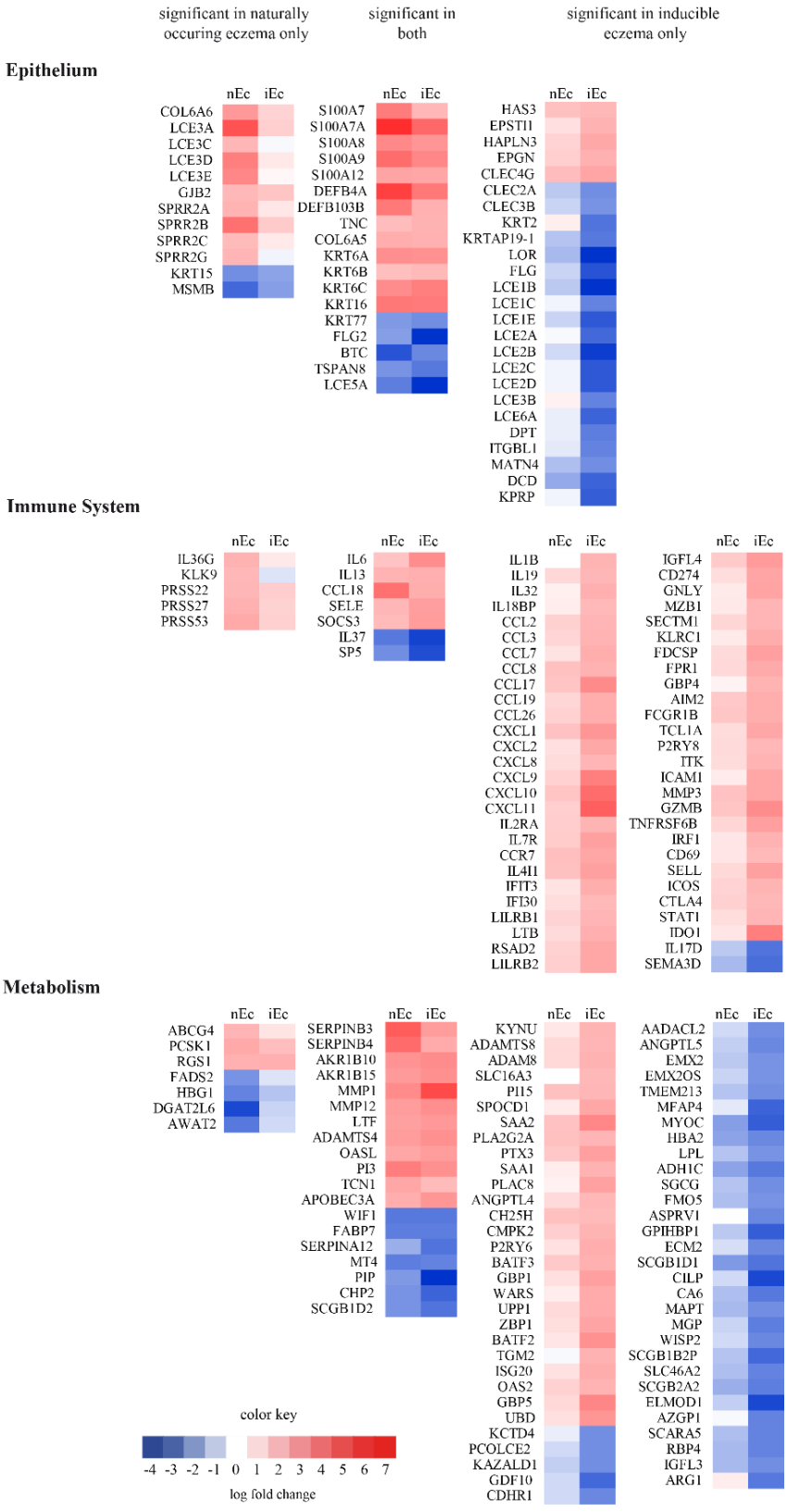


Figure 22: Regulated genes in naturally occurring eczema (nEc) and/or ACD (iEC)

Heat map of all genes significantly regulated in naturally occurring eczema only (left side) and ACD (right side) or both (middle) compared to non-involved skin. Histogram indicates the color code for $\log_2$ fold induction. Modified from Quaranta et al., 2014.

According to the found top hits, GO term analysis for the terms “biological process”, “cellular component” and “molecular function” unraveled that e.g. the pathways “inflammatory response”, “chemokine activity,” and “extracellular space” were significant in ACD and not in naturally occurring eczema. In contrast, the GO term “cornified envelope” was identified within the naturally occurring eczema lesions only (Figure 23).

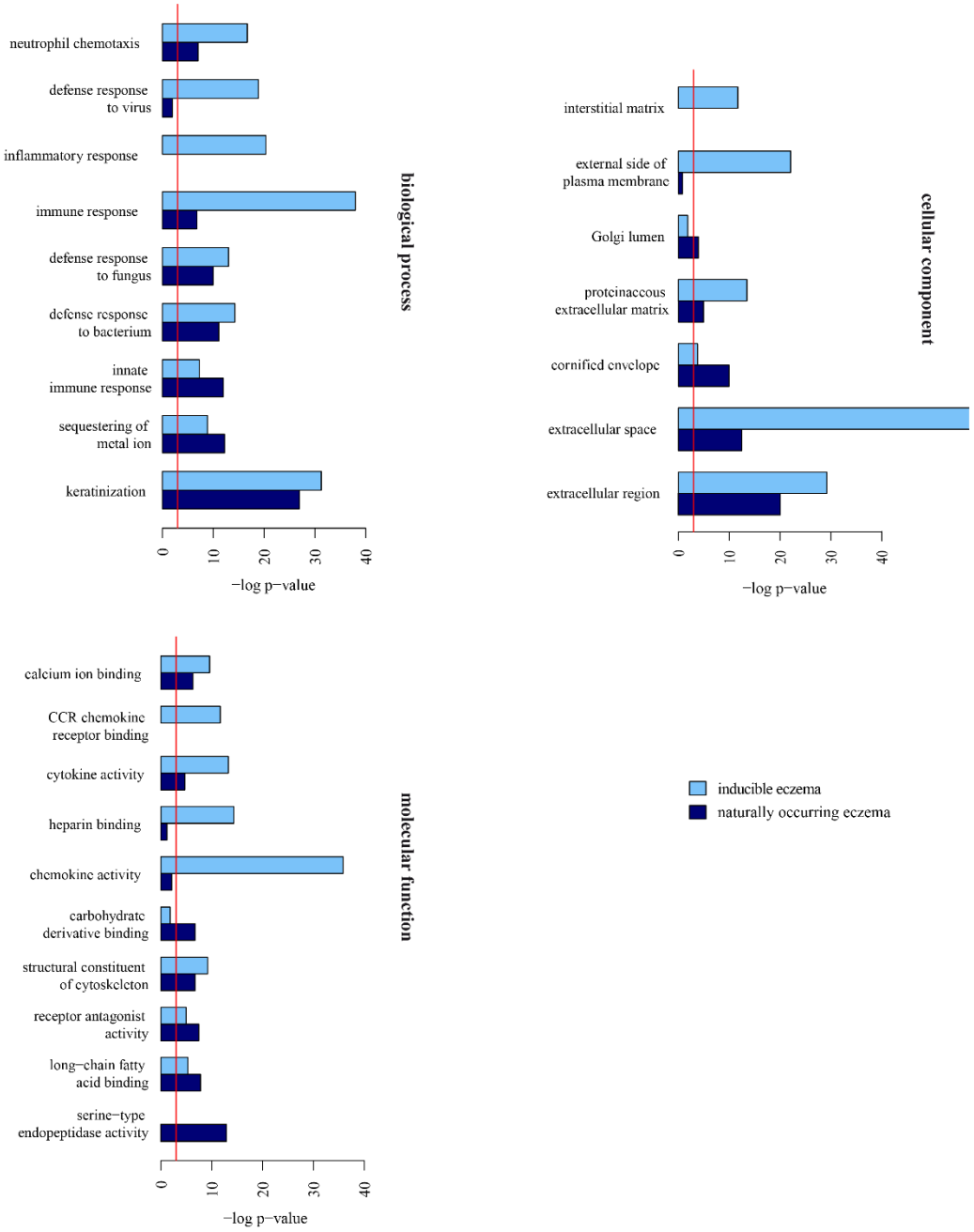


Figure 23: Signaling pathway analysis of naturally occurring and induced eczema.

The most significant hits for the GO terms “biological process”, “cellular component” and “molecular function” for both natural occurring eczema and inducible eczema (ACD) are depicted. The bar size indicated the level of significance (negative log p value); the vertical line shows the 0.05 significance level. Modified from Quaranta et al., 2014.

### 3.3.2 Intraindividual comparison of molecular signatures of psoriasis and eczema

After analyzing the heterogeneity within eczema subtypes we aimed to perform an intra-individual comparison of the molecular signatures of psoriasis and eczema in patients affected by both psoriasis and atopic or non-atopic eczema simultaneously (n = 24) using whole-genome expression arrays of lesional and autologous non-lesional skin.

#### 3.3.2.1 Significantly regulated genes in psoriasis and/or eczema

The number of genes that were significantly differentially regulated in psoriatic and eczematous skin, respectively, compared to non-involved skin in each patient was evaluated. It could be shown that among the 24 patients with coexisting psoriasis and either atopic, non-atopic or contact eczema 101 (77 upregulated, 24 downregulated) genes were exclusively expressed in psoriatic plaques, 39 (25 upregulated, 14 downregulated) genes were significantly regulated in eczematous, but not psoriatic skin, and 45 (28 upregulated, 17 downregulated) genes were regulated in both diseases (Figure 24).

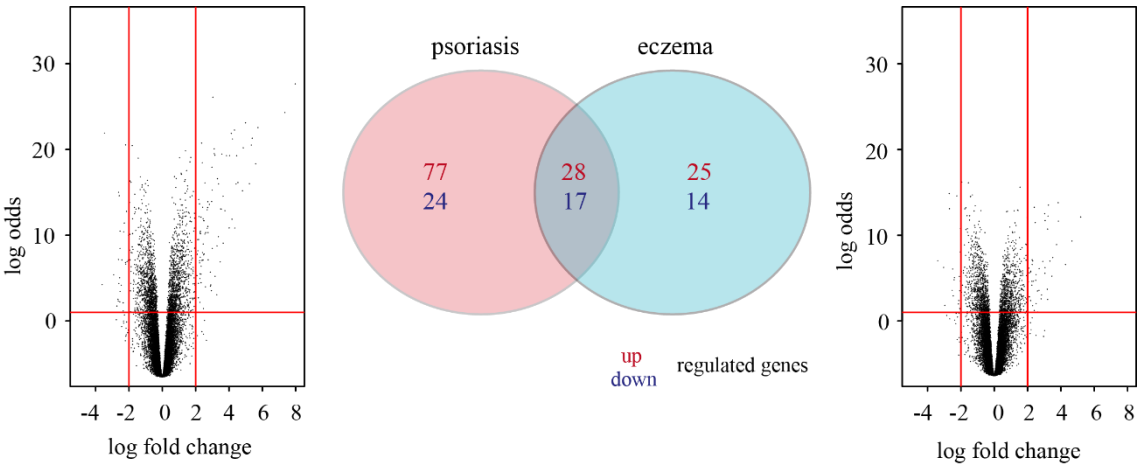


Figure 24: Intraindividual comparison of gene expression in psoriasis and eczema

Number of genes significantly up- or downregulated compared to autologous non-involved skin in patients suffering from psoriasis and a variant of eczema simultaneously (n = 24), as illustrated using Venn diagrams and Volcano plots. Modified from Quaranta et al., 2014.

#### 3.3.2.2 Function of significantly regulated genes and distinct and common signaling pathways

Genes significantly regulated in psoriatic as well as in eczematous skin as compared to non-involved skin were categorized into three groups: “immune system”, “epidermal

component" and "metabolism". The full list of genes significantly regulated in either psoriasis or eczema or regulated in both diseases is depicted in Figure 25. For each category, the most significant findings are outlined: Briefly, it could be demonstrated for the category of "immune system" that psoriasis was characterized by upregulation of genes encoding for cytokines of the IL-10 family (IL-19, IL-20) as well as genes encoding for IL-36G and IL-36A. Besides a trend for higher induction of Th17 associated cytokines such as IL-17A, IL-17F and IL-22 was observed (data not shown). Genes encoding for cytokines that were exclusively induced in eczema were IL-6 and the Th2 cytokine IL-13 (Figure 25), with a trend for a higher induction of other Th2 cytokines (IL-4, IL-5, and IL-10) than in psoriasis (data not shown).

For the compartment of "epithelium" for example, numerous antimicrobial peptides (AMPs) were found to be upregulated in both psoriasis and eczema such as the defensins *DEFB4* and *DEFB103B* and the S100 proteins *S100A7A, S100A7, S100A8, S100A9*, and *S100A12*. For the compartment of "metabolism" interestingly many genes involved in glucose, lipid, and amino acid metabolism were exclusively regulated in psoriatic, but not in eczematous skin, for example the phospholipase *PLA2G4D* and nitric oxide synthase 2 (iNOS or *NOS2* were upregulated in psoriatic plaques only (Figure 25). Subsequent pathway analysis using the Gene Ontology (GO) terms similarly showed commonly regulated pathways such as "innate immune response", "leukocyte cell-cell adhesion" and "defense response to bacteria and fungi", whereas pathways in the context of metabolism such as "serine type endopeptidase activity" where exclusively regulated in psoriasis (data not shown).

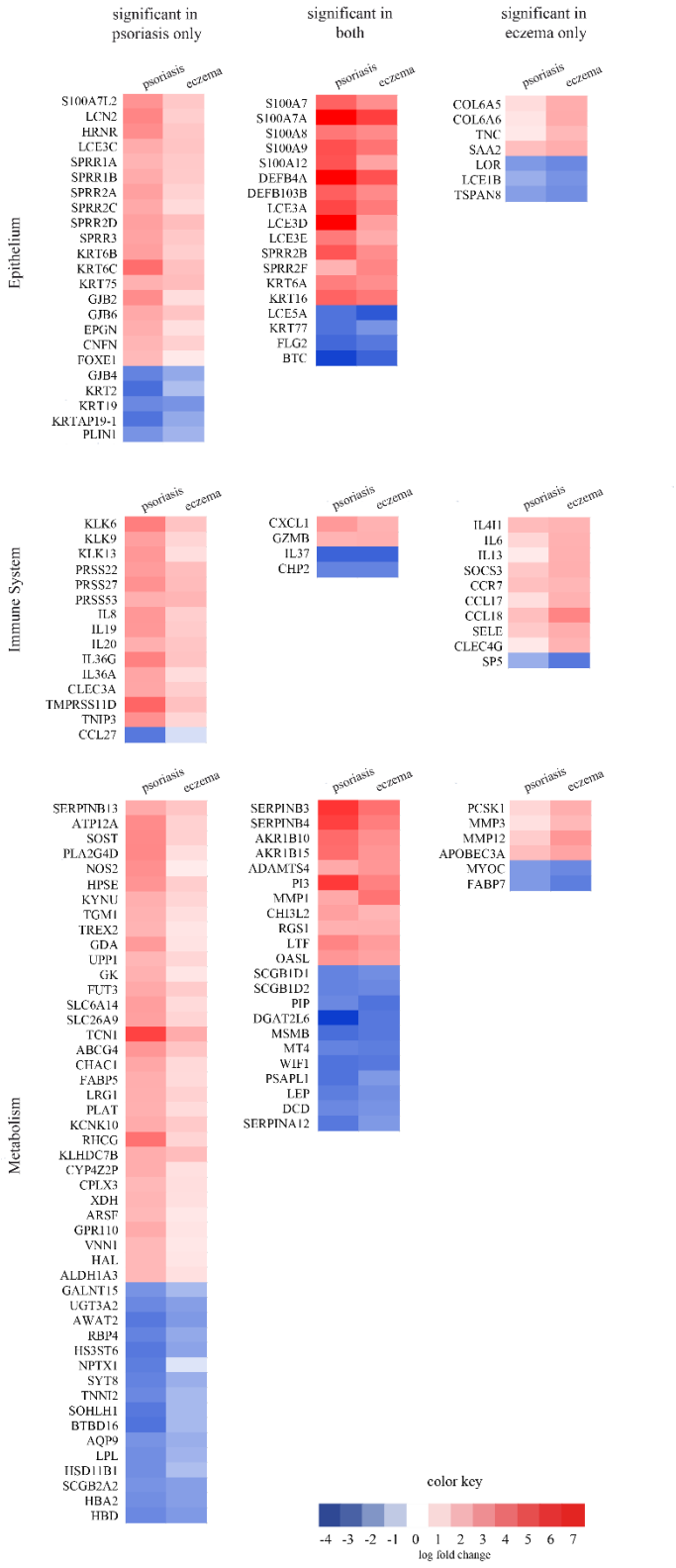


Figure 25: Genes up- or downregulated in psoriasis and/or eczema

Heat map of all genes significantly regulated in psoriasis (left side) and eczema (right side) or both (middle) within the same patients compared to non-involved skin. Histogram indicates the color code for $\log_2$ fold induction. Modified from Quaranta et al., 2014.

### 3.3.3 Establishing a disease classifier to distinguish psoriasis and eczema

Based on the different disease signatures between psoriasis and eczema variants on single gene and signaling pathway level we sought to translate these results into a molecular disease classifier (MC) that would be able to distinguish psoriasis from eczema. We pre-selected 15 genes according to the degree of significantly different expression (log fold induction as first criterion, followed by p value) in the whole-genome expression arrays throughout the 24 patients (Table 10, Figure 26 A).

Table 10: Preselection of the 15 most significantly differentially regulated genes between psoriasis and eczema

Genes that are chosen for the final molecular classifier (MC) are highlighted in red. Depicted are the full names, main function and assignment to the rough categories of "metabolism", "immune system" and "epidermis". Table modified from Quaranta et al., 2014.

| Target | Full name | Function | Category |
|---|---|---|---|
| *SOST* | Sclerostin | Inhibition of Wnt signaling | Metabolism |
| *PLA2G4D* | Phospholipase A2, group IVG | Widespread metabolic functions | Metabolism |
| *IL36G* | Interleukin-36G | Induces epidermal proliferation and AMPs | Immune System |
| *NOS2* | Inducible nitric oxide synthase | Stress-induced molecule with multiple functions on immune and metabolic processes | Metabolism, Immune System |
| *KLK13* | Kallikrein-related peptidase 13 | Induction of AMPs in the skin | Immune System |
| *GDA* | Guanine deaminase | Involved in purine metabolism | Metabolism |
| *IL36A* | Interleukin-36A | Induces epidermal proliferation and AMPs | Immune System |
| *TGM1* | Transglutaminase 1 | Formation of the cornified envelope | Epidermis |
| *NPTX1* | Neuronal pentraxin 1 | Involved in neuronal metabolism and damage | Metabolism |

| | | | |
|---|---|---|---|
| *CCL27* | Chemokine (C-C motif) ligand 27 | Binds to CCR10, promotes lymphocytes migration into the skin | Epidermis, Immune System |
| *CLEC4G* | C-type lectin family member 4 | Inhibits activation of $CD4^+$ T cells | Immune system |
| *IL-13* | Interleukin-13 | Acts on epithelium (inhibition of AMPs, induction of fibrosis, induction of chemokines) and on macrophages | Immune system |
| *TCN1* | Transcobalamin 1 | Vitamin B12 binding | Metabolism |
| *TMPRSS11D* | Serine transmembrane protease 11D | Preform of macrophage activating molecule | Immune System |
| *RHCG* | RH family, C glycoprotein | Ammonia transporter | Metabolism |

An independent cohort of 53 patients suffering from either psoriasis (n = 25) or eczema (n = 28) was established to train and test the classifier (Figure 27). For the training cohort of 19 patients (n = 9 for psoriasis and n = 10 for eczema), expression of the selected 15 genes was detected using real-time PCR (RT-PCR) in all 19 patients. Then a two-sample, two-sided Welch's t-test on the log-transformed measurements was performed, followed by a Bonferroni p value correction to assign each of the 15 genes with a p value. The primer sequences used for real-time PCR are given in Table 6. Amongst all possible combinations of the minimum of genes needed for correct classification, *CCL27* and *NOS2* were the genes with lowest adjusted p values (for significantly up- and downregulation, respectively, of psoriasis vs eczema). Based on these two genes, a classifier was eventually trained using a 10-fold cross-validation and support vector machines (SVMs). An average accuracy of 100% was achieved (Figure 26 B). With an independent third cohort (34 patients in total; 16 psoriasis patients and 18 eczema patients), the classifier was tested and could classify 33 out of 34 patients as predicted from clinical and histological evaluation (kappa = 0.94; Figure 26 C, Figure 27). Prediction probabilities of the patients are listed in Table 11.

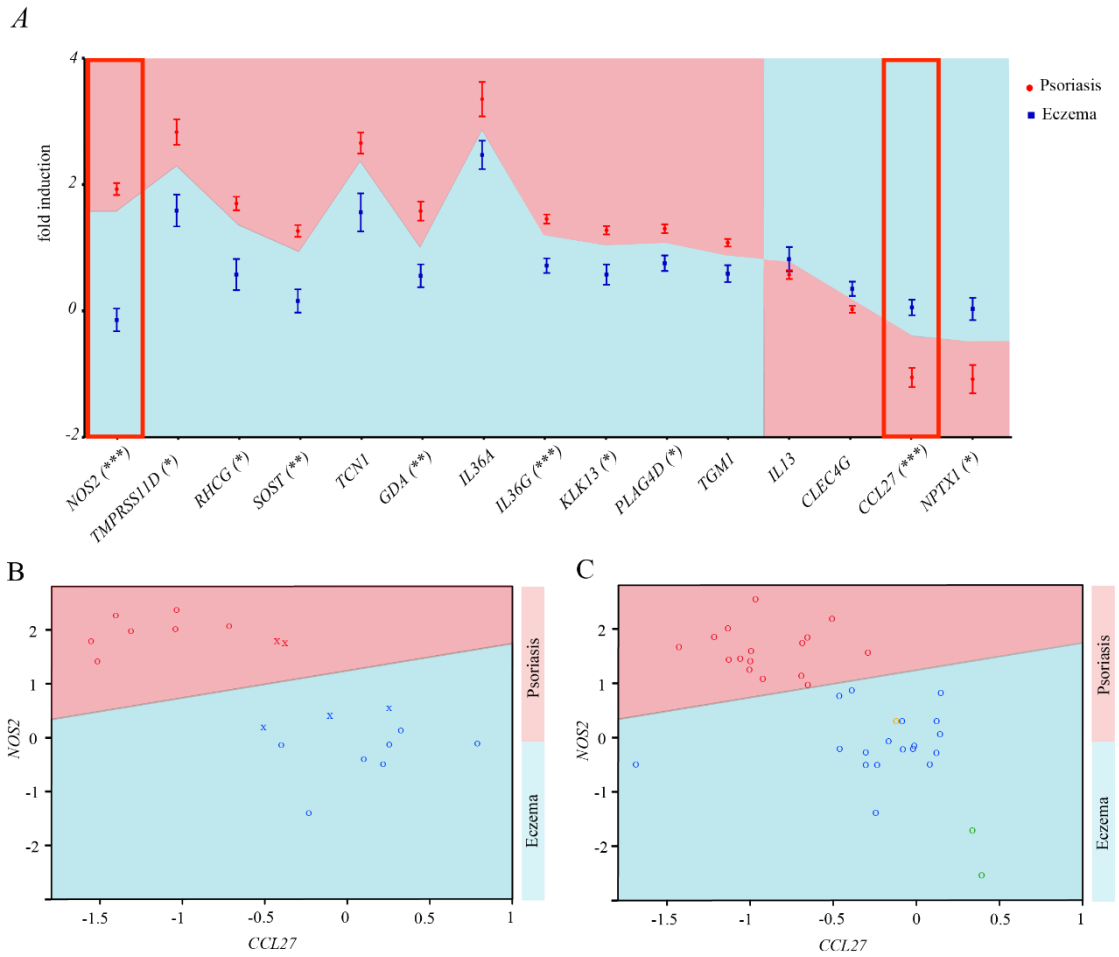


Figure 26: Establishing a molecular disease classifier (MC) for psoriasis and eczema

(A) The 15 most significantly differentially expressed genes between psoriasis end eczema were validated via real-time PCR in an independent training cohort of 9 psoriasis patients and 10 eczema patients. The curve indicates the cutoff value between the two groups for each gene. Red frames indicate the two genes chosen for the final classifier. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Values were log2 transformed. (B) A MC consisting of *NOS2* (y-axis) and *CCL27* (x-axis) accurately separates psoriasis and eczema patients in a training cohort consisting of 19 patients. Shown are data samples of the training set after log transformation and scaling. The crosses indicate the support vectors, the circles indicate the remaining data samples of the training set. (C) Performance of the MC in an independent test cohort (16 psoriasis patients and 18 eczema patients). The yellow circle shows one initially misclassified patient X, the green circles the clinically and histologically unclear patient Y illustrated in Figure 28 and Figure 29. Figure modified from Quaranta et al., 2014.

Table 11: Probabilities of the patients in the independent test cohort for the diagnosis of psoriasis or eczema according to the MC (n = 34)

Thirty-three patients were assigned to the assumed disease class (= pre-diagnosed disease). The case of inconsistency (highlighted in red) of the given diagnosis and the classifier diagnosis revealed that the initial diagnosis of psoriasis was most likely incorrect in this patient X (see also Figure 28). Y1 and Y2 (highlighted in green) indicate biopsies of a clinically and histologically unclear case Y (see also Figure 29). Table modified from Quaranta et al., 2014.

| Patient | Probability for Psoriasis | Probability for Eczema | Pre-diagnosed disease |
|---|---|---|---|
| 10 | 0.73454455126492 | 0.26545544873508 | Psoriasis |
| 11 | 0.815619318704725 | 0.184380681295275 | Psoriasis |
| 12 | 0.810770319202224 | 0.189229680797776 | Psoriasis |
| 13 | 0.902998777176718 | 0.0970012228232821 | Psoriasis |
| 14 | 0.782497003248794 | 0.217502996751206 | Psoriasis |
| 15 | 0.915904631532405 | 0.0840953684675953 | Psoriasis |
| 16 | 0.61874127772914 | 0.38125872227086 | Psoriasis |
| 17 | 0.96389272593258 | 0.0361072740674205 | Psoriasis |
| 18 | 0.715822983274895 | 0.284177016725106 | Psoriasis |
| 19 | 0.644981151651768 | 0.355018848348232 | Psoriasis |
| 20 | 0.914479385240275 | 0.0855206147597249 | Psoriasis |
| 21 | 0.931622478313302 | 0.0683775216866978 | Psoriasis |
| 31 | 0.00754799261752247 | 0.992452007382478 | Eczema |
| 32 | 0.152019527916118 | 0.847980472083881 | Eczema |
| 33 | 0.0865743764376922 | 0.913425623562308 | Eczema |
| 34 | 0.080652648492756 | 0.919347351507244 | Eczema |
| 35 | 0.0434395162155441 | 0.956560483784456 | Eczema |
| 36 | 0.0414636845956101 | 0.95853631540439 | Eczema |
| 37 | 0.044145491857433 | 0.955854508142567 | Eczema |
| 38 | 0.0302034132547937 | 0.969796586745206 | Eczema |
| 39 | 0.124359060796823 | 0.875640939203177 | Eczema |
| 41 | 0.0650797973346789 | 0.934920202665321 | Eczema |
| 42 | 0.0578485262412057 | 0.942151473758794 | Eczema |
| 43 | 0.0648233797155669 | 0.935176620284433 | Eczema |
| 44 | 0.277486993300571 | 0.722513006699429 | Eczema |
| X | 0.15255171664836 | 0.84744828335164 | Psoriasis |
| 57 | 0.0835325407704469 | 0.916467459229553 | Eczema |
| 58 | 0.526929416716535 | 0.473070583283465 | Psoriasis |

| | | | |
|---|---|---|---|
| 59 | 0.41419704296405 | 0.58580295703595 | Eczema |
| 60 | 0.840339816073202 | 0.159660183926798 | Psoriasis |
| Y1 | $1.5*10^{-9}$ | 0.999984800373679 | Eczema |
| Y2 | $5.42*10^{-7}$ | 0.999999457894581 | Psoriasis |
| 64 | 0.388592367461531 | 0.611407632538469 | Eczema |
| 65 | 0.861489182344632 | 0.138510817655368 | Psoriasis |
| 66 | 0.0594755619039556 | 0.940524438096044 | Eczema |
| 68 | 0.14892583189241 | 0.85107416810759 | Eczema |

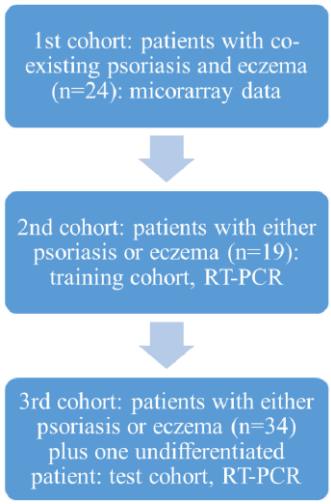


Figure 27: Flow chart of patient cohorts used for microarray data, classifier training and classifier testing. Figure taken from Quaranta et al., 2014.

One patient X was classified as eczema with a probability of 0.85, although the initial given diagnosis was psoriasis (Table 11). However, when back-tracing clinical and histological features of this patient, it became clear that the initial diagnosis psoriasis was most likely not correct. Clinically, the 54 year-old patient presented with disseminated, demarcated eczema-like skin lesions with centripetal desquamation that had erupted two months before (Figure 28 A-C). Histological evaluation revealed neutrophil micro-abscesses, spongiosis, single cell necrosis in the epidermis and an epidermotropism of immune cells (Figure 28 D and E). Other hallmarks for psoriasis such as acanthosis and epidermal thinning above dermal papillae containing dilated and tortuous capillaries were not observed. In line with that observation, the patient did not respond well to dithranol,

a classical topical psoriasis treatment. Furthermore, skin lesions did not relapse after remission. Retrospectively, other diagnoses like pityriasis rosea, eczema or pityriasis lichenoides chronica were clearly to be favored in this patient.

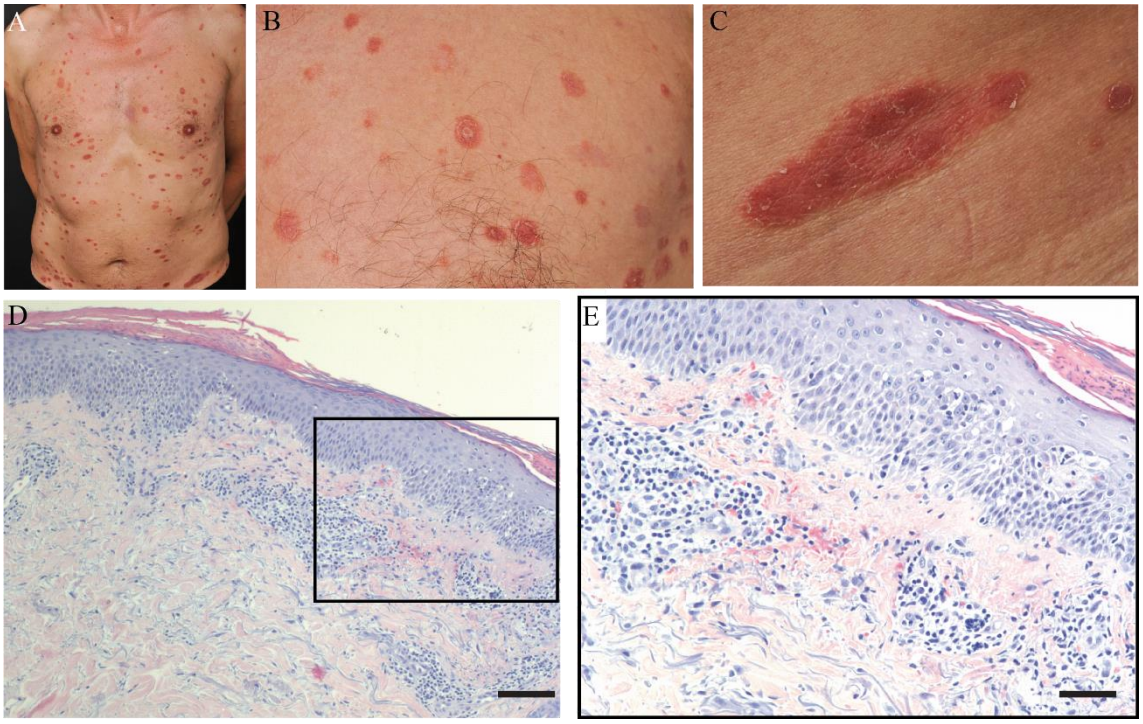


Figure 28: Initially misdiagnosed patient X detected by the MC

Clinical and histological presentation of a patient initially misdiagnosed as psoriasis as detected by the MC. The clinical picture (A-C) did not show typical psoriasiform plaques with scaling. Histological examination (D, E) did not reveal psoriasis specific features such as epidermal hyperplasia and epidermal thinning above dermal papillae. Instead, spongiosis of epidermis dominates the histological picture. (Scale bar in D 100 µm (overview) and in E 50 µm (inset)). Figure modified from Quaranta et al., 2014.

Apart from testing patients for which a clear diagnosis based on clinical or histological picture has been made, one patient was tested with the MC where the gold standard methods of clinical eye and histopathology could not distinguish between psoriasis and eczema (Figure 29). The female 53 year-old patient has suffered from skin lesions for years. Eczema could have been favored because she also had allergic asthma, and mildly elevated IgE levels (108 IU/ml; reference value: < 100 IU/ml) and the lesions were itchy. However, the family history for psoriasis was positive and the stationary plaques at extensor surfaces were rather typical for psoriasis. In addition, the histological evaluation was conflicting: On the one hand, there was plump acanthosis, partially missing stratum granulosum and parakeratosis rather accounting for psoriasis. On the other hand, T cell epidermotropism and very few neutrophils with missing microabscesses were more typical for

eczema. When this patient was tested in the classifier, two biopsies from two different lesion sites were classified as eczema with a probability above 99% (Table 11), indicating the classifier might be superior to current gold standard diagnostic tools.

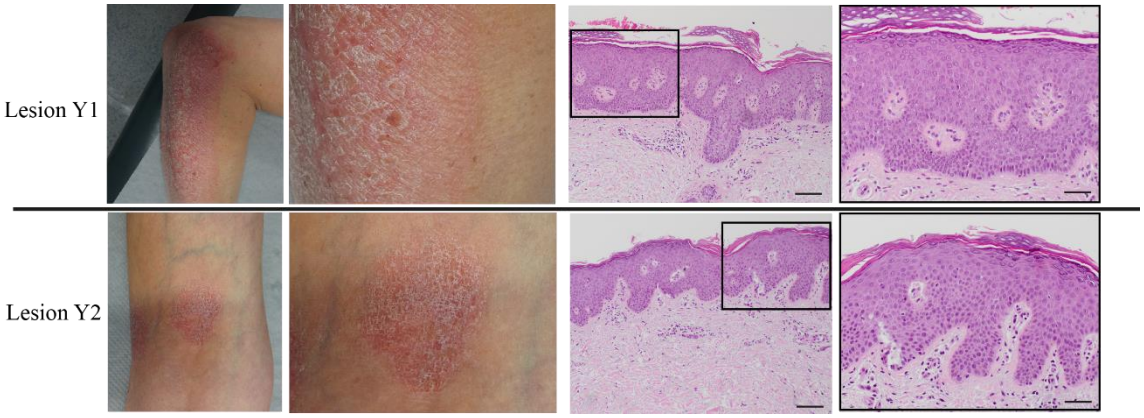


Figure 29: Clinically and histologically unclear patient Y tested by the MC

Clinical presentation of an unclear patient showing both features of psoriasis (Y1) and eczema (Y2). Histological examination for both specimens showed hallmarks of psoriasis such as parakeratosis and hypogranulosis as well as classical features of eczema such as T cell epidermotropism and missing neutrophils. (Scale bars 100 µm (overview) and 50 µm (inset)). Figure modified from Quaranta et al., 2014.

#### 3.3.3.1 The molecular disease classifier (MC) consisting of *NOS2* and *CCL27* precisely separates clear cases of psoriasis and eczema in a larger cohort

To validate our molecular classifier for future clinical use we applied it to a new, larger follow-up cohort of 85 patients suffering from clear psoriasis (44 patients, 45 samples) or eczema (41 patients, 44 samples). In this cohort, also samples from other clinics were included to test the classifier for reproducibility under different conditions (examiners, biopsy acquisition technique etc.). To refine probability prediction for single patients logistic regression instead of the R package "e1071" using support vector machines (SVM) was applied to the RT-PCR data set to retrain the MC. For each sample, disease probabilities for both the diagnosis of psoriasis and eczema were calculated. A cut-off probability value of 55% for clear prediction was chosen and eventually 87 out of 89 samples were assigned to the correct diagnosis. Test specificity for psoriasis (eczema) was 100% (97.7%), sensitivity was 97.7% (100%) and the AUC (area under the ROC curve) was 0.9929. The MC was then tested for robustness using a 10-fold cross validation yielding a specificity for psoriasis (eczema) of 100% ± 0% (96% ± 8.4 %), a sensitivity of 96% ± 8.4% (100% ± 0%) and an AUC of 99% ± 3.2%. One psoriasis patient was misclassified as eczema and another psoriasis patient was assigned to the "grey zone" of diagnostic

reliability with a probability of 45% for psoriasis (Figure 30 A and B). Prediction probabilities of all samples are listed in Table 12. As autologous healthy skin cannot be routinely obtained in clinical practice, we tested if the MC would also perform with high sensitivity and specificity without autologous healthy skin. Therefore, we calculated a mean Ct value for *18S*, *NOS2* and *CCL27* from non-lesional skin samples of all patients and used these values as calibrators for relative quantification of *NOS2* and *CCL27* transcripts. The MC based on pooled healthy skin ($MC_{pooled}$) still performed with a comparable test sensitivity of 97.8% and a specificity of 97.8% for both the diagnosis of psoriasis and eczema (prediction probabilities not shown). Apart from the 85 patients, 3 additional patients (in total n = 88) without autologous healthy skin could be diagnosed correctly with $MC_{pooled}$ (Figure 30 C and D).

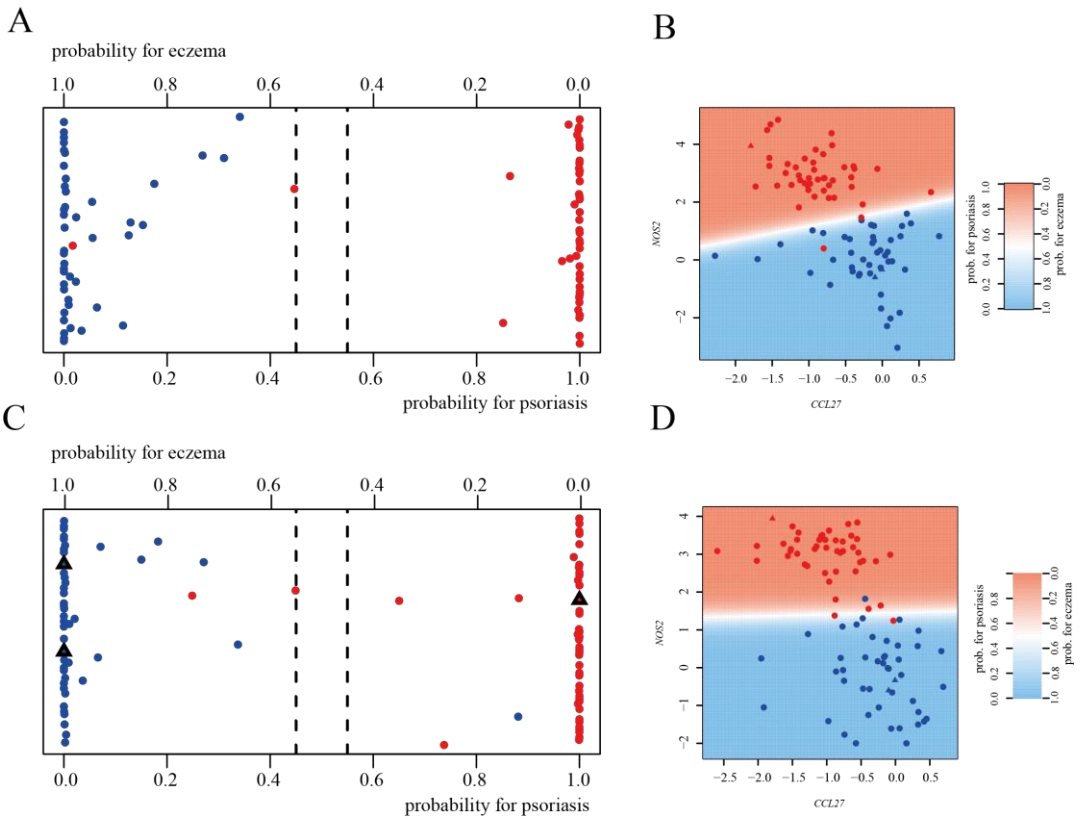


Figure 30: The MC tested on clear cases of psoriasis and eczema

The molecular classifier (MC) was applied to clear cases of psoriasis and eczema. In (A and B) RT-PCR values of *CCL27* and *NOS2* were normalized to autologous healthy skin (n = 89 samples), whereas in (C and D) values were normalized to pooled healthy skin (mean Ct values of *CCL27*, *NOS2* and 18S, n = 92 samples). Samples with corresponding healthy skin are represented as circles (blue = eczema, red = psoriasis), while samples with missing corresponding autologous skin in (C) are depicted as triangles. Prediction probabilities for both diseases are indicated on upper and lower sides of each graph. The scatterplots in (B) and (D) show the gene expression of *NOS2* and *CCL27*, dots represent patients, color-coded according to disease state.

Table 12: Probabilities for the diagnosis of psoriasis and eczema given by MC for the validation cohort

According to MC 87 out of 89 samples (n samples = 89, n patients = 85) were assigned to the assumed disease class. One misdiagnosed patient (labeled in red) and one undefined patient (labeled in blue) was found in the cohort of psoriasis.

| ID | Label | Pred. label | Prob. psoriasis | Prob. eczema |
|---|---|---|---|---|
| 1 | AE | AE | 0.02372685 | 0.97627315 |
| 2 | Pso | Pso | 0.99342309 | 0.00657691 |
| 3 | AE | AE | 0.00365157 | 0.99634843 |
| 4 | Pso | Pso | 0.99998358 | 1.6422E-05 |
| 5 | AE | AE | 5.3303E-08 | 0.99999995 |
| 6 | AE | AE | 0.02321239 | 0.97678761 |
| 7 | Pso | Pso | 0.99999998 | 2.3153E-08 |
| 8.1 | AE | AE | 0.26880659 | 0.73119341 |
| 8.2 | AE | AE | 0.06406988 | 0.93593012 |
| 9 | Pso | Pso | 0.99999995 | 5.1618E-08 |
| 10 | AE | AE | 0.05467448 | 0.94532552 |
| 11 | AE | AE | 2.8136E-08 | 0.99999997 |
| 12 | AE | AE | 2.0289E-10 | 1 |
| 13 | AE | AE | 4.7659E-05 | 0.99995234 |
| 14 | AE | AE | 0.00371276 | 0.99628724 |
| 15 | AE | AE | 9.5207E-05 | 0.99990479 |
| 16 | Pso | Pso | 0.99961475 | 0.00038525 |
| 17 | Pso | Pso | 0.99912289 | 0.00087711 |
| 18 | Pso | Pso | 0.98090588 | 0.01909412 |
| 19 | AE | AE | 0.00027062 | 0.99972938 |
| 20 | Pso | Pso | 0.99999999 | 1.3834E-08 |
| 21 | AE | AE | 0.0128862 | 0.9871138 |
| 22 | Pso | Pso | 0.99979259 | 0.00020741 |
| 23 | AE | AE | 0.00140237 | 0.99859763 |
| 24 | AE | AE | 0.00085995 | 0.99914005 |
| 25 | AE | AE | 0.00028964 | 0.99971036 |
| 26 | Pso | Pso | 0.96570363 | 0.03429637 |
| 27 | AE | AE | 0.01174046 | 0.98825954 |
| 28 | Pso | Pso | 0.9985979 | 0.0014021 |
| 29 | Pso | Pso | 0.99944595 | 0.00055405 |
| 30 | Pso | Pso | 0.99977722 | 0.00022278 |
| 31 | Pso | Pso | 0.99986835 | 0.00013165 |

| | | | | |
|---|---|---|---|---|
| 32 | Pso | Pso | 0.99956666 | 0.00043334 |
| 33 | Pso | Pso | 0.99890079 | 0.00109921 |
| 34.1 | Pso | Pso | 0.99974511 | 0.00025489 |
| 34.2 | Pso | Pso | 0.99999938 | 6.2375E-07 |
| 35 | AE | AE | 1.8548E-07 | 0.99999981 |
| 36 | AE | AE | 0.00013636 | 0.99986364 |
| 37 | Pso | Pso | 0.97826746 | 0.02173254 |
| 38 | AE | AE | 0.00030296 | 0.99969704 |
| 39 | AE | AE | 0.34112635 | 0.65887365 |
| 40 | Pso | Pso | 0.99923957 | 0.00076043 |
| 41 | Pso | AE | 0.44665496 | 0.55334504 |
| 42 | Pso | Pso | 0.99998204 | 1.7959E-05 |
| 43 | Pso | Pso | 0.99998583 | 1.4173E-05 |
| 44 | AE | AE | 0.17525914 | 0.82474086 |
| 45 | AE | AE | 1.7639E-06 | 0.99999824 |
| 46 | Pso | Pso | 0.99990659 | 9.3408E-05 |
| 47 | AE | AE | 0.00084759 | 0.99915241 |
| 48 | AE | AE | 0.12923772 | 0.87076228 |
| 49 | Pso | Pso | 0.99995938 | 4.0616E-05 |
| 50 | AE | AE | 0.03426869 | 0.96573131 |
| 51 | AE | AE | 0.15311791 | 0.84688209 |
| 52 | Pso | Pso | 0.86483136 | 0.13516864 |
| 53 | AE | AE | 0.00210424 | 0.99789576 |
| 54 | AE | AE | 0.05572508 | 0.94427492 |
| 55 | AE | AE | 7.5461E-05 | 0.99992454 |
| 56 | AE | AE | 4.072E-05 | 0.99995928 |
| 57 | AE | AE | 7.5142E-05 | 0.99992486 |
| 58 | Pso | Pso | 0.99492332 | 0.00507668 |
| 59.1 | AE | AE | 0.114528 | 0.885472 |
| 59.2 | AE | AE | 0.00051142 | 0.99948858 |
| 60 | AE | AE | 0.31031725 | 0.68968275 |
| 61 | AE | AE | 0.00175779 | 0.99824221 |
| 62 | Pso | Pso | 0.99994862 | 5.1379E-05 |
| 63 | Pso | Pso | 0.99953221 | 0.00046779 |
| 64 | Pso | Pso | 0.99931906 | 0.00068094 |
| 65 | AE | AE | 0.00861326 | 0.99138674 |

| | | | | |
|---|---|---|---|---|
| 66 | Pso | Pso | 0.9999946 | 5.4009E-06 |
| 67 | AE | AE | 0.00228706 | 0.99771294 |
| 68 | Pso | Pso | 0.99997663 | 2.3367E-05 |
| 69 | Pso | Pso | 0.99711005 | 0.00288995 |
| 70 | Pso | Pso | 0.99999499 | 5.0132E-06 |
| 71 | AE | AE | 0.00034714 | 0.99965286 |
| 72 | Pso | Pso | 0.99930238 | 0.00069762 |
| 73 | Pso | Pso | 0.99861272 | 0.00138728 |
| 74 | Pso | Pso | 0.99870089 | 0.00129911 |
| 75 | Pso | Pso | 0.99928673 | 0.00071327 |
| 76 | Pso | Pso | 0.99921049 | 0.00078951 |
| 77 | Pso | Pso | 0.99967241 | 0.00032759 |
| 78 | Pso | AE | 0.01709895 | 0.98290105 |
| 79 | Pso | Pso | 0.98988636 | 0.01011364 |
| 80 | Pso | Pso | 0.85144383 | 0.14855617 |
| 81 | Pso | Pso | 0.99999561 | 4.3858E-06 |
| 82.1 | AE | AE | 0.00929111 | 0.99070889 |
| 82.2 | AE | AE | 9.13E-09 | 0.99999999 |
| 83 | AE | AE | 0.12567759 | 0.87432241 |
| 84 | AE | AE | 0.00017518 | 0.99982482 |
| 85 | Pso | Pso | 0.99744343 | 0.00255657 |

#### 3.3.3.2 MC identifies subtypes of psoriasis and eczema

After having successfully tested patients suffering from clear classical plaque psoriasis on the MC, we next applied the MC on patients with nummular eczema (n = 8), psoriatic and eczematous hand, foot or scalp lesions (n = 6), erythroderma (n = 2), guttate psoriasis (n = 6) and inverse psoriasis (n = 3). Besides, patients suffering from both psoriatic and eczematous lesions concomitantly (n = 6) were tested to confirm co-existence of both diseases. Twenty-nine out of 31 patients were assigned to the correct diagnosis with the MC trained on autologous healthy skin (Figure 31, prediction probabilities not shown). One patient with hand eczema could not be given an accurate diagnosis (probability for psoriasis/eczema = 0.49/0.51) and two more patients (one patient with guttate psoriasis and one patient with coexistent psoriasis and eczema) were not correctly classified according to clinical and histolopathological presentation.

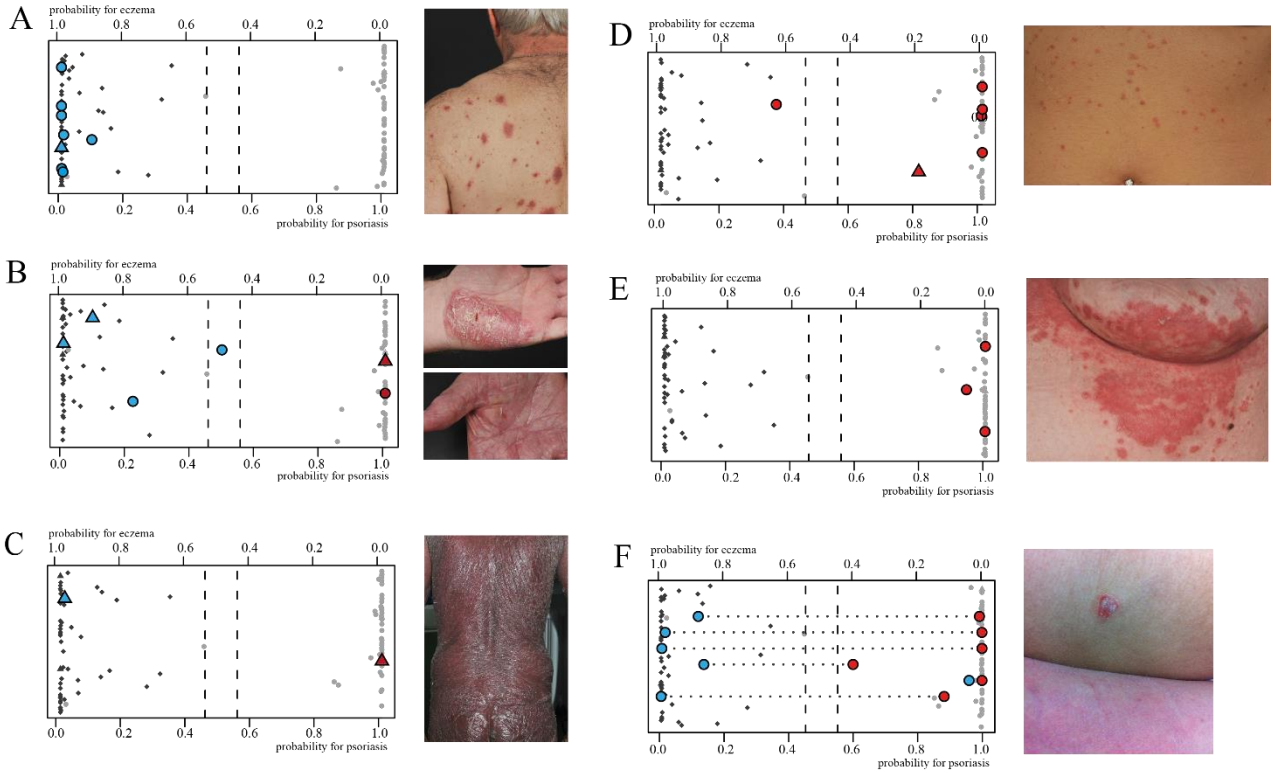


Figure 31: Application of the MC on subtypes of psoriasis and eczema

Subtypes of psoriasis (indicated in red) and eczema (indicated in blue) including nummular eczema (A), hand/feet and scalp lesions (B), erythroderma (C), guttate psoriasis (D), inverse psoriasis (E) and patients suffering from coexisting psoriasis and eczema (F) were tested on the MC. Each sample is represented as a colored circle; samples without corresponding autologous skin are depicted as colored triangles. Grey dots in the background represent the clear cases of psoriasis and eczema from Figure 30.

#### 3.3.3.3 Psoriasis and eczema are distinguishable by the MC on protein level using immunofluorescence stainings

We could show that classification of psoriasis and eczema on the level of mRNA by RT-PCR was feasible without the prerequisite of comparing the expression of *NOS2* and *CCL27* to their corresponding baseline levels in autologous healthy skin. Thus, we subsequently followed the hypothesis that the MC would not only work on RNA level but also on protein level. Therefore, we established immunofluorescence stainings on formalin-fixed paraffin-embedded (FFPE) sections for the corresponding proteins iNOS and CCL27. In total, 41 FFPE sections of lesional skin from clear patients suffering from psoriasis and eczema that have already been tested in previous experiments for *NOS2* and *CCL27* in RT-PCR experiments were randomly picked and stained for iNOS and CCL27. Immunofluorescence was visualized using an inverted epifluorescence microscope (green channel: iNOS, red channel: CCL27, blue channel: DAPI). After normalization to unspecific background fluorescence, eczema samples were characterized by lower iNOS signal

as compared to psoriasis samples (intensity of green fluorescence: $4.05x10^6 \pm 2.1x10^5$ in psoriasis; $3.24x10^6 \pm 1.4x10^5$ in eczema, $p = 0.0047$, Figure 32 and Figure 33). In contrast to iNOS showing quantitative difference of expression between psoriasis and eczema, CCL27 was demonstrated to show qualitative expression differences: Here, CCL27 protein was rather detected in the nucleus in eczema samples, whereas in psoriasis CCL27 protein was characterized by cytoplasmic distribution (Figure 32 and Figure 33). Both quantitative and morphological findings were quantified by an image analysis program based on the three criteria of a) mean intensity of green fluorescence, b) Fourier transformation analysis in the red and blue channel and c) convolution analysis using the signals from the red and blue channel. Samples were plotted in three-dimensional space for visualization of the two separate diagnostic groups of psoriasis and eczema (Figure 33). Two out of 18 eczema samples were plotted in the "psoriasis cloud" and one psoriasis sample out of 23 was plotted in the "eczema cloud", resulting in a test specificity for psoriasis (eczema) of 88.9% (95.7%) and a test sensitivity for psoriasis (eczema) of 95.7% (88.9%).

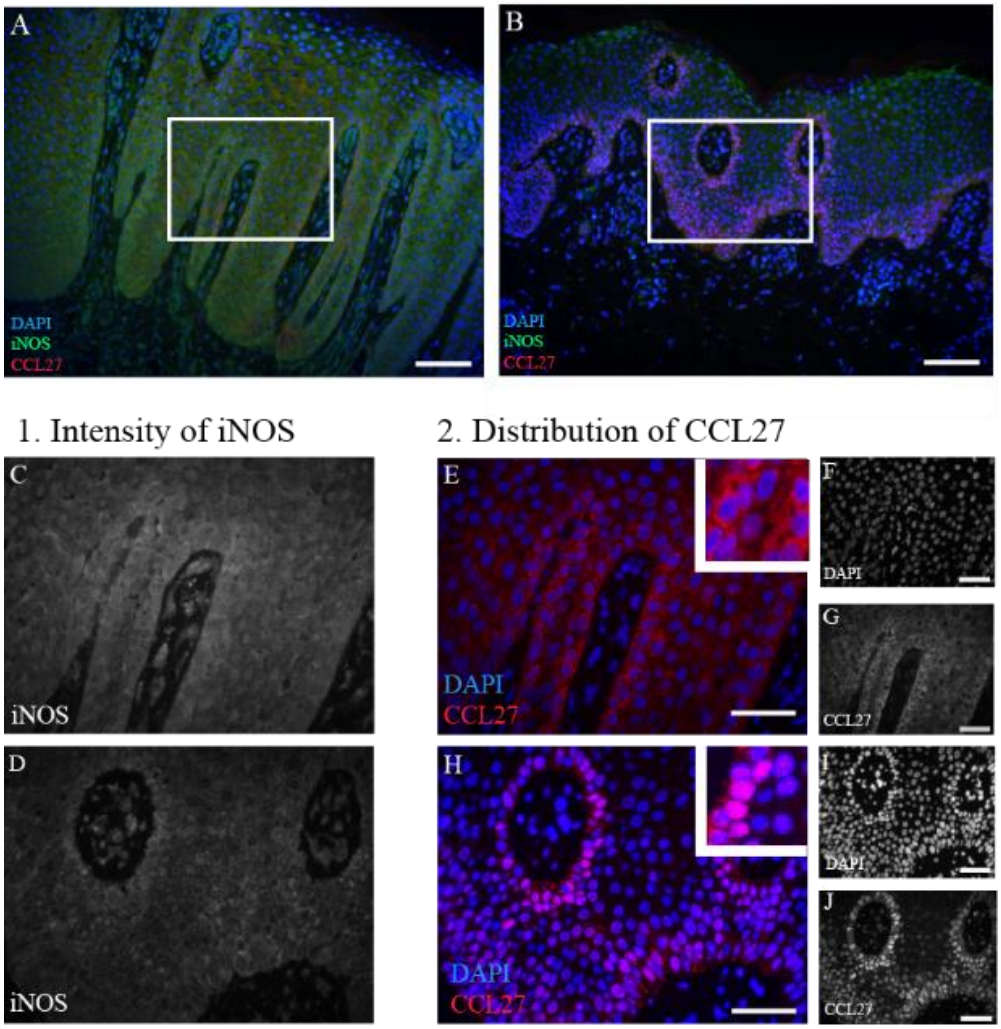


Figure 32: Immunofluorescent stainings of the two markers iNOS and CCL27 on FFPE sections of psoriasis and eczema

Immunofluorescent stainings of iNOS (green) and CCL27 (red) on FFPE sections of psoriasis (A) and eczema (B). Higher magnification view of boxes in (A) and (B) are depicted in (C-J). Compared to eczema, n = 18, (D), iNOS expression is more prominent in psoriasis, n = 23, (C). CCL27 shows rather nuclear localization in eczema (H-J) in comparison to psoriasis where CCL27 is distributed in the cytoplasm (E-G). Insets in (E) and (H) highlight distribution differences of CCL27 and are at a magnification of 2x. (Scale bars in A and B: 100µm. Scale bars in C-J: 50 µm).

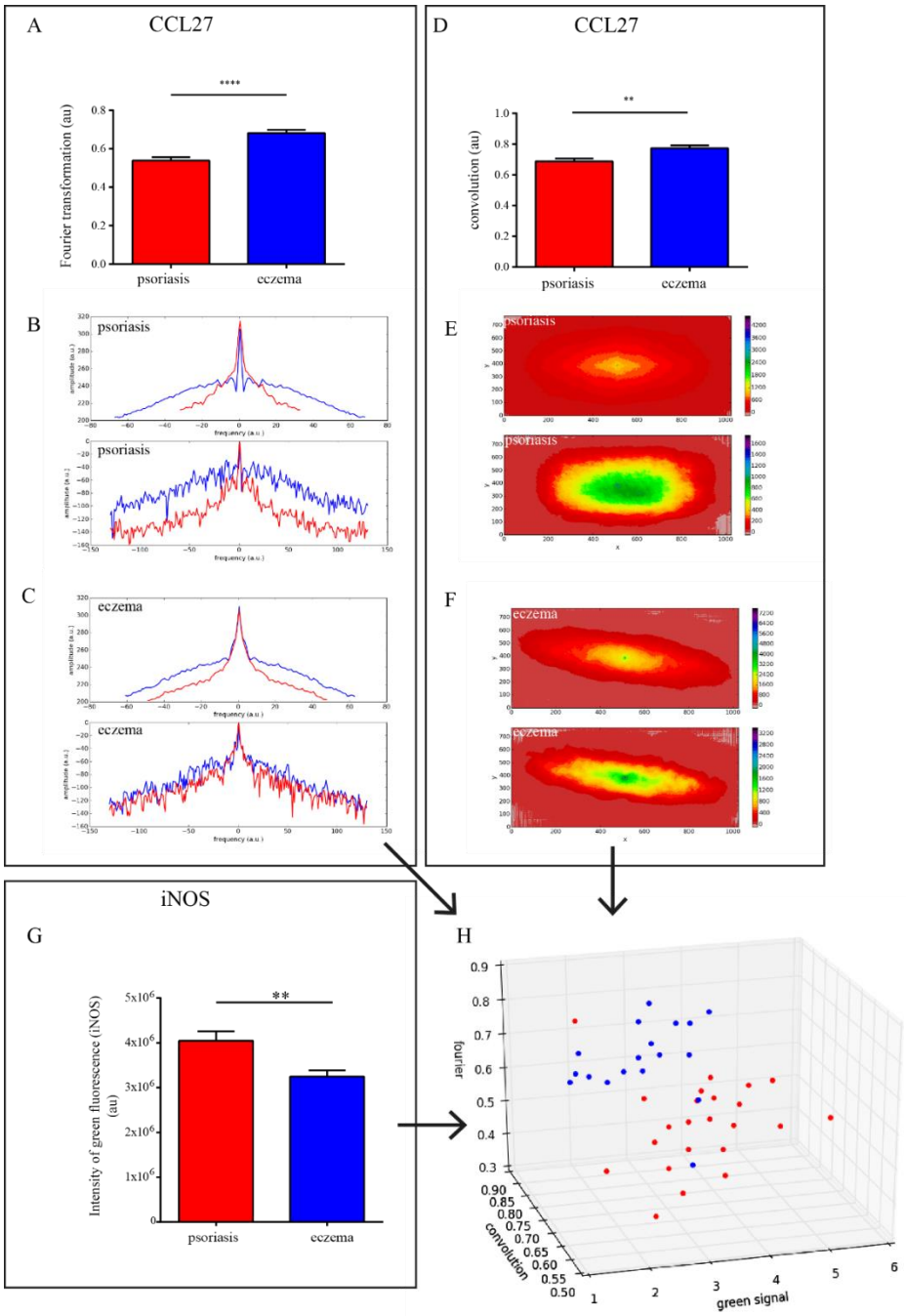


Figure 33: Multi-step image analysis of immunofluorescent stainings of FFPE sections of psoriasis (n = 23) and eczema (n = 18).

Nuclear staining pattern of CCL27 in eczema in contrast to the rather cytoplasmic distribution of CCL27 in psoriasis is captured by Fourier transformation (A-C) and convolution (D-F). Eczema shows similar frequency distribution of signals in the DAPI and CCL27 channel in contrast to psoriasis resulting in higher values for the parameter of Fourier transformation in eczema (0.68 ± 0.02) (C) as compared to psoriasis (0.54 ± 0.02) (B). In addition, convolution of DAPI and CCL27 channel is higher in eczema (0.77 ± 0.02) (F) than in psoriasis (0.68 ± 0.02), (E). Intensity of green fluorescent signal and therefore iNOS expression ($\sum m_{green}$) is more prominent in psoriasis ($4.05x10^6 \pm 2.1x10^5$ ) than in eczema ($3.24x10^6 \pm 1.4x10^5$) (G). Means and SEM are plotted in (A), (D) and (G). Results from all three operations reflecting the qualitative and quantitative staining patterns for psoriasis and eczema were consolidated in an image program resulting in a 3D plot visualizing the psoriasis (red) and eczema (blue) cloud (H).

#### 3.3.3.4 *NOS2* and *CCL27* correlate with hallmarks of psoriasis and eczema

We could successfully demonstrate that the MC enabled clear separation between psoriasis and eczema for classical types and subtypes of psoriasis and eczema on the level of mRNA and validated the relevance of both markers on protein level. Thus, the next obvious hypothesis to be proven was if the markers also correlate with well-established hallmarks of psoriasis and eczema, thus highlighting their biological relevance. We validated the discriminatory power of *NOS2* and *CCL27* in separating psoriasis from eczema by using anamnestic, clinical, histopathological and laboratory characteristics of psoriasis and eczema. Forty-two parameters (11 clinical, nine anamnestic, 15 histological and seven laboratory characteristics) from all patients were examined for association or correlation with mRNA levels of *NOS2* and *CCL27* (Figure 34, Table 13). *NOS2* levels were shown to be significantly associated with histological parameters assigned to psoriasis whereas the most significant ones were hypogranulosis ($p = 1.65x10^{-7}$), microabscess ($p = 4.02x10^{-6}$) and dilated dermal capillaries ($p = 4.93x10^{-5}$). Moreover, there was a marked positive association of *NOS2* with clinical parameters such as BMI ($p = 0.012$) and infect associated exacerbation ($p = 0.07$). In contrast, *CCL27* was negatively associated with BMI ($p = 0.029$), positively associated with allergic rhinoconjunctivitis ($p = 0.009$) and on histological level negatively associated with dilated dermal capillaries ($p = 0.005$). Apart from *NOS2* levels which showed a trend to be positively associated with PASI (p = n.s.), there was no further association of *NOS2* and *CCL27* with disease scores (Figure 35).

Table 13: Clinical and histological attributes significantly associated with *NOS2* and *CCL27* expression

| *NOS2* | | *CCL27* | |
|---|---|---|---|
| Parameter | p value | Parameter | p value |
| Infect associated exacerbation | 0.074686364 | Allergic rhinoconjunctivitis | 0.009166069 |
| Allergic rhinoconjunctivitis | 0.021569178 | Asthma | 0.086713857 |
| Scalp involved | 0.074686364 | Nails involved | 0.086713857 |
| BMI | 0.013816891 | BMI | 0.029097858 |
| Pruritus | 0.02229509 | Hypogranulosis | 0.037396988 |
| Papillomatosis | 0.006610122 | Dilated dermal capillaries | 0.004955004 |
| Hyperkeratosis | 0.074686364 | Microabscess | 0.086713857 |
| Microabscess | 4.02112E-06 | | |
| Neutrophils | 0.00148334 | | |
| Eosinophils | 0.009759676 | | |
| S. *aureus* | 0.059292868 | | |

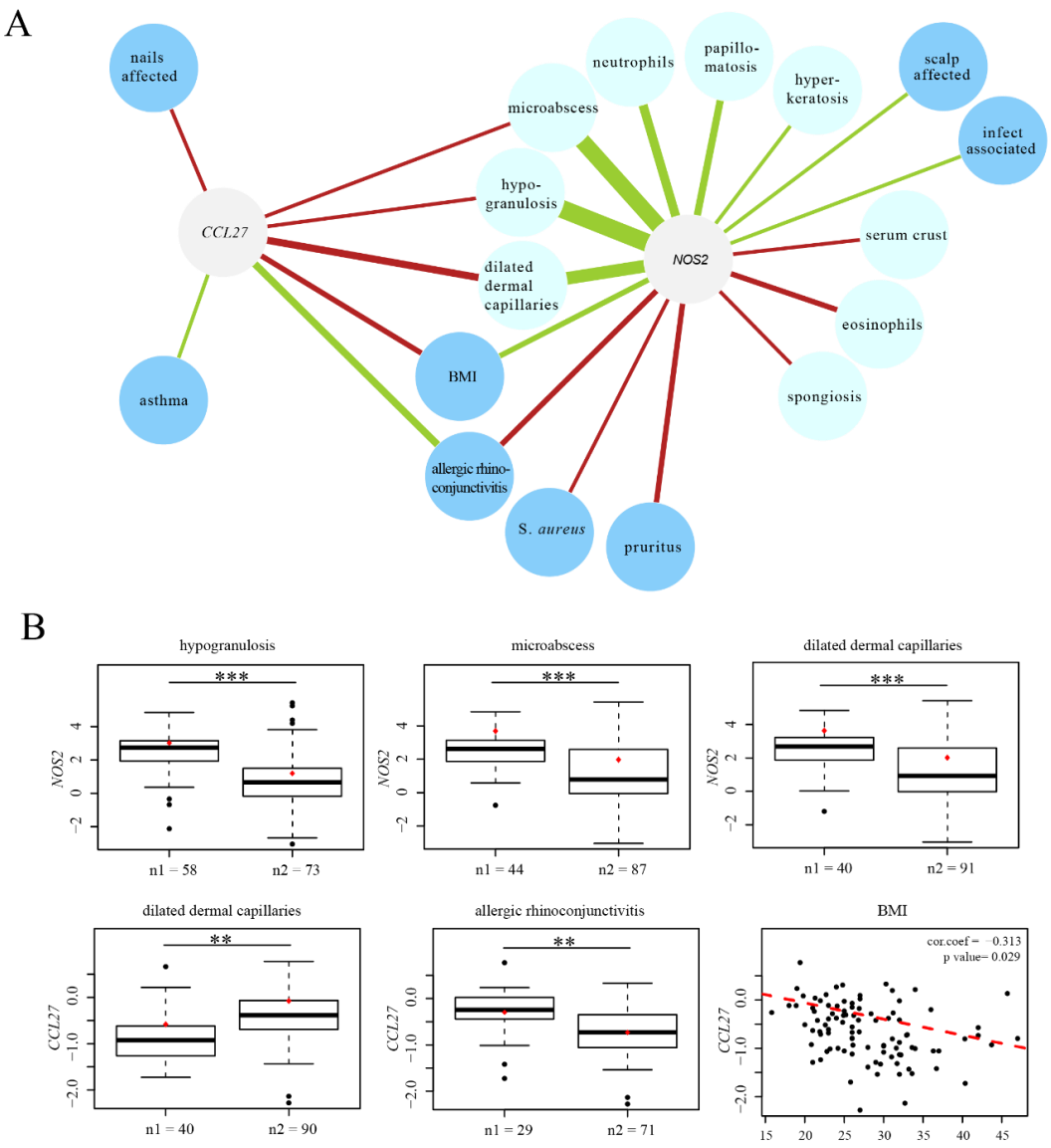


Figure 34: Correlation of *NOS2* and *CCL27* with hallmarks of psoriasis and eczema

Validation of *NOS2* and *CCL27* using anamnestic, clinical and laboratory parameters (indicated in dark blue) as well as histological parameters (indicated in light blue) (A). Green lines indicate positive associations, red lines negative association. Levels of significance are represented by the size of lines with thick lines indicating high significance levels. n1 = feature present, n2 = feature not present. The most significant associations for both *CCL27* and *NOS2* are shown in (B).

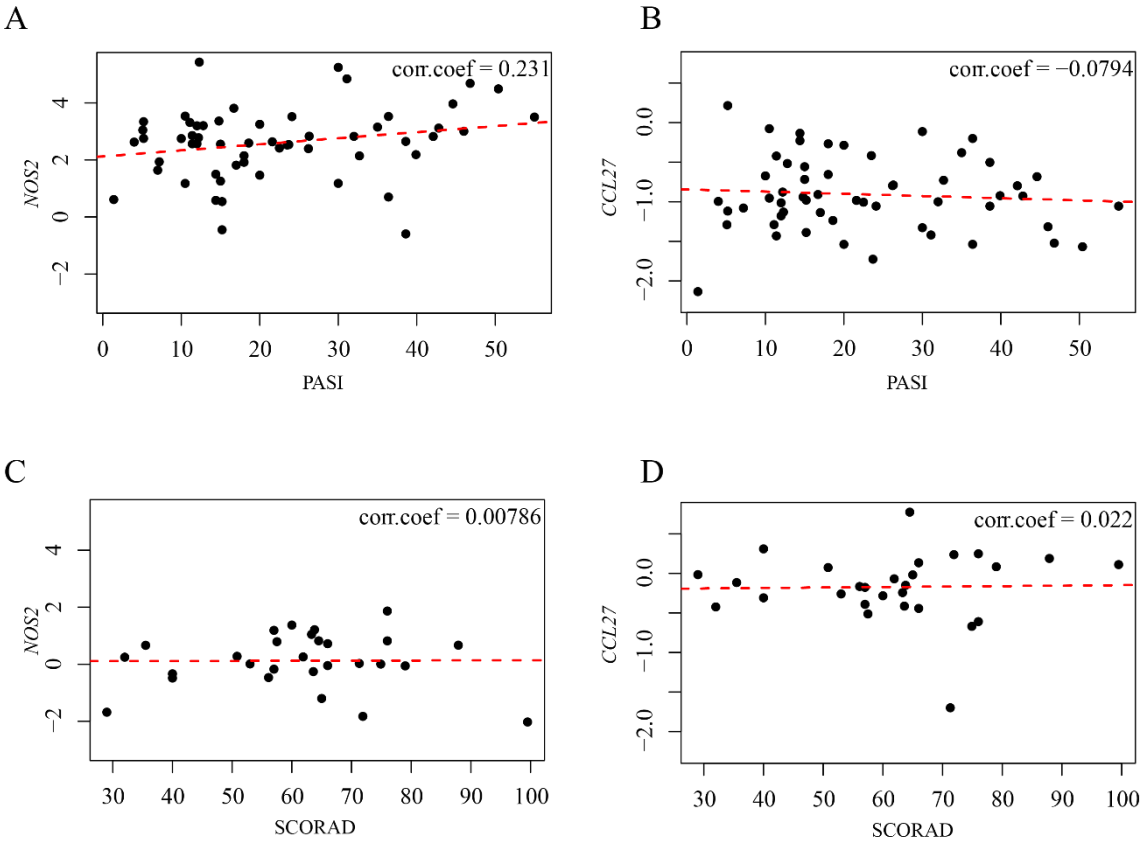


Figure 35: Correlation of *NOS2* and *CCL27* expression with the disease scores PASI and SCORAD

Apart from a slight but not significant positive correlation of *NOS2* with PASI (p = 0.237) (A), there are no further significant correlations of *NOS2* and SCOARD (p = 1) (C) or *CCL27* with PASI (p = 0.799) (B) or SCORAD (p = 1) (D). PASI scores could be obtained from 60 patients and SCORAD scores from 29 patients.

#### 3.3.3.5 Validation of the MC as a diagnostic tool for patients remaining unclear by standard diagnostic means

In a final step, the capacity of the MC as new diagnostic tool was tested in a cohort of patients who initially presented for a diagnostic workup and remained unclear based on clinical picture and dermatohistopathology. The first cohort comprised five patients who presented with a conflicting picture of clinical phenotype (unequivocal picture of psoriasis) and histopathological picture (diagnosis of eczema in histopathology). During the course of disease, all five patients eventually showed good therapeutic response to typical psoriasis treatments such as topical calcipotriol/steroid combinations and infliximab on systemic level and as such endorsing the initial clinical diagnosis. The MC assigned all five cases to the diagnosis of psoriasis and confirmed the decision for psoriasis treatment (Figure 36) indicating that the MC could avoid future diagnostics based on therapy outcome.

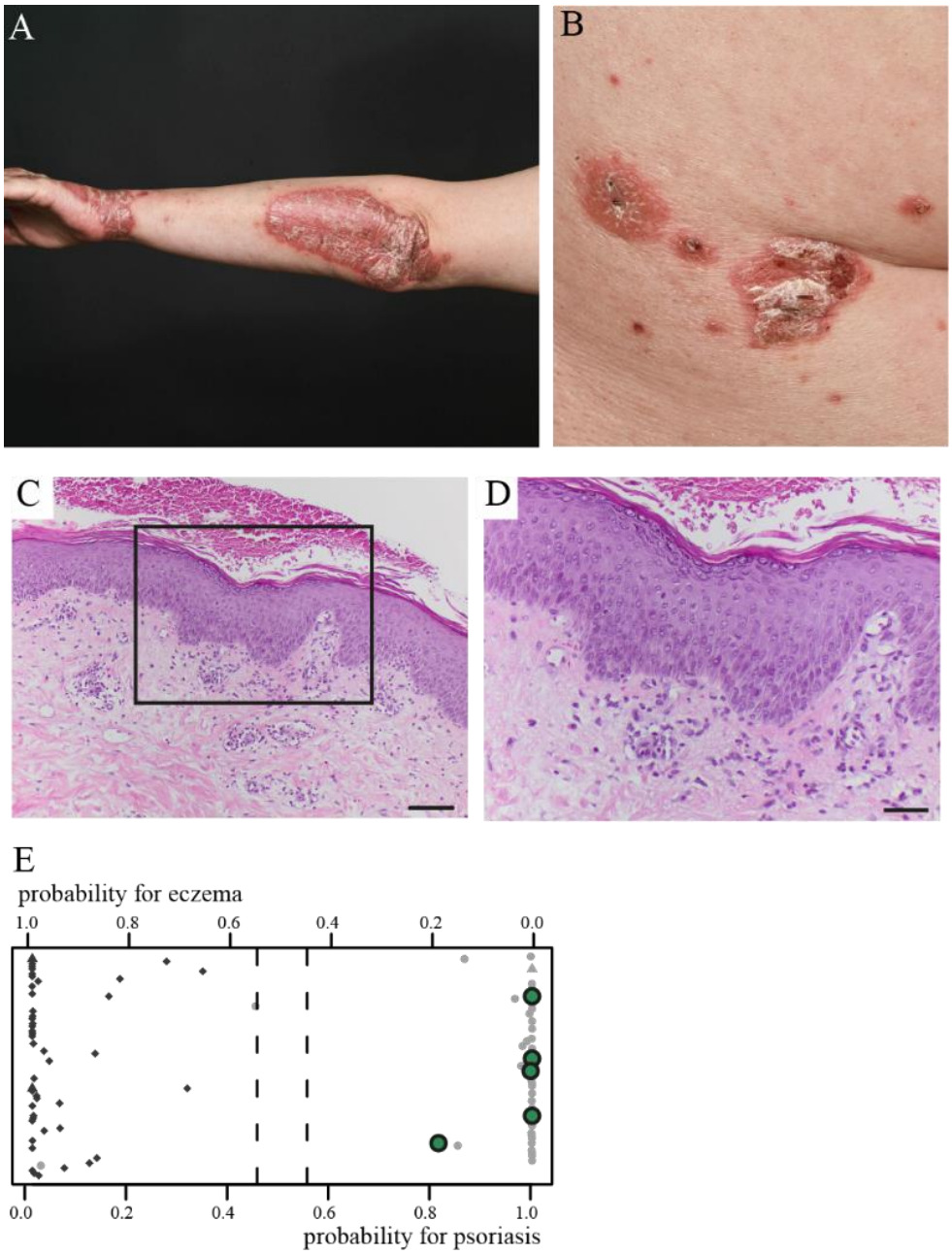


Figure 36: The MC tested on patients with conflicting clinical and histopathological picture

Patients presenting with classical phenotype of psoriasis (A and B) were diagnosed as eczema in histopathology (C) and (D) due to classical findings such as irregular acanthosis, normal composition of granular layer and serum crust. The MC clearly assigned all cases (indicated as green circles) to the diagnosis of psoriasis, which was later confirmed by therapeutic response to psoriasis treatment (E). (Scale bars in C: 100 µm and in D: 50 µm).

The second cohort comprised five patients who remained unclear by both clinical and histological means (Figure 37- Figure 39 Table 14). Patient 129 (Table 14, Figure 37) was a 20 year old man with slightly itchy skin lesions since early childhood. Besides, he

suffered from allergic asthma and allergic rhinoconjunctivitis favoring eczema as underlying diagnosis of the skin disease. However, the localization of the lesions on extensor rather than on flexural surfaces, missing colonization by *Staphylococcus aureus* and the positive family history for psoriasis would have rather favored the diagnosis of psoriasis. Moreover, histology was clearly consistent with psoriasis showing hyperparakeratosis, acanthosis, psoriasis-like papillomatosis, hypogranulosis, and dilated dermal capillaries. Under the diagnosis of psoriasis, the patient was prescribed fumaric acid, a well-established psoriasis treatment. However, under this therapy the lesions worsened and only improved when – under the new working diagnosis of eczema – alitretinoin was started. According to the therapeutic response, the patient reached > 99% probability for eczema in our MC and mapped in the field of eczema on the immunofluorescence based classifier.

Patient 127, a 78 year old male (Figure 38, Table 14), presented with itchy nummular plaques on feet and arms. Histology showed eczema with the classical findings of hyperparakeratosis, hypo and - hypergranulosis, focal spongiosis and a mixed cellular infiltrate. Stable improvement of disease could not be achieved by topical steroids. However, when starting a systemic therapy with fumaric acid, the patient's lesions improved quickly. In line with this, both immunofluorescence and RT-PCR based MC clearly assigned the patient to the diagnosis of psoriasis (97.9% probability on RT-PCR based MC).

The remaining three patients 128, 125 and 126 are presented in Table 14 and Figure 39 and have been correctly assigned to the respective diagnosis as the two patients in detail presented here.

Table 14: Characteristics of patients tested on the MC who remained unclear after routine diagnostics

| **Characteristic** | **Patient 129** | **Patient 127** | **Patient 128** | **Patient 125** | **Patient 126** |
|---|---|---|---|---|---|
| **Sex** | Male | Male | Male | Male | Male |
| **Age (in years)** | 20 | 78 | 52 | 78 | 22 |
| **Onset of disease (age in years)** | Early childhood | 76 | 20 | 77 | Early childhood |
| **Nail involvement** | Yes | No | Yes | No | Yes |
| **Pruritus** | 3/10 | 7/10 | 8/10 | 8/10 | 7/10 |
| **Histological diagnosis** | Psoriasis | Eczema | Eczema (hyperparakeratosis, acanthosis, perivascular immune infiltrate consisting of lymphocytes, macrophages and eosinophils) | Hyperparakeratosis, focal parakeratosis, pronounced spongiosis, acanthosis, mixed infiltrate of lymphocytes, macrophages , neutrophils and eosinophils | Eczematized psoriasis (hypogranulosis, epidermal thinning, spongiosis, some eosinophils |
| **Comorbidities** | Asthma, allergic rhinoconjunctivitis | Arterial hypertension, diabetes mellitus, | Asthma, rheumatoid arthritis | No | Asthma, allergic rhinoconjunctivitis |
| **Family history** | Positive (mother with psoriasis) | Negative | Negative | Negative | Negative |
| **Smoker** | Yes | Yes | Yes | No | No |
| **BMI** | 27 | 30 | 25 | 26 | 23 |
| **Skin colonization** | No | No | S. *aureus* | No | No |
| **Total IgE** | n.d. | 13.1 IU/ml | 6955 IU/ml | 185 IU/ml | 954 IU/ml |
| **Antigen (IgE class)** | n.d. | no specific IgE in standard panel | *P.pratense* (1), *D. pteronyssinus* (6) | Chicken protein (1), milk protein (2) | Cod (1), *A. vulgaris* (2), *P.pratense* (3), *D. pteronyssinus* (6), |
| **Therapeutic outcome** | Worsening under fumaric acid, improvement under altiretinoin | Stable improvement under fumaric acid | Stable improvement under methotrexate + ustekinumab, no improvement under cyclosporine A, infliximab and adalimumab | No improvement under topical steroid and UV therapy, improvement under neotigason and fumaric acid | Stable improvement under methotrexate, refractory to topical steroids |

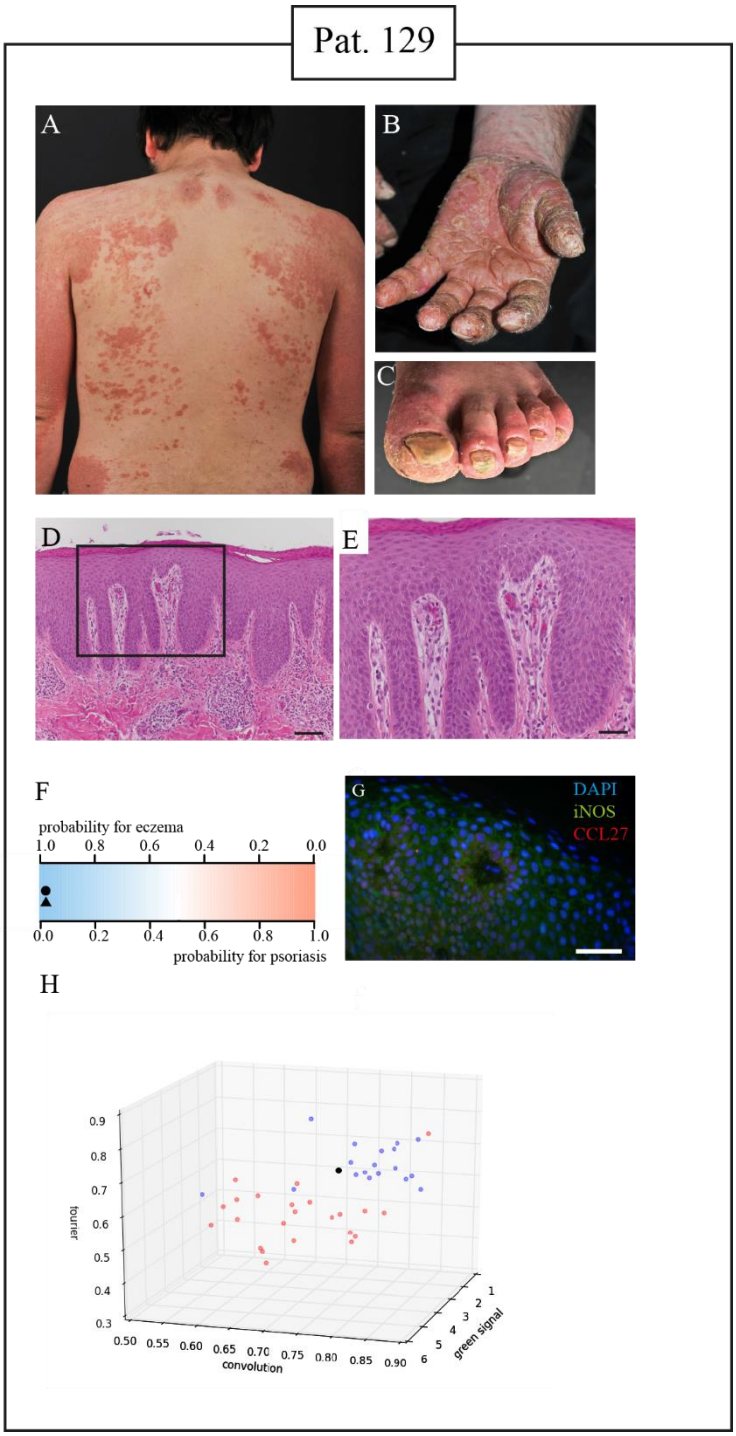


Figure 37: Application of the MC on clinically and histologically unclear patient 129

Patient 129 was diagnosed as psoriasis based on clinical picture (A-C) and histopathology (D, E). However, both RT-PCR (F) and immunofluorescence based MC (G, H) diagnosed eczema in this patient which was consistent with the patient's therapeutic course. The circle in (F) indicates the patient's probability when the MC trained on autologous healthy skin was applied, the triangle indicate the patient's probability on the $MC_{pooled}$. (Scale bars: 100 µm in D, 50 µm in E, G).

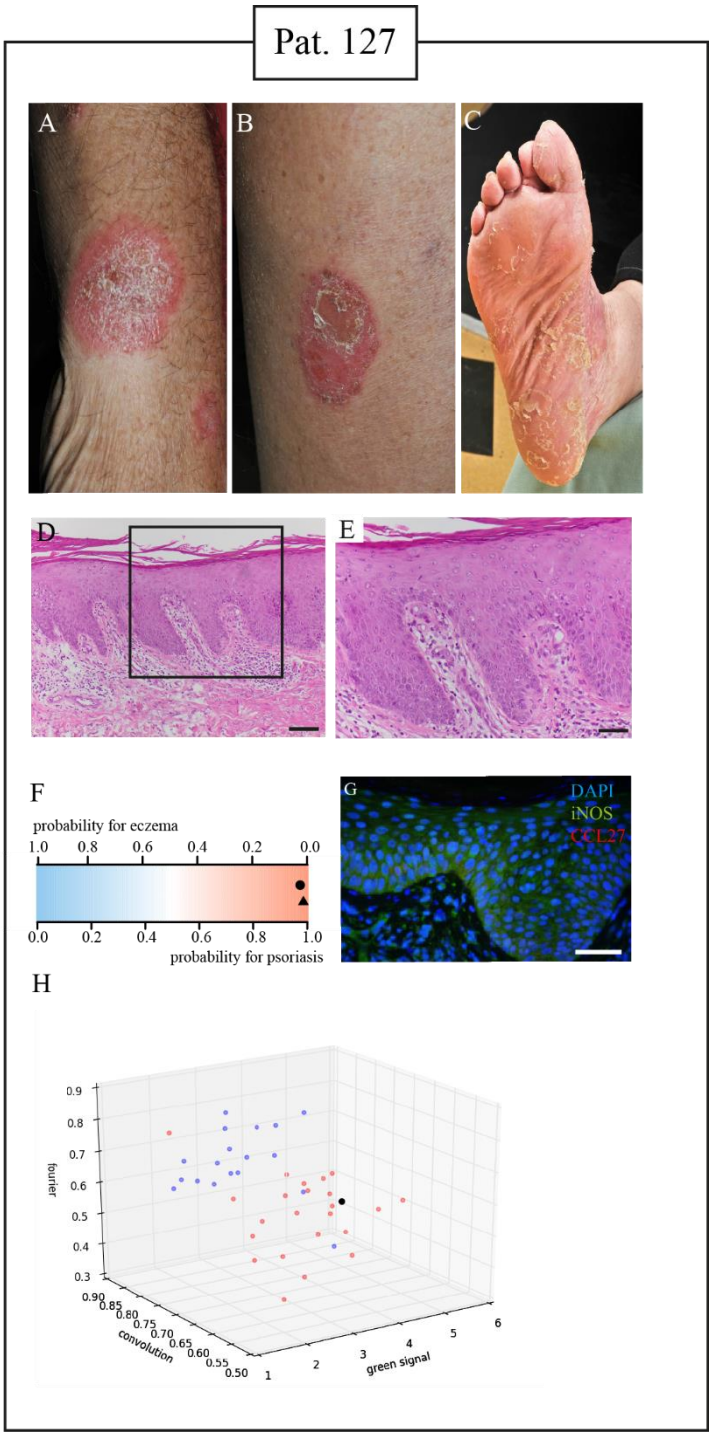


Figure 38: Application of the MC on clinically and histologically unclear patient 127

Patient 127 was diagnosed as eczema based on clinical picture (A-C) and histopathology (D, E). However, both RT-PCR (F) and immunofluorescence based MC (G, H) diagnosed psoriasis in this patient which was consistent with the patient's therapeutic response to fumaric acid. The circle in (F) indicates the patient's probability when the MC trained on autologous healthy skin was applied, the triangle indicate the patient's probability on the $MC_{pooled}$. (Scale bars: 100 µm in D, 50 µm in E, G).

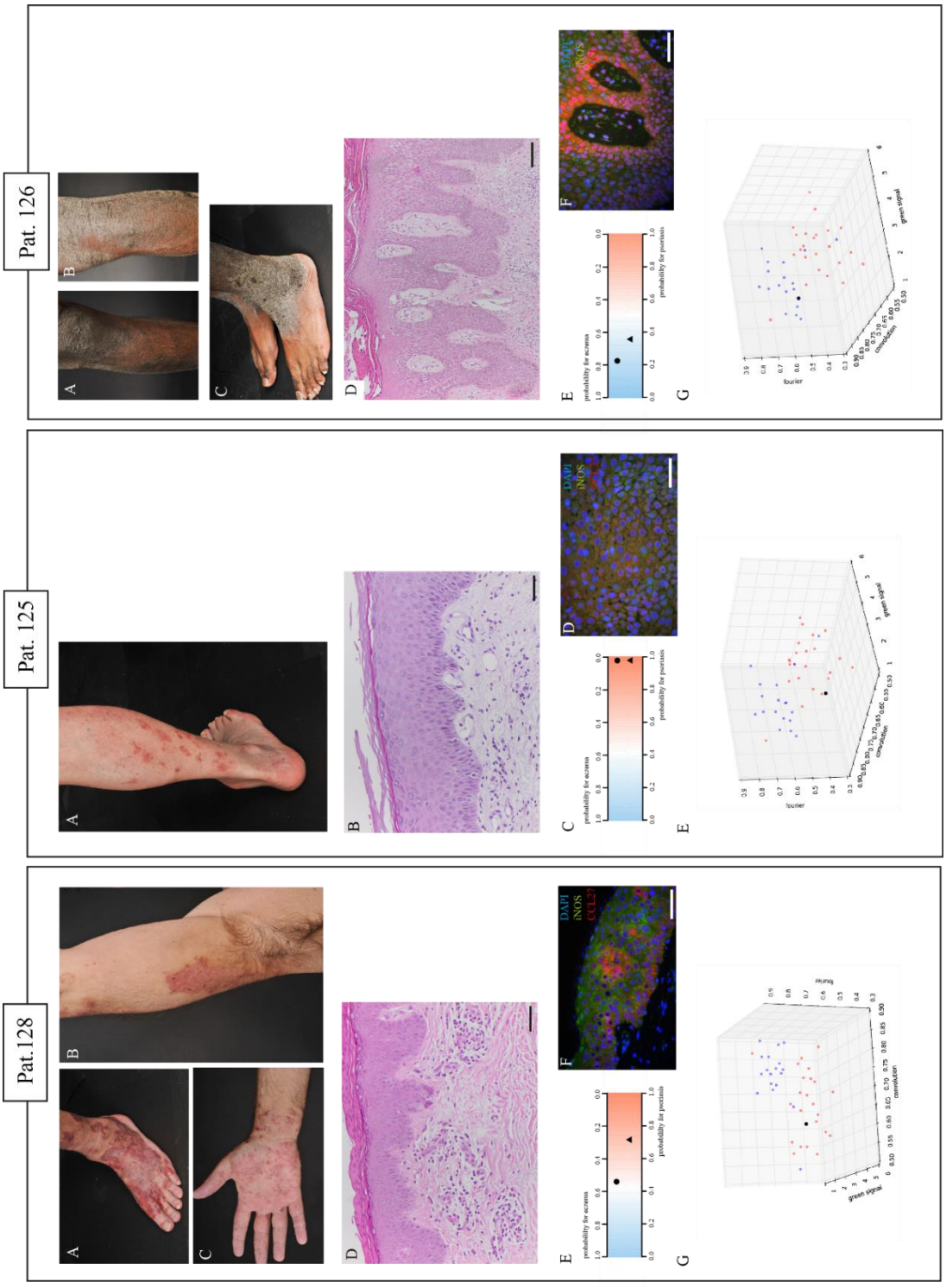


Figure 39: Application of the MC on unclear patients 128, 125 and 126.

Patient 128 (A-C) was diagnosed as eczema histologically (D), initially confirmed by worsening of lesions upon therapy with the TNF-α inhibitor infliximab. However, he eventually improved under ustekinumab (IL-12/IL-23 blocker), a classical psoriasis drug, in combination with methotrexate. The MC (E) assigned the patient to the diagnosis of psoriasis with a probability of 45% (indifferent diagnostic grey area) but clear prognosis was reached when using pooled healthy skin as calibrator (probability for psoriasis: 70%). In addition, immunofluorescence was consistent with psoriasis (F, G). Patient 125 (A) was diagnosed as eczema (B); however, clinical improvement could only be achieved under therapy with fumaric acid. Our

RT-PCR based MC clearly assigned this patient to the diagnosis of psoriasis (probability for both autologous and pooled MC > 99.9% (C)) which was confirmed by the immunofluorescence based MC (D, E). Patient 126 suffered from eczematous plaques on both flexor and extensor parts of the body (A-C). Histology was consistent with psoriasis (D), but the MC assigned him to the diagnosis of eczema (78% probability for eczema on the MC based on autologous healthy skin and 65% probability for eczema on the $MC_{pooled}$) (E). In addition, immunofluorescence was in line with eczema diagnosis (F, G). Circles indicate the patients' probability when the MC trained on autologous healthy skin was applied; triangles indicate the patients' probability on the $MC_{pooled}$. (Scale bars in D, Pat. 128 and B, Pat. 125: 50 µm. Scale bar in D, Pat. 126: 100 µm. Scale bars in F, Pat.128 and 126 and D, Pat.125: 50 µm).

#### 3.3.3.6 The MC as a reliable tool for other inflammatory skin diseases?

The MC based on solely two genes showed to reliably predict the two diagnoses of psoriasis and eczema. Concerning the specificity of the two genes for the diagnostic labels of "eczema" or "psoriasis" the question arises if the MC in its current form would be able to distinguish between eczema, psoriasis and other inflammatory skin diseases (e.g. lichen planus) or if for three diseases a third gene would need to be introduced to the MC.

To answer this question 10 additional patients suffering from lichen planus that were neither under topical nor systemic therapy were tested on the MC. All ten patients were assigned to the diagnosis of eczema indicating that using only two markers three diseases cannot be distinguished (Table 15).

Table 15: Probabilities of the 10 patients suffering from lichen planus for the diagnosis of psoriasis or eczema according to the MC

All 10 patients were assigned to the disease class of eczema showing that the MC in its current form cannot distinguish other diseases than psoriasis and eczema.

| Patient | Probability for Psoriasis | Probability for Eczema | Predicted by MC | Pre-diagnosed disease (True class) |
|---|---|---|---|---|
| 45 | 0.057 | 0.943 | Eczema | Lichen |
| 46 | 0.071 | 0.929 | Eczema | Lichen |
| 47 | 0.050 | 0.950 | Eczema | Lichen |
| 48 | 0.039 | 0.961 | Eczema | Lichen |
| 49 | 0.055 | 0.945 | Eczema | Lichen |
| 50 | 0.149 | 0.851 | Eczema | Lichen |
| 51 | 0.068 | 0.932 | Eczema | Lichen |
| 52 | 0.145 | 0.855 | Eczema | Lichen |
| 53 | 0.070 | 0.930 | Eczema | Lichen |
| 54 | 0.061 | 0.939 | Eczema | Lichen |

To explore the possibility if the classifier could be expanded to lichen planus by adding another gene marker, we a) screened the literature for possible additional gene markers exclusively regulated in lichen planus and b) performed additional whole-genome expression arrays of four lichen planus patients to gain our own data set. Based on the proposed classifier of Wenzel et al. for lichen planus (Wenzel *et al.*, 2008) and according to most significantly differentially regulated genes between lichen planus, psoriasis and eczema in our cohort, following candidate genes were chosen: Type I interferon related genes *IFI44L (*Interferon-induced protein 44-like*)*, *IFIT (*Interferon-induced protein with tetratricopeptide repeats*)* and *MX1* (MX dynamin-like GTPase 1) as well as *IDO* (Indoleamine 2,3-Dioxygenase 1) and *CXCL9* (Chemokine (C-X-C motif) ligand 9*)*. Regarding each of the markers separately on the level on mRNA by performing RT-PCR, none of them turned out to separate lichen planus from psoriasis and eczema in our data set (Figure 40).

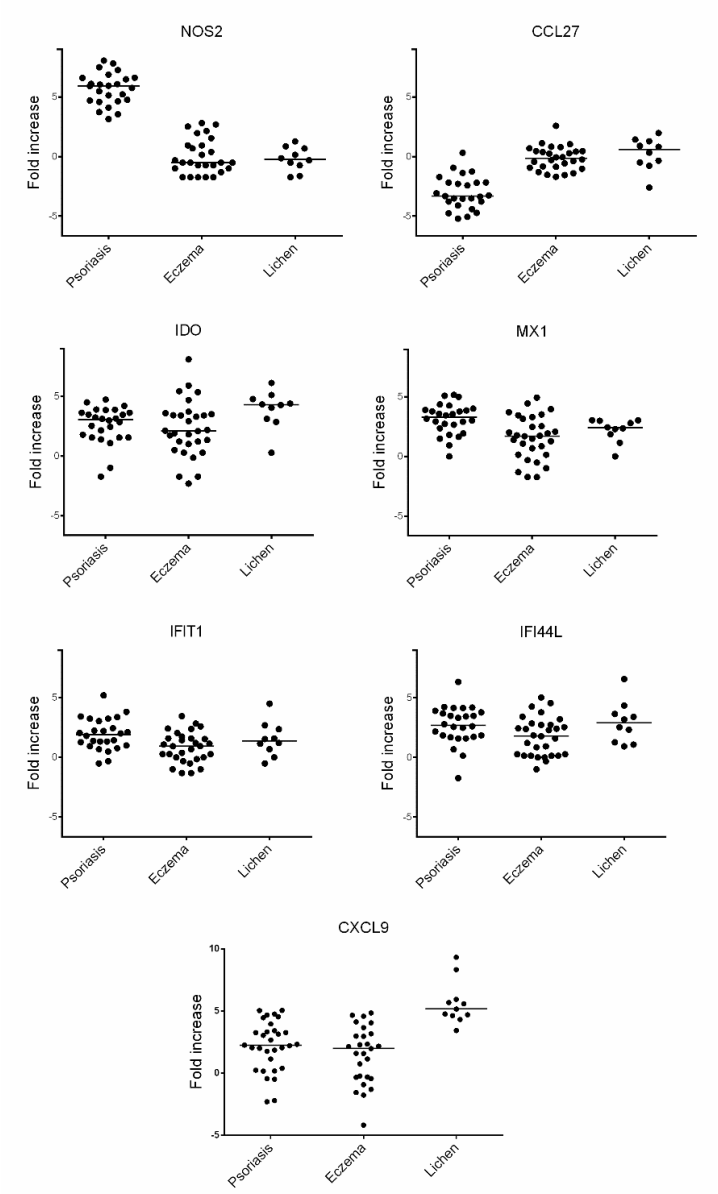


Figure 40: RT-PCR validation of additional candidate genes to separate the three skin diseases of psoriasis, eczema and lichen planus

RT- PCR analysis of the candidate genes *IDO*, *MX1*, *IFI1*, *IFI44L* and *CXCL9* in lesional skin of psoriasis (n = 26), eczema (n = 28), and lichen planus (n = 10). Shown is the log fold induction as compared to autologous non-involved skin.

However, when expanding our classifier of *NOS2* and *CCL27* with *CXCL9*, the marker which was previously published to separate lichen planus from psoriasis as well as from eczema (Wenzel *et al.*, 2008), acceptable but not complete performance could be achieved (Table 16). Three of 13 eczema patients classified as lichen planus and one of four lichen planus patients classified as eczema (total accuracy 96%, kappa = 0.78).

Table 16: An expanded diseases classifier for psoriasis, eczema and lichen planus

The expanded disease classifier based on RNA level by RT-PCR consisting of *NOS2*, *CCL27* and *CXCL9* classified 26 out of 30 patients correct (classifier was trained with a radial kernel function with gamma = 1 and cost = 10 and a 10 fold cross validation; accuracy 96%, kappa = 0.78). Shown are given probabilities.

| Patient | Probability for Psoriasis | Probability for Eczema | Probability for Li-chen | Predicted by MC | Pre-diagnosed disease (True class) |
|---|---|---|---|---|---|
| 10 | 0.80 | 0.13 | 0.07 | Pso | Pso |
| 11 | 0.89 | 0.07 | 0.04 | Pso | Pso |
| 12 | 0.87 | 0.08 | 0.05 | Pso | Pso |
| 13 | 0.90 | 0.06 | 0.04 | Pso | Pso |
| 14 | 0.81 | 0.12 | 0.07 | Pso | Pso |
| 15 | 0.93 | 0.04 | 0.03 | Pso | Pso |
| 16 | 0.62 | 0.29 | 0.09 | Pso | Pso |
| 17 | 0.87 | 0.07 | 0.06 | Pso | Pso |
| 18 | 0.73 | 0.20 | 0.07 | Pso | Pso |
| 19 | 0.62 | 0.25 | 0.13 | Pso | Pso |
| 20 | 0.66 | 0.21 | 0.13 | Pso | Pso |
| 21 | 0.90 | 0.06 | 0.05 | Pso | Pso |
| 31 | 0.05 | 0.82 | 0.13 | AE | AE |
| 32 | 0.21 | 0.37 | 0.41 | Li | AE |
| 33 | 0.04 | 0.88 | 0.08 | AE | AE |
| 34 | 0.02 | 0.73 | 0.24 | AE | AE |
| 35 | 0.04 | 0.86 | 0.10 | AE | AE |
| 36 | 0.03 | 0.84 | 0.13 | AE | AE |
| 37 | 0.04 | 0.88 | 0.09 | AE | AE |
| 38 | 0.05 | 0.83 | 0.12 | AE | AE |
| 39 | 0.02 | 0.17 | 0.81 | Li | AE |
| 41 | 0.02 | 0.74 | 0.24 | AE | AE |
| 42 | 0.10 | 0.27 | 0.63 | Li | AE |
| 43 | 0.02 | 0.90 | 0.08 | AE | AE |
| 44 | 0.12 | 0.67 | 0.21 | AE | AE |
| 45 | 0.03 | 0.17 | 0.80 | Li | Li |
| 46 | 0.05 | 0.07 | 0.88 | Li | Li |
| 47 | 0.04 | 0.51 | 0.45 | AE | Li |
| 48 | 0.03 | 0.22 | 0.74 | Li | Li |

# 4 Discussion

## 4.1. The complexity and heterogeneity of psoriasis and eczema: Major obstacles for research success

The heterogeneity of psoriasis and in particular of eczema is obvious when regarding the clinical and histological variety of phenotypes (1.3.2 Clinical variety of psoriasis and eczema; 1.3.3 Histopathological variety of psoriasis and eczema). However, also on molecular level this heterogeneity is striking. In our study, psoriasis signatures were compared with eczema signatures, but also chronic naturally occurring forms of eczema such as nummular eczema, dyshidrotic eczema and atopic eczema with self-limited variants of eczema such as acute contact dermatitis to nickel.
We found that the more homogeneous a disease is, the more genes will be found to be uniquely upregulated or downregulated within a cohort of patients affected by this disease. By our intraindividual comparison of gene expression profiles in the cohort of patients suffering from both psoriasis and eczema we found 101 genes exclusively expressed in psoriasis compared to 39 genes significantly regulated in eczema but not in psoriasis. This matches the fact, that different variants of eczema, namely atopic, non-atopic and contact eczema have been included resulting in higher heterogeneity as compared to the psoriasis cohort where only cases of plaque psoriasis have been included.

This is in line with previous reports, which found many more genes upregulated and downregulated in psoriasis than in atopic eczema. When comparing psoriasis and eczema Guttman-Yassky et al. found 1989 genes exclusively regulated in psoriasis compared to only 896 genes exclusively regulated in eczema (Guttman-Yassky *et al.*, 2009). These results clearly hint at a higher homogeneity within the disease of psoriasis than within the cohort of eczema– provided that patients with classical plaques psoriasis and not pustular psoriasis were included.

Expanding our analysis within the cohort of eczema by comparing acute contact dermatitis to nickel (inducible eczema) with naturally occurring eczema (atopic eczema, nummular and dyshidrotic eczema) we found an even higher discrepancy of the numbers of genes regulated: 172 genes were exclusively up – and downregulated in acute contact dermatitis whereas only 28 genes were exclusively regulated in the cohort of naturally

occurring eczema. This finding highlights again the fact that contact dermatitis to nickel is a relatively homogeneous condition, which - due to the sole trigger nickel – proceeds in well-characterized subsequent disease stages and thus enables sampling of skin biopsies at the same stage of the disease. According to the literature (Martin *et al.*, 2011), we found a broad activation of immune-response including innate immune mechanisms and signaling pathways such as inflammasome activation (*AIM2*, *IL1B* etc.) which are known to be involved in defending microbial pathogens (Man *et al.*, 2016). In contrast, the variability within the group of naturally occurring eczema was higher due to different possible triggers (allergens, genetic barrier defects etc.), different courses of the diseases and other mechanisms not yet elucidated (Quaranta *et al.*, 2014b).

Novel diagnostic markers and tools in the field of inflammatory skin diseases have been broadly hampered by the complexity and the heterogeneity of these diseases. To further advance in the field and to once being able to not only develop but also to choose the best currently available therapy for individual patients, different approaches have been increasingly followed over the past years. The awareness that individual characterization on molecular level is necessary to achieve these goals has not least been fueled by the continuous news of success from the melanoma research field, which has proven a cutting-edge role over the past years. Tumor tissues are screened for BRAF V600 mutations and patients may be considered for therapy with the BRAF inhibitor vemurafenib when results are positive. Dummer et al. explicitly state that in cases where results for this mutation are negative, "…further molecular testing can be carried out for NRAS, c-Kit, GNA11 or GNAQ; this helps to direct patients to the appropriate targeted treatment or clinical trial." (Dummer *et al.*, 2015). According to the authors patients carrying NRAS mutations may benefit from MEK kinase-inhibitor therapy and analysis of PDL-1 expression would help to select patients for anti-PD1 treatment (Dummer *et al.*, 2015). This prime example shows that therapy for subgroups can be provided, once the subgroup-specific disease mechanisms have been unraveled.

Several attempts of stratification that have been undertaken in the field of inflammatory skin diseases are outlined in the following: To start, Suarez-Farinas et al.. stratified patients with atopic eczema in intrinsic and extrinsic form while only the latter one is characterized by high serum IgE. The fact that the clinical and histological phenotype of intrinsic and extrinsic eczema are indistinguishable from each other was reflected by equal amounts of infiltrates of T cells, dendritic cells and corresponding epidermal alterations.

However, on the molecular level, they could show that, overall, lesional skin in intrinsic atopic eczema showed similar or even higher Th2 and Th1 activity but a significantly more prominent Th22 and Th17 immune response compared to extrinsic atopic eczema. In contrast, only extrinsic atopic eczema showed positive correlations between SCORAD and Th2-cytokines such as IL-4 and IL-5 (Suarez-Farinas *et al.*, 2013b). This finding might support clinicians filtering patients for anti-IL-4 treatment or also for anti-IL-17 treatment currently only approved for psoriasis.
Another recent study shed light on ethnical differences that may be relevant in disease pathogenesis of eczema by comparing the Asian eczema phenotype to the European American eczema phenotype. Using principal component analysis of real-time PCR data, they found that the Asian atopic eczema phenotype clustered between the European American atopic eczema phenotype and psoriasis phenotypes with increased Th17 activation, but also a strong Th2 component. The psoriasis-similar phenotype was also reflected by increased hyperplasia and parakeratosis in Asian patients (Noda *et al.*, 2015).

Although these two studies exemplify that stratification of patients according to ethnical, clinical and molecular characteristics is a prerequisite for investigating the complex diseases of psoriasis and eczema, the heterogeneity of chronic inflammatory skin diseases is not sufficiently acknowledged in most studies investigating the pathogenesis of these diseases. Novel approaches are needed to achieve a substantial scientific break-through.

In this work, three of such approaches are investigated:

a) The alopecia areata (AA) model as a model to investigate gene-environment interaction in the pathogenesis of chronic inflammatory skin diseases: Due to their specific background AA patients are prone to develop various inflammatory skin diseases such as psoriasis and eczema depending on individual susceptibility and environmental triggers
b) The imiquimod model in humans which provides a homogeneous standardized psoriasis-like skin reaction in humans via patch-test application and thus represents a possible model for some aspects of psoriasis and eczema
c) Patients suffering from both psoriasis and eczema simultaneously represent an ideal model to compare distinct inflammatory diseases within the same patient regardless of the underlying genetic background and environmental influences,

which typically increase complexity and inaccuracy of big cohort studies. Like in the AA model, depending on the specific immunological trigger at the respective lesion site inflammatory responses are driven into different directions resulting in distinct clinical, histological and molecular phenotypes.

## 4.2 Patients with AA and coexisting inflammatory skin diseases: A model to study the interplay of genes and environment in the pathogenesis of chronic inflammatory skin diseases

### 4.2.1 Proof of concept: AA patients are prone to develop inflammatory skin diseases depending on individual susceptibility and environmental triggers

Alopecia areata has been shown to be associated with atopy and in particular atopic eczema and the rates of coincidence of both diseases are comparable to our study, in which we found 15.1% of all AA patients to suffer from atopic eczema (Chu *et al.*, 2011; Goh *et al.*, 2006; Guzman-Sanchez *et al.*, 2007; Villasante Fricke and Miteva, 2015). Increased prevalence rates of psoriasis and vitiligo as compared to the normal population have also been reported for AA patients in large cohort studies (Chu *et al.*, 2011; Goh *et al.*, 2006). However, in our study the co-occurrence of AA and psoriasis and AA and vitiligo was more frequent with 7.1% compared to 3.9% for vitiligo and 5.3% compared to 4.3% for psoriasis (Goh *et al.*, 2006). The discrepancy may be explained by the lower number of patients included in our study but generally, our data supports previous findings and thus a quantitative bias towards patients with coexisting inflammatory skin diseases could be ruled out. Whereas the prevalence rate for lichen planus with < 1% was not increased in the AA cohort compared to the normal population of approximately 1% (Le Cleach and Chosidow, 2012) we could validate the finding that other inflammatory disorders such as psoriasis and atopic eczema are more frequent in AA patients than in the normal population. In addition, we could confirm that severe AA subtypes are associated with earlier age of onset (Goh *et al.*, 2006). Apart from the epidemiological data revealing a high frequency of coexisting inflammatory skin diseases in our cohort of AA patients, we found a broad immune response reflected in the abundance of TNF-α, IFN-γ, IL-4 and IL-17 producing T cell subsets infiltrating AA lesions. Both facts argue for generalized

overactivation of T cells in the skin of AA patients. This concept is buttressed by genetic studies which showed that alopecia areata is among others associated with a disruption of genes and pathways controlling the TGF-ß/Tregs pathway and the activity of cytotoxic T cells (Betz *et al.*, 2015; Petukhova *et al.*, 2010; Xing *et al.*, 2014). Associated loci outside immune response pathways comprise genes expressed in the hair follicle (Betz *et al.*, 2015). This implies that on the basis of genetically determined deviation of immune response, the T cell response is not genetically determined but shaped by the condition of the respective structure, for example the combination of overactivated T cell and the instability of the hair follicle (autophagy, oxidative stress) elicit the phenotype of alopecia areata. Nevertheless, at the same time, for example, eczema may also occur in AA patients where filaggrin mutations and overactivated T cells converge. In line with this, Betz et al. could show that filaggrin mutations were significantly associated with the presence of atopic eczema among AA patients (Betz et al., 2007). AA may thus be regarded a model disease for overactivation of T cells in the skin where a genetically primed organism and specific triggers (environmental stimuli, antigens…) coincide. Depending on the specific stimulus or antigen, distinct cutaneous inflammation are switched on and may develop in parallel and independent of each other. Showing that coexisting inflammatory skin diseases are clinically and histologically typical with distinct T cell infiltrates not altered by the AA background, we support the idea of AA as a model to study coexisting inflammatory skin diseases and eventually decipher the local trigger in the future. If so, the nature of the antigen determines the outcome of an immune response, in the case of AA patients on the genetically fixed basis of higher susceptibility towards cutaneous inflammation.

### 4.2.2 Patients with AA and coexisting inflammatory skin diseases: A prime example for the need of individual therapeutic decisions

In our study, we presented AA patients and showed their individual skin comorbidities. Already here, at the stage of clinical routine diagnostics, the standard techniques of performing clinical and histopathological examinations provide useful information in the decision-process for optimal individual therapeutic regimens. Topical administration of corticosteroids is the gold standard therapy of AA (Gilhar et al., 2012; Mancuso et al., 2003). For more severe cases, when hair loss is acute and rapidly progressing or when extensive hair loss may cause psychosocial problems, systemic therapies are initiated. Steroids are again preferentially chosen (Gilhar et al., 2012), alternatives are immunosuppressive

drugs such as cyclosporine (Acikgoz et al., 2014) or methotrexate (Hammerschmidt and Mulinari Brenner, 2014). Knowing, however, that patients with atopic eczema or psoriasis frequently suffer from severe disease exacerbation after discontinuing treatments with systemic steroids (Augustin et al., 2011; Bieber, 2008; Nestle et al., 2009), one should consider alternative substances in AA patients suffering in parallel from eczema and/or psoriasis that are more appropriate for the coexisting skin disease. Here, methotrexate which is also effective in atopic eczema (Bieber, 2008) or psoriasis (Boehncke and Schon, 2015) may be the better option. In patients similar to ours were both AA and atopic eczema were shown to be characterized by relative high amounts of IL-4 producing T cells, dupilumab, a monoclonal antibody blocking IL-4 and IL-13, could be a therapeutic option (Beck et al., 2014). Recently, a prominent interferon signature has been discovered for AA and in this context, AA patients were shown to benefit from therapies targeting the Janus kinase (JAK) pathway such as the JAK1/2 inhibitor baricitinib (Jabbari et al., 2015; Xing et al., 2014). This is of particular interest for those AA patients suffering concomitantly from psoriasis, as JAK inhibitors such as tofacitinib have also been shown therapeutic efficacy in clinical studies for psoriasis patients (Chiricozzi et al., 2015; Papp et al., 2015a). AA patients at risk to develop coexisting psoriasis may optimally be treated with such a regime. As more and more specific immune-modulating therapies are brought to the market, individualized target oriented medicine could be established for AA when taking into account the patients' specific comorbidities.

## 4.3 The imiquimod model: A model for inflammatory skin diseases in humans

Investigating patients with a specific background like patients suffering from AA offers a model to decipher interactions between genetics and environmental triggers in the pathogenesis of inflammatory skin diseases. However, to gain functional insights and perform interventional studies, novel approaches to imitate the complex inflammatory phenomena in proper models are needed. Due to the lack of adequate mouse models and missing models in humans for inflammatory diseases, investigators are facing major obstacles to exceed the purely descriptive level of analyzing the pathogenesis of psoriasis and eczema in humans.

In our study, we investigated if application of imiquimod cream in humans would be a suitable model to mimic (at least) some aspects of psoriasis and eczema. In case some signaling pathways were reflected by the imiquimod model, one could imagine to further perform interventional studies (e.g. drug tests) using imiquimod-induced inflammation.

The imquimod mouse model has been evolved as a well-established animal model to study psoriasis. It fulfills more or less all criteria for an ideal psoriasis model such as acanthosis, hyperparakeratosis, partial loss of the stratum granulosum, papillomatosis, presence of inflammatory cells (amongst others T cells, dendritic cells and neutrophils), a functional role for T cells, neoangiogenesis and response to anti-psoriatic drugs (van der Fits *et al.*, 2009). However, papillomatosis is observed sometimes but not always and the effect of anti-psoriatic drugs has not yet been fully demonstrated: A study investigating the effects of topical anti-psoriatic treatments such as steroid, calcipotriol and tazarotene has shown a beneficial effect for clobetasol but the other drugs failed to show inhibitory effects on epidermal hyperproliferation and inflammation (Sun *et al.*, 2013). Another group demonstrated successful treatment of lesions in the imiquimod model when applying a combination of glucosamine, an immunomodulatory agent, in combination with cyclosporine A (Kim *et al.*, 2015). However, both steroids and cyclosporine are also used for the treatment of eczema in humans (Lauffer and Ring, 2016) indicating that the imiquimod model may rather represent a model for psoriasis-like dermatitis than psoriasis. The fact that imiquimod-induced skin inflammation decreases during the eighth day when imiquimod is given to wild type and not genetically modified mice (Wang *et al.*, 2015) may corroborate the idea that imiquimod rather induces acute self-limited dermatitis than chronic psoriasis-like phenotypes. However, independently of histological and clinical aspects, it remains unclear if psoriasis might still be reflected by the imiquimod model. In mice, dual inhibition of IL-17 and IL-23 – key cytokines of also human psoriasis – in the imiquimod model have efficaciously reduced disease score indicating that on molecular level similarities with psoriasis might still be of relevance (Mangan *et al.*, 2015).

On gene expression level, other mouse models for psoriasis have proven to mimic gene expression patterns typical for human psoriasis better than the imiquimod model in terms of psoriasis-typical immune mechanisms. Here, e.g. imiquimod-induced inflammation had less overlap with human psoriasis in terms of immune system related pathways and

is characterized by less induction of TNF-α and IFN-γ, key cytokines of psoriatic inflammation (Swindell *et al.*, 2011). These findings were supported by an analysis of Lowes et al. who found – when performing enrichment analysis for cytokine-related inflammatory pathways on a selection of widely accepted mouse models for psoriasis – that the imiquimod mouse model represents less pathways of human psoriasis as compared to other mouse models (Lowes *et al.*, 2014). In our study, we also measured cytokine production of isolated T cells from PL (pseudolymphoma-like inflammation induced by imiquimod cream in our study) but also from psoriasis and eczema and found lower levels of TNF-α in PL than in psoriasis corroborating the findings from the mouse model. For IFN-γ statistical significance could not be reached.

In our study, we could show that on the level of histopathology imiquimod cream rather induced eczema than psoriasis as hallmarks of psoriasis such as microabscess, parakeratosis and hypogranulosis were absent, instead spongiosis and other characteristics of eczema were found. However, on molecular level, the overlap between naturally occurring eczema (nE) including atopic eczema and imiquimod cream induced inflammation was significantly lower than for example the overlap between acute contact dermatitis (ACD) and nE. This indicates that imiquimod cream induced inflammation does not mimic eczema in general as it does not reflect a) the chronicity of naturally occurring eczema and b) the heterogeneity of naturally occurring eczema. In contrast to nE, which can be triggered by many environmental influences and genetic variances, imiquimod cream was shown to induce a uniform inflammation pattern in patients with eczema and/or psoriasis but also in healthy volunteers independent of their genetic background and was self-limited after cessation of treatment. Thus, only few top hits genes were regulated in common in both nE and PL and among those genes many were regulated also in ACD and psoriasis indicating markers of general skin inflammation. Among those “unspecific” commonly regulated genes of first-line inflammation in the skin are lactotransferrin *(LTF*) and members of the S100 family exerting antimicrobial functions as well as the proinflammatory chemokine *CXCL1* attracting neutrophils (Eckert *et al.*, 2004; Ward *et al.*, 2002; Zaja-Milatovic and Richmond, 2008).

With respects to the clinical course characterized by induction of inflammation upon a defined stimulus and rapid clearance of lesions after cession of imiquimod cream, the similarity between imiquimod cream induced inflammation and ACD is obvious. Like in

imiquimod cream induced inflammation ACD is triggered by a particular stimulus, nickel in our cases, and resolves by itself when the allergen is taken off. In line with this, we found the highest overlap in overall disease signature between ACD and imiquimod cream induced inflammation (correlation coefficient of 0.78). Within the list of significantly regulated top hit genes, a high number of commonly regulated genes between ACD and PL was found within the category of immune system. In-depth analysis of pathways revealed that both PL and ACD were characterized by activation of the NF-κB signaling. This results does not seem surprising when considering the fact that a) nickel can directly activate TLR4 (Martin, 2012; Schmidt *et al.*, 2010) and b) considering that all TLR signaling pathways including TLR4 and TLR7 in the imiquimod cream culminate in the activation of NF-κB which controls the transcription of many inflammatory cytokine genes (Kawai and Akira, 2007). Activation of TLR4 as well as activation of TLR7 has been shown to activate an intracellular signaling pathway leading to the recruitment of MyD88 and interleukin-1 receptor-associated kinase 1 (IRAK) which in turn – via TNF receptor-associated factor 6 (TRAF6) and IκB kinase (IKK) complexes – activates the NF-κB signaling cascade.

TLR7 activation in addition can also lead to activation of interferon regulatory factor 7 (IRF-7) which regulates the expression of IFN-α and IFN-β (Kawai and Akira, 2007; Li and Verma, 2002). NF-κB signaling is a major signaling pathway of innate immune system and required for rapid induction of expression of acute-phase antimicrobial defense genes in response to pathogens which fits well to the rapid clinical response seen in both imiquimod and nickel treated skin. However, this signaling pathway is also essential for the adaptive immune system, in particular for the development of Th1-type responses (Li and Verma, 2002). In line with this, IRF-1 activated by NF-κB not only induces apoptosis via caspase 8 as demonstrated in our pathway analysis but also promotes the differentiation of Th1 cells (Dou *et al.*, 2014). Besides, in both ACD and PL IL-2/IL-2R signaling and granzyme B (*GZMB*) which is crucial for the induction of target cell apoptosis by cytotoxic T lymphocytes has shown to be of relevance in our pathway analysis. Also our immunohistochemical staining with CD4/CD8 T cell ratio showed a higher proportion of $CD8^+$ T cells for PL (1.06 ± 0.12) than for atopic eczema (3.67 ± 1.61). A direct link between TLR7 and contact hypersensitivity has been shown by Thatcher et al. In their study, they demonstrate that topical imiquimod treatment prevented UV-light induced

loss of contact hypersensitivity by preserving Th1 cytokines and the function of Langerhans cells (Thatcher *et al.*, 2006).

Though similarities between imiquimod-induced skin reaction and acute contact dermatitis are obvious, we also found unique similarities between imiquimod-induced skin inflammation and psoriasis indicating that the reaction is more complex than expected from first glance. As in psoriasis, T cell infiltrate in PL was characterized by higher amounts of $CD8^+$ T cells in psoriasis ($1.44 \pm 0.34$ for psoriasis vs. $1.06 \pm 0.12$ in PL) as compared to eczema. In addition, neutrophils, hallmarks of psoriatic inflammatory infiltrate, were shown to be more frequent in PL and psoriasis than in eczema. More importantly, we found that plasmacytoid dendritic cells (pDCs) are significantly upregulated in PL as compared to eczema and a trend for higher amounts of pDCs in psoriasis than in eczema was seen. The effects of imiquimid cream have mainly been attributed to the activation of TLR7 on pDCs and in both human and mice pDCs were shown to be increased and are thought to play a decisive role in particular for the initiation of disease (Patel *et al.*, 2011). Albanesi et al. could demonstrate that pDC infiltration in human psoriasis correlated with the expression of markers typical of early phases of psoriasis, whereas it was almost absent in long-lasting lesions (Albanesi *et al.*, 2009). Gilliet et al. have worked out one of the most accepted models for the initiation phase of psoriasis (Ganguly *et al.*, 2009; Lowes *et al.*, 2014; Nestle *et al.*, 2005). According to this model, antimicrobial peptides, self-DNA and self-RNA are released upon cell death of keratinocytes (initial trauma of the skin). They activate TLR7 and TLR9 on pDCs, which in turn produce vast amounts of IFN-α and instigate T cell activation. The prominent IFN-γ signature as well as TLR7 activation could not be observed in our study for psoriasis as only chronic forms of plaque psoriasis were included into our study. However, for PL we could clearly corroborate the findings of Dickson et al. (Dickson *et al.*, 2015). They investigated TLR7 activation in healthy human skin using tape stripping and imiquimod cream and found a prominent increase in type 1 IFN regulated genes such as *CXCL10*, *CXCR3*, MX dynamin-like GTPase 1(*MX1)*, *MX2*, interferon-induced protein 44 (*IFI44)*, 2'-5'-oligoadenylate synthetase (*OAS)*, signal transducer and activator of transcription 1 (*STAT1)* and *STAT2*. All listed genes apart from *STAT1* have also been found to be upregulated in our PL cohort, with *CXCL10* even ranging at position number one concerning fold induction (< 7.5 log fold induction, data not shown).

When looking at pathways shared between psoriasis in the chronic situation – from which samples were obtained in our study – and PL we found a common significance of the IL-23 pathway. This is in line with a study of Vinter et al. who showed similar overexpression of IL-23p19 in in imiquimod-treated and lesional psoriatic skin (Vinter et al., 2015).

For the imiquimod mouse model it could be early proven that imiquimod-induced inflammation is mainly mediated by the IL-23/IL-17 axis (van der Fits *et al.,* 2009). Besides, a mouse model where psoriasis is induced by intradermal injection of IL-23 in the murine dorsum has been shown the greatest overall resemblance to human psoriasis (Lowes *et al.,* 2014; Suarez-Farinas *et al.,* 2013a). However, the relevance of the IL-23 pathway has become more and more evident for human psoriasis: IL-23 was found to be highly expressed in skin lesions of psoriasis patients and to be mainly produced by dendritic cells (Lee *et al.,* 2004; Piskin *et al.,* 2006). The discovery of the novel Th17 population and its relevance for the pathogenesis of psoriasis as well as the finding that IL-23 promotes the expansion of Th17 cells producing IL-17A and TNF-α – key cytokines of psoriasis – led to the acceptance of the IL-23A/IL-17 axis involved in the acute and chronic phase of psoriasis. Not least the great efficacy of the anti-IL-12/IL-23 monoclonal antibody ustekinumab in psoriasis treatment has shown the prominent role of this pathway (Di Cesare *et al.,* 2009; Di Meglio and Nestle, 2010). As summarized by Lowes et al. the role of IL-23 in the acute and chronic phase of psoriasis could be pictured as shown in Figure 41.

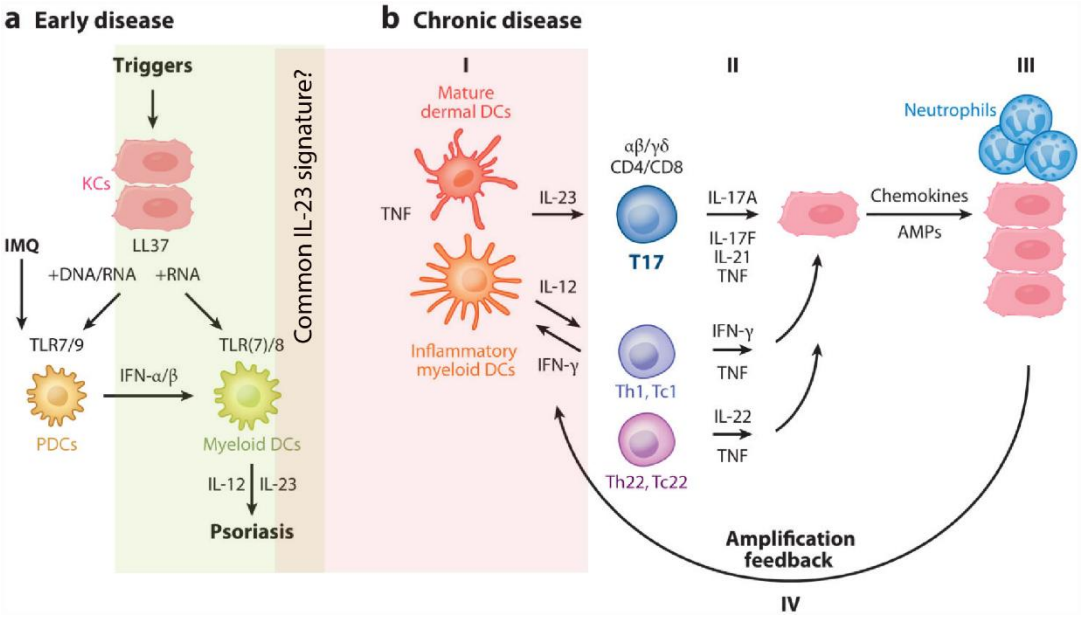


Figure 41: The role of IL-23 during initiation and maintenance of psoriatic inflammation

During early disease (a) imiquimod (IMQ) or a complex of antimicrobial peptides and self-DNA/self-RNA activates TLR7/9 receptors on plasmacytoid dendritic cells (pDCs) which in turn produce interferons (IFN). LL-37/RNA complexes can also activate resident myeloid DCs to produce IL-12 and IL-23which induce Th17 activation and thus initiate disease. During chronic disease (b) mature dermal DCs and inflammatory myeloid DCs produce IL-23 and IL-12 (I) which activate T17 (Th17 and Tc17), Th1 and Th22 (II) cells to contribute to the cytokine milieu and to act on keratinocytes (III). Keratinocytes in turn produce chemokines and antimicrobial peptides (AMPs) to amplify cutaneous immune responses (IV). Thus, IL-23 contributes to the expansion and survival of T17 cells producing IL-17. Modified from Lowes et al., 2014.

Taken together these findings, one might assume that the common IL-23 signature found in PL and Psoriasis is mainly based on the fact that IL-23 plays a role in both initial and chronic phase of psoriasis. This is supported by the missing hallmarks psoriasis architecture such as hyperparakeratosis, neoangiogenesis and papillomatosis in PL samples.

However, due to missing alternatives for human models of psoriasis, imiquimod cream induced skin inflammation could at least mirror the early phase of psoriasis and might thus serve as a model to study initiation of psoriatic disease in humans. However, it remains to be elucidated if the IL-23 pathway is indeed mimicked as supposed in Figure 41. IL-23 production could also be a mere "side-product" of overshooting NF-κB activation, that via activation of IL-1 induces IL-23 as suggested by our pathway analysis and published data (Cogswell *et al.*, 1994; Liu *et al.*, 2007). Further in vitro experiments and in vivo experiments would have to clarify this possibility in more detail.

## 4.4 Patients affected by both psoriasis and eczema – a model to study disease-specific signatures

In our study, the human imiquimod model has been shown to reflect aspects of psoriasis and eczema and might be useful for further investigating particular phases of disease evolvement and for studying particular pathways such as the IL-23 cytokine pathway. However, to develop disease-specific classifiers, which at their best allow predicting the best therapeutic option for individuals, there is no way around to study both psoriasis and eczema at their more complex acute and chronic form occurring in "real-life-patients".

### 4.4.1 Intraindividual comparison of psoriasis and eczema

Modern high-throughput technologies analyzing the full transcriptome of diseased skin have enabled research to generate big data sets for inflammatory skin diseases.

However, the methods are vulnerable and interindividual differences as well as distinct experimental set-ups have eventually resulted in only little overlap between differentially expressed genes across different studies (Ewald *et al.*, 2015). Using the random-effects model, Ewald et al. combined microarray data from independent but similarly designed studies for meta-analysis. They could show that using this method, the reproducibility and the power of the transcriptome of atopic eczema could be increased as inflammatory and barrier pathways were more significantly enriched compared to individual studies (Ewald *et al.*, 2015). This approach may indeed help to carve out the most prominent players by correcting for interstudy differences, however, as only one condition per patient, namely eczema, is studied, mechanisms of general cutaneous inflammation may not be separated from disease-specific signatures. In line with this, Nomura et al. observed that when comparing psoriasis and eczema signatures using microarrays that the expression of most of the more than 12,000 genes examined were similar in psoriasis and atopic eczema. They and others who took advantage of the approach of interdisease comparison found differential expression of genes, for example genes encoding for antimicrobial peptides such as β defensing 2 (*HBD2)* and elafin. However, the broad overlap of genes between both conditions hints at the presence of disease-unspecific inflammatory background noise (de Jongh *et al.*, 2005; Guttman-Yassky *et al.*, 2008; Nomura *et al.*, 2003a).

With our model of intraindividual disease-comparison, we can kill two birds with one stone and combine the two presented approaches: First, the comparison of different inflammatory diseases allows deciphering disease-specific mechanisms independent of general inflammatory background processes. Second, examining the two conditions in the same patients limits interindividual differences such as alterations in environmental circumstances prior to sampling, genetic heterogeneity and differences in experimental procedures. Thus, all observed regulated genes shown in our study were not driven by the genetic background, but depend exclusively on the local stimulus that drives the inflammation towards the clinical phenotype of psoriasis or eczema, respectively. The fact that psoriasis, eczema and non-lesional skin do not cluster according to the skin condition when using principal component analysis, but rather in patient-wise clustering (data not shown), highlights the presence of interindividual differences that mask disease-specific genes. In contrast, when only genes significantly up- or downregulated as compared to non-involved skin were taken into account, a disease-related grouping was observed which confirms our approach (Quaranta *et al.*, 2014b). In summary, we could show that although psoriasis and eczema do share common signaling pathways and top hit genes, they are characterized by distinct mechanisms. For the epidermal compartment all forms of naturally occurring eczema for example share severe defects in epidermal cornification and barrier, whereas psoriasis is characterized by altered epidermal development and differentiation (genes of the *SPRR* family and the *LCE* family involved in epidermal differentiation are upregulated in psoriasis) (Quaranta *et al.*, 2014b). In terms of immune system, we could show that all eczema variants are characterized by significant upregulation of Th2 cytokines (IL-13, IL-10, IL-4 and IL-5), whereas in psoriasis Th17 responses, IL-10 family cytokines and IL-36 signatures dominate (Quaranta *et al.*, 2014b). Regarding the domain of metabolism, only psoriasis but not eczema was shown to upregulate genes encoding for proteins involved in glucose and lipid metabolism (e.g. genes encoding for iNOS, aldo-keto reductases and phospholipases) clearly showing that psoriasis is not only an inflammatory but also a metabolic disease (Quaranta *et al.*, 2014b).

The data resulting from this intraindividual disease comparison enabled us to develop a small-size disease classifier, a novel diagnostic tool that is of increasing relevance for daily clinical practice.

### 4.4.2 A disease classifier for psoriasis and eczema

Though main hallmarks of both psoriasis and eczema have been discovered, the underlying pathomechanisms are complex and are due – to their heterogeneity – of different priority in individual patients. This is reflected by the fact that even when applying the most specific therapies so far, namely the anti-IL-17 antibody secukinumab in psoriasis and anti-IL4 and anti-IL-13 antibody dupilumab in atopic eczema, only around 80% of psoriasis patients will show at least 75% improvement (Langley *et al.*, 2014) and only around 70% of eczema patients are therapy-responders (Beck *et al.*, 2014). Up to date, the therapeutic response cannot be predicted because reliable biomarkers and diagnostic tests do not exist for psoriasis and eczema. Thus, characterizing the disease-specific signatures of psoriasis and eczema has incited researches to design disease classification systems that would be able to distinguish both diseases with the smallest set of genes possible.

One of the earliest approaches was presented by Wenzel et al. who designed a skin-specific array and – due to its common and distinct features with psoriasis and eczema – lichen planus was chosen as model disease. Using unclassified clustering they could clearly distinguish lichen planus from psoriasis and eczema (Wenzel *et al.*, 2008). However, the minimum amount of genes needed for classification was not determined. By comparing disease signatures of psoriasis, eczema and healthy skin on mRNA level, Guttman-Yassky et al. found an "immune-signature" class prediction comprising 10 genes among those *IL-15, IL-17A* and *NOS2A* that classified all cases included into the study accurately. Also when focusing on genes from the domain of epidermal differentiation disease classification was possible: Based on the finding that keratinocyte terminal differentiation was oppositely polarized in psoriasis and eczema, a class prediction with 13 significantly differently expressed genes was created and predicted the correct diagnosis (Guttman-Yassky *et al.*, 2009). A broad approach covering a multitude of skin diseases has been recently presented by Inkeles et al.: Using microarray data from 16 different skin diseases and more than 300 samples a multi-disease classifier could be created which diagnosed with 93% accuracy and predicted the eventual diagnosis in an undifferentiated patient (Inkeles *et al.*, 2014).

However, reducing the size of classifiers has remained a main goal not least as implementation into daily clinical practice would require manageable methods. The first single-

molecule based classifier using IL-36γ as marker was, however, only able to classify 15 out of 21 samples leaving a substantial diagnostic gap (D'Erme *et al.*, 2015).

Using our approach of performing intraindividual disease comparison, we were able to downsize the list of interesting candidate genes. With our classifier only based on the expression of *NOS2* and *CCL27*, we could show robust classification and demonstrated that these two markers successfully passed several validation levels. Beginning with its application in a large cohort of clear patients, going on to a cohort of subclasses and indistinct cases to its application on protein level, the classifier predicted with high sensitivity and specificity.

The high sensitivity and specificity of our molecular test is based on its two components which – working as mutually complementing couple – mirror the complex disease signatures of psoriasis and eczema. *NOS2* is known to be a key player for metabolic and inflammatory processes in its function as NO producer (Kanwar *et al.*, 2009). We corroborated these findings as *NOS2* expression in lesional skin significantly correlated positively with patients' BMI. Moreover, in line with the findings that *NOS2* is significantly upregulated in psoriatic lesional skin compared to healthy skin or eczema (Bruch-Gerharz *et al.*, 1996; Guttman-Yassky *et al.*, 2009; Nomura *et al.*, 2003a; Ormerod *et al.*, 1998), we showed that *NOS2* expression was highly associated with hallmarks of psoriasis such as hypogranulosis and neutrophils, but negatively associated with eosinophils and spongiosis as characteristics of eczema. In contrast, levels of *CCL27* were shown to be positively associated with asthma and allergic rhinoconjunctivitis. This finding strengthens previous results showing significantly lower expression of *CCL27* in psoriasis skin compared with atopic eczema and normal control skin (Nomura *et al.*, 2003a). Besides, it is in line with the finding that *CCL27* serum levels correlate with clinical severity score of eczema (Kakinuma *et al.*, 2003) and the fact of enhanced contact hypersensitivity to Th2, but not Th1 stimuli in CCL27-transgenic mice (Kagami *et al.*, 2008).

As, however, our markers have succeeded in giving the correct prognosis for clinically and histologically unclear markers, *NOS2* and *CCL27* have proven to represent disease-specific parameters beyond established clinical criteria and histological parameters. Thus, the presented diagnostic tool is robust and at the same time superior than current gold standard diagnostic methods in giving the correct diagnosis for overlapping phenotypes. Compared to the above-described classifiers of other groups, we could show that ours is

the first on the level of PCR characterized by both small size and high test sensitivity and specificity.

We showed that in the case of patients who display features of both psoriasis and eczema a probability to suffer from either the one or the other disease could be given by our classifier and the patient may be directly subjected to the adequate therapy. If the classifier had been available already at the time of diagnosis of one of our index patients (3.3.3.5 Validation of the MC as a diagnostic tool for patients remaining unclear by standard diagnostic means), the psoriasis treatment with fumaderm could have been avoided in this patient, as well as the subsequent exacerbation of disease. Our classifier clearly suggested eczema as underlying diagnosis of the skin condition in this patient. Thus, when established in clinical daily practice our classifier may prevent eczema patients – when misdiagnosed as psoriasis – from experiencing impairment of disease if for example a TNF-α inhibitor is chosen (Jacobi *et al.*, 2005). Moreover, it may prevent unnecessary detention of psoriasis patients – when misdiagnosed as eczema – from early induction of specific systemic therapy with, for example, a TNF-α inhibitor. The classifier's impact is not limited to the improvement of the individual's disease course. It may also have economic implications for the health system: The annual costs for psoriasis per patient may rise up to 24,000 USD when the TNF-α inhibitor infliximab is applied (Beyer and Wolverton, 2010), whereas the annual costs for medium potent topical steroids for an eczema patient are in the range of 120 USD (Green *et al.*, 2005).

With the results from the classifier, a clinician can be supported while making the general therapeutic decision between "eczema therapy" and "psoriasis therapy". However, as the classifier is only based on two markers it cannot provide further differentiated information concerning the detailed therapeutic regimen. However, questions often arise when the concrete substances among the many available ones have to be chosen. For example, how would a clinician know if a TNF- α inhibitor would be more effective in a patient than for example an IL-17 blocker once it is obvious that the patients need systemic treatment? Up to date, no classifier can give information about the risk for chronicity, progression and development of comorbidities, not to speak about the optimal therapeutic regimen. To answer these questions novel innovative approaches for patient stratification need to be followed. Section 4.5 will highlight some of the possible new approaches.

In addition, any patient needs to be pre-evaluated by a clinician before being tested by the classifier. When testing lichen planus on our two genes based classifier (section 3.3.3.6 The MC as a reliable tool for other inflammatory skin diseases?), it was diagnosed as eczema showing that it seems impossible to distinguish three diseases with only two gene markers. The price for a highly robust classifier performance in combination with simple experimental design is that a specific clinical question needs to be defined. Our classifier answers the clinically relevant question how to distinguish psoriasis from eczema. A less defined clinical question (e.g. „Is this phenotype psoriasis, eczema, or lichen planus? “) requires additional gene markers. To distinguish three diseases at least three genes are needed and the experienced eye of a clinician is not fully dispensable. Expanding our classifier based on *NOS2* and *CCL27* to *CXCL9,* we could classify 26 out of 30 patients suffering from either lichen planus, psoriasis or eczema correctly. As the marker was chosen based on other publications and our own data did not reach statistical significance (only four microarrays on lichen planus samples included), a better marker combination for higher sensitivity and specificity seems possible.

## 4.5 Opening new horizons for inflammatory skin diseases: Summary, outlook and perspectives

For my studies, I used three different models to investigate psoriasis and eczema and each model has proven its advantages and disadvantages:

The alopecia areata model provides a unique platform to study the interplay of genes and environment. We could verify that AA patients do have a background that predisposes to also develop inflammatory skin diseases and that depending on the stimulus, inflammation can be driven towards specific inflammatory patterns which resemble typical disease patterns seen in single affected patients. However, results of e.g. gene expression analyses have not been provided by our analysis and it remains unclear how reliably results gained from this specific cohort can be transferred to general questions regarding psoriasis and eczema. Besides, the heterogeneity within the respective inflammatory skin diseases is high, as – due to limited AA patient numbers – no further stratification within the inflammatory skin diseases has been performed.

This is in contrast with the second model I studied, the imiquimod-induced skin inflammation. Here the induced reaction has been shown to be homogeneous in all patients and healthy volunteers independent of the genetic background. Besides, this reaction could be easily induced by simple topical patch test application. However, these advantages are contrasted by inaccuracy in terms of adequately reflecting one specific skin disease. We could show that the imiquimod-induced skin reaction not only mimics characteristics of acute contact dermatitis but also reflects important pathways of psoriasis such as the IL-23 pathway. It remains unclear how this model could find its implementation as a proper human model and questions to be studied most likely need to be explicitly defined.

The third model represented by the cohort of patients both affected by psoriasis and eczema simultaneously, faces the same disadvantage as the AA model. Though intraindividual comparison provides the big advantage of investigating diseases independent of genetic background and environmental factors such as short-term environmental differences before material sampling, heterogeneity within this group is still high. In contrast to the AA study, however, the number of patients within this study was high enough to at least differentiate between naturally occurring eczema and acute contact dermatitis and thus to reduce heterogeneity.

Moving the borders of knowledge in inflammatory skin diseases further, the models I investigated could be integrated with novel models to come in order to investigate functional relationships. On clinical level, patients need to be stratified and on experimental level, techniques need to be refined, different technical approaches should be combined and data from mouse models need to be carefully validated at an early stage in humans. Here, the role of computational biology becomes more and more fundamental, as the big data sets generated need to be integrated with each other.

### 4.5.1 Combination of novel techniques and individual parameters

High throughput technologies such as microarray analyses and RNA sequencing have mainly contributed to our understanding of the pathogenesis of psoriasis and eczema and the discovery of main key mediators and therapeutic targets. However, their power alone is limited, as for example the contribution of the different compartments of the skin to the molecular events in diseased skin cannot be disclosed. To overcome this problem, Esaki et al. for example performed laser capture microdissection to separate the epidermis and

dermis of lesional and non-lesional skin from patients with atopic eczema and normal skin from healthy controls. The different compartments were subjected to microarrays and real-time PCR analyses as well as immunostaining studies. Using this approach, they found a multitude of gene products usually not detected on arrays, for example IL-22 and TSLP. Besides, they could localize disease-specific barrier or immune molecules to the respective compartments, which may help in elucidating the detailed interactions of key players (Esaki *et al.*, 2015).

An example of a successful integrative biology approach in psoriasis is the study of Perera et al. analyzing the role of IL-22 as a model cytokine. Injecting IL-22 in human skin grafts of mice led to a psoriasis-like phenotype in mice, which could be blocked by an IL-22 neutralizing antibody. Integrating subsequently both the murine IL-22 and anti-IL-22 cytokine transcriptomes and mapping them onto a human psoriasis gene-coexpression-network, they identified key cytokine-dependent hub genes. One of the hub genes, the so far unexplored serine/threonine kinase PIM1 was found to be a possible critical checkpoint for human skin inflammation (Perera *et al.*, 2014). This study shows how crucial network-based strategies are. In tumor research, this approach has already been carried further: Using unsupervised network-based stratification (NBS), somatic tumor genomes can be integrated with gene networks allowing for stratification of cancer into informative subtypes. Here, patients with mutations in similar network regions clustered together in subgroups which in turn were predictive of clinical outcomes such as patient survival, response to therapy or tumor histology (Hofree *et al.*, 2013). The success in the tumor field is certainly based on the advantage that here, researchers focus on tumor mutations which are relatively stable in comparison to transcriptomics and proteomics data which are relevant for inflammatory skin diseases. RNA and proteins are constantly exposed to degradation processes leading to the situation that only a “snap-shot picture” from the disease is captured. This of course contributes to the higher intervariability within the same but also different data types. As a result, transcriptome and proteome datasets usually show only weak positive correlation. However, in their study “Integrated network analysis of transcriptome and proteomic data in psoriasis” Piruzian et al. show that if more precise computational methods are applied such as topology and biological networks obstacles may be overcome. They state that “…for example, topology methods such as "hidden nodes" can identify and rank the upstream regulatory genes responsible for expression and protein level alterations while network tools help to uncover functional modules most

affected in the datasets, identify the most influential genes/proteins within the modules and suggest how specific modules contribution to clinical phenotype…" (Piruzian *et al.*, 2010). Another difference between inflammatory skin diseases and tumors is that tumors develop when – due to genetic predisposition and environmental influences – a particular "mutational overload" is reached. This means that the endpoint of many investigations are the tumor-underlying mutations that can eventually be captured by tumor genome sequencing and then related to clinical information for establishment of subgroups. Thus, targeting the endpoint, which is the tumor mutation, has already proven great success. But what are the "endpoint candidates" for psoriasis and eczema? Somatic mutations within the diseased skin do not play a role and metastases to indicate system contribution do not occur. Instead, psoriasis and eczema are equally "intrinsic and extrinsic" diseases, meaning that environment and genes, epidermis and immune system are constantly interacting leading to undulant disease courses with phases of exacerbation and improvement, whereas tumor is usually characterized by constant progression. The interplay of environment and skin has been of broad interest over the past years: Many studies have focused on characterizing the microbiome as interaction between skin commensals and skin organ is increasingly regarded as crucial part within the pathogenesis (Blaser, 2014; Grice *et al.*, 2009). The human microbiome is even considered as "second genome" (Grice and Segre, 2012). In line with this, Kong et al. showed that temporal shifts in the skin microbiome were associated with disease flares in children with atopic dermatitis (Kong *et al.*, 2012). Going beyond the purely descriptive level, early mouse studies have started revealing functional relationships between microbiome and disease: Kobayashi et al. demonstrated differential contributions of bacterial species during development of eczema. *Staphylococcus aureus* was shown to drive eczema formation, whereas *Corynebacterium bovis* induced robust Th2 responses (Kobayashi *et al.*, 2015). The numbers of studies focusing on diverse molecular aspects of psoriasis and atopic eczema could be continued infinitely, comprising studies investigating metabolomics (Armstrong *et al.*, 2014; Huang *et al.*, 2014) and epigenetics (Quraishi *et al.*, 2015; Roberson *et al.*, 2012). But not only research technologies are advancing. Also modern telecommunication technologies are in the forefront, enabling broad progress in the field of telemedicine which in turn may support basic research. Online-tools to monitor and advice patients to strengthen their compliance have been successfully implemented in the field of diabetes mellitus and heart insufficiency (Comin-Colet *et al.*, 2015; Deacon and Edirippulige,

2015). In 2015, 94% of all households in Germany use at least one mobile phone (Statistisches Bundesamt Pressemitteilung Nr. 172 vom 12.05.2015) and thus it is not surprising that the use of mobile technologies such as Apps are rapidly expanding practice to track and improve health outcomes (Boudreaux *et al.*, 2014). The possibility to collect data via these technologies for researches are unbounded: Individual disease courses could be tracked via regular self-monitoring by the patients themselves and tailored life-style consults could be given independent of doctoral visits. These data could then be integrated with laboratory and clinical data collected at the hospital and in the lab. The challenge now is to integrate and validate the multidimensional data derived from many disciplines of research that are constantly accumulating.

### 4.5.2 Leaving the track of antique disease nomenclature

Integrating information with novel complex computational biology approaches is one thing. The other fundamental prerequisite for successful multidimensional data analysis is precise information about what data exactly is subjected to the "black box" of computational data analysis. The fact that many disease classifiers are of mediocre sensitivity and specificity is mainly due to the scarce amount of clinical information that comes along with each sample. Apart from sex and age, other clinical information such as duration of the lesion at time of sampling, comorbidities and family history are not considered. Thus, the pitfall of most basic research studies is that patients included to the respective study are not deeply phenotyped, but rather given a rough label such as "eczema" and "psoriasis". Doing so, the heterogeneity of both diseases is already disregarded at the earliest stage of work- up (Figure 42) and strongly reduces the possibilities of computational biology in terms of deciphering subtypes of disease. Dermatology may be in particular affected by the system of purely description-based diagnoses: Dermatological nomenclature and disease systematics has been accrued from historical observations focusing on clinical morphology rather than on underlying disease mechanisms. Psoriasis and pityriasis rosea for example are both listed in the category of erythrosquamous diseases as the clinical feature of both diseases are comprised of the efflorescences erythema, plaques and scales. However, their pathogenesis and clinical course are entirely different (Watanabe *et al.*, 2002). On the other hand, especially the armamentarium of dermatological assessment offers the unique possibility of newly categorizing diseases provided

that it is not limited to it. The idea would be to label patients with a variety of clinical, histological, laboratory and anamnestic attributes rather than just the coarse disease label. For psoriasis, Guinot et al. have realized that psoriasis is not one homogeneous entity but rather a collection of many different phenotypes resulting in distinct treatment-responses. Using a hypothesis-free approach, they performed a cluster analysis on a cohort of more than 1000 psoriasis patients according to defined clinical characteristics (e.g. clinical aspect of plaque, individual daily life factors, comorbidities, body sites affected) assessed for each patient. At least six different clusters and thus six relatively distinct clinical phenotypes could be found (Guinot *et al.*, 2009). Performing genetic and pharmacological studies within these more homogeneous subgroups seem promising. However, pure phenotyping on clinical aspects may be limited and the goal should be to not only label patients with a number of attributes but also tag this "clinical phenotype fingerprint or barcode" to molecular data comprising date about genome, transcriptome and microbiome. For example one eczema patient would undergo molecular analysis labeled with the attributes of "papules", "erythema", "spongiosis", "elevated IgE level" and "acute onset" while another patient would undergo the molecular analysis with the attributes of "plaques", "hyperkeratosis", "erythema" and "colonization with *Staphylococcus aureus*" (Figure 42).

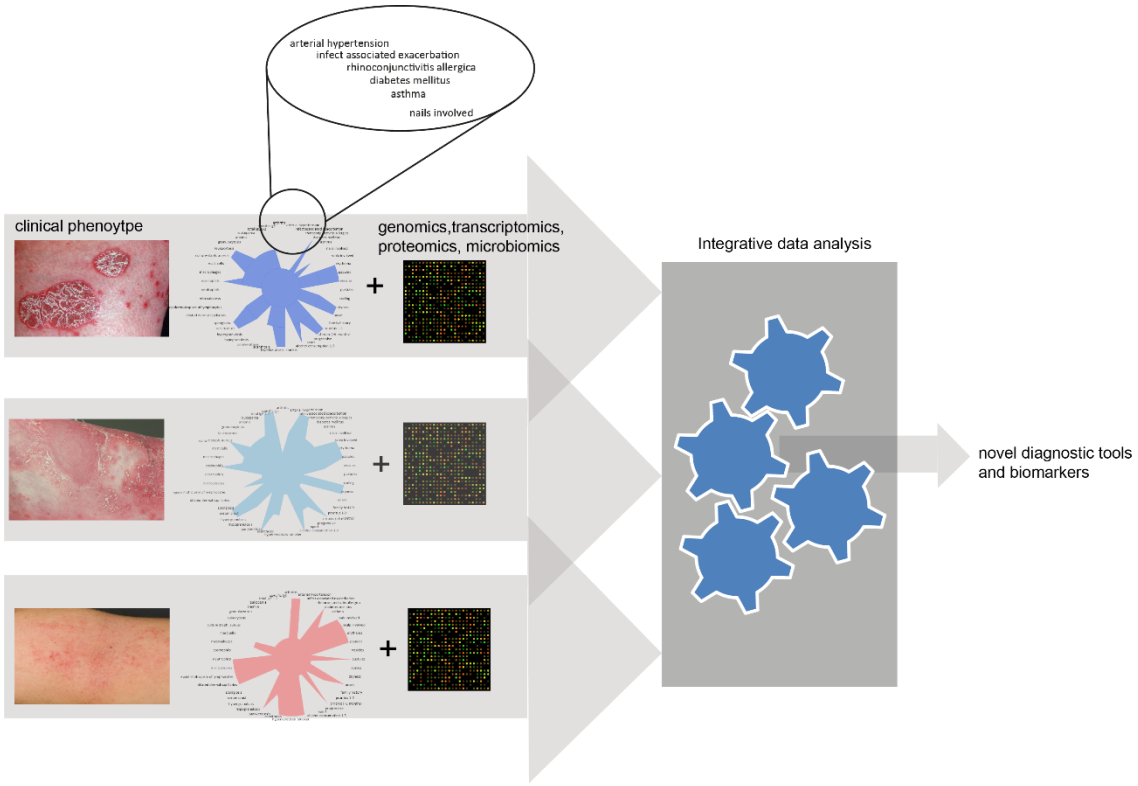


Figure 42: Workflow of multimodal data acquisition and integration to define new diagnostic tools and biomarkers

In our classifier study, we used 46 attributes to characterize the patients included. We then associated the attributes with the expression of *NOS2* and *CCL27* levels. Doing so, we could associate our markers to classical hallmarks of the disease. However, the study size was too small considering a) the rather incomplete information about therapies and b) the many combinations of possible therapeutic options to find correlations of *NOS2* and *CCL27* expression with therapeutical outcome. With a larger, fully described cohort available, one could imagine that for example the level of *NOS2* expression would predict the optimal therapy. To carry this idea a bit further one could imagine that skin diseases are not purely classified by the rough diagnostic name any more but rather by their molecular signature resulting for example in the subgroups of "low *NOS2*" and "high *NOS2*" level psoriasis patients with implications for therapeutic outcome and risk for comorbidities. In our study, we could show that high *NOS2* levels significantly correlated with higher BMI and thus high *NOS2* levels might be correlated with a higher risk to develop metabolic diseases. This indicates that *NOS2* could be a representative of such as a new wave of biomarkers.

# References

Acikgoz G, Caliskan E, Tunca M, *et al.* (2014) The effect of oral cyclosporine in the treatment of severe alopecia areata. *Cutaneous and ocular toxicology* 33:247-52.

Ahlehoff O, Skov L, Gislason G, *et al.* (2012) Pharmacological undertreatment of coronary risk factors in patients with psoriasis: observational study of the Danish nationwide registries. *PloS one* 7:e36342.

Albanesi C, Scarponi C, Pallotta S, *et al.* (2009) Chemerin expression marks early psoriatic skin lesions and correlates with plasmacytoid dendritic cell recruitment. *The Journal of experimental medicine* 206:249-58.

Armstrong AW, Wu J, Johnson MA, *et al.* (2014) Metabolomics in psoriatic disease: pilot study reveals metabolite differences in psoriasis and psoriatic arthritis. *F1000Res* 3:248.

Armstrong EJ, Harskamp CT, Armstrong AW (2013) Psoriasis and major adverse cardiovascular events: a systematic review and meta-analysis of observational studies. *Journal of the American Heart Association* 2:e000062.

Atwan A, Ingram JR, Abbott R, *et al.* (2015) Oral fumaric acid esters for psoriasis. *Cochrane Database Syst Rev* 8:CD010497.

Augustin M, Kruger K, Radtke MA, *et al.* (2008) Disease severity, quality of life and health care in plaque-type psoriasis: a multicenter cross-sectional study in Germany. *Dermatology* 216:366-72.

Augustin M, Schafer I, Reich K, *et al.* (2011) Systemic treatment with corticosteroids in psoriasis--health care provision far beyond the S3-guidelines. *J Dtsch Dermatol Ges* 9:833-8.

Aydin O, Engin B, Oguz O, *et al.* (2008) Non-pustular palmoplantar psoriasis: is histologic differentiation from eczematous dermatitis possible? *Journal of cutaneous pathology* 35:169-73.

Barker J (2014) Psoriasis heritability: 125 years and counting. *The British journal of dermatology* 171:3-5.

Bauer S, Gagneur J, Robinson PN (2010) GOing Bayesian: model-based gene set analysis of genome-scale data. *Nucleic Acids Res* 38:3523-32.

Beattie PE, Lewis-Jones MS (2006) A comparative study of impairment of quality of life in children with skin disease and children with other chronic childhood diseases. *The British journal of dermatology* 155:145-51.

Beck LA, Thaci D, Hamilton JD, *et al.* (2014) Dupilumab treatment in adults with moderate-to-severe atopic dermatitis. *N Engl J Med* 371:130-9.

Betz RC, Petukhova L, Ripke S, *et al.* (2015) Genome-wide meta-analysis in alopecia areata resolves HLA associations and reveals two new susceptibility loci. *Nature communications* 6:5966.

Betz RC, Pforr J, Flaquer A, *et al.* (2007) Loss-of-function mutations in the filaggrin gene and alopecia areata: strong risk factor for a severe course of disease in patients comorbid for atopic disease. *The Journal of investigative dermatology* 127:2539-43.

Beyer V, Wolverton SE (2010) Recent trends in systemic psoriasis treatment costs. *Archives of dermatology* 146:46-54.

Bieber T (2008) Atopic dermatitis. *N Engl J Med* 358:1483-94.

Blaser MJ (2014) The microbiome revolution. *The Journal of clinical investigation* 124:4162-5.

Blauvelt A, Armstrong AW, Krueger GG (2015) Essential Truths for the Care and Management of Moderate-to-Severe Psoriasis. *Journal of drugs in dermatology : JDD* 14:805-12.

Boehncke WH, Boehncke S, Tobin AM, *et al.* (2011) The 'psoriatic march': a concept of how severe psoriasis may drive cardiovascular comorbidity. *Experimental dermatology* 20:303-7.

Boehncke WH, Schon MP (2015) Psoriasis. *Lancet* 386:983-94.

Boudreaux ED, Waring ME, Hayes RB, *et al.* (2014) Evaluating and selecting mobile health apps: strategies for healthcare providers and healthcare organizations. *Transl Behav Med* 4:363-71.

Boyman O, Hefti HP, Conrad C, *et al.* (2004) Spontaneous development of psoriasis in a new animal model shows an essential role for resident T cells and tumor necrosis factor-alpha. *The Journal of experimental medicine* 199:731-6.

Brenninkmeijer EE, Schram ME, Leeflang MM, *et al.* (2008) Diagnostic criteria for atopic dermatitis: a systematic review. *The British journal of dermatology* 158:754-65.

Brezinski EA, Dhillon JS, Armstrong AW (2015) Economic Burden of Psoriasis in the United States: A Systematic Review. *JAMA Dermatol* 151:651-8.

Bruch-Gerharz D, Fehsel K, Suschek C, *et al.* (1996) A proinflammatory activity of interleukin 8 in human skin: expression of the inducible nitric oxide synthase in psoriatic lesions and cultured keratinocytes. *The Journal of experimental medicine* 184:2007-12.

Carroll CL, Balkrishnan R, Feldman SR, *et al.* (2005) The burden of atopic dermatitis: impact on the patient, family, and society. *Pediatric dermatology* 22:192-9.

Chen H, Poon A, Yeung C, *et al.* (2011) A genetic risk score combining ten psoriasis risk loci improves disease prediction. *PloS one* 6:e19454.

Chiricozzi A, Faleri S, Saraceno R, *et al.* (2015) Tofacitinib for the treatment of moderate-to-severe psoriasis. *Expert review of clinical immunology* 11:443-55.

Christophers E (2001) Psoriasis--epidemiology and clinical spectrum. *Clin Exp Dermatol* 26:314-20.

Chu SY, Chen YJ, Tseng WC, *et al.* (2011) Comorbidity profiles among patients with alopecia areata: the importance of onset age, a nationwide population-based study. *Journal of the American Academy of Dermatology* 65:949-56.

Cogswell JP, Godlevski MM, Wisely GB, *et al.* (1994) NF-kappa B regulates IL-1 beta transcription through a consensus NF-kappa B binding site and a nonconsensus CRE-like site. *J Immunol* 153:712-23.

Comin-Colet J, Enjuanes C, Verdu-Rotellar JM, *et al.* (2015) Impact on clinical events and healthcare costs of adding telemedicine to multidisciplinary disease management programmes for heart failure: Results of a randomized controlled trial. *Journal of telemedicine and telecare*.

D'Erme AM, Wilsmann-Theis D, Wagenpfeil J, *et al.* (2015) IL-36gamma (IL-1F9) is a biomarker for psoriasis skin lesions. *The Journal of investigative dermatology* 135:1025-32.

Dalgard FJ, Gieler U, Tomas-Aragones L, *et al.* (2015) The psychological burden of skin diseases: a cross-sectional multicenter study among dermatological out-patients in 13 European countries. *The Journal of investigative dermatology* 135:984-91.

de Jongh GJ, Zeeuwen PL, Kucharekova M, *et al.* (2005) High expression levels of keratinocyte antimicrobial proteins in psoriasis compared with atopic dermatitis. *The Journal of investigative dermatology* 125:1163-73.

Deacon AJ, Edirippulige S (2015) Using mobile technology to motivate adolescents with type 1 diabetes mellitus: A systematic review of recent literature. *Journal of telemedicine and telecare*.

Deckers IA, McLean S, Linssen S, *et al.* (2012) Investigating international time trends in the incidence and prevalence of atopic eczema 1990-2010: a systematic review of epidemiological studies. *PloS one* 7:e39803.

Deckert S, Kopkow C, Schmitt J (2014) Nonallergic comorbidities of atopic eczema: an overview of systematic reviews. *Allergy* 69:37-45.

Di Cesare A, Di Meglio P, Nestle FO (2009) The IL-23/Th17 axis in the immunopathogenesis of psoriasis. *The Journal of investigative dermatology* 129:1339-50.

Di Meglio P, Nestle FO (2010) The role of IL-23 in the immunopathogenesis of psoriasis. *F1000 Biol Rep* 2.

Dickson MC, Ludbrook VJ, Perry HC, *et al.* (2015) A model of skin inflammation in humans leads to a rapid and reproducible increase in the interferon response signature: a potential translational model for drug development. *Inflamm Res* 64:171-83.

Diepgen TL (2003) Occupational skin-disease data in Europe. *Int Arch Occup Environ Health* 76:331-8.

Diepgen TL, Scheidt R, Weisshaar E, *et al.* (2013) Cost of illness from occupational hand eczema in Germany. *Contact Dermatitis* 69:99-106.

Dou L, Liang HF, Geller DA, *et al.* (2014) The regulation role of interferon regulatory factor-1 gene and clinical relevance. *Hum Immunol* 75:1110-4.

Dummer R, Hauschild A, Lindenblatt N, *et al.* (2015) Cutaneous melanoma: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol* 26 Suppl 5:v126-32.

Eckert RL, Broome AM, Ruse M, *et al.* (2004) S100 proteins in the epidermis. *The Journal of investigative dermatology* 123:23-33.

Ellinghaus D, Baurecht H, Esparza-Gordillo J, *et al.* (2013) High-density genotyping study identifies four new susceptibility loci for atopic dermatitis. *Nature genetics* 45:808-12.

Esaki H, Ewald DA, Ungar B, *et al.* (2015) Identification of novel immune and barrier genes in atopic dermatitis by means of laser capture microdissection. *The Journal of allergy and clinical immunology* 135:153-63.

Ewald DA, Malajian D, Krueger JG, *et al.* (2015) Meta-analysis derived atopic dermatitis (MADAD) transcriptome defines a robust AD signature highlighting the involvement of atherosclerosis and lipid metabolism pathways. *BMC Med Genomics* 8:60.

Eyerich K, Novak N (2013) Immunology of atopic eczema: overcoming the Th1/Th2 paradigm. *Allergy* 68:974-82.

Eyerich S, Onken AT, Weidinger S, *et al.* (2011) Mutual antagonism of T cells causing psoriasis and atopic eczema. *N Engl J Med* 365:231-8.

Feldman SR, Krueger GG (2005) Psoriasis assessment tools in clinical trials. *Annals of the rheumatic diseases* 64 Suppl 2:ii65-8; discussion ii9-73.

Flutter B, Nestle FO (2013) TLRs to cytokines: Mechanistic insights from the imiquimod mouse model of psoriasis. *European journal of immunology*.

Ganguly D, Chamilos G, Lande R, *et al.* (2009) Self-RNA-antimicrobial peptide complexes activate human dendritic cells through TLR7 and TLR8. *The Journal of experimental medicine* 206:1983-94.

Garzorz N, Eyerich K (2015) NOS2 and CCL27: clinical implications for psoriasis and eczema diagnosis and management. *Expert review of clinical immunology* 11:167-9.

Genetics EA, Lifecourse Epidemiology Eczema C, Australian Asthma Genetics C, *et al.* (2015) Multi-ancestry genome-wide association study of 21,000 cases and 95,000 controls identifies new risk loci for atopic dermatitis. *Nature genetics*.

Gilhar A, Etzioni A, Paus R (2012) Alopecia areata. *N Engl J Med* 366:1515-25.

Gilhar A, Paus R, Kalish RS (2007) Lymphocytes, neuropeptides, and genes involved in alopecia areata. *J Clin Invest* 117:2019-27.

Goh C, Finkel M, Christos PJ, *et al.* (2006) Profile of 513 patients with alopecia areata: associations of disease subtypes with atopy, autoimmune disease and positive family history. *J Eur Acad Dermatol Venereol* 20:1055-60.

Gordon KB, Duffin KC, Bissonnette R, *et al.* (2015) A Phase 2 Trial of Guselkumab versus Adalimumab for Plaque Psoriasis. *N Engl J Med* 373:136-44.

Gordon KB, Langley RG, Leonardi C, *et al.* (2006) Clinical response to adalimumab treatment in patients with moderate to severe psoriasis: double-blind, randomized controlled trial and open-label extension study. *Journal of the American Academy of Dermatology* 55:598-606.

Gottlieb AB, Chamian F, Masud S, *et al.* (2005) TNF inhibition rapidly down-regulates multiple proinflammatory pathways in psoriasis plaques. *J Immunol* 175:2721-9.

Gottlieb AB, Dann F (2009) Comorbidities in patients with psoriasis. *The American journal of medicine* 122:1150 e1-9.

Gowda S, Goldblum OM, McCall WV, *et al.* (2010) Factors affecting sleep quality in patients with psoriasis. *Journal of the American Academy of Dermatology* 63:114-23.

Green C, Colquitt JL, Kirby J, *et al.* (2005) Topical corticosteroids for atopic eczema: clinical and cost effectiveness of once-daily vs. more frequent use. *The British journal of dermatology* 152:130-41.

Grice EA, Kong HH, Conlan S, *et al.* (2009) Topographical and temporal diversity of the human skin microbiome. *Science* 324:1190-2.

Grice EA, Segre JA (2012) The human microbiome: our second genome. *Annu Rev Genomics Hum Genet* 13:151-70.

Griffiths CE, Reich K, Lebwohl M, *et al.* (2015) Comparison of ixekizumab with etanercept or placebo in moderate-to-severe psoriasis (UNCOVER-2 and UNCOVER-3): results from two phase 3 randomised trials. *Lancet* 386:541-51.

Griffiths CE, Strober BE, van de Kerkhof P, *et al.* (2010) Comparison of ustekinumab and etanercept for moderate-to-severe psoriasis. *N Engl J Med* 362:118-28.

Gudjonsson JE, Johnston A, Dyson M, *et al.* (2007) Mouse models of psoriasis. *The Journal of investigative dermatology* 127:1292-308.

Guinot C, Latreille J, Perrussel M, *et al.* (2009) Psoriasis: characterization of six different clinical phenotypes. *Experimental dermatology* 18:712-9.

Guttman-Yassky E, Lowes MA, Fuentes-Duculan J, *et al.* (2008) Low expression of the IL-23/Th17 pathway in atopic dermatitis compared to psoriasis. *J Immunol* 181:7420-7.

Guttman-Yassky E, Nograles KE, Krueger JG (2011a) Contrasting pathogenesis of atopic dermatitis and psoriasis--part I: clinical and pathologic concepts. *The Journal of allergy and clinical immunology* 127:1110-8.

Guttman-Yassky E, Nograles KE, Krueger JG (2011b) Contrasting pathogenesis of atopic dermatitis and psoriasis--part II: immune cell subsets and therapeutic concepts. *The Journal of allergy and clinical immunology* 127:1420-32.

Guttman-Yassky E, Suarez-Farinas M, Chiricozzi A, *et al.* (2009) Broad defects in epidermal cornification in atopic dermatitis identified through genomic analysis. *The Journal of allergy and clinical immunology* 124:1235-44 e58.

Guzman-Sanchez DA, Villanueva-Quintero GD, Alfaro Alfaro N, *et al.* (2007) A clinical study of alopecia areata in Mexico. *International journal of dermatology* 46:1308-10.

Hammerschmidt M, Mulinari Brenner F (2014) Efficacy and safety of methotrexate in alopecia areata. *Anais brasileiros de dermatologia* 89:729-34.

Hanifin JM, Reed ML, Eczema P, *et al.* (2007) A population-based survey of eczema prevalence in the United States. *Dermatitis* 18:82-91.

Haroon M, Kirby B, FitzGerald O (2013) High prevalence of psoriatic arthritis in patients with severe psoriasis with suboptimal performance of screening questionnaires. *Annals of the rheumatic diseases* 72:736-40.

Hay RJ, Johns NE, Williams HC, *et al.* (2014) The global burden of skin disease in 2010: an analysis of the prevalence and impact of skin conditions. *The Journal of investigative dermatology* 134:1527-34.

Helling B, Konig M, Dalken B, *et al.* (2015) A specific CD4 epitope bound by tregalizumab mediates activation of regulatory T cells by a unique signaling pathway. *Immunol Cell Biol* 93:396-405.

Hofree M, Shen JP, Carter H, *et al.* (2013) Network-based stratification of tumor mutations. *Nature methods* 10:1108-15.

Hrehorow E, Salomon J, Matusiak L, *et al.* (2012) Patients with psoriasis feel stigmatized. *Acta dermato-venereologica* 92:67-72.

Hsu L, Armstrong AW (2014) JAK inhibitors: treatment efficacy and safety profile in patients with psoriasis. *J Immunol Res* 2014:283617.

Huang Y, Chen G, Liu X, *et al.* (2014) Serum metabolomics study and eicosanoid analysis of childhood atopic dermatitis based on liquid chromatography-mass spectrometry. *J Proteome Res* 13:5715-23.

Inkeles MS, Scumpia PO, Swindell WR, *et al.* (2014) Comparison of Molecular Signatures from Multiple Skin Diseases Identifies Mechanisms of Immunopathogenesis. *The Journal of investigative dermatology*.

Irvine AD, McLean WH, Leung DY (2011) Filaggrin mutations associated with skin and allergic diseases. *N Engl J Med* 365:1315-27.

Jabbari A, Dai Z, Xing L, *et al.* (2015) Reversal of Alopecia Areata Following Treatment With the JAK1/2 Inhibitor Baricitinib. *EBioMedicine* 2:351-5.

Jacobi A, Antoni C, Manger B, *et al.* (2005) Infliximab in the treatment of moderate to severe atopic dermatitis. *Journal of the American Academy of Dermatology* 52:522-6.

Kagami S, Saeki H, Tsunemi Y, *et al.* (2008) CCL27-transgenic mice show enhanced contact hypersensitivity to Th2, but not Th1 stimuli. *European journal of immunology* 38:647-57.

Kakinuma T, Saeki H, Tsunemi Y, *et al.* (2003) Increased serum cutaneous T cell-attracting chemokine (CCL27) levels in patients with atopic dermatitis and psoriasis vulgaris. *The Journal of allergy and clinical immunology* 111:592-7.

Kanwar JR, Kanwar RK, Burrow H, *et al.* (2009) Recent advances on the roles of NO in cancer and chronic inflammatory disorders. *Current medicinal chemistry* 16:2373-94.

Karimkhani C, Silverberg JI, Dellavalle RP (2015) Defining intrinsic vs. extrinsic atopic dermatitis. *Dermatol Online J* 21.

Kawai T, Akira S (2007) Signaling to NF-kappaB by Toll-like receptors. *Trends Mol Med* 13:460-9.

Kim CH, Kim JY, Lee AY (2015) Therapeutic and immunomodulatory effects of glucosamine in combination with low-dose cyclosporine a in a murine model of imiquimod-induced psoriasis. *Eur J Pharmacol* 756:43-51.

Kimball AB, Szapary P, Mrowietz U, *et al.* (2012) Underdiagnosis and undertreatment of cardiovascular risk factors in patients with moderate to severe psoriasis. *Journal of the American Academy of Dermatology* 67:76-85.

Kobayashi T, Glatz M, Horiuchi K, *et al.* (2015) Dysbiosis and Staphylococcus aureus Colonization Drives Inflammation in Atopic Dermatitis. *Immunity* 42:756-66.

Kong HH, Oh J, Deming C, *et al.* (2012) Temporal shifts in the skin microbiome associated with disease flares and treatment in children with atopic dermatitis. *Genome research* 22:850-9.

Krueger JG, Ferris LK, Menter A, *et al.* (2015a) Anti-IL-23A mAb BI 655066 for treatment of moderate-to-severe psoriasis: Safety, efficacy, pharmacokinetics, and biomarker results of a single-rising-dose, randomized, double-blind, placebo-controlled trial. *The Journal of allergy and clinical immunology* 136:116-24 e7.

Krueger JG, Suarez-Farinas M, Cueto I, *et al.* (2015b) A Randomized, Placebo-Controlled Study of SRT2104, a SIRT1 Activator, in Patients with Moderate to Severe Psoriasis. *PloS one* 10:e0142081.

Langley RG, Elewski BE, Lebwohl M, *et al.* (2014) Secukinumab in plaque psoriasis--results of two phase 3 trials. *N Engl J Med* 371:326-38.

Lauffer F, Ring J (2016) Target-oriented therapy: Emerging drugs for atopic dermatitis. *Expert Opin Emerg Drugs*.

Le Cleach L, Chosidow O (2012) Clinical practice. Lichen planus. *N Engl J Med* 366:723-32.

Lebwohl M, Strober B, Menter A, *et al.* (2015) Phase 3 Studies Comparing Brodalumab with Ustekinumab in Psoriasis. *N Engl J Med* 373:1318-28.

Lebwohl MG, Bachelez H, Barker J, *et al.* (2014) Patient perspectives in the management of psoriasis: results from the population-based Multinational Assessment of Psoriasis and Psoriatic Arthritis Survey. *Journal of the American Academy of Dermatology* 70:871-81 e1-30.

Lee CH, Maibach HI (1995) The sodium lauryl sulfate model: an overview. *Contact Dermatitis* 33:1-7.

Lee E, Trepicchio WL, Oestreicher JL, *et al.* (2004) Increased expression of interleukin 23 p19 and p40 in lesional skin of patients with psoriasis vulgaris. *The Journal of experimental medicine* 199:125-30.

Leonardi CL, Powers JL, Matheson RT, *et al.* (2003) Etanercept as monotherapy in patients with psoriasis. *N Engl J Med* 349:2014-22.

Li Q, Verma IM (2002) NF-kappaB regulation in the immune system. *Nat Rev Immunol* 2:725-34.

Liu FL, Chen CH, Chu SJ, *et al.* (2007) Interleukin (IL)-23 p19 expression induced by IL-1beta in human fibroblast-like synoviocytes with rheumatoid arthritis via active nuclear factor-kappaB and AP-1 dependent pathway. *Rheumatology* 46:1266-73.

Lowes MA, Suarez-Farinas M, Krueger JG (2014) Immunology of psoriasis. *Annu Rev Immunol* 32:227-55.

Man SM, Karki R, Kanneganti TD (2016) AIM2 inflammasome in infection, cancer, and autoimmunity: Role in DNA sensing, inflammation, and innate immunity. *European journal of immunology* 46:269-80.

Mancuso G, Balducci A, Casadio C, *et al.* (2003) Efficacy of betamethasone valerate foam formulation in comparison with betamethasone dipropionate lotion in the treatment of mild-to-moderate alopecia areata: a multicenter, prospective, randomized, controlled, investigator-blinded trial. *Int J Dermatol* 42:572-5.

Mangan PR, Su LJ, Jenny V, *et al.* (2015) Dual Inhibition of Interleukin-23 and Interleukin-17 Offers Superior Efficacy in Mouse Models of Autoimmunity. *J Pharmacol Exp Ther* 354:152-65.

Mansouri Y, Guttman-Yassky E (2015) Immune Pathways in Atopic Dermatitis, and Definition of Biomarkers through Broad and Targeted Therapeutics. *J Clin Med* 4:858-73.

Martin PE, Eckert JK, Koplin JJ, *et al.* (2015) Which infants with eczema are at risk of food allergy? Results from a population-based cohort. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* 45:255-64.

Martin SF (2012) Allergic contact dermatitis: xenoinflammation of the skin. *Curr Opin Immunol* 24:720-9.

Martin SF, Esser PR, Weber FC, *et al.* (2011) Mechanisms of chemical-induced innate immunity in allergic contact dermatitis. *Allergy* 66:1152-63.

McDonagh AJ, Tazi-Ahnini R (2002) Epidemiology and genetics of alopecia areata. *Clin Exp Dermatol* 27:405-9.

McElwee KJ, Gilhar A, Tobin DJ, *et al.* (2013) What causes alopecia areata? *Exp Dermatol* 22:609-26.

McKeage K (2014) A review of CT-P13: an infliximab biosimilar. *BioDrugs* 28:313-21.

Mease P, Genovese MC, Gladstein G, *et al.* (2011) Abatacept in the treatment of patients with psoriatic arthritis: results of a six-month, multicenter, randomized, double-blind, placebo-controlled, phase II trial. *Arthritis Rheum* 63:939-48.

Mehta NN, Li R, Krishnamoorthy P, *et al.* (2012) Abnormal lipoprotein particles and cholesterol efflux capacity in patients with psoriasis. *Atherosclerosis* 224:218-21.

Mrowietz U, Kragballe K, Reich K, *et al.* (2011) Definition of treatment goals for moderate to severe psoriasis: a European consensus. *Archives of dermatological research* 303:1-10.

Murray CJ, Vos T, Lozano R, *et al.* (2012) Disability-adjusted life years (DALYs) for 291 diseases and injuries in 21 regions, 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet* 380:2197-223.

Nakamura Y, Oscherwitz J, Cease KB, *et al.* (2013) Staphylococcus delta-toxin induces allergic skin disease by activating mast cells. *Nature* 503:397-401.

Nast A, Boehncke WH, Mrowietz U, *et al.* (2012) S3 - Guidelines on the treatment of psoriasis vulgaris (English version). Update. *Journal der Deutschen Dermatologischen Gesellschaft = Journal of the German Society of Dermatology : JDDG* 10 Suppl 2:S1-95.

Nelson PA, Kane K, Chisholm A, *et al.* (2015) 'I should have taken that further' - missed opportunities during cardiovascular risk assessment in patients with psoriasis in UK primary care settings: a mixed-methods study. *Health expectations : an international journal of public participation in health care and health policy*.

Nestle FO, Conrad C, Tun-Kyi A, *et al.* (2005) Plasmacytoid predendritic cells initiate psoriasis through interferon-alpha production. *The Journal of experimental medicine* 202:135-43.

Nestle FO, Kaplan DH, Barker J (2009) Psoriasis. *N Engl J Med* 361:496-509.

Noda S, Suarez-Farinas M, Ungar B, *et al.* (2015) The Asian atopic dermatitis phenotype combines features of atopic dermatitis and psoriasis with increased TH17 polarization. *The Journal of allergy and clinical immunology* 136:1254-64.

Nomura I, Gao B, Boguniewicz M, *et al.* (2003a) Distinct patterns of gene expression in the skin lesions of atopic dermatitis and psoriasis: a gene microarray analysis. *The Journal of allergy and clinical immunology* 112:1195-202.

Nomura I, Goleva E, Howell MD, *et al.* (2003b) Cytokine milieu of atopic dermatitis, as compared to psoriasis, skin prevents induction of innate immune response genes. *J Immunol* 171:3262-9.

Nutten S (2015) Atopic dermatitis: global epidemiology and risk factors. *Ann Nutr Metab* 66 Suppl 1:8-16.

Oji V, Luger TA (2015) The skin in psoriasis: assessment and challenges. *Clinical and experimental rheumatology* 33:14-9.

Oranje AP, Glazenburg EJ, Wolkerstorfer A, *et al.* (2007) Practical issues on interpretation of scoring atopic dermatitis: the SCORAD index, objective SCORAD and the three-item severity score. *The British journal of dermatology* 157:645-8.

Ormerod AD, Weller R, Copeland P, *et al.* (1998) Detection of nitric oxide and nitric oxide synthases in psoriasis. *Archives of dermatological research* 290:3-8.

Papp K, Pariser D, Catlin M, *et al.* (2015a) A phase 2a randomized, double-blind, placebo-controlled, sequential dose-escalation study to evaluate the efficacy and safety of ASP015K, a novel Janus kinase inhibitor, in patients with moderate-to-severe psoriasis. *The British journal of dermatology* 173:767-76.

Papp K, Reich K, Leonardi CL, *et al.* (2015b) Apremilast, an oral phosphodiesterase 4 (PDE4) inhibitor, in patients with moderate to severe plaque psoriasis: Results of a phase III, randomized, controlled trial (Efficacy and Safety Trial Evaluating the Effects of Apremilast in Psoriasis [ESTEEM] 1). *Journal of the American Academy of Dermatology* 73:37-49.

Papp K, Thaci D, Reich K, *et al.* (2015c) Tildrakizumab (MK-3222), an anti-interleukin-23p19 monoclonal antibody, improves psoriasis in a phase IIb randomized placebo-controlled trial. *The British journal of dermatology* 173:930-9.

Patel U, Mark NM, Machler BC, *et al.* (2011) Imiquimod 5% cream induced psoriasis: a case report, summary of the literature and mechanism. *The British journal of dermatology* 164:670-2.

Perera GK, Ainali C, Semenova E, *et al.* (2014) Integrative biology approach identifies cytokine targeting strategies for psoriasis. *Science translational medicine* 6:223ra22.

Petukhova L, Duvic M, Hordinsky M, *et al.* (2010) Genome-wide association study in alopecia areata implicates both innate and adaptive immunity. *Nature* 466:113-7.

Piruzian E, Bruskin S, Ishkin A, *et al.* (2010) Integrated network analysis of transcriptomic and proteomic data in psoriasis. *BMC Syst Biol* 4:41.

Piskin G, Sylva-Steenland RM, Bos JD, *et al.* (2006) In vitro and in situ expression of IL-23 by keratinocytes in healthy skin and psoriasis lesions: enhanced expression in psoriatic skin. *J Immunol* 176:1908-15.

Prey S, Paul C, Bronsard V, *et al.* (2010) Cardiovascular risk factors in patients with plaque psoriasis: a systematic review of epidemiological studies. *Journal of the European Academy of Dermatology and Venereology : JEADV* 24 Suppl 2:23-30.

Quaranta M, Eyerich S, Knapp B, *et al.* (2014a) Allergic contact dermatitis in psoriasis patients: typical, delayed, and non-interacting. *PloS one* 9:e101814.

Quaranta M, Knapp B, Garzorz N, *et al.* (2014b) Intraindividual genome expression analysis reveals a specific molecular signature of psoriasis and eczema. *Science translational medicine* 6:244ra90.

Quraishi BM, Zhang H, Everson TM, *et al.* (2015) Identifying CpG sites associated with eczema via random forest screening of epigenome-scale DNA methylation. *Clin Epigenetics* 7:68.

Qureshi AA, Choi HK, Setty AR, *et al.* (2009) Psoriasis and the risk of diabetes and hypertension: a prospective study of US female nurses. *Archives of dermatology* 145:379-82.

Rapp SR, Feldman SR, Exum ML, *et al.* (1999) Psoriasis causes as much disability as other major medical diseases. *Journal of the American Academy of Dermatology* 41:401-7.

Reich K, Nestle FO, Papp K, *et al.* (2005) Infliximab induction and maintenance therapy for moderate-to-severe psoriasis: a phase III, multicentre, double-blind trial. *Lancet* 366:1367-74.

Reich K, Ortonne JP, Gottlieb AB, *et al.* (2012) Successful treatment of moderate to severe plaque psoriasis with the PEGylated Fab' certolizumab pegol: results of a phase II randomized, placebo-controlled trial with a re-treatment extension. *The British journal of dermatology* 167:180-90.

Roberson ED, Liu Y, Ryan C, *et al.* (2012) A subset of methylated CpG sites differentiate psoriatic from normal skin. *The Journal of investigative dermatology* 132:583-92.

Rodriguez TA, Fernandes KE, Dresser KL, *et al.* (2010) Concordance rate of alopecia areata in identical twins supports both genetic and environmental factors. *J Am Acad Dermatol* 62:525-7.

Roosta N, Black DS, Peng D, *et al.* (2010) Skin disease and stigma in emerging adulthood: impact on healthy development. *J Cutan Med Surg* 14:285-90.

Schmidt M, Raghavan B, Muller V, *et al.* (2010) Crucial role for human Toll-like receptor 4 in the development of contact allergy to nickel. *Nature immunology* 11:814-9.

Schmitt J, Langan S, Deckert S, *et al.* (2013) Assessment of clinical signs of atopic dermatitis: a systematic review and recommendation. *The Journal of allergy and clinical immunology* 132:1337-47.

Schmitt J, Rosumeck S, Thomaschewski G, *et al.* (2014) Efficacy and safety of systemic treatments for moderate-to-severe psoriasis: meta-analysis of randomized controlled trials. *The British journal of dermatology* 170:274-303.

Schmitt J, Schwarz K, Baurecht H, *et al.* (2015) Atopic dermatitis is associated with an increased risk for rheumatoid arthritis and inflammatory bowel disease, and a decreased risk for type 1 diabetes. *The Journal of allergy and clinical immunology*.

Schon MP (2008) Animal models of psoriasis: a critical appraisal. *Experimental dermatology* 17:703-12.

Silverberg JI, Garg NK, Paller AS, *et al.* (2015) Sleep disturbances in adults with eczema are associated with impaired overall health: a US population-based study. *The Journal of investigative dermatology* 135:56-66.

Spergel JM (2010) From atopic dermatitis to asthma: the atopic march. *Ann Allergy Asthma Immunol* 105:99-106; quiz 7-9, 17.

Suarez-Farinas M, Arbeit R, Jiang W, *et al.* (2013a) Suppression of molecular inflammatory pathways by Toll-like receptor 7, 8, and 9 antagonists in a model of IL-23-induced skin inflammation. *PloS one* 8:e84634.

Suarez-Farinas M, Dhingra N, Gittler J, *et al.* (2013b) Intrinsic atopic dermatitis shows similar TH2 and higher TH17 immune activation compared with extrinsic atopic dermatitis. *The Journal of allergy and clinical immunology* 132:361-70.

Sun J, Dou W, Zhao Y, *et al.* (2013) A comparison of the effects of topical treatment of calcipotriol, camptothecin, clobetasol and tazarotene on an imiquimod-induced psoriasis-like mouse model. *Immunopharmacology and immunotoxicology*.

Swindell WR, Johnston A, Carbajal S, *et al.* (2011) Genome-wide expression profiling of five mouse models identifies similarities and differences with human psoriasis. *PloS one* 6:e18266.

Szepietowski JC, Reich A (2015) Pruritus in psoriasis: An update. *Eur J Pain*.

Thaci D, Simpson EL, Beck LA, *et al.* (2015) Efficacy and safety of dupilumab in adults with moderate-to-severe atopic dermatitis inadequately controlled by topical treatments: a randomised, placebo-controlled, dose-ranging phase 2b trial. *Lancet*.

Thatcher TH, Luzina I, Fishelevich R, *et al.* (2006) Topical imiquimod treatment prevents UV-light induced loss of contact hypersensitivity and immune tolerance. *The Journal of investigative dermatology* 126:821-31.

Tsoi LC, Spain SL, Ellinghaus E, *et al.* (2015) Enhanced meta-analysis and replication studies identify five new psoriasis susceptibility loci. *Nature communications* 6:7001.

Tsoi LC, Spain SL, Knight J, *et al.* (2012) Identification of 15 new psoriasis susceptibility loci highlights the role of innate immunity. *Nature genetics* 44:1341-8.

Vaclavkova A, Chimenti S, Arenberger P, *et al.* (2014) Oral ponesimod in patients with chronic plaque psoriasis: a randomised, double-blind, placebo-controlled phase 2 trial. *Lancet* 384:2036-45.

van der Fits L, Mourits S, Voerman JS, *et al.* (2009) Imiquimod-induced psoriasis-like skin inflammation in mice is mediated via the IL-23/IL-17 axis. *J Immunol* 182:5836-45.

Vanderpuye-Orgle J, Zhao Y, Lu J, *et al.* (2015) Evaluating the economic burden of psoriasis in the United States. *Journal of the American Academy of Dermatology* 72:961-7 e5.

Verboom P, Hakkaart-Van L, Sturkenboom M, *et al.* (2002) The cost of atopic dermatitis in the Netherlands: an international comparison. *The British journal of dermatology* 147:716-24.

Villanova F, Di Meglio P, Nestle FO (2013) Biomarkers in psoriasis and psoriatic arthritis. *Annals of the rheumatic diseases* 72 Suppl 2:ii104-10.

Villasante Fricke AC, Miteva M (2015) Epidemiology and burden of alopecia areata: a systematic review. *Clin Cosmet Investig Dermatol* 8:397-403.

Vinter H, Iversen L, Steiniche T, *et al.* (2015) Aldara(R)-induced skin inflammation: studies of patients with psoriasis. *The British journal of dermatology* 172:345-53.

Wang X, Sun J, Hu J (2015) IMQ Induced K14-VEGF Mouse: A Stable and Long-Term Mouse Model of Psoriasis-Like Inflammation. *PloS one* 10:e0145498.

Ward PP, Uribe-Luna S, Conneely OM (2002) Lactoferrin and host defense. *Biochem Cell Biol* 80:95-102.

Wasserman D, Guzman-Sanchez DA, Scott K, *et al.* (2007) Alopecia areata. *Int J Dermatol* 46:121-31.

Watanabe T, Kawamura T, Jacob SE, *et al.* (2002) Pityriasis rosea is associated with systemic active infection with both human herpesvirus-7 and human herpesvirus-6. *The Journal of investigative dermatology* 119:793-7.

Weidinger S, Novak N (2015) Atopic dermatitis. *Lancet*.

Wenzel J, Peters B, Zahn S, *et al.* (2008) Gene expression profiling of lichen planus reflects CXCL9+-mediated inflammation and distinguishes this disease from atopic dermatitis and psoriasis. *The Journal of investigative dermatology* 128:67-78.

Wolk K, Mallbris L, Larsson P, *et al.* (2009) Excessive body weight and smoking associates with a high risk of onset of plaque psoriasis. *Acta dermato-venereologica* 89:492-7.

Xing L, Dai Z, Jabbari A, *et al.* (2014) Alopecia areata is driven by cytotoxic T lymphocytes and is reversed by JAK inhibition. *Nat Med* 20:1043-9.

Yi S, Kim SE, Park MK, *et al.* (2012) Comparative pharmacokinetics of HD203, a biosimilar of etanercept, with marketed etanercept (Enbrel(R)): a double-blind, single-dose, crossover study in healthy volunteers. *BioDrugs* 26:177-84.

Yin X, Cheng H, Lin Y, *et al.* (2015) A weighted polygenic risk score using 14 known susceptibility variants to estimate risk and age onset of psoriasis in Han Chinese. *PloS one* 10:e0125369.

Yoon SY, Park HS, Lee JH, *et al.* (2013) Histological differentiation between palmoplantar pustulosis and pompholyx. *Journal of the European Academy of Dermatology and Venereology : JEADV* 27:889-93.

Zaja-Milatovic S, Richmond A (2008) CXC chemokines and their receptors: a case for a significant biological role in cutaneous wound healing. *Histol Histopathol* 23:1399-407.

# Publications resulting from this thesis

**Garzorz N***, Alsisi M*, Todorova A, Atenhan A, Thomas J, Lauffer F et al. Dissecting susceptibility from exogenous triggers: The model of alopecia areata and associated inflammatory skin diseases. Journal of the European Academy of Dermatology and Venereology : JEADV. 2015 Sep 28. * = both authors contributed equally.

**Garzorz N**, Eyerich K. NOS2 and CCL27: clinical implications for psoriasis and eczema diagnosis and management. Expert Rev Clin Immunol. 2015 Feb;11(2):167-9.

Quaranta M*, Knapp B*, **Garzorz N**, Mattii M, Pullabhatla V, Pennino D, et al. Intraindividual genome expression analysis reveals a specific molecular signature of psoriasis and eczema. Science translational medicine. 2014 Jul 9;6(244):244ra90. * = both authors contributed equally.

# Acknowledgements

I would like to thank all members of the Center of Allergy and Environment (ZAUM) in particular the members and former members of our group (AG Eyerich) Dr. Jenny Thomas, Dr. Felix Lauffer, Anne Atenhan, Jana Sänger, Dr. Martina Mattii and Dr. Maria Quaranta who spent me great support and help throughout all projects. My thank goes to Prof. Dr. Dr. Johannes Ring and Prof. Carsten Schmidt-Weber for their support as members of my PhD committee.

I would like to thank all those colleagues from the clinic who helped me with the acquisition of biological material and patient data, in particular Dr. Antonia Todorova and Dr. Mohammed Al-Asisi. I would like to thank our collaborators from the Institute of Computational Biology (Helmholtz Center Munich) Linda Krause, Dr. Bettina Knapp, Dr. Nikola Müller and Prof. Dr. Dr. Fabian Theis who have contributed bioinformatical analyses to both the imiquimod and the classifier study.

I would like to express my gratitude to my supervisors PD Dr. Stefanie Eyerich and Prof. Dr. Dr. Kilian Eyerich who gave me the unique chance to combine clinical and scientific work in exciting translational dermatological research and always found the time to discuss data and give fruitful advice.

I would like to thank all my friends, and especially my parents, sister and Sebastian Paul for their advice, help and emotional support at all times.

# Curriculum Vitae

**Personal details**

Nationality: German
Date of birth: June 3, 1986
Place of birth: Aalen, Germany

| | | |
|---|---|---|
| **Work experience** | 09/2012 to present | Resident and Research Fellow at the Department of Dermatology and Allergy at the Technical University of Munich, Munich |
| | 02/2011-12/2011 | Sub-Internships at the Weill Cornell Medical College (New York), at the Klinikum Rechts der Isar (Munich), at the Massachusetts Eye and Ear Infirmary Hospital (Boston, Harvard Medical School) and at the Regionalspital Prättigau (Switzerland) |
| **Education** | | |
| ***University*** | 05/2013 | Medical Doctor (MD); Doctoral thesis: "The role of signal transducer and activator of transcription 3 (STAT3) in central axonal regeneration", LMU Munich<br>-Grade: summa cum laude (1,0) , top grade |
| | 05/2012 | ***Approbation*** (Licence to practise medicine) |
| | 04/2012 | ***Zweiter Abschnitt der Ärztlichen Prüfung*** (final medical examination)<br>- Grade: excellent: 1,5 |
| | 2007 -2012 | Medical studies (clinic) at the Technical University of Munich |
| | 04/2007 | ***Erster Abschnitt der Ärztlichen Prüfung*** (preliminary medical examination)<br>- Grade: excellent: 1,5 |
| | 2005-2007 | Medical studies (preclinic) at the Eberhard-Karls-University Tübingen |
| ***Gymnasium (high school)*** | 1996 – 2004 | Theodor- Heuss-Gymnasium (high school)<br>- Completed ***Abitur***<br>Graduated with a grade of 1,0 (top grade) |

**Awards**

Awarded the Psoriasisbund-Preis 2016

Member of the ESDR Academy for Future Leaders in Dermatology 2015

Awarded the Almirall Förderpreis 2015

Awarded a travel grant for poster presentation and oral presentation at the 44th Annual *ESDR* Meeting Copenhagen

Awarded a scholarship from the German National Academic Foundation (Studienstiftung des deutschen Volkes) from April 2005 to 2012

**Publications**

**Garzorz N**, Alsisi M, Todorova A, Atenhan A, Thomas J, Lauffer F et al. Dissecting susceptibility from exogenous triggers: The model of alopecia areata and associated inflammatory skin diseases. Journal of the European Academy of Dermatology and Venereology : JEADV. 2015 Sep 28.

**Garzorz N**, Eyerich K. NOS2 and CCL27: clinical implications for psoriasis and eczema diagnosis and management. Expert Rev Clin Immunol. 2015 Feb;11(2):167-9.

**Garzorz N**, Papanagiotou V, Atenhan A, Andres C, Eyerich S, Eyerich K, et al. *Pyoderma gangrenosum, acne, psoriasis, arthritis and suppurative hidradenitis (PAPASH)-syndrome: a new entity within the spectrum of autoinflammatory syndromes?* Journal of the European Academy of Dermatology and Venereology : JEADV. 2014 Jul 28.

Quaranta M, Eyerich S, Knapp B, Nasorri F, Scarponi C, Mattii M, **Garzorz N**, et al. Allergic contact dermatitis in psoriasis patients: typical, delayed, and non-interacting. PloS one. 2014;9(7):e101814.

Quaranta M*, Knapp B*, **Garzorz N**, Mattii M, Pullabhatla V, Pennino D, et al. Intraindividual genome expression analysis reveals a specific molecular signature of psoriasis and eczema. Science translational medicine. 2014 Jul 9;6(244):244ra90. * = both authors contributed equally.

Bareyre FM*, **Garzorz N*,** Lang C, Misgeld T, Buning H, Kerschensteiner M. *In vivo imaging reveals a phase-specific role of STAT3 during central and peripheral nervous system axon regeneration.* Proceedings of the National Academy of Sciences of the United States of America. 2011 Apr 12;108(15):6282-7. * = both authors contributed equally

**Garzorz, N**.: Basics Neuroanatomie. Munich: Urban & Fischer, 1.Edition 2008; 176 Seiten. ISBN-10: 3437424564 (Lehrbuch)