RESEARCH ARTICLE

# Interleukin-17A blockade with secukinumab results in decreased neutrophil infiltration in psoriasis: minimally-invasive measurement by tape stripping

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**Abstract**: Psoriasis is a well characterized interleukin (IL)-17A-driven skin disease with neutrophil infiltration and epidermal hyperkeratosis. Several biomarkers, most prominently β-defensin-2 (BD-2), have been identified using local and systemic invasive measurements as responsive markers of IL-17A-driven skin pathology. We sought to determine whether measurements of epidermal proteins by tape stripping could offer a minimally-invasive method to assess treatment responses. We compared the expression of 170 proteins in the epidermis (tape stripping) and dermis (open flow microperfusion) of 8 psoriatic subjects before and after administration of a single dose of subcutaneous (s.c.) anti-IL-17A mAb secukinumab. Proteomic analyses of tape strips revealed a >3-fold decrease in 32 epidermal and inflammatory cell proteins in response to secukinumab. The epidermal proteins with the largest (>10-fold) decreases were: matrix metalloproteinase-8 (MMP-8, 15.68-fold, $p<0.05$); myeloperoxidase (MPO, 14.72-fold, $p<0.005$); IL-8 (11.93-fold, $p<0.05$); MMP-9 (10.81-fold, $p<0.005$); and IL-1β (10.35-fold, $p<0.05$). For these proteins, greater-fold protein changes were detected in the epidermis compared to dermis. Immunohistochemical analysis confirmed that neutrophils are the predominant cell type in psoriatic skin lesions that express MPO, MMP-8 and MMP-9, and that secukinumab treatment dramatically decreases neutrophil accumulation. Thus, tape stripping may be used to assess epidermal neutrophils, and protein biomarker responses to anti-IL-17A therapy in psoriasis.

**Keywords**: biomarker, IL-17, psoriasis, tape-stripping, epidermis

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**Received:** July 8, 2016; **Accepted:** July 30, 2016; **Published Online:** September 28, 2016

**Citation:** Loesche C, Kolbinger F, Valentin M A, *et al*. 2016, Interleukin-17A blockade with secukinumab results in decreased neutrophil infiltration in psoriasis: minimally-invasive measurement by tape stripping. *Advances in Precision Medicine*, vol.1(2): 1–9. http://dx.doi.org/10.18063/APM.2016.02.003.

## Introduction

Plaque psoriasis is a chronic inflammatory skin disorder characterized by infiltration of activated T lymphocytes, neutrophils, mast cells, and dendritic cells leading to epidermal keratinocyte hyperproliferation[1,2] affecting approximately 2%–3% of the general population[3]. Psoriasis treatment has recently been revolutionized with the introduction of interleukin (IL)-17A targeted therapies[4] achieving

rapid and lasting significant decrease in psoriatic plaques (dramatic response in psoriasis area severity index [PASI] reduction). Much has been known about psoriasis pathogenesis and IL-17A biology by studying gene and protein expression in skin biopsies before and after IL-17A-targeted therapies[5-9], including a prominent role of neutrophils in both processes[10]. These studies have identified β-defensin-2 (BD-2) encoded by the gene *DEFB4A*, as a biomarker of anti-IL-17A response in psoriasis. However, BD-2 measurement has only been validated by invasive methods such as skin biopsy and blood sampling, and most recently by minimal-invasive open flow microperfusion (OFM) to measure proteins in dermal interstitial fluid (dISF)[6].

We sought to determine if therapeutic responses in psoriasis could be measured in a minimally-invasive manner by measuring epidermal proteins in the upper stratum corneum by skin tape stripping, and if additional insights into psoriasis pathogenesis could be gained by comparing proteins in dermal and epidermal skin compartments. In this study, we examined 170 proteins in the epidermis of eight psoriatic subjects before and 2 weeks after a single dose of the fully human, IL-17A-selective monoclonal antibody secukinumab (300 mg subcutaneous [s.c.]), and identified that neutrophil proteins were abundant and easily detectable in psoriasis epidermis, and that they rapidly and strongly decreased after secukinumab treatment. While neutrophil protein changes were detectable in dISF, they were more readily and robustly detected in the epidermis. In contrast, BD-2 changes were larger in dISF than in the epidermis. Our findings shed new insights into the inflammatory pathways active in specific layers of psoriatic skin and reveal the proximal impact of IL-17A blockade on these pathways.

## Materials and Methods

### Study Design and Objectives

This study was conducted as a part of a larger, previously reported single-center, open-label exploratory study (ClinicalTrials.gov identifier NCT01539213); the details of which have been previously described[6,11]. Specific to this study, eight subjects with moderate to severe psoriasis plaques who received a single 300 mg s.c. injection of secukinumab on study day 1 also underwent epidermal sampling by tape stripping before secukinumab treatment (Day 1) and on study day 15 (D15, 14 days after secukinumab treatment). OFM was used to sample the dISF.

## Ethics

The NCT01539213 study was carried out at the Clinical Research Center of the Medical University in Graz, Austria, after being approved by the ethics committee and the Austrian Agency for Health and Food Safety (AGES). It was conducted according to the principles of Good Clinical Practice (GCP) and the Declaration of Helsinki. Written informed consent was obtained from each subject prior to any study specific procedures.

In addition, skin biopsies from patients with psoriasis sampled in a previous multicenter trial NCT-00805480, conducted after the approval of ethics committees and health authority, have been analyzed.

### Tape Stripping and Protein Measurement

At baseline (Day 1, D1) and Day 15 (D15), 10 consecutive skin tape strip samples were collected after ethanol cleaning (CuDerm; D-Squame Standard Sampling Discs; 22mm diameter; catalogue number D100) on skin from a representative psoriatic lesion in otherwise healthy subjects. Proteins from skin tape strips were extracted for 2h at 4°C in 0.5 mL of assay buffer (50 mM Tris/HCl, 150 mM NaCl, 0.05% (v/v) CHAPS; adjusted to pH 7.6).

With the exception of IL-17A, the chemiluminescent multiplex enzyme immunoassay platform from Aushon BioSystems (Billerica, MA, USA) was used to profile and quantify the levels of 170 proteins distributed over 43 panels as previously described[6]. Free IL-17A was quantified using microparticle-based fluorescent sandwich immunoassays based on Erenna technology validated in human serum (Singulex® IL-17A Human Immunoassay kit, Cat No.: 03-0017- 05). Free IL-17A levels in normal skin using tape strips were determined in healthy subjects (Novartis internal process). The lower limit of quantification (LOQ) for IL-17A was 0.096pg/mL. For comparison, dISF was sampled using OFM as previously described[11].

### Immunohistochemistry

Three micrometer thick paraffin tissue sections from human skin biopsies were stained by immunohistochemistry using Ventana Discovery XT stainer (Roche Diagnostics, Switzerland) and following primary antibodies: rabbit anti-MPO, marker of neutrophils (polyclonal, Dako, Denmark), rabbit anti-human MMP-8 (clone EP1252Y, Abcam, UK), and rabbit anti-human MMP-9 (clone EP1254, Abcam, UK). Negative con-

trol staining was performed with matched concentrations of either mouse, rabbit, and goat isotype controls (MAB002, R&D Systems, UK, ab172730 and ab 27478, Abcam, UK and AB-108-C, R&D Systems, UK) instead of the primary antibodies.

### Statistical Analysis and Hierarchical Clustering

The PASI changes from baseline were analyzed using the Wilcoxon rank sum test as the number of subjects is small and the PASI clinical scale is ordinal and not linear.

Serum and tape strip data from each subject was summarized using log (baseline/D15) or log fold decreases. Summary statistics on the log fold decrease scale have been back transformed to the fold change (increase or decrease) dependent on whether the log fold change summary statistics is positive or negative. If log (fold decrease) is negative, then we have a fold decrease and if log (fold decrease) is positive then we have a fold increase. Fold increases are usually understood but fold decreases can be confusing. For example, a sevenfold decrease from baseline indicates that the D15 values were 1/7 of the baseline value. The logfold decreases were analyzed using the Wilcoxon rank sum test.

All concentrations below LOQ were imputed as LOQ. One serum BD-2 concentration was above the upper LOQ and this was imputed as the upper LOQ. If concentrations were below LOQ at both BL and D15 then that subject was excluded from the analysis. This approach for handling below LOQ or above the upper limit of quantification is conservative and produces the minimum log fold decreases.

All tests were performed one-sided, either looking for a reduction from baseline which was the case for PASI or log fold decreases from baseline. No adjustment was made for multiple testing. If one-sided p-values are less than 0.05, then they would be considered statistically significant decreases or log fold decreases from baseline. All analysis was performed using SAS version 9.3.

The hierarchical clustering of the protein data and resulting heatmap and dendrogram graphic were generated using the “gplots” package of the R statistical platform (R version 3.2.2 and gplots version 2.17.0).

## Results

### Differential Protein Changes in Epidermis and Dermis After Anti-IL-17A Treatment

We have previously reported that serum and dISF BD-2 protein levels are robust biomarkers of IL-17A driven skin pathology[6]. Both of these fluids require invasive sampling methods, and we sought to determine if non-invasive tissue sampling of the epidermis would be a reliable method to measure anti-IL-17A response at a molecular level. We sampled the upper stratum corneum of the epidermis before and 2 weeks after secukinumab treatment by tape stripping (Figure 1), and measured 170 proteins using the same multiplexed chemiluminescent assay system as with dISF. In comparing the protein changes in epidermis vs. dermis, many differences were notable, including the levels of many proteins changed more dramatically in the epidermis vs. the dermis (Figure 2). Using a 3-fold change from baseline as a threshold, 32 proteins were shown to be down-regulated in the epidermis (Table 1)

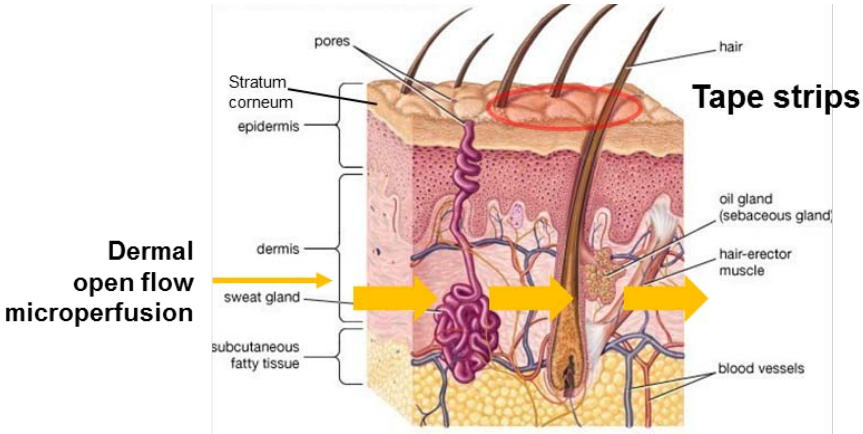


**Figure 1.** Schematic representation of skin sampling methods used. Diagram showing the three structural layers of the skin: epidermis, dermis, and subcutaneous fatty tissue as well as blood vessels, hair follicles, sebaceous and sweat glands within the different layers of the skin. Compartments captured by tape stripping (red circle) and OFM (yellow arrows) are depicted on top. *By courtesy of Encyclopaedia Britannica, Inc., copyright 2010; adapted with permission.*

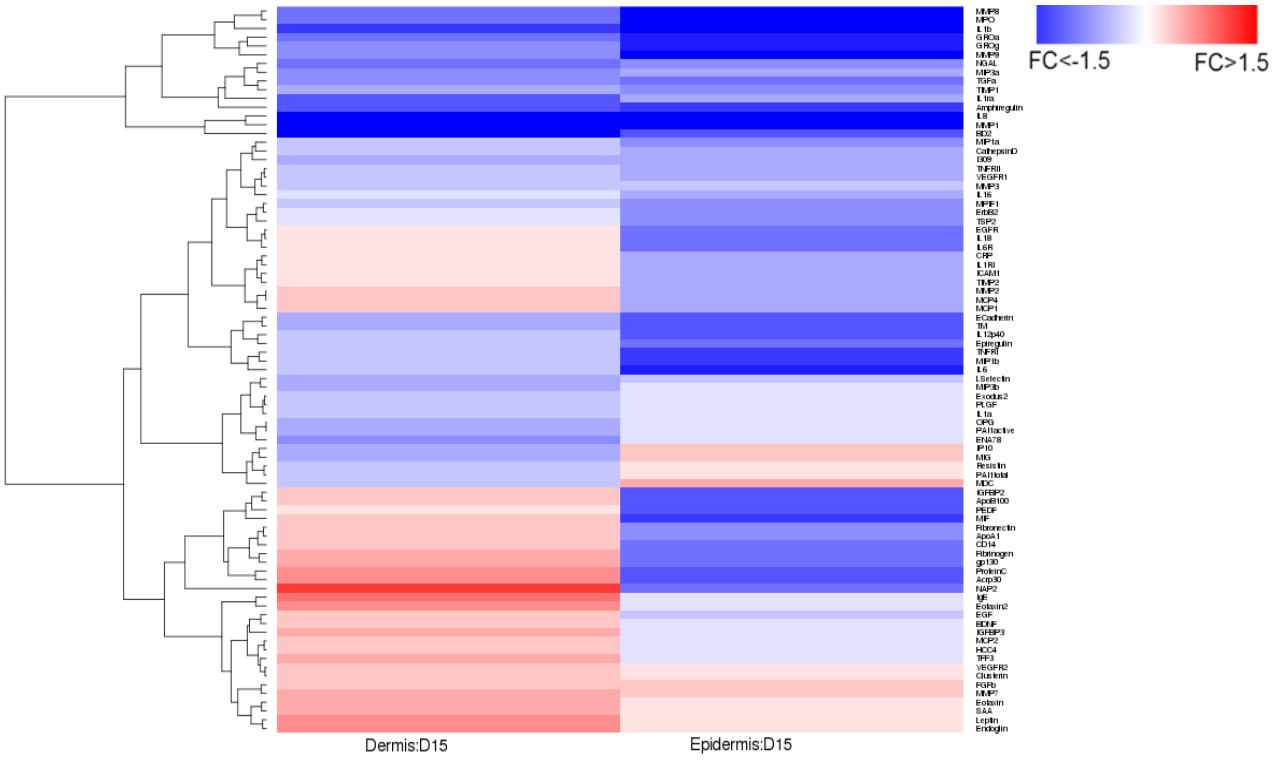


**Figure 2.** Proteomic analysis of different skin layers after IL-17A blockade reveal early changes in the epidermal compartment. Heatmap of 83 proteins with at least a 1.5 fold change (FC) at D15 compared to baseline in both, the epidermis and dermis, of psoriatic subjects treated at D1 with secukinumab. Epidermal tape strip samples and dISF samples were taken from lesional skin. Hierarchical clustering was performed on the fold-change of protein concentration to show changes in patterns of expression of related proteins in different compartments at two time points. Blue represents down-regulation from baseline and red indicates up-regulation.

**Table 1.** Proteins with the greatest fold reduction (<−3.00) in the epidermis in comparison to the dermis of subjects with psoriasis after treatment with a single dose of secukinumab

| Protein | Epidermis (tape strip) | Dermis (dISF) | Protein | Epidermis (tape strip) | Dermis (dISF) |
|---|---|---|---|---|---|
| MMP-8 | –15.68 | –3.42 | Thrombomodulin | –4.30 | –1.97 |
| MPO | –14.72 | –3.20 | IL-12p40 | –4.06 | –1.47 |
| IL-8 | –11.93 | –16.03 | α2–Macroglobulin | –4.05 | ND |
| MMP-9 | –10.81 | –2.22 | Acrp30 | –4.01 | 2.72 |
| IL-1β | –10.35 | –5.47 | PEDF | –3.84 | 1.15 |
| MMP-1 | –7.90 | –15.19 | ApoB100 | –3.82 | 1.55 |
| GRO-α | –7.29 | –3.13 | IGFBP2 | –3.76 | 1.42 |
| IL-6 | –6.92 | –1.35 | RBP4 | –3.62 | ND |
| GRO-γ | –6.28 | –2.20 | Fibrinogen | –3.51 | 1.69 |
| MIF | –5.57 | 1.31 | VEGF | –3.49 | ND |
| MIP-1β | –5.18 | –1.32 | IL-17 | –3.48 | ND |
| TNF-RI | –4.86 | –1.50 | Epiregulin | –3.42 | –1.46 |
| Amphiregulin | –4.69 | –4.42 | TGF-α | –3.12 | –2.25 |
| E–cadherin | –4.59 | –2.08 | gp130 | –3.11 | 1.82 |
| ProteinC | –4.36 | 2.21 | EGFR | –3.07 | 1.03 |
| BD-2 | –4.31 | –32.20 | IL-18 | –3.00 | 1.04 |

compared with 8 in the dISF. While levels of BD-2, an antimicrobial peptide known to be a good IL-17A pathway marker, did decrease in the epidermis by 4.3-fold at D15 ($p<0.05$, Figure 3) in parallel to improvements of disease activity (Figure 3), the change was not as high as in the dISF (32.2-fold), where BD-2 was the protein with the highest fold-change (Table 1)[6]. Other proteins showed larger changes in the epidermis. IL-17A in tape strips from psoriasis subjects was not at levels high enough for detection by the Aushon system, but they were high enough to be detected using the Singulex system, with low but detectable levels in all 8 psoriasis subjects before secukinumab, and undetectable levels in healthy volunteers (Figure 3).

## IL-17A Blockade Down-regulates Leukocyte Proteases, Chemoattractants and Markers of Inflammation

The four proteins with the greatest-fold change in the epidermis by tape stripping were MMP-8, MPO, IL-8 (CXCL8), and MMP-9 (Table 1 and Figure 4). In contrast, all of the proteins except IL-8 (which was not evaluable due to missing data) had much lower levels of change in the dISF, suggesting compartmental differences in pathophysiology. To better understand the differences and define the source of these proteins, we performed immunohistochemistry on skin biopsies obtained before and 2 weeks after secukinumab therapy.

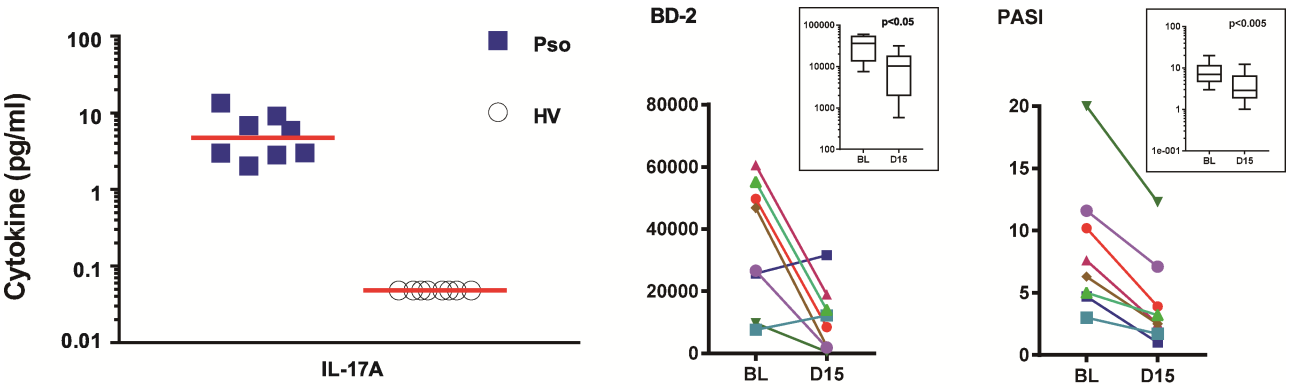


**Figure 3.** Baseline epidermal protein levels of BD-2 decline rapidly in parallel to disease activity (PASI) upon secukinumab treatment.(Upper panel) baseline human IL-17A levels in tape strip samples of psoriasis lesional and healthy volunteer skin; data below LOQ are shown as open symbols; (Lower panel, left) human BD-2 levels in tape strip samples of psoriasis lesional skin before and 14 days after a single dose of secukinumab; (Lower panel, left) PASI scores before and 14 days after a single dose of secukinumab. Data for all 8 psoriasis patients are shown. Inserts show 25$^{th}$ and 75$^{th}$ percentiles, and vertical lines depict minimum and maximum values; the horizontal line depicts the Median value. Statistical significance was calculated using the Wilcoxon rank sum test (one-sided). Values below LOQ were included as LOQ.

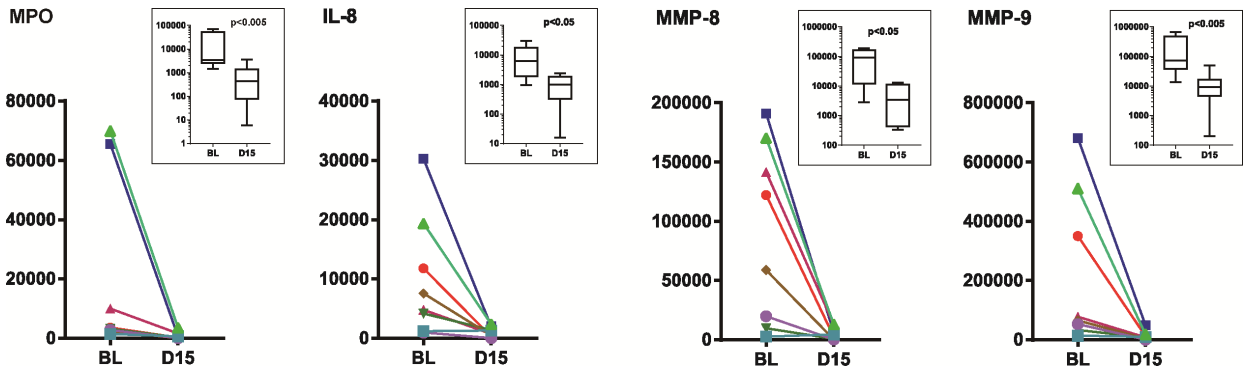


**Figure 4.** Leukocyte proteases and neutrophil-associated proteins are significantly reduced in the epidermis following secukinumab treatment. Protein levels in tape strip samples of psoriasis lesional skin before and 14 days after a single dose of secukinumab. Data for all 8 psoriasis patients are shown. Inserts show 25$^{th}$ and 75$^{th}$ percentiles and vertical lines depict minimum and maximum values; the horizontal line depicts the Median value. Statistical significance was calculated using the Wilcoxon rank sum test (one-sided). Values below LOQ were included as LOQ.

In the epidermis, the expression of MMP-8/MPO/MMP-9 proteins was most abundant in Munro's abscesses filled with neutrophils, with lesser but evident neutrophil infiltration and MMP-8/MPO/MMP-9 expression in the dermis (Figure 5). Not only neutrophil numbers were reduced and neutrophil-produced proteins down-regulated after secukinumab, but also protein levels of neutrophil-attracting chemokines other than IL-8, including CXCL1 (GRO-α), CXCL3 (GRO- γ), and CXCL7 (NAP2) were decreased in the epidermis after the treatment (Table 1 and data not shown). While neutrophil-associated proteins represented the majority of chemo-attractants down-regulated, IL-17A blockade also strongly down-regulated MIF[12,13], MIP-1α (CCL3), and MIP-1β (CCL4)[14,15], chemokines that may be involved in attracting and activating Th17, dendritic cells, and macrophages in lesional skin. Secukinumab also modulated the expression of the pro-inflammatory cytokines IL-1β, IL-6, IL-12p40, and IL-18 (Figure 6).

### IL-17A Blockade Results in Downregulation of Markers of Keratinocyte Proliferation

Examination of the numerous changes in expression of proteins in the dermis and epidermis prompted us to examine whether specific pathways were strongly represented in these profiles. At the protein level, members of the epidermal growth factor (EGF)-family of autocrine keratinocyte growth factors, such as amphiregulin, epiregulin, and transforming growth factor alpha (TGF-α) were down-regulated, particularly within the epidermis after administration of secukinumab, suggesting that these ligands were driving keratinocyte hyperproliferation (Figure 7). In contrast, protein levels of EGF were unchanged or changed only slightly over time.

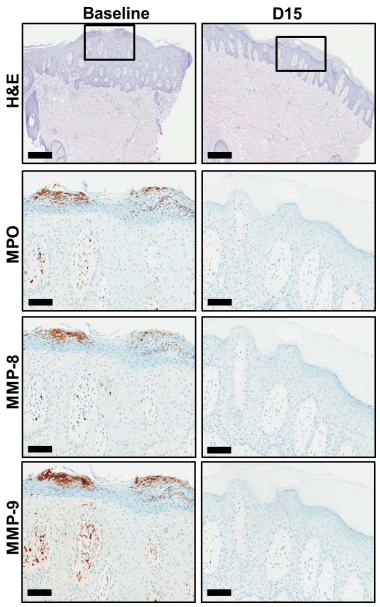


**Figure 5.** Histopathology and immunohistochemistry of skin biopsies at baseline and on D15 after treatment with secukinumab. Left panel: representative example of skin biopsy with characteristic psoriatic pathology of acanthosis, parakeratosis, presence of Munro's abscesses (MPO-positive), and dermal inflammatory infiltrates. Positive immune-staining for MMP-8 and MMP-9 was found predominantly in neutrophils including Munro's abscesses. Right panel: two weeks after treatment with a single dose of 10 mg secukinumab i.v., normalization of skin pathology, absence of Munro's abscesses, and a marked reduction of dermal inflammatory infiltrates was observed. No MMP-8 and MMP-9 staining was observed on D15. Scale bars represent 400 µm (H&E) and 100 µm (IHC images).

## Discussion

In this study, we wanted to explore a non-invasive method, using easy to handle and commercially available tape strips, to detect relevant disease markers and assess anti-IL-17A treatment responses in the epidermis on a molecular basis and to compare these effects to dermal changes. Previously, tape stripping has been employed to detect proteolytic activities[16,17],

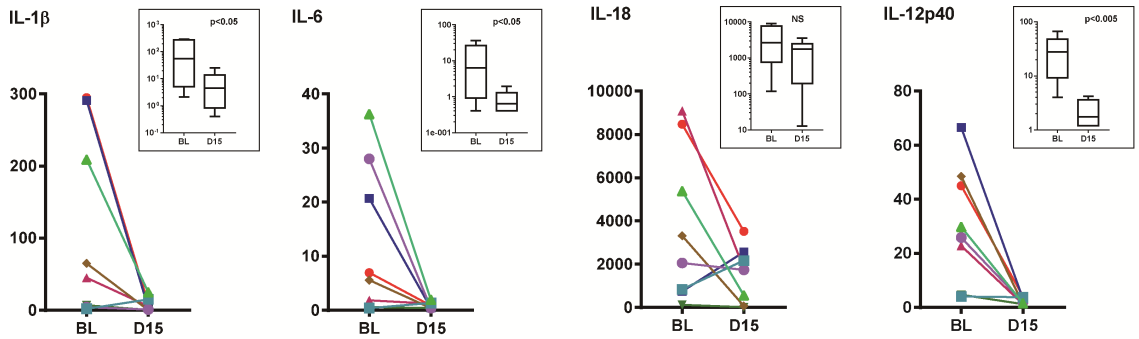


**Figure 6.** IL-17A inhibition leads to reduction of pro-inflammatory cytokines in the epidermis of psoriasis patients. Cytokine protein levels in tape strip samples of psoriasis lesional skin before and 14 days after a single dose of secukinumab. Data for all 8 psoriasis patients are shown. Inserts show 25$^{th}$ and 75$^{th}$ percentiles and vertical lines depict minimum and maximum values; the horizontal line depicts the median value. Statistical significance was calculated using the Wilcoxon rank sum test (one-sided). Values below LOQ were included as LOQ. NS= not significant (p>0.05).

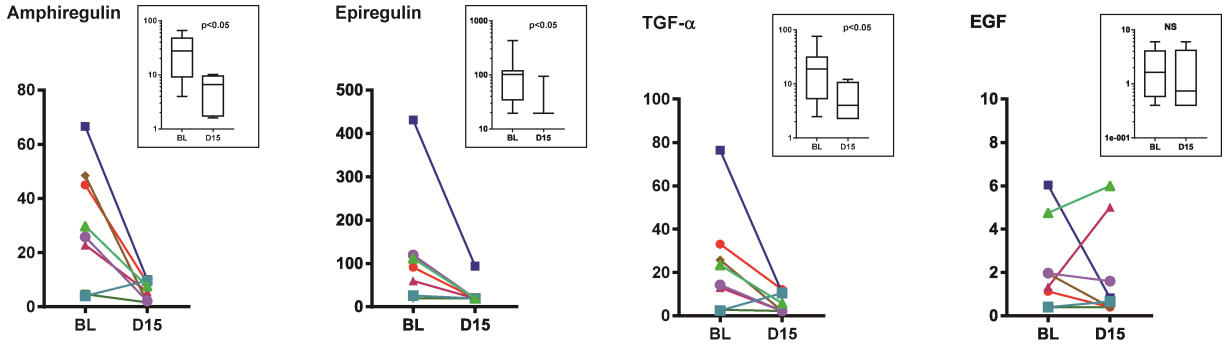


**Figure 7.** Reduction of keratinocyte growth factors in the epidermis of psoriasis patients upon secukinumab treatment. Protein levels of members of the EGF-family of growth factors in tape strip samples of psoriasis lesional skin before and 14 days after a single dose of secukinumab. Data for all 8 psoriasis patients are shown. Inserts show 25$^{th}$ and 75$^{th}$ percentiles and vertical lines depict minimum and maximum values; the horizontal line depicts the median value. Statistical significance was calculated using the Wilcoxon rank sum test (one-sided). Values below LOQ were included as LOQ. NS=not significant (p>0.05).
An early effect on keratinocyte proliferation by IL-17A blockade was confirmed by a reduction of Ki67 and keratin 16, direct and indirect markers of keratinocyte proliferation, by IHC[8,10] and by gene expression analysis of biopsy samples[10] (Data not shown).

disrupt the skin barrier before immunization[18], and to evaluate drug concentrations in skin[19,20]. To the best of our knowledge, de Jongh (2008) was one of the first to quantify cytokines (IL-1α) in diseased lesional stratum corneum from atopic dermatitis patients using this non-invasive technique. More recently, as protein detection methods have improved, tape stripping has been applied to measure stratum corneum concentrations of single proteins, primarily in atopic dermatitis. Indeed, stratum corneumBD-2, IL-8, thymus and activation-regulated chemokine (TARC), and thymic stromal lymphoprotein (TSLP) have been individually measured by tape stripping and correlated with disease activity in atopic dermatitis[21–24]. Stratum corneum protein levels can be influenced by genetic polymorphisms, suggesting that they are responsive and relevant to biological influences[25–28]. Undeniably, responses to drug therapy for example doxycycline in rosacea may also be monitorable[29]. In this study, we were able to measure and quantify protein levels of a large number of proteins simultaneously in a multiplexed format from tape strips using a highly sensitive chemiluminescent multiplex enzyme immunoassay. While some proteins like IL-17A were difficult to detect with this system, they were detectable with sufficiently sensitive assays like Singulex, tested individually.

We have recently shown that BD-2, measured in either serum or dISF, was a robust biomarker of psoriasis disease activity that responds rapidly to IL-17A blockade with secukinumab[6]. We also suggested that serum BD-2 may be a surrogate for IL-17A mediated skin disease activity that could be used to monitor responses to anti-IL-17A targeted therapies. However, this would require invasive monitoring by venipuncture. We showed here that BD-2 can also be monitored by tape stripping. However, the fold-change in stratum corneum BD-2 levels was smaller than in dISF, but similar to that in serumat D15[6]. Due to the turnover of the epidermal layers, we may hypothesize that it may take longer to detect bigger changes in the epidermis than in serum. However, BD-2 measurement by tape stripping may be valuable to monitor anti-IL-17A responses in psoriasis, as we did not detect significant side effects of the technique in our study. We have shown that therapeutically relevant dermal concentrations of secukinumab are detected in these 8 psoriasis patients at D8 and D15[11] and thus, a direct local effect on dermis and epidermis may be possible. While BD-2 may not be a unique marker of response to anti-IL-17A therapies, as BD-2 was qualitatively shown to be reduced by immunohistochemical staining following anti-TNFα treatment using etanercept[30], our data indicated that BD-2 measurements in serum[6] or by tape stripping may be useful in monitoring residual disease activity or early responses to different therapies in psoriasis. However, an early complete clearance of skin lesions remains the most important and clinically relevant marker for response to therapy. Whether BD-2 may also be useful in predicting response to therapy or strength of therapeutic response (e.g., PASI90) remains an open question and would require larger prospective clinical studies. The proteins with the highest fold-change in stratum corneum in response to secukinumab were MMP-8, MPO, IL-8, MMP-9, and IL-1β. MMP-8, MPO, and

MMP-9 were consistently detectable at high levels in psoriasis stratum corneum while the cytokines IL-1β and IL-8 were present at slightly lower but consistently detectable levels. All five proteins responded well to IL-17A blockade in a statistically significant manner. Immunohistochemistry showed that the proteins were expressed primarily by neutrophils. Since MPO is a specific marker of neutrophils, and MMP-8 and MMP-9 are neutrophil-associated proteases, their localization to neutrophils was not surprising. Munro originally identified the association between epidermal neutrophil microabscesses and psoriasis[31], and others have highlighted their role in IL-17A responses[10,32]. These findings have led to a resurgence in the interest of neutrophils in psoriasis. Our study also highlights the importance of neutrophils to psoriasis pathogenesis and response to IL-17A based therapies.

Another novel finding of this study was the observation that protein levels of three EGF-family ligands (amphiregulin, epiregulin, and TGF-α) were rapidly down-regulated upon IL-17A inhibition by secukinumab. These findings are consistent with earlier transcriptional data where amphiregulin, epiregulin, and TGF-α mRNA were found to be overexpressed in psoriatic epidermis and confined to the spinous layer but not the basal layer of psoriatic skin[33,34]. These three proteins are produced and secreted by human keratinocytes and act as autocrine growth factors[35]. Based on these data, we hypothesize that amphiregulin, epiregulin, and TGF-α may be important in driving the epidermal hyperproliferation seen in psoriasis. Down-regulation of amphiregulin has been demonstrated to be linked to epidermal remodeling and keratinocyte migration with shedding of membrane-bound E-cadherin being increased in amphiregulin transgenic mice displaying psoriasis-like skin alterations[36]. Interestingly, we found that soluble E-cadherin, which is believed to reduce keratinocyte cell-cell adhesion and thereby facilitate increased keratinocyte motility and neutrophil migration in the highly proliferative lesional psoriatic skin, was down-regulated in psoriatic lesions.

## Conclusion

In summary, this exploratory study has shown that minimally invasive tape stripping provides a useful way to assess disease pathomechanisms as well as response to a targeted therapy at the protein level directly in diseased skin. While providing further details on mechanisms of IL-17A targeting therapies such as secukinumab in psoriasis, tape stripping combined with proteomic analysis should also be useful in assessing disease pathomechanisms and activated pathways in other skin diseases.

## Conflict of Interest and Funding

All authors but FS and BA are full time employees of Novartis Institutes of BioMedical Research.

## Acknowledgments

This clinical trial was sponsored by Novartis Pharma AG. The authors would like to thank the subjects who participated in the study; Julia Mader for help in monitoring patients; Christian Dragatin, Maria Ratzer, and Karin Tiffner for OFM and sample management, sample analysis, and technical assistance; Xiaoyu Jiang (current affiliation, Merrimack Pharmaceuticals) for statistical help; and Dominic Ehrismann for administrative and editorial assistance.

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