

KEYWORDS

asthma, IL-33, murine models, nonsteroidal anti-inflammatory drug, protease allergen

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Biomarkers detected in dried blood spots from atopic dermatitis patients strongly correlate with disease severity

To the Editor,

Assessment of disease severity in atopic dermatitis (AD) is essential for monitoring treatment effects and for comparison of clinical trials investigating new treatment options. Although serum biomarkers

offer an objective outcome measure for disease severity in AD,¹ a disadvantage is the need for a venipuncture, which requires trained personnel and immediate processing of blood to prevent degradation of biomarkers. Since biomarker levels may be influenced by

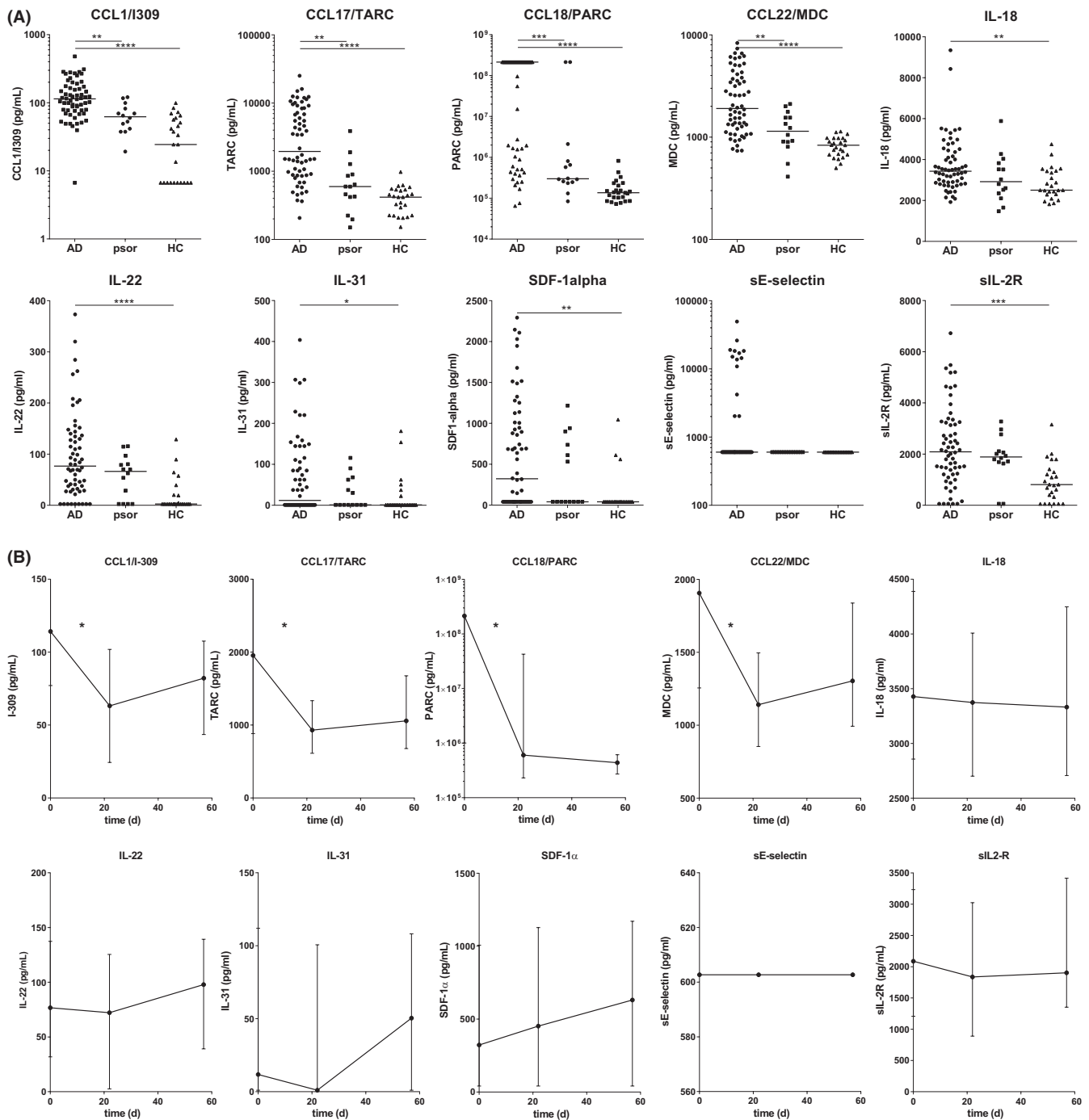


FIGURE 1 A, Biomarker levels in dried blood spots (DBS) from atopic dermatitis (AD) patients, patients with psoriasis, and healthy controls. Differences in biomarker levels between 65 AD patients, 14 psoriasis patients, and 26 healthy controls at baseline were analyzed by ANOVA testing. Horizontal bars represent median biomarkers levels. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. AD, atopic dermatitis; HC, healthy controls; psor, psoriasis. B, Biomarker levels measured in DBS during treatment of AD patients. Error bars represent 95% confidence intervals. Linear mixed models were used to model the changes over time of biomarkers after treatment initiation with topical steroids. P -values are shown in Table S3

processing, time, and temperature, strict protocolized handling is needed. This can be challenging, for example, in multicentre trials. Dried blood spots (DBS) are a convenient alternative to venipuncture. DBS can be obtained using a simple, minimally invasive, nearly painless procedure by the patients themselves, outside medical institutions. The aim of this study was to investigate whether biomarkers

determined in DBS offer a reliable tool for the assessment of disease severity in AD.

Sixty-five adult AD patients (median age 32 years (interquartile range (IQR) 23-47, median Eczema Area and Severity Index (EASI) score 17.4, IQR 10.0-25.7), 14 psoriasis patients, and 26 nonatopic (healthy) controls (HC) were included in a prospective cohort study

TABLE 1 Correlations between Eczema Area and Severity Index (EASI), serum, and dried blood spots (DBS) biomarkers

	EASI vs serum biomarker	EASI vs DBS biomarker	serum vs DBS biomarker
I-309	0.41***	0.52****	0.86****
log TARC	0.58****	0.68****	0.90****
log PARC	0.40**	0.38**	0.50****
log MDC	0.41***	0.51****	0.84****
IL-18	0.01	0.13	-0.04
IL-22	0.34**	0.34**	0.27*
IL-31	0.06	0.13	0.30*
SDF-1 α	-0.08	0.17	0.22
log sE-selectin	0.39**	0.23	0.32*
sIL-2R	0.08	0.20	0.28*

Pearson correlations between disease severity measured by EASI and biomarkers measured in serum and DBS from 65 AD patients.

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$.

**** $P < 0.0001$.

(Table S1; serum biomarker levels of these AD patients were previously reported in another study).² At t_0 , patients started with daily application of topical steroids (European and US classification class III), which was tapered to what we consider a safe maintenance scheme between t_1 and t_2 . After treatment initiation, disease severity significantly decreased to a median EASI score of 3.3 (IQR 1.2-5.6) at t_1 , and remained stable until t_2 (median EASI score 2.7, IQR 1.0-4.6), resulting in a median percentage change in EASI score from baseline to t_1 of -81% and -84% at t_2 . The Patient-Oriented Eczema Measure (POEM) and pruritus according to the visual analog scale (VAS) showed a similar pattern (Figure S2).

At baseline (t_0), t_1 , and t_2 , serum and DBS were collected for biomarker measurement. DBS were obtained by a fingerprick lancet (Super Blade, 1.5 mm blade/penetration depth 1.6 mm, Sarstedt) from digitus III or IV and spotted onto filter paper (Whatman 903 Proteinsaver; GE Healthcare) to create DBS (following the protocol published by Ostler et al) (Figure S1B).³ Spotted filter papers were dried horizontally overnight at room temperature and subsequently stored with desiccant in individual air tight polyethylene bags at -80°C under constant monitoring of humidity levels until analysis.

For elution of DBS, two 3.2 mm disks (containing approximately 3 μ L of whole blood each) were punched from the central part of each spot into a MultiScreen 96-well filter plate well (hydrophilic, low protein binding Durapore Membrane, Merck Chemicals) and 100 μ L elution buffer was added per well (PBS containing 5 mL/L Tween-20, 10 g/L bovine serum albumin, and complete protease inhibitor cocktail with EDTA (complete ULTRA tablets, Roche, one tablet per 25 mL buffer)). Plates were sealed and placed overnight at 4°C on a microshaker set to 600 rpm to extract the analytes. Finally, plates were centrifuged at 2100 g for 2 minutes. Biomarkers in both

serum and eluted DBS were measured by multiplex technology (see Data S1). At baseline, levels of IL-18, IL-22, IL-31, sIL-2R, and stromal cell-derived factor 1 α (SDF-1 α) measured in DBS from AD patients were significantly higher than in HC ($P < 0.05$), but similar to patients with psoriasis (Figure 1A). Levels of I-309, thymus and activation-regulated chemokine (TARC), pulmonary and activation-regulated chemokine (PARC), and macrophage-derived chemokine (MDC) in DBS at t_0 were significantly upregulated in AD patients compared to patients with psoriasis and HC ($P < 0.01$) (Figure 1A). TARC showed the strongest correlation (Pearson) with disease severity ($r = 0.68$, $P < 0.0001$), followed by I-309 ($r = 0.52$, $P < 0.001$), MDC ($r = 0.51$, $P < 0.001$), and PARC ($r = 0.38$, $P < 0.01$). (Tables 1 and S1). TARC, PARC, I-309, and MDC, members of the CC-chemokine family, are involved in inflammation and recruitment of T cells into the skin and play a role in the pathogenesis of AD.⁴ The other biomarker levels did not correlate with disease severity.

Thymus and activation-regulated chemokine, I-309, and MDC levels in DBS highly correlated with serum levels ($r = 0.90$, $P < 0.0001$; $r = 0.86$ and $r = 0.84$, respectively; $P < 0.0001$, Table 1). PARC, IL-22, IL-31, sE-selectin, and sIL-2R in DBS and serum showed significant correlations but showed lower r values (varying from $r = 0.27$ to $r = 0.50$, Table 1). Interestingly, TARC DBS levels showed a stronger correlation with EASI than TARC serum levels ($r = 0.68$ vs $r = 0.58$, respectively; Table 1). The strong correlation of TARC levels in DBS with disease severity is in accordance with observations in serum.⁵ The stronger correlation with disease severity when measured in DBS compared to serum may be explained by higher recovery levels in DBS than in serum due to a faster release of intracellular components in DBS.⁶ An additional explanation might be that these biomarkers are expressed at different concentrations in capillary blood (DBS) and venous blood (traditional blood draw).

In a linear mixed model approach modeling, EASI score changes over time using a combination of biomarkers (described in Data S1) showed that I-309, TARC, PARC, and MDC decrease significantly during daily treatment with topical steroids (t_0 - t_1), and then remain stable during intermittent treatment with topical steroids (t_1 - t_2) (Figure 1B, Table S3). The biomarkers IL-18, IL-22, IL-31, sIL-2R, and SDF-1 α did not change during treatment. Remarkably, using only TARC levels resulted in the best model for prediction of EASI scores, both at baseline (ie, before treatment initiation) and after treatment initiation (Table S4). After adjusting for gender and age, these results remained unchanged.

Since TARC level in DBS was the strongest predictor of disease severity measured by EASI, we investigated the stability of TARC levels in DBS for the application in future clinical trials, daily practice, and biobanking purposes. TARC levels were stable after storage at room temperature for 7 days (Figure S3A), or after storage at -80°C for 34 months (Figure S3B) and after three freeze/thaw cycles. (Figure S3C).

An advantage of DBS over venipuncture is the simplicity of the minimally invasive and nearly painless procedure. While venipuncture requires trained personnel, DBS can be performed by patients themselves, outside medical institutions. DBS only require a very small

amount of blood ($\pm 10 \mu\text{L}$), which is a particular advantage in studies in newborns and infants. DBS also offer a huge simplification in storage (ambient temperature) and transport (eg, by mail), which reduces costs and simplifies logistic harmonization between centers participating in clinical trials. In a small subset of patients, we confirmed that TARC DBS levels are stable when stored at room temperature for up to 7 days after collection, and levels are not influenced by repeated freeze/thawing. Moreover, when DBS are stored at -80°C , TARC levels remain stable up to 34 months. Earlier reports have shown that cytokine levels in DBS remain stable up to 23 years when DBS are stored at -24°C .⁷ This makes TARC measured in DBS a very reliable and cost-effective biomarker for disease severity in AD.

Dried blood spots also offer the possibility of measuring drug concentrations of new biological agents⁸ and current drugs that are frequently used in AD treatment, including cyclosporin A,⁹ mycophenolic acid, and tacrolimus.⁹ Combining measurement of drug concentrations in DBS with disease severity biomarkers would be a major step forward in clinical care and trials.

In conclusion, we have shown that in a population of mild to severe adult AD patients TARC, PARC, I-309, and MDC levels measured in DBS highly correlate with disease severity and significantly decrease during effective treatment with topical steroids. DBS offer a simple and minimally invasive method for measurement of biomarkers and provide a tool for objective severity outcome measurements in AD that can easily be applied in clinical trials and daily practice.

CONFLICTS OF INTEREST

Judith Thijs, Renée Fiechter, Barbara Giovannone, Edward Knol, Stefan Nierkens Carla Bruijnzeel-Koomen, DirkJan Hijnen: no conflicts of interest. Marjolein de Bruin-Weller: consultant for Regeneron Pharmaceuticals, Inc, Sanofi Genzyme; an advisory board member for AbbVie, Regeneron Pharmaceuticals, Inc, Sanofi Genzyme; and principal investigator for AbbVie, Regeneron Pharmaceuticals, Inc, Roche, Sanofi Genzyme.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

EASI
POEM
VAS pruritus



Daily treatment with topical steroids
3 weeks

EASI
POEM
VAS pruritus



Intermittent treatment with topical steroids
3-9 weeks

EASI
POEM
VAS pruritus



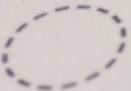
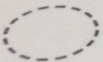
t_0

t_1

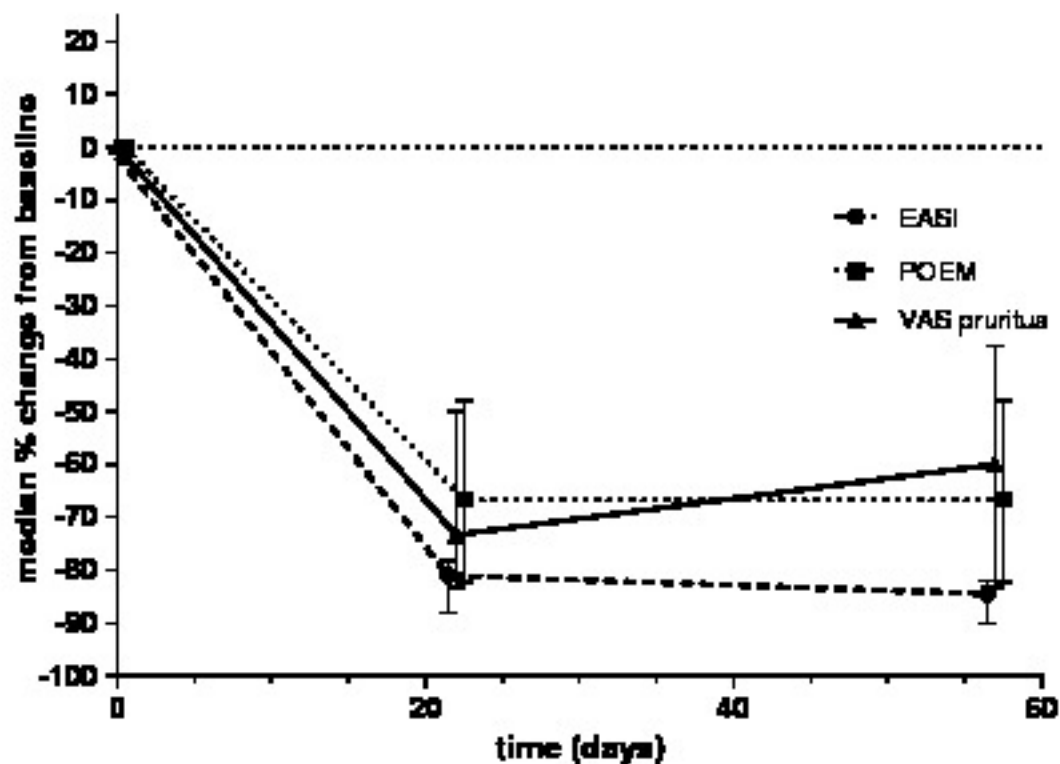
t_2



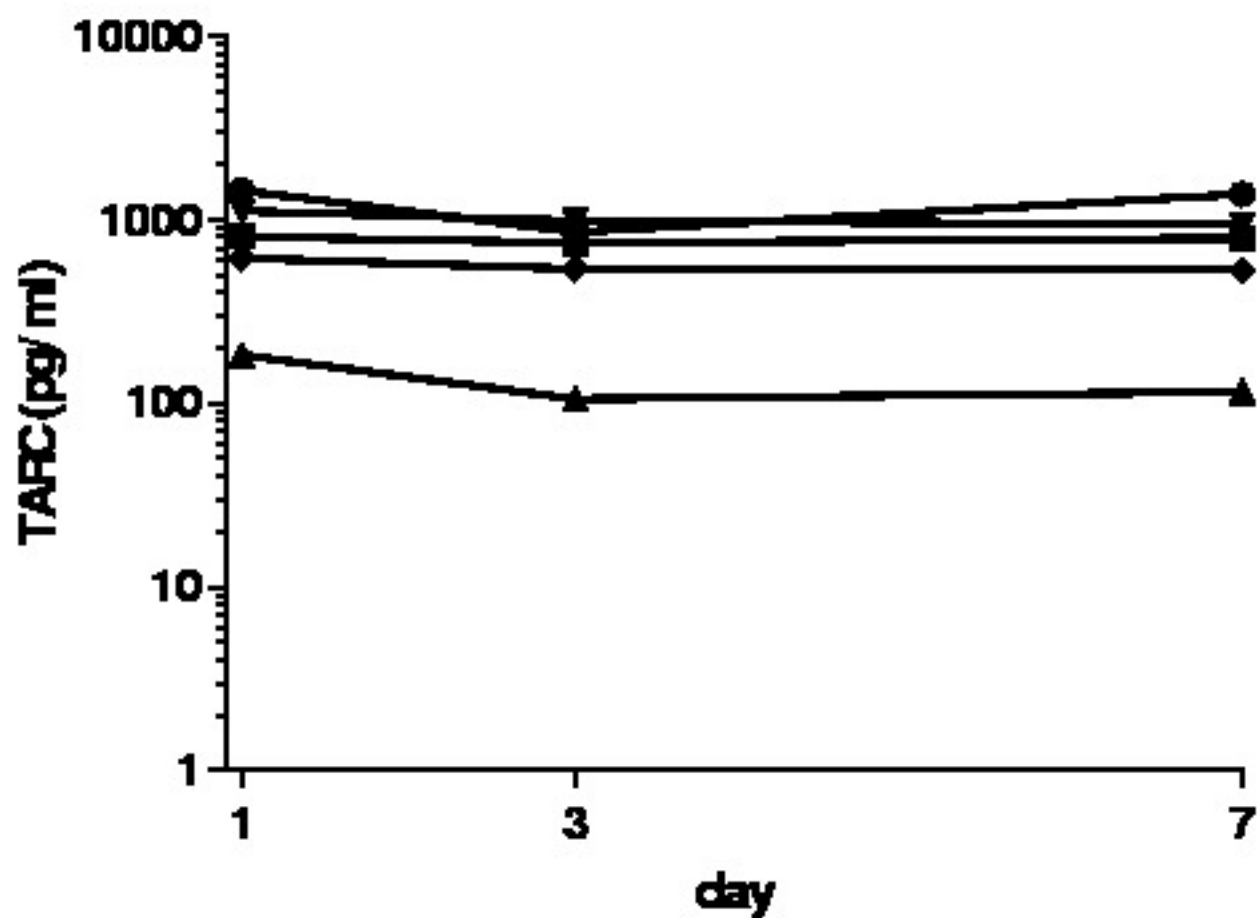
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Huispostnummer F03.722
Heidelberglaan 100
3584 CX Utrecht



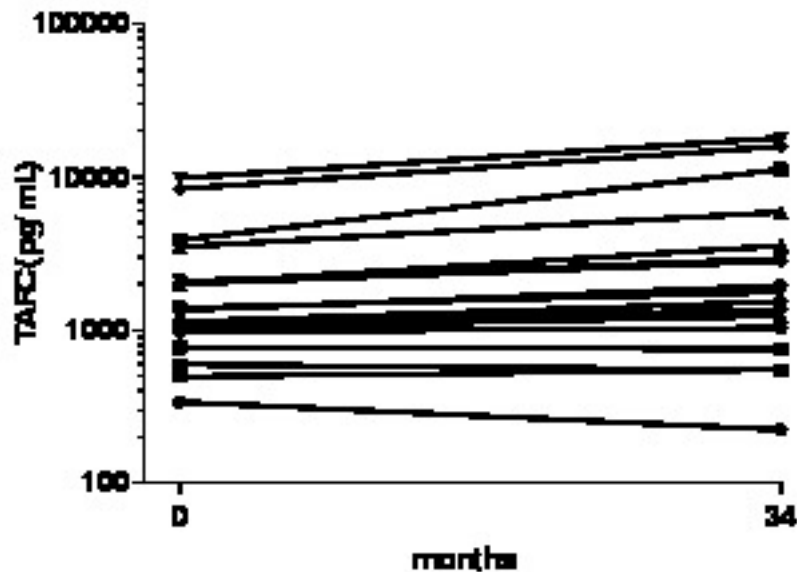
Median % change in disease severity



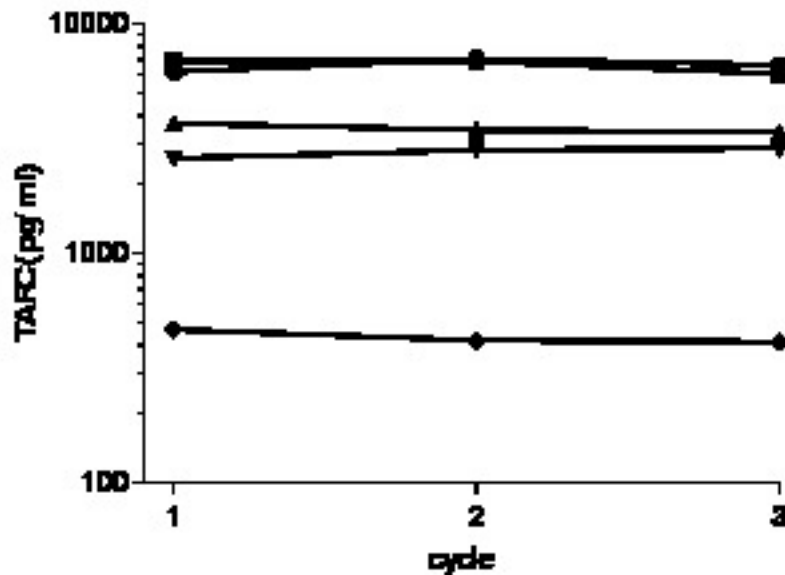
Stability at room temperature DBSTARC



Long term storage DBS TARC



Stability DBS TARCFreeze/thaw cycles



- Supplementary data -

Biomarkers detected in dried blood spots from atopic dermatitis patients strongly correlate with disease severity.

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Methods

Patients and controls

A cohort of 65 adult AD patients, as defined by the criteria of Hanifin and Rajka,¹ was recruited at the outpatient clinic of the University Medical Center Utrecht. Patients using oral immunosuppressive drugs or UV-therapy within three months before baseline were excluded from the study. Patients were followed for approximately two months. Sample size was calculated using correlation coefficients from a previously conducted study,² applying a Fisher's z-test for Pearson correlation in SAS version 9.2 (SAS Institute, Cary, NC, USA) with the following parameters: one-sided, $\alpha=0.05$, and power=0.8, resulting in 60 patients.

During the first visit (t_0) serum and DBS were collected and all patients started with daily application of potent topical steroids (European classification class III: fluticasone furoate, mometasone furoate or betamethasone) after instruction by a specialized nurse. During a second visit (t_1) after three weeks, serum and DBS were collected, and patients were instructed to taper the frequency of topical corticosteroid application. Collection of serum and DBS was repeated after 6 to 12 weeks (t_2) of treatment. The outline of the study protocol is summarized in **Figure E1A**.

Fourteen psoriasis patients were included as disease controls during a visit to the outpatient clinic and were all treated with emollients and topical steroids only. Serum and DBS were collected on a single time point. A second control group consisted of 26 non-atopic healthy controls. The healthy controls were age- and sex-matched to the AD patients. The protocols of this study were approved by the Institutional Review Board of the University Medical Center Utrecht (Utrecht, The Netherlands), adhering to the Declaration of Helsinki Principles and all patients provided written informed consent.

Disease severity

As suggested by HOME, we used the eczema area and severity index (EASI)³ and the patient oriented eczema measure (POEM).⁴ In addition we used a Visual Analogue Scale (VAS) for pruritus.⁵ Disease severity in psoriasis patients was assessed by the Psoriasis Area Severity Index (PASI),⁶ the self-administered psoriasis area and severity index (SA-PASI),⁷ and VAS pruritus.

Stability of biomarkers in DBS

After collection, DBS are dried for 2-3 hours at room temperature. After drying, DBS can be transported at ambient temperature. To investigate DBS biomarker level stability over time, we stored DBS from 5 AD patients at room temperature and measured biomarker levels in DBS 1, 3, and 7 days after collection. To study long-term stability in DBS, we included DBS from 17 AD patients that were stored at -80°C . Biomarker levels were measured after collection and after 34 months of storage. The influence of freeze thaw cycles on biomarker levels in DBS were also studied. DBS from 5 AD patients were thawed and frozen 3 times; biomarker levels were measured after each cycle.

Measurement of analytes in serum and DBS

A recently published study showed that the biomarkers TARC, MDC, PARC, IL-22, sE-selectin, sIL-2R, IL-18, I-309, and SDF-1 α showed good correlation with disease severity when measured in serum.⁹ We therefore measured these biomarkers both in serum and in DBS using a Luminex platform. Multiplex immunoassays were performed at the MultiPlex Core Facility of the Laboratory of Translational Immunology (UMC Utrecht, The Netherlands) Uniquely color-coded magnetic beads (MagPlex Microspheres, Luminex, Austin, Texas) were conjugated to antibodies specific for the reported analytes and incubated with 50 μ L of standard dilutions or sample for 1 hour (continuous shaking at room temperature in the dark). Samples were diluted in High Performance Elisa buffer (HPE; Sanquin, The Netherlands). Pre-treatment of samples included filtration (0.22 μ m pore size) and incubation with HeteroBlock to prevent interference by binding of heterophilic antibodies. Plates were washed (Bio-Plex Pro II Wash Station; Bio-Rad, Hercules, CA, USA) and a corresponding cocktail of biotinylated detection antibodies was added for 1 hour (continuous shaking at room temperature in the dark). Repeated washings were followed by a 10-minute streptavidin-phycoerythrin (PE) incubation (continuous shaking at room temperature in the dark). Fluorescence intensity of PE was measured using a Flexmap 3D system (Luminex) and analysed using BioPlex Manager software version 6.1; (Bio-Rad, Hercules, CA, USA) using 5-parameter curve fitting. Data was analyzed by 5-parametric curve fitting using Bio-Plex Manager software, version 6.1 (Biorad, Hercules, CA). Multiplex immunoassays were in-house validated and show an intra- and inter assay variability of less than 5% and 20%, respectively, and recovery values of spikes proteins in serum of 99-102%.¹⁰

Statistical analysis

Patient demographics and sample characteristics were compared by unpaired t-tests. Analyte measurements above or below the assay limits of detection were given values equivalent to the upper and lower limits respectively. TARC, PARC, MDC, and sE-selectin levels in serum and DBS were normalized by log-transformation. Correlation coefficients were tested using Pearson correlation tests. Levels of biomarkers between groups were compared with ANOVA. Linear mixed models were used to model the change over time of biomarkers after treatment initiation. This kind of model generalizes regression models by taking into account correlation between measurements in longitudinal data. We investigated the effects of the different biomarkers on EASI scores using a piecewise linear mixed model. For all AD patients, two slopes were considered: one for the first treatment period (between t_0 and t_1) and one for the second treatment period (between t_1 and t_2). The time for the slope's change ($t = 22$ days) was based on the median duration of the first treatment period. Correlation between individual baseline values and the subsequent slopes was handled through the unstructured covariance matrix of random effects. Models were adjusted for age and gender. To test if a combination of biomarkers improves prediction of EASI compared to a single biomarker, an EASI prediction model was built using the Akaike Information Criteria (the lower the better) that quantifies the quality of the fit to the data. The Akaike criteria were used to identify the model that best described the behavior of EASI within the AD patients. Differences in biomarker levels in the stability tests were compared by a t-test in case of two measurements and by repeated measures ANOVA in case of more than two measurements. P-values < 0.05 were considered statistically significant. All statistical analyses were conducted using SAS version 9.4 (SAS Institute, Cary, North Carolina, USA) and Prism (version 6; Graphpad).

Table E1. Baseline characteristics

	AD patients (n=65)	Psoriasis patients (n=14)	Healthy controls (n=26)
Age, yrs	31.6 (22.6-46.7)	39.4 (27.6-67.5)	36.5 (29.5-54.3)
Male, n (%)	31 (47)	8 (67)	13 (50)
EASI	17.4 (10.0-25.7)	N/A	N/A
POEM	21.0 (17.0-25.0)	N/A	N/A
PASI	N/A	4.2 (2.3-7.5)	N/A
SA-PASI	N/A	7.8 (4.8-17.6)	N/A
VAS pruritus	7.5 (6.0-8.0)	5.0 (0.0-7.0)	N/A

Categorical variables are presented as counts and percentages; continuous variables are presented as median (InterQuartileRange). ANOVA testing revealed no significant differences in age or gender between the groups. N/A; not available.

Table E2. Correlation matrix at baseline

	POEM		VAS pruritus		EASI	
	r	p-value	r	p-value	r	p-value
IL-18	-0,03	0,80	0,02	0,87	0,13	0,32
IL-22	0,09	0,51	0,24	0,09	0,34	0,01
IL-31	0,03	0,82	-0,25	0,08	0,13	0,33
ccl1/I309	0,38	0,00	0,11	0,45	0,52	0,00
sIL-2R	0,03	0,81	-0,18	0,21	0,21	0,11
sdf1alpha	-0,02	0,86	-0,06	0,67	0,17	0,19
log TARC	0,30	0,02	0,03	0,85	0,68	0,00
log MDC	0,25	0,05	0,19	0,20	0,50	0,00
Log sE-selectin	0,08	0,54	-0,04	0,79	0,23	0,07
log PARC	0,08	0,53	0,07	0,62	0,38	0,00

Pearson correlations between disease severity measured by POEM, VAS pruritus, and EASI, and biomarkers measured in DBS from 65 AD patients

114 **Table E3. Changes over time of DBS biomarkers.**

Biomarker	Estimate (95%CI)	P-value
Baseline		
I-309 (pg/ml)	130.17 (110.00, 150.35)	116
Log TARC (pg/ml)	3.37 (3.24, 3.49)	117
Log PARC (pg/ml)	7.31 (7.00, 7.63)	
IL-18 (pg/ml)	3674.08 (3337.49, 4010.67)	118
IL-22 (pg/ml)	93.60 (73.17, 114.02)	
IL-31 (pg/ml)	66.35 (43.36, 89.34)	119
Log MDC (pg/ml)	3.33 (3.25, 3.40)	120
sIL-2R (pg/ml)	2316.19 (1920.67, 2711.71)	
Log sE-selectin (pg/ml)	3.05 (2.91, 3.19)	121
SDF-1 α (pg/ml)	628.21 (460.86, 795.57)	122
Change/week (between t₀ and t₁)		
I-309 (pg/ml)	-19.49 (-26.69, -12.29)	<.0001 ¹²³
Log TARC (pg/ml)	-0.13 (-0.17, -0.10)	<.0001 ¹²⁴
Log PARC (pg/ml)	-0.35(-0.48, -0.23)	<.0001 ¹²⁴
IL-18 (pg/ml)	-72.15 (-161.57, 17.28)	0.11 ¹²⁵
IL-22 (pg/ml)	-4.63 (-12.83, 3.57)	0.26
IL-31 (pg/ml)	-0.09 (-9.37, 9.20)	0.99 ¹²⁶
Log MDC (pg/ml)	-0.08 (-0.10, -0.06)	<.0001 ¹²⁷
sIL-2R (pg/ml)	-94.48 (-211.77, 22.80)	0.11 ¹²⁷
Log sE-selectin (pg/ml)	-0.05 (-0.11, 0.01)	0.08 ¹²⁸
SDF-1 α (pg/ml)	-22.21 (-82.38, 37.96)	0.46 ¹²⁹
Change/week (between t₁ and t₂)		
I-309 (pg/ml)	0.52 (-1.28, 2.32)	0.56 ¹³⁰
Log TARC (pg/ml)	0.004 (-0.005, 0.01)	0.39
Log PARC (pg/ml)	0.02 (-0.02, 0.05)	0.37 ¹³¹
IL-18 (pg/ml)	15.41 (-12.025, 42.85)	0.26 ¹³²
IL-22 (pg/ml)	1.18 (-0.94, 3.30)	0.27
IL-31 (pg/ml)	-0.29 (-2.85, 2.28)	0.82 ¹³³
Log MDC (pg/ml)	0.001 (-0.004, 0.007)	0.61
sIL-2R (pg/ml)	13.88 (-19.54, 47.30)	0.41 ¹³⁴
Log sE-selectin (pg/ml)	0.01 (0.002, 0.03)	0.03 ¹³⁵
SDF-1 α (pg/ml)	11.53 (-5.96, 29.02)	0.19

136 Linear mixed models were used to model the changes over time of biomarkers measured in DBS after
137 treatment initiation with topical steroids. At t₀ patients started with daily application of topical steroids,
138 which was tapered to what we consider a safe maintenance scheme between t₁ and t₂. The biomarkers I-
139 309, TARC, PARC, and MDC showed a significant decrease during daily treatment with topical steroids
140 (between t₀ and t₁) and then remained at stable levels during intermittent treatment with topical steroids
141 (between t₁ and t₂).

Table E4. Results linear mixed model analysis for prediction of EASI scores

Effect	Estimate	Standard Error	P value
Model at baseline			
TARC (per increase of 1 log)	16.07	5.02	<.0001
Model during treatment			
TARC (per increase of 1 log)	4.86	1.42	0.0015

Akaike information criteria were used to identify the best model for the prediction of EASI scores. The best model for EASI changes over time only included TARC measured in DBS. This resulted in the following equations: EASI (before treatment i.e at baseline) = $-36.9104 + 16.0686 \times \text{LogTARC}$; EASI (during treatment) = $-9.9958 + 4.8570 \times \text{LogTARC}$.

Figure E1. Study design.

A. Outline of the study protocol

Patients were included during their first visit to the outpatient clinic (t_0). After t_0 all patients were treated with daily application of potent topical steroids (European classification class III; US classification class III). After three weeks (t_1) the use of topical corticosteroids was tapered. Disease severity was assessed by EASI, POEM, and VAS pruritus at all three timepoints (t_0 , t_1 , t_2); DBS and serum were collected at the same timepoints.

EASI; Eczema Area and Severity Index, POEM; Patient-Oriented Eczema Measure, VAS; Visual Analogue Scale.

B. Obtaining a dried blood spot

Figure E2. Changes in EASI, POEM, and VAS pruritus scores in AD patients during treatment with topical steroids.

Median percentage change from baseline for EASI, POEM and VAS pruritus in 65 AD patients during treatment with local topical steroids. EASI; Eczema Area and Severity Index, POEM; Patient-Oriented Eczema Measure, VAS; Visual Analogue Scale.

Figure E3A-C. Stability of TARC in DBS from atopic dermatitis patients.

(A) TARC levels in DBS from 5 AD patients were stable for 1, 3 and 7 days after collection and storage at room temperature. Repeated measures ANOVA did not show statistically significant differences between TARC levels after 1, 3 or 7 days of storage. (B) TARC levels measured in DBS from 17 AD patients after collection and after 34 months of storage at -80°C . A t-test showed no differences between TARC levels at 0 and 34 months. (C) TARC levels measured in DBS from 5 AD patients after repeated freeze-thawing. Repeated measures ANOVA did not show statistically significant differences between TARC levels after 1, 2 or 3 freeze-thaw cycles.

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