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Detection of IL-36y via non-invasive tape stripping reliably discriminates psoriasis from atopic eczema

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Conflict of Interest
None of the Authors declares a conflict of interest.

Clinical Implications
A non-invasive, easy to perform diagnostic to verify psoriatic inflammation is of great value for lesions and anatomical locations where morphology is atypical or limited.

Capsule summary
This report demonstrates that sampling and detection of IL-36γ protein by non-invasive tape stripping of skin lesion provides a highly sensitive and selective diagnostic for psoriatic inflammation.
Key words
Psoriasis, Atopic Dermatitis, Eczema, IL-36, diagnostic, biomarker
To the Editor:

Inflammatory skin reactions, regardless of their distinct underlying pathophysiological mechanisms can often present with similar morphology. Whilst typical eczema and plaque psoriasis lesions are easily distinguishable by experienced dermatologists, these two diseases often cause diagnostic difficulties where inflammation is minimal or located in certain anatomical regions including flexures, scalp, auricular or palmoplantar areas. In these cases diagnosis can be problematic, in particular in the primary care setting where misdiagnosis may lead to delayed appropriate treatment and/or overuse of antibiotics. Histopathology, which is the current gold standard in diagnosing challenging cases is invasive, costly, and often unavailable in the primary care setting. Furthermore, histopathologic differentiation of psoriasis from eczema in some anatomical localisations, such as palmoplantar, is difficult. Unfortunately, there are also no reliable blood-derived diagnostic biomarkers that cover the wide range of clinical phenotypes and disease severities, and although RNA signatures from lesional skin biopsies have been described for atopic dermatitis (AD) and psoriasis, mRNA analysis can be costly and labour intensive. A simple, non-invasive and reliable diagnostic approach would therefore be of great clinical benefit. To address this need, this study uses a non-invasive, tape-stripping and ELISA based approach to investigate potential protein biomarkers that are able to discriminate eczematous from psoriatic inflammation presenting with a range of severities.

As the epidermis is a significant source of chemokines, inflammatory lesions from psoriatic and AD patients were initially sampled via tape-stripping and analysed for neutrophil-recruiting chemokines CXCL1, IL-8 as well as CCL20 which recruits IL-17/IL-22 producing cells (please refer to the Online Repository for detailed methodology). Although these chemokines were found at significantly higher levels in tape stripping samples from psoriasis lesions compared to AD, ROC curve analysis indicated that they would not be ideal as strong discriminators of the two conditions (IL-8: Area under the curve (AUC) 0.83, SE (Standard Error) 0.0523, 95% CI 0.726 to 0.931; CXCL1: AUC 0.796, SE 0.049, 95% CI 0.7 to 0.891 (Figure 1B); CCL20: AUC 0.82, SE 0.045, 95% CI 0.731 to 0.905). For all three chemokines no or very low levels of protein were detected in healthy and non-lesional samples.

As high levels of IL-36γ mRNA and protein have been reported in psoriatic lesions, IL-36γ from tape samples was quantitated through use of a novel in-house sandwich ELISA (Online Repository). IL-36γ showed a trend for increased levels in AD patients compared to non-lesional or healthy skin (mean: 71 / 13.5 / 57.5 pg/µg total protein, respectively). However, IL-36γ levels within psoriatic lesions were significantly greater than in AD lesions (mean, 719 vs 71 pg/µg total protein, Figure 1A). No difference in IL-36γ expression was observed in healthy versus non-lesional psoriatic skin taken from ventral forearm areas. A ROC curve (AUC 0.987, SE 0.0114, 95% CI 0.965 to 1.01) was plotted to determine sensitivity and specificity of IL-36γ in a diagnostic approach (Figure 1A). At an optimal cut off level of 214 pg/µg total protein (Youden’s Index 0.944) IL-36γ specificity was calculated as 100% (95% CI 90-100%) and sensitivity as 94.44% (95% CI 84.61% - 98.84%). This suggests that IL-36γ has an excellent potential as a diagnostic marker for psoriatic inflammation. When compared with CXCL1, the increased expression of IL-36γ is far more consistent, being found across all psoriasis patients. We also measured IL-36γ expression in a range of other skin pathologies including fungal infection, lichen planus, systemic, subacute cutaneous and chronic discoid lupus erythematosus all of which showed expression levels below the cut off level of 214 pg/µg total protein (data not shown).

Both CCL27 and CCL17 have been suggested as potential biomarkers for AD. CCL17 preferentially attracts IL-4/IL-13 producing lymphocytes whereas CCL27 is involved in the homing of memory T cells to the skin. Epidermal tape stripping demonstrated CCL17 but
not CCL27 to be of value to identify AD (Figure 1C). CCL27 was increased in both psoriatic and AD lesions. In those cases were CCL17 was detectable it pointed to an underlying AD inflammation (AUC 0.79, SE 0.058, 95% CI 0.676 to 0.903). However, unlike IL-36γ which was detectable in all psoriatic lesions tested, a significant number of AD samples (25%) as well as 57.4% of psoriatic samples failed to show any measurable levels of CCL17.

The patient cohort (supplementary Table 1) included in our analysis showed prototypic plaque psoriasis or AD. However, to further illustrate the potential of tape collected IL-36γ as a diagnostic approach for psoriasis, we investigated clinical cases of unclear diagnosis. In each case the diagnostic value of IL-36γ was correct when including dermatohistopathological results. As an example, we show 2 clinical cases (for details: Online Repository). The first case was clinically diagnosed with and treated for palmoplantar eczema in our dermatology department. However, analysis of tape stripping showed levels of IL-36γ at 960 pg/µg total protein, indicative of psoriasis. This fact along with subsequent dermatohistopathology analysis and changes in clinical features were supportive of a corrected diagnosis to that of psoriasis (Figure 2A). The other case was a patient with symptoms of joint pain in addition to erythrosquamous skin lesions. For the referring rheumatologist a confirmation of these skin lesions as psoriasis would guide future diagnostic and treatment pathways in the direction of psoriatic arthritis. However, tape stripping failed to confirm psoriatic inflammation and the diagnosis for the skin lesions was confirmed to be chronic eczema (Figure 2B).

Numerous soluble mediators, cell surface molecules and intracellular proteins have previously been described to be upregulated in psoriatic lesions. In our non-invasive approach we could confirm that neutrophil chemoattractants IL-8 and CXCL1 as well as the chemoattractant for IL-17 producing cells, CCL20, are elevated in psoriatic as compared to eczema lesions. Contrary to work showing reduced expression of CCL27 in psoriasis and value of CCL27 as a biomarker for AD, we did not find significant differences in the amount of this chemokine. The differences between our findings and that of some others is likely to be due to the method of sampling.

To conclude, although the effects of other factors such as systemic and topical therapeutics, age, sun exposure, and lesion chronicity need to be investigated in future studies, the results presented here confirm IL-36γ to be a robust, specific and reliable biomarker for psoriatic inflammation which out-performs previously reported biomarkers and is likely to withstand all the challenges in real life primary and secondary dermatology care.

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References
Figure legends

Figure 1
Comparison of IL-36γ, CXCL1, and CCL17 levels in tape samples from psoriatic and atopic eczema lesions.
Tape stripping was performed from healthy volunteers and lesional as well as non-lesional (NL) skin from patients with clinically diagnosed atopic dermatitis (AD) or psoriasis (Pso). Tape derived cytokine levels were normalised to total protein content. ROC curves are depicted for all 3 parameters comparing the eczema and psoriasis samples.

Figure 2
Tape stripping for diagnosis of difficult clinical cases.
Shown are pictures of 2 clinical presentations where the clinical diagnosis was unclear or challenging. Based on an IL-36γ cut-off level of 214 pg/µg total protein, tape stripping results indicated psoriatic inflammation for case A but not B. Histopathological examination confirmed the results. A. Palmar psoriasis initially misdiagnosed as hand eczema; B. Chronic eczema.

Supplementary Tables 1 and 2
Patient demographics and Local Lesion Scores.
Summary of patient demographics and the local lesion scores as determined by experienced dermatologists for all eczema and psoriasis patients included in the study.
Supplementary Materials and Methods

Patients included. Patients with the clinical diagnosis of plaque psoriasis or AD presenting with “typical” morphology as diagnosed by consultant dermatologists were included into the study. Hanifin and Rajka diagnosis criteria were followed for eczema and diagnosis of plaque psoriasis was based on traditional pattern recognition combined with patient history. Patient demographics are shown in Supplementary Table 1. This study was approved by NRES Committee North East-York (ILTIPP study: REC 14/NE/1199, ALPHA study: REC 14/YH/1259) and for recruitment of volunteers by the ethical committee of the University of Leeds (BIOSCI 14-001). All participants provided written informed consent prior to participation. Overall diseases activity differed significantly and was not collected for analysis as the focus was on the individual lesion. A local lesion severity score was assessed based on Psoriasis Activity and Severity Index (PASI) items (covering the range of 0-4) by specialist dermatologists prior to sample collection (Supplementary Table 2). Patients receiving systemic immunomodulatory therapy or biologics were excluded apart from 1 patient on anti-TNFα therapy (Etanercept) which had lost efficacy and one patient on low dose acitretin therapy. Where possible preference was given to patients who have not used topical corticosteroids in the sampled area for at least 48 hours. In the eczema group patients were considered atopic if presenting with clinical symptoms of immediate type reactions to allergens and/or the presence of specific IgE as measured by routine radioallergosorbent test in serum samples.

Samples. Tape stripping was performed from lesional and non-lesional skin (ventral forearm) at routine dermatology and combined dermatology/rheumatology clinics and processed using an improved tape stripping protocol based on previously published methodology E1,E2. D-Squame adhesive discs of 3.8 cm² (CuDerm corporation, Dallas, USA) were used. Sample site was guided by location of lesions, but preference was given to the hands, arms and upper trunk area. Only non-erosive, non-oozing lesions were tape stripped. The first tape was discarded and 10 subsequent tapes from the same location were collected and immediately stored on dry ice for transportation or stored at -80°C until protein was extracted.

Protein extraction and concentration determination. Frozen tapes were transferred into 1.5 ml of lysis buffer (20 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 1X protease inhibitor cocktail (Roche, Welwyn Garden City, UK). Tubes containing the tapes were left on ice for 30 minutes prior to sonication cycles of 3 times 20 seconds with 20 seconds interval on ice between each sonication. The extracts were centrifuged at 15,000g for 10 minutes and protein concentration of each sample was determined using bicinchoninic acid assay (Life Technologies Ltd, Paisley, UK).

Generation of human IL-36γ ELISA and measurement of soluble mediators. To generate the monoclonal antibodies against human IL-36γ, C57B/6 mice or Sprague Dawley rats were immunised twice with recombinant IL-36γ Ser18-Asp169 first in complete Freund’s adjuvant (FA) and subsequently incomplete FA. The aqueous boosts 3-4 days prior to fusions were intra-peritoneally with the same protein in phosphate buffered saline (PBS). Spleen cells were fused with myeloma cell lines Y3-AG 1.2.3 (rat) or SP2/O-Ag14 (mouse) as appropriate, using a method similar to the original described by Kohler and Milstein in 1975 E3. Hybridoma supernatants were screened against IL-36γ - or appropriate control - coated immunosorbent plates and selected fusion wells were cloned twice to ensure monoclonality. Characterisation of purified monoclonal antibodies indicated that the antibody generated by mouse hybridoma B5A2 performed effectively as a capture antibody, and the antibody generated by rat hybridoma HCL17 performed effectively as a detection antibody by sandwich ELISA. Antibodies were subsequently purified using either protein A or protein G affinity chromatography. The antibody HCL17 was then biotinylated using EZ link NHS-LC-biotin (Thermo Scientific) following the manufacturer’s instructions.

ELISA. IL-36γ ELISA: Immunosorbent 96-well ELISA plates (Nunc. Life Technologies, Paisley, UK) were coated with 2 µg/ml B5A2 capture antibody in PBS at 4°C overnight. Plates were then washed
3 times with 0.1% Tween-20/PBS and blocked for 1 hour in 2% bovine serum albumin in 0.1% Tween-20/PBS. Samples were subsequently incubated for 1 hour at room temperature, before washing and incubation with 1 μg/ml HCL17 biotinylated detection antibody for 1 hour. Plates were then washed and incubated with streptavidin-HRP (Biolegend London, UK) for 20 minutes. After washing TMB was used as chromogenic substrate (Thermo Scientific). The reaction was stopped using 2N H₂SO₄ and optical density measured at 450 nm. A standard curve was obtained from a 7 point serial dilution of protein standard and used to calculate IL-36γ concentrations. CCL27 from the tape stripping samples was quantified by ELISA (Bio-Techne Ltd, Abingdon, UK), according to the manufacturers’ protocol.

**Multiplex bead-based quantification assays.** Cytokines (CCL20, CXCL1, IL-8) from the tape stripping samples were measured using bead-based immunoassay purchased from Biolegend. The assay was carried out following the manufacturer’s instructions and fluorescent beads were acquired by flow cytometry (LSRII, BD Bioscience, UK). The results were analysed using LEGENDplex analysis software (Biolegend).

**Histopathology.** Routine diagnostic punch biopsies were taken from lesional skin, fixed in 10% formaldehyde, embedded in paraffin, stained with haematoxylin-eosin and evaluated by experienced dermatopathologists.

**Statistical Analysis.** Results were analysed using GraphPad Prism version 7.00. Results for each group are depicted with mean ± SD. Data were analysed using one-way ANOVA followed by Tukey multiple comparison test to determine statistically significant differences between groups. Sensitivity and specificity analysis was performed by receiver operating characteristics (ROC) curve analysis. The optimal cut of level was determined according to Youden’s Index. * stands for p < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001.

**Clinical Cases.** A: 61 years old male patient had been diagnosed with chronic palmoplantar eczema based on clinical appearance and symptoms (Figure 2A). There were no signs of eczema or psoriasis elsewhere on the body. The patient had no personal history of atopy; family history was positive for allergic asthma and negative for psoriasis. Patch testing revealed a positive reaction to Methylchloroisothiazolinone, the exposure to which the patient subsequently avoided. Total IgE level was 767 kU/L, without detectable specific IgE to inhaled allergen mix. Mycology microscopy and culture were negative for fungal infection. The patient very little no benefit from immersion PUVA therapy and presented little response to oral altretinoin 30 mg/day. During the disease course, lesions gained a slightly different appearance as coarser scales developed. Tape stripping samples were taken, before a diagnostic biopsy from a right palmar lesion. Results were highly suggestive of the diagnosis of psoriasis; with significantly enhanced levels of IL-36γ (IL-36γ = 959.3 pg/µg, CXCL1 = 15.9 pg/µg, IL-8 = 29.2 pg/µg, CCL17 was not detected). Histopathological examination from the same site confirmed the diagnosis of psoriasis. Subsequently re-examination of the patient reveal some nail pitting (Figure 2A), a symptom associated with psoriatic inflammation. Case B was referred by rheumatology presented with unclassified polyarthralgia and some skin symptoms. The presence of relatively well circumscribed margins and a thick, adherent scale included psoriasis as differential diagnosis (Figure 2B). However, low expression of IL-36γ in tape stripping samples failed to suggest psoriasis (IL-36γ = 25.2 pg/µg, CXCL1 = 0.12 pg/µg, IL-8 = 0.25 pg/µg, CCL17 was below detection levels). Subsequent histopathological examination confirmed the diagnosis of eczema.

**Detailed Legend of Figure 2.** A, upper hand pictures: Typical clinical signs of eczema: palmar erythema with moderate infiltration of the skin, deeply seated vesicles with discrete erosions and fine scaling associated with intense itchiness. A, lower hand pictures: The same patient 3 months later presented a change in clinical phenotype with the presence of rare intact pustules accompanied by accentuated scaling and typical nail changes showing pitting and ridging. Histopathological examination shows regular epidermal hyperplasia, with clubbing and anastomosis of the elongated rete ridges associated with suprapapillary plate thinning. There is mild, patchy,
perivascular infiltrate composed of lymphocytes, without eosinophils and mild exocytosis. The granular layer is still present. There is, however, some parakeratosis in which collections of neutrophils are identified. (Hematoxylin-eosin stain; high power magnification x400).

B, upper pictures: Relatively well demarcated, slightly erythematous, intensely itchy lesion covered by thick, adherent scales in the left lateral supramalleolar localisation of a 51 years old female patient. Histopathological examination shows irregular epidermal hyperplasia in association with hyperkeratosis and hypergranulosis. There is patchy epidermal spongiosis with focal lymphocytic exocytosis. There is no club-shaped elongation of the rete ridges and neutrophils are not identified. The features are in favour of those of a lichenified eczema. (Hematoxylin-eosin stain; high power magnification x400)

References


### Table 1. Demographic Characteristics of Patients

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### Table 2. Comparison of Local Severity Scores of patients with Atopic Dermatitis (AD) and Psoriasis

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Supplementary Tables