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INVESTIGATIVE REPORT

IL-20, IL-21 and p40: Potential Biomarkers of Treatment Response for Ustekinumab

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Although biological drugs in psoriasis treatment show clinical efficacy, there are still a proportion of patients in whom little treatment response is obtained. The aim of this study was to identify molecular biomarkers for treatment response and to investigate the molecular effects of ustekinumab treatment of psoriasis. The mRNA expression of various genes in skin biopsies was analysed by quantitative polymerase chain reaction (qPCR). At baseline, there was no significant clinical difference between responders and non-responders. Ten patients were clinical responders, with a mean baseline Psoriasis Area and Severity Index (PASI) score of 15.4 and a mean percentage improvement of 89.6%. No significant reduction in PASI during treatment was seen among the 5 non-responders. In the responder group, ustekinumab therapy reduced the mRNA expression of the majority of the studied genes in lesional psoriatic skin. IL-20, IL-21 and p40 mRNA expression in lesional psoriatic skin at baseline were significantly upregulated by factors of 2.7, 2.4 and 2.3, respectively, among non-responders compared with responders. The mRNA levels of p40, IL-20 and IL-21 at baseline may serve as potential predictors of treatment response to ustekinumab treatment. ***Key words: psoriasis; cytokines; ustekinumab; biomarkers.***

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Biologic drugs targeting specific cytokines in the inflammatory cascade are effective in the treatment of moderate to severe psoriasis and have led to better understanding of the immunology and pathophysiology of the disease (1–4). Systemic therapies, such as cyclosporine and methotrexate, were the first to implicate that immunological pathways play a pivotal role in the pathogenesis of psoriasis (5). However, with biologics came a group of drugs with much greater selectivity and therefore fewer side-effects (6, 7). Ustekinumab is the most recently registered biologic for the treatment of psoriasis.

Ustekinumab is a human monoclonal $IgG_{1\kappa}$ antibody that binds with high affinity and specificity to the shared p40 subunit of interleukin (IL)-12 and IL-23, thereby blocking their interaction with the receptor IL-12Rβ1 (8). IL-12 and IL-23 are proinflammatory cytokines that play a pivotal role in Th1 differentiation and Th17 proliferation, respectively (9), and therefore, treatment with ustekinumab represents a way of studying the effects of simultaneous inhibition of the IL-12/Th1 and IL-23/Th17 axis in psoriasis (10). Ustekinumab has demonstrated significant clinical efficacy and safety in several clinical trials (11–15).

Anti-tumour necrosis factor (TNF)-α antibodies are another group of biological drugs used in the treatment of psoriasis vulgaris. These drugs also demonstrate clinical efficacy and a good safety profile (2, 3, 16, 17).

Based on increasing knowledge of the immunological basis of psoriasis, partly due to the development of these therapies, increasing numbers of targeted therapies are emerging in the treatment of psoriasis. Evidence points to a pivotal role of IL-17A and IL-22 in the pathogenesis of psoriasis, and drugs blocking these cytokines are currently undergoing clinical trials (18, 19).

With the many alternatives in biological therapy the quest for biomarkers of treatment response for the individual biological drugs seems warranted in order to individualize patient care. Also, the high cost of biologics makes it important to identify treatment responders. In this context, biomarkers could be various cytokines predicting responders to a given drug.

In this study, we intended to investigate the molecular effects of ustekinumab in lesional psoriatic skin, and the expression of the following genes was studied: p40, p19, IL-17A, IL-17C, IL-20, IL-21, IL-22, IL-1β, IL-6, IL-8, TNF-α, IFN-α, hBD2, IRF-7, IL-23R and IL-12Rβ1. Increased expression of several of the above-mentioned genes has previously been shown in lesional psoriatic skin (20–23). The aim of the present study was to identify potential predictors of treatment response with ustekinumab treatment and to determine changes in gene expression in lesional psoriatic skin during ustekinumab treatment.

MATERIALS AND METHODS

Study population and ustekinumab treatment

A total of 18 adult patients with moderate to severe, chronic plaque psoriasis were included in the study. The patients had not received any topical treatment for 2 weeks and no systemic or ultraviolet B (UVB) treatment for 6 weeks prior to inclusion. Pa-

tients treated with another biological were left without treatment for at least 5 times the terminal half-life of the previous therapy. Ustekinumab (STELARA™, Centocor Ortho Biotech Services, Horsham, PA, USA) was administered subcutaneously on day 0 and subsequently on days 28 and 112. The dosage was either 45 or 90 mg, based on the baseline body weight (45 mg < 100 kg; 90 mg ≥ 100 kg) of the patient. Each patient was followed-up by a dermatologist, who collected the biopsies and evaluated the clinical appearance, using the Psoriasis Area and Severity Index (PASI) score, and the safety of the treatment.

Biopsies

At baseline, 4-mm punch biopsies were taken from non-lesional and lesional plaque-type psoriatic skin. Punch biopsies from the same lesion were obtained again from lesional skin 4, 28 and 112 days after treatment start. Thus, 5 sets of 3 punch biopsies were collected. For each patient, biopsies were taken from only one anatomical site and the non-lesional biopsies were taken at a distance of at least 5 cm from a lesional plaque. The set of biopsies used for immunohistochemistry were fixed in 3.7% paraformaldehyde and embedded in paraffin wax. The other set of biopsies used for quantitative reverse transcription-polymerase chain reaction (qRT-PCR) were instantly snap-frozen in liquid nitrogen and stored until further use.

Histology

Sections of paraffin-embedded tissue samples, 4 µm thick, from non-lesional and lesional psoriatic skin at baseline and lesional psoriatic skin 4, 28 and 112 days after treatment start were stained with haematoxylin and eosin and evaluated by light microscopy.

Immunofluorescence

The immunofluorescence procedure on paraffin-embedded tissue samples were conducted as described previously (17). Briefly, anti-cytokeratin 16 antibody (K16) was obtained from Abcam, Cambridge, UK (ab53117). The samples were incubated with K16 at 4ºC in Tris-buffered saline with 0.1% triton (TBST) blocking buffer overnight. The samples were then washed and incubated with AlexaFlour® 488 secondary antibody (Invitrogen, Carlsbad, CA, USA) for 1 h. Samples were evaluated by epifluorescence microscopy (Leica, Heidelberg, Germany).

Quantitative polymerase chain reaction

RNA from punch biopsies was isolated as previously described (24). For reverse transcription Taqman Reverse Transcription reagents (Applied Biosystems, Foster City, CA, USA) were used. For quantitative PCR (qPCR) we used Platinum® qPCR SuperMix-UDG (Invitrogen, Carlsbad, CA, USA), and primers and probes that were Taqman 20X Assays-On-Demand (FAM-labelled MGB-probes) gene expression assay mix (Applied Biosystems). Each sample was loaded as triplets and analysed on a Rotorgene-3000 real-time PCR machine (Corbett Research, Cambridge, UK). Relative gene expression levels were determined by using the relative standard curve method, as outlined in User Bulletin 2 (ABI PRISM 7700 sequencing detection system, Applied Biosystems). Briefly, a standard curve for each gene was made of 5-fold serial dilutions of total RNA from a punch biopsy from lesional psoriatic skin. The curve was then used to calculate relative amounts of target mRNA in the samples. As housekeeping gene ribosomal protein, large, P0 (RPLP0) was used. Assay ID for the primers and probes used in this study were as follows: p40 (Hs01011518_m1); p19 (Hs00413259_m1); IL-20 (Hs00218888_m1); IL-21 (Hs00222327_m1); IL-22 (Hs00220924_m1); IL-1β (Hs00174097_m1); IL-6 (Hs00985639_m1); IL-8 (Hs00174103_m1); TNF-α (Hs00174128_m1); IL-17A (Hs00936345_m1); IL-17C (Hs00171163_m1); hBD2 (Hs00823638_m1); IFN-α (Hs00855471_g1); IRF-7 (Hs00185375_m1); IL-23R (Hs00332759_m1); IL-12Rβ1 (Hs01106578_m1); and RPLP0 (Hs99999902_m1).

Statistical analysis

Results are expressed as dot plots, and the horizontal line expresses the mean value. In the biopsy study demonstrating the effect of ustekinumab treatment statistical analysis was carried out using a one-way repeated measures analysis of variance (ANOVA) followed by a Student Newman–Keuls test. Statistical analysis for potential biomarkers was assessed by a Student's *t*-test. $p < 0.05$ was regarded as statistically significant.

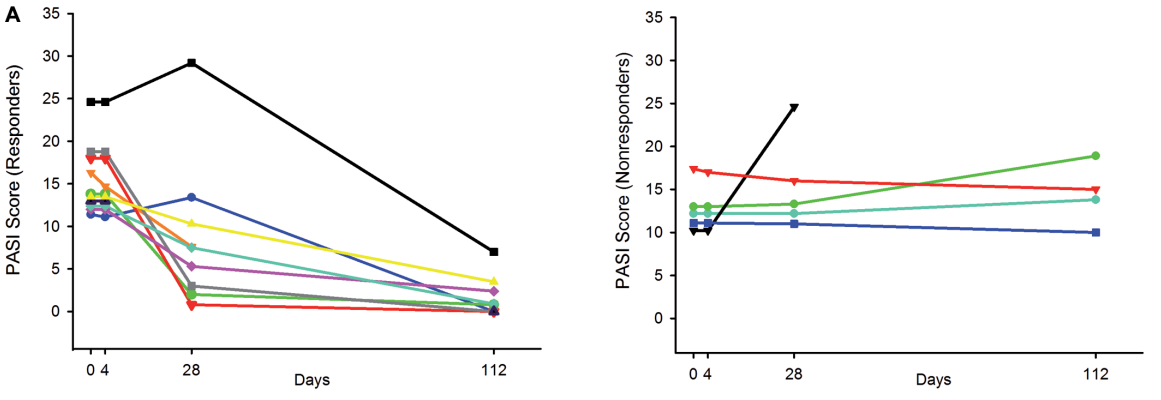
RESULTS

Patient characteristics: responders vs. non-responders

In this study, 18 patients were given either 45 or 90 mg ustekinumab subcutaneously on days 0, 28 and 112. The mean PASI score for all patients was 14.4 (range 10.2–24.6; standard error of the mean (SEM) = 3.6) at baseline. The patients were divided into 3 groups, responders, partial responders and non-responders, based on their PASI score at day 112 (Fig. 1B). As responders, a reduction in PASI score of at least 70% at day 112 was chosen as clinical end-point, and as non-responders a reduction in PASI of no more than 30% was chosen as clinical end-point. Two patients were withdrawn from the study on day 28 due to side-effects of the treatment (1 patient had a severe flare-up and the other patient developed pancytopaenia, which was probably unrelated to ustekinumab treatment), but data up until these events were included.

Three patients (16.7%) with a reduction in PASI score of between 30% and 70% were considered partial responders and excluded from the study results. The partial responders did not tend to differ from the responders and non-responders except for their treatment response to ustekinumab. They had a baseline PASI score of between 10 and 20 and, similar to the responder and non-responder groups, no clinical change was seen at day 4 (Fig. 1A). Thus, the partial responders responded to ustekinumab treatment, but at day 112 the PASI reduction compared with baseline was only between 30% and 70%.

At baseline, there was no significant difference in PASI score between responders and non-responders, and no correlation was seen between the 2 dosages used and the outcome of treatment response, as already demonstrated in a large-scale clinical trial (14). Clinical improvement was evaluated on days 4, 28 and 112 after the start of ustekinumab treatment. Ten patients were clinical responders with a mean baseline PASI score of 15.4 (range 11.4–24.6; SEM = 4.1) and a mean improvement of 89.6% (range 71.5–100%; SEM = 2.4) at day 112. At day 4 after the start of therapy, no clinical improvements were observed in the responder group



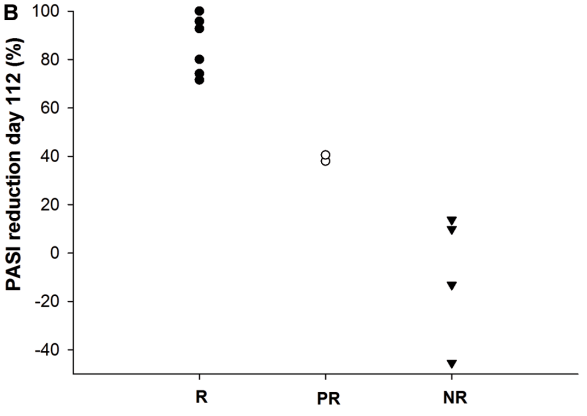
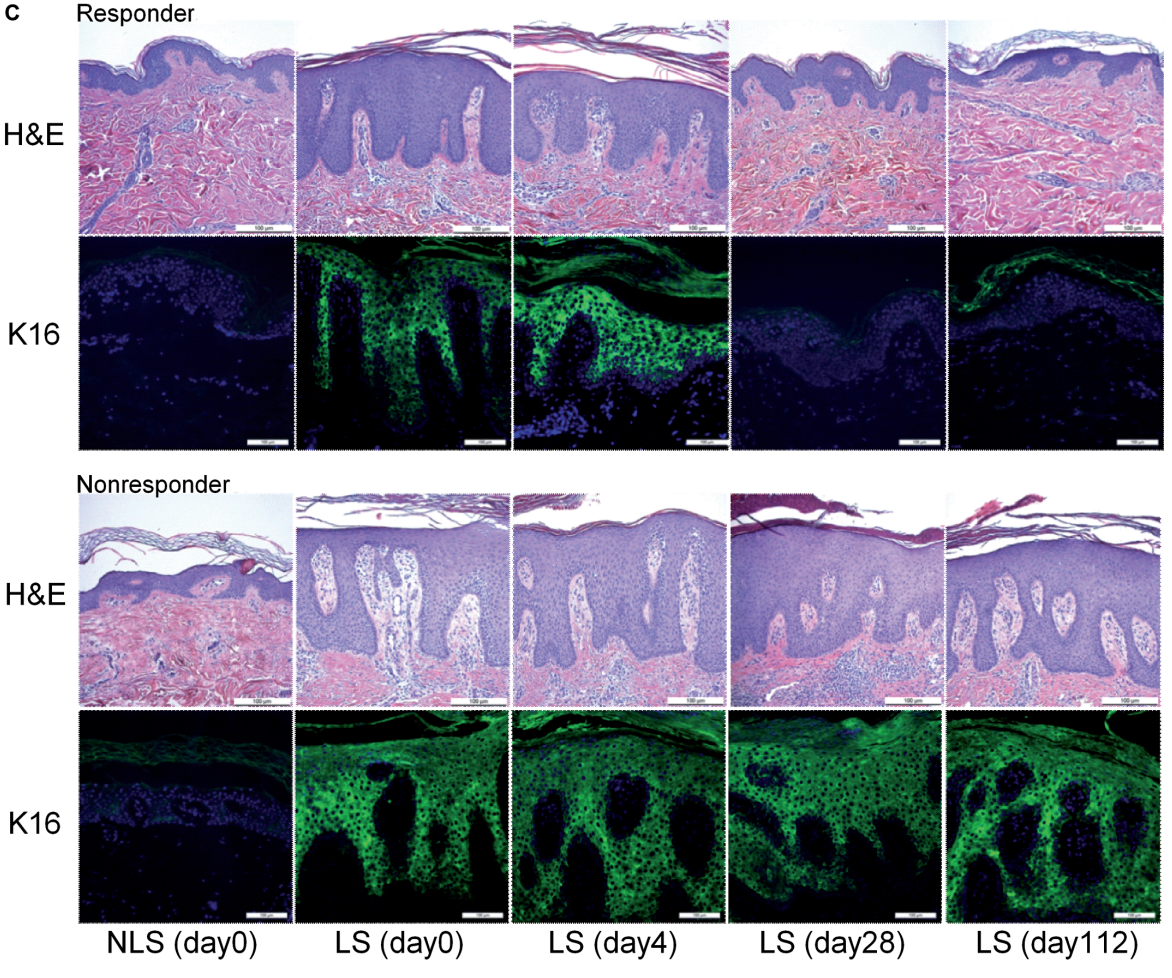


Fig. 1. Clinical and histological responses to ustekinumab treatment. (A) Individual Psoriasis Area and Severity Index (PASI) scores of 10 responders (left) and 5 non-responders (right) before (day 0) and at fixed days following ustekinumab therapy. Partial responders are not depicted. Each patient is represented by one colour. (B) The PASI score reduction at day 112 in percentages. The patients were divided into 3 groups, responders (R), partial responders (PR) and non-responders (NR), based on their reduction in PASI score at day 112. (C) Haematoxylin and eosin (H&E) staining and keratin 16 (K16) immunofluorescence staining of the 4-µm sections of paraffin-embedded tissue samples of non-lesional skin (NLS) and lesional skin (LS) before (day 0) and of LS at fixed days after the start of ustekinumab therapy. Stained sections of punch biopsies from one representative responder and non-responder are displayed. The H&E staining was evaluated by light microscopy. K16 was evaluated by epifluorescence microscopy and is represented by the green colour (AlexaFlour® 488); the blue colour (4',6-diamidino-2-phenylindole) demonstrates the cell nuclei. Scale bar = 100 µm.



(Fig. 1A). However, from day 28 clinical improvement was observed in 8 out of 10 patients in the responder group, and at day 112 the psoriasis had almost cleared in all 10 responders. The reduction in PASI score at day 112 was significant ($p = 0.000003$) compared with baseline. Five patients were considered as clinical non-responders with a mean baseline PASI score of 12.8 (range 10.2–17.4; SEM = 2.8) and a mean exacerbation of 12.9% in total PASI (range 9.9–45.4%; SEM = 3.7) at day 112 compared with baseline (Fig. 1A).

The effects of ustekinumab treatment on epidermal thickness, expression of keratin 16 and psoriasis histopathology in the responder and non-responder group are illustrated in Fig. 1C. At baseline, there was no visible difference in the histological appearance between responders and non-responders. Haematoxylin and eosin staining and keratin 16 immunofluorescence staining of the paraffin-embedded tissue sections supported the clinical improvements. In the responder group, reduction in epidermal thickness and hyperproliferation were seen at day 28. After 112 days of treatment, epidermal thickness and keratinocyte differentiation were normalized in 9 out of the 10 clinical responders (1 patient stopped after day 28). No or little changes in histopathology, epidermal thickness and expression of keratin 16 were observed in the non-responder group.

IL-20, IL-21 and p40: potential biomarkers of treatment response for ustekinumab

At present, no studies have been able to identify reliable biomarkers of treatment response for biological drugs (25–29). In an attempt to identify potential predictors of treatment response for ustekinumab, the mRNA expression at baseline of the various studied genes was compared between responders and non-responders.

At baseline, IL-20, IL-21 and p40 mRNA expression levels in lesional psoriatic skin were significantly upregulated by a factor 2.7, 2.4 and 2.3 among non-responders compared with responders, respectively ($p < 0.05$; Fig. 2). No significant differences in mRNA expression of the remaining 13 genes were seen at baseline between non-responders and responders (data not shown).

Ustekinumab: effects on mRNA expression of various proinflammatory genes

The mRNA expression of the following genes was studied by qPCR during ustekinumab treatment: p40, p19, IL-17A, IL-17C, IL-20, IL-21, IL-22, IL-1β, IL-6, IL-8, TNF-α, IFN-α, hBD2, IRF-7, IL-23R and IL-12Rβ1. In accordance with previous studies (20–23), we found an increased expression level of the studied genes in lesional psoriatic skin compared with non-lesional skin, except for TNF-α (Fig. S1 (available from http://www.medicaljournals.se/acta/content/?doi=10.2340/00015555-1440); data for the non-responders and the partial responders are shown in Fig. S2 and S3; available from http://www.medicaljournals.se/acta/content/?doi=10.2340/00015555-1440) and IFN-α (data not shown).

At day 4, the expression of the examined genes was unchanged compared with baseline in lesional psoriatic skin in the responder group, demonstrating that ustekinumab had no immediate effect on the mRNA expression level. At day 28, the mRNA expression of p40, p19, IL-17A, IL-17C, IL-22, IL-1β, IL-6, IL-8, hBD2, IRF-7, IL-23R and IL-12Rβ1 was reduced compared with baseline in lesional psoriatic skin in the responder group. The down-modulation was even more pronounced at day 112, reaching the level of non-lesional skin for most of the examined genes. The mRNA level of IL-6 was not reduced any further from day 28 to day 112 (Fig. S1). We were not able to detect any IL-21 mRNA in non-lesional psoriatic skin in this study. No significant changes in IL-20 and IL-21 mRNA expression were seen at day 28 compared with baseline in lesional psoriatic skin, whereas the level of these 2 genes was reduced at

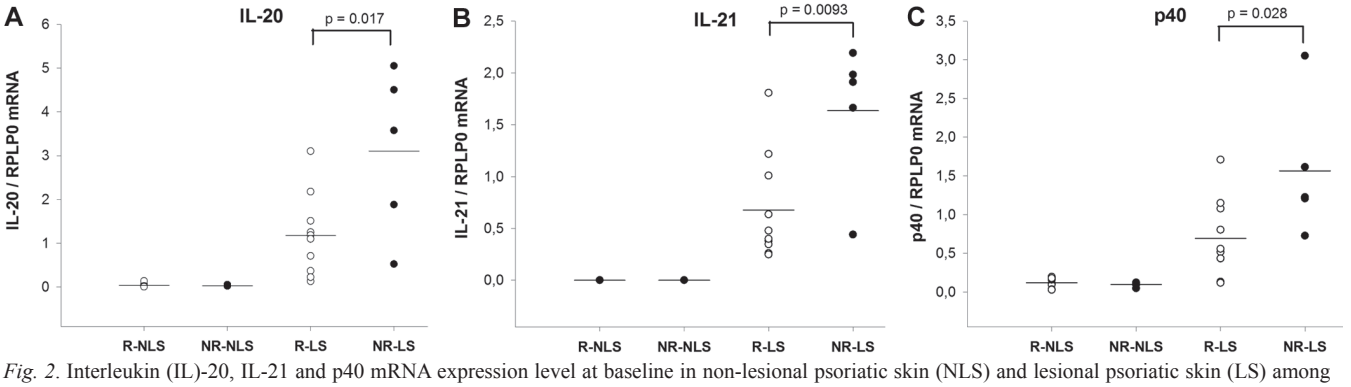


Fig. 2. Interleukin (IL)-20, IL-21 and p40 mRNA expression level at baseline in non-lesional psoriatic skin (NLS) and lesional psoriatic skin (LS) among non-responders (NR) compared with responders (R). The isolated RNA from the punch biopsies in LS was analysed by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and normalized to ribosomal protein, large, P0 (RPLP0); all samples were loaded as triplets. Results are presented as dot plots with the mean shown for 10 R and 5 NR and each patient is represented by 1 symbol (open circles = responders, filled circles = non-responders. Baseline LS values were compared between R and NS and $p < 0.05$ was considered significant. (A) mRNA expression for IL-20. (B) mRNA expression for IL-21. (C) mRNA expression for p40.

day 112, for IL-20 mRNA expression reaching the level of non-lesional skin (Fig. S1).

Among the responders, the level of IFN-α in lesional psoriatic skin did not change during ustekinumab treatment (data not shown). However, we found a reduction in the level of IRF-7 mRNA expression, an IFN-α inducible gene (22), during ustekinumab treatment. In accordance with previous studies (17, 30), we found no difference in TNF-α mRNA expression between non-lesional and lesional psoriatic skin. Furthermore, we found no change in the mRNA level of TNF-α in lesional skin during ustekinumab treatment (Fig. S1).

In the non-responder group, the expression of the studied genes was largely unchanged in lesional psoriatic skin from day 0 to day 112 (data shown in Fig. S2).

DISCUSSION

In this study we have investigated the molecular effects of ustekinumab treatment of psoriasis and we have identified potential biomarkers that may serve as predictors of treatment response.

At present, 5 biological drugs are approved for the treatment of psoriasis vulgaris: 3 anti-TNF-α agents (infliximab, adalimumab, etanercept), 1 anti-IL-12/23 agent (ustekinumab) and 1 T-cell modulating agent (alefacept, in European Union (EU) only approved in Switzerland). With the evolving knowledge of the immunological basis of psoriasis, more and more targeted therapies, such as, for example, anti-IL-17A and anti-IL-22 targeting antibodies will emerge (18, 19). Although the biological drugs have significant clinical efficacy (2, 3, 11–17, 31), there are still a proportion of patients in whom no or little treatment response to the individual biological therapies are obtained. In addition, these treatments are expensive and associated with potential side-effects. Therefore, the quest for biomarkers is needed to predict treatment outcomes for the biological therapies and to individualize care (29).

Here we have demonstrated a significant upregulation of IL-20, IL-21 and p40 mRNA expression at baseline in lesional psoriatic skin among non-responders compared with responders. Furthermore, we showed that the decline in mRNA expression of the majority of the studied genes accompanied the clinical improvement.

Previous attempts to identify biomarkers of treatment response in psoriasis have been sparse and unsuccessful and only a few studies have dealt with biomarkers in the skin (25–29). Peripheral blood mononuclear cells (PBMCs) have been studied for differences between the responders and non-responders (26, 27). Others have investigated the relationships between genetic polymorphisms and treatment response in an attempt to identify potential biomarkers (29). Thus, a more functional approach, such as analysis of RNA expression using qRT-PCR and RNA microarray analysis for the identification of biomarkers, has also been suggested (29).

We suggest that a difference in the mRNA expression level of specific genes at baseline between responders and non-responders could serve as a general marker to predict whether or not a patient will respond to ustekinumab therapy. A few other studies have identified potential biomarkers of treatment response by means of functional approaches (29). One of these studies uses gene expression in PBMCs at baseline for comparison of the difference between the responders and non-responders (26). The remaining studies compare the 2 groups at different time-points after treatment start (25, 27, 29). In this study, we demonstrate a difference in the mRNA level of IL-20, IL-21 and p40 at baseline between the responders and the non-responders. However, to make clinical use of this difference at baseline in 2 otherwise identical groups of psoriasis patients, we need to set a threshold value in the mRNA expression of IL-20, IL-21 and p40, separating the non-responders from the responders, and this is still not possible based on the available data.

In the responder group, ustekinumab therapy reduced the mRNA expression of the majority of the studied genes in lesional psoriatic skin at day 28, which was in parallel with the observed clinical improvements. Previously, adalimumab has been shown to inhibit p38 mitogen-activated protein kinase (MAPK) activity as early as day 4 after treatment start in lesional psoriatic skin, which precedes clinical improvement (17). Moreover, another study demonstrated that specific p38 MAPK-regulated genes, such as IL-1β, IL-8, IL-17C and IL-20, were normalized by adalimumab as early as 4 days after the start of treatment (32). In contrast, we were not able to identify immediate early responding genes to ustekinumab treatment, demonstrating that adalimumab and ustekinumab mediate their anti-psoriatic effects by different mechanisms.

Patients with a high mRNA expression level of IL-20, IL-21 and p40 in their lesional psoriatic skin seem to constitute a subgroup of ustekinumab-insensitive psoriatic patients. The high level of p40 could reflect a group of patients in which the current dosage of administered ustekinumab is simply too low. At present, IL-20 and IL-21 have not been demonstrated as being downstream genes from p40, but our findings indicate that there could be a correlation. However, the pathophysiological explanation of our observation is still unknown and further studies are needed in order to verify this.

Our results do not identify a threshold value of IL-20, IL-21 and p40 mRNA expression that can be used to separate responders to ustekinumab treatment from non-responders. Therefore, further studies are needed before our findings can be used in the daily clinical situation.

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The authors declare no conflicts of interest.

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