ORIGINAL ARTICLE

Effect of immunosuppressive treatment on biomarkers in adult atopic dermatitis patients

E. Roekevisch,[1,†] K. Szegedi,[1,†] D.P. Hack,[1] M.E. Schram,[1] P.C.J.M. Res,[1] J.D. Bos,[1] M.M.G. Leeflang,[2] R.M. Luiten,[1] S. Kezic,[3] P.I. Spuls,[1] M.A. Middelkamp-Hup[1,*]

[1]Department of Dermatology, Amsterdam University Medical Center, Location Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

[2]Department of Clinical Epidemiology, Biostatistics and Bioinformatics, Amsterdam University Medical Center, Location Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

[3]Coronel Institute of Occupational Health, Amsterdam Public Health Research Institute, Amsterdam University Medical Center, Location Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

*Correspondence: M.A. Middelkamp-Hup. E-mail: m.a.middelkamphup@amsterdamumc.nl

Abstract

Background Biomarkers to objectively measure disease severity and predict therapeutic responses are needed in atopic dermatitis (AD).

Objective Primary aim: To identify biomarkers reflecting therapeutic response in patients with AD treated systemically. Secondary aims: (i) To identify a biomarker pattern predicting responsiveness to systemic treatment. (ii) To identify differences in expression of biomarker in filaggrin gene (*FLG*) mutation carriers vs. non-*FLG* mutations carriers.

Methods Thirty-eight severe AD patients treated with methotrexate or azathioprine participated. Serum levels of a proliferation-inducing ligand, B-cell activating factor of the TNF family, thymus and activation-regulated chemokine (chemokine (C-C motif) ligand 17) (TARC (CCl-17)), interleukin-1 receptor antagonist (IL-1RA), interleukin-1 bèta, IL-4, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-18, IL-31, interferon gamma, tumour necrosis factor alpha, vascular endothelial growth factor (VEGF), monokine induced by interferon gamma (chemokine (C-X-C motif) ligand 9), interferon gamma-induced protein 10 (C-X-C motif chemokine Ligand 10), monocyte chemoattractant protein-1 (chemokine (C-C Motif) ligand 2), macrophage inflammatory protein-1 beta (chemokine (C-C motif) ligand 4), regulated on activation, normal T cell expressed and secreted (chemokine (C-C motif) ligand 5), Cutaneous T-cell-attracting chemokine (chemokine (C-C motif) ligand 27) (CTACK (CCL-27)), thymic stromal lymphopoietin, IL-5, interleukin-1 alpha and granulocyte-colony stimulating factor were analysed by ELISA and Luminex. The primary outcomes were differences in mean absolute change of SCORing Atopic Dermatitis (SCORAD) between groups after 12 weeks compared with baseline. Responders to treatment were defined by a SCORAD reduction in ≥50%. Buccal mucosa swabs were collected to determine *FLG* genotype status.

Results Thymus and activation-regulated chemokine, CTACK, IL-13 and VEGF showed a significant decrease after treatment with methotrexate or azathioprine. However, no decrease in individual cytokine levels was significantly correlated with a change in any of the outcome parameters. In addition, baseline biomarker levels were not significantly different between responders and non-responders, and *FLG* and non-*FLG* mutants showed similar biomarker profiles.

Conclusion Thymus and activation-regulated chemokine and CTACK were confirmed as potential biomarkers. VEGF and IL-13 have a potential value as well. Biomarkers could not be used to discriminate at baseline between responders and non-responders, or *FLG* genotype status.

Received: 25 April 2019; Accepted: 17 October 2019

Conflicts of interest

PS: Consultancies in the past for Sanofi 111017 and AbbVie 041217 (unpaid). Independent research grants in the past >5 years ago. Contract support: involved in performing clinical trials with many pharmaceutical industries that manufacture drugs used for the treatment of e.g. psoriasis and atopic dermatitis for which we get financial compensation paid to the department/hospital. MAMH: consultant to Sanofi and Pfizer.

Funding source

None.

© 2019 The Authors. *Journal of the European Academy of Dermatology and Venereology* published by John Wiley & Sons Ltd on behalf of European Academy of Dermatology and Venereology

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

Introduction

Atopic dermatitis (AD) is characterized by an epidermal barrier dysfunction and an immune dysregulation favouring a Th2 cellular response.[1] A method to objectively measure disease severity and to predict therapeutic responses in AD is wanted, and much research is being done to evaluate biomarkers in AD. Biomarkers may play an important role in personalized treatment of AD in the near future, since it is assumed that treatments will be more effective when targeting the patient's specific biological signature as determined by biomarkers. A systematic review and meta-analysis[2] showed that serum thymus and activation-regulated chemokine (TARC) to date are considered the best biomarker for assessing disease severity. T-cell-attracting chemokine (CTACK), sE-selectin, macrophage-derived chemokine (MDC), lactate dehydrogenase (LDH) and interleukin (IL)-18 could be useful as severity biomarkers as well, but need to be validated in additional studies. Three other biomarkers are B-cell activating factor of the TNF family (BAFF), a proliferation-inducing ligand (APRIL) and IL-31, but contradictory experimental findings make their importance relatively unclear.[3–12] IL-4 and IL-13 are believed to have a role in AD pathogenesis, because the frequencies of IL-4 and IL-13-producing cells, among them CD4+ and CD8+ T-cell subsets, are significantly higher in AD patients than in healthy subjects.[13] In addition, Dupilumab, which blocks IL-4 and IL-13, has shown efficacy in patients with AD.[14]

Although some promising results on potential biomarkers for AD have been published, more research is needed to find a reliable biomarker profile for disease severity and especially for predicting responsiveness to immunosuppressive treatment, which may result in tailor-made clinical treatment. To date, data on this topic are sparse. In the present study, the primary aim was to identify biomarkers that reflect the therapeutic response in AD patients treated with methotrexate (MTX) or azathioprine (AZA). Secondary aims were (i) to identify a biomarker predicting responsiveness to systemic immunosuppressive treatment, and (ii) to identify differences in biomarker profiles in filaggrin gene (*FLG*) mutation carriers vs. non-carriers.[15]

Materials and methods

The methods of the original RCT, containing inclusion and exclusion criteria, concealment of treatment and treatment regimens have been published previously.[15]

Study population and treatment protocol

This is a sub-study of patients participating in a single blind RCT evaluating efficacy and safety of MTX vs. AZA over a 12-week period.[15] All 42 randomized patients were asked to donate blood for biomarker determinations. Healthy volunteers were also recruited. Inclusion criteria for healthy volunteers were no personal or family history of AD, asthma and allergic rhinoconjunctivitis.

Primary outcomes of treatment were differences in mean absolute change of SCORing Atopic Dermatitis (SCORAD) between groups after 12 weeks compared with baseline. The SCORAD was also subdivided in objective SCORAD. Secondary outcomes were the absolute change of Eczema Area and Severity Index (EASI), pruritus and sleeplessness scores on a visual analogue scale (VAS, range 0–10). EASI and SCORAD assessments were provided by a blinded assessor. VAS-itch and sleeplessness was provided by the patient.

As both MTX and AZA induced a significant reduction in SCORAD at 12 weeks without a significant difference in effect between treatments,[15] all patients were pooled together and divided into responders and non-responders. Responders to treatment were defined as patients with a SCORAD reduction in 50% or more 12 weeks after baseline (SCORAD50), and biomarkers of responders and non-responders were compared at baseline. This study was conducted with institutional research ethics committee approval (Academic Medical Center Amsterdam), and all patients and healthy volunteers signed informed consent which complied with all the Declaration of Helsinki Principles.

Blood sample collection

Peripheral serum blood samples were collected before starting treatment (week 0, baseline) and 12 weeks after using MTX or AZA. Peripheral serum blood samples of 18 healthy volunteers were collected at one not further specified time point.

Filaggrin genotyping

A buccal swab was taken to investigate *FLG* mutation status (R501X, 2282del4, R2447X, S3247X and 3321delA mutations). Patients were pooled together and divided into *FLG* mutation carriers and non-*FLG* mutation carriers. During the RCT, no one was aware of the mutation status. The methods have been published previously.[16]

Cytokine and chemokine analyses

IL-31, APRIL and BAFF in serum samples were measured using specific ELISA kits. IL-31 measurement (R&D Systems Europe Ltd., Abingdon, UK) was performed according to manufacturer's instructions with the following modifications: diluent was composed of PBS (Fresenius Kabi, Zeist, The Netherlands) supplemented with 50% fetal bovine serum (Lonza Verviers, Sprl., Verviers, Belgium) and used for dilution of standard and detection antibody. Blocking step was performed with 3% milk (Nutricia Nederland BV, Zoetermeer, The Netherlands) in PBS. Capture antibody was diluted in a working concentration of 1.6 μg/mL instead of 0.8 μg/mL. Streptavidin-HRP was diluted to a working concentration of 1 : 300 instead of 1 : 200. The limit of detection was 63–4000 pg/mL.

© 2019 The Authors. *Journal of the European Academy of Dermatology and Venereology* published by John Wiley & Sons Ltd on behalf of European Academy of Dermatology and Venereology

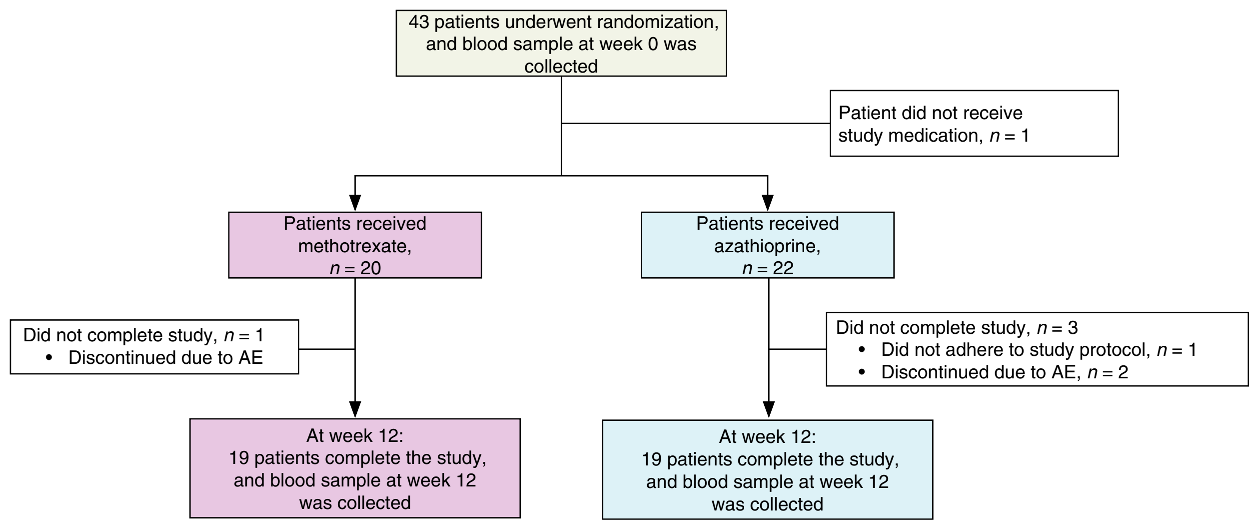

Figure 1 Flow chart of the randomization, treatment and blood sample collection of study participants. AE, adverse event.

A proliferation-inducing ligand (eBioscience, Hatfield, UK) measurement was performed according to the manufacturer's instructions. The limit of detection was 780–25 000 pg/mL. BAFF (Antigenix America Inc., Huntington Station, NY, USA) assay was conducted according to the manufacturer's instructions with the following modification: the blocking step was performed with 3% milk in PBS. The limit of detection was 156–10 000 pg/mL. For all ELISAs, the stop solutions were added after monitoring the colour development at 620 nm. The substrate reaction was stopped when the highest standard had reached an OD of 9.0–9.5.

Levels of TARC were assessed using a Luminex-based multiplex system (Millipore BV, Amsterdam, The Netherlands), according to the manufacturer's instructions. The limit of detection was 9.8–10.000 pg/mL.

A Luminex-based multiplex system (Bio-Rad Laboratories BV, Veenendaal, The Netherlands) was used to determine the levels of interleukin-1 alpha (IL-1α), interleukin-1 receptor antagonist (IL-1RA), interleukin-1 bèta (IL-1β), IL-4, IL-5, IL-6, IL-7, IL-8,

Table 1 Patients' baseline characteristics

	Total (*n* = 38)	MTX (*n* = 19)	AZA (*n* = 19)	Responders† (*n* = 15)	Non-responders (*n* = 23)	*FLG* mutation carriers (*n* = 12)	Non-*FLG* mutation carriers (*n* = 22)
Age (year), mean (SD)	39.8 (14.2)	41.5 (13.7)	38.0 (14.9)	39.3 (13.1)	40.0 (15.2)	50.6 (12.9)	34.8 (12.4)
Male sex (%)	21 (55%)	10 (53%)	11 (58%)	8 (53%)	13 (57%)	7 (58%)	13(59%)
Presence of asthma or allergic rhinitis (%)	35 (92%)	18 (95%)	17 (89%)	14 (93%)	21 (91%)	12 (100%)	19 (86%)
Received MTX (%)	19 (50%)	19 (100%)	–	7 (47%)	12 (52%)	6 (50%)	11 (50%)
Received AZA (%)	19 (50%)	–	19 (100%)	8 (53%)	11 (48%)	6 (50%)	11 (50%)
Presence of allergen-specific IgE (%)	38 (100%)	19 (100%)	19 (100%)	15 (100%)	23 (100%)	12 (100%)	22 (100%)
Duration of AD (year), mean (SD)	35.9 (16.6)	38.3 (15.1)	33.5 (18.1)	34.9 (14.7)	36.6 (18.1)	50.3 (13.1)	28.8 (14.0)
Outcome							
Total SCORAD, mean (SD)	57.8 (11.4)	57.1 (12.2)	58.4 (10.8)	59.8 (11.1)	56.4 (11.6)	59.7 (13.2)	57.6 (10.7)
Objective SCORAD, mean (SD)	45.4 (10.1)	45.0 (10.4)	45,6 (10.3)	47.9 (9.9)	43.8 (10.2)	49.6 (11.3)	43.9 (9.5)
EASI, mean (SD)	28.2 (13.4)	27.6 (12.6)	30.1 (14.6)	30.7 (13.9)	27.6(13.4)	33.6 (14.1)	26.6 (13.7)
VAS-itch, mean (SD)	7.4 (1.7)	7.2 (1.8)	7,6 (1.6)	7,8 (1,0)	7.1 (2.0)	6.7 (2.4)	7.7 (1.2)
VAS-sleep loss, mean (SD)	5.5 (2.6)	4.9 (2.7)	6.0 (2.5)	5.3 (2.3)	5.5 (2.8)	5.0 (2.8)	5.9 (2.3)

†Responders to treatment were defined by a SCORAD reduction in ≥50% 12 weeks after baseline.
Non-responders: SCORAD <50% 12 weeks after baseline.
AZA, azathioprine; FLG, filaggrin gene; MTX, methotrexate; SD, standard deviation.

© 2019 The Authors. *Journal of the European Academy of Dermatology and Venereology* published by John Wiley & Sons Ltd on behalf of European Academy of Dermatology and Venereology

Table 2 Serum levels of cytokines/chemokines under systemic treatment with MTX and AZA

	Week 0	Week 12	*P*-value	Mean difference between week 12 and week 0	*P*-value MTX vs. AZA of the absolute difference between week 12 and week 0
APRIL (pg/mL), median (IQR)					
Total AD group (*n* = 38)	1391 (1109–1920)	1397 (953–1986)	0.68	28.0 (−2993 to 124.8)	0.72
MTX (*n* = 19)	1430 (863–1921)	1397 (911–1970)	0.98	36.0 (−198.0–123.0)	
AZA (*n* = 19)	1360 (1120–1920)	1397 (962–2002)	0.65	20.0 (−335.0 to 143.0)	
BAFF (pg/mL), median (IQR)					
Total AD group (*n* = 35)	96.0 (26.0–364.0)	222.0 (42.0–410.0)	0.47	0.0 (−44.0 to 106.0)	0.06
MTX (*n* = 17)	70.0 (29.0–212.0)	192.0 (33.0–282.0)	0.07	84.0 (−26.0 to 194.0)	
AZA (*n* = 18)	160.0 (26.0–670.0)	248.0 (46.50–557.0)	0.46	0.0 (−151.5 to 38.50)	
TARC (pg/mL), median (IQR)					
Total AD group (*n* = 38)	1254 (895.4–2455)	827.2 (479.9–1733)	**0.0001**	−403.8 (−734.5 to −53.72)	**0.02**
MTX (*n* = 19)	2166 (1142–2809)	828.3 (492.5–1717)	0.004	−700.0 (−1595 to −125.0)	
AZA (*n* = 19)	1038 (604.6–2113)	826.1 (381.0–2098)	0.03	−231.9 (−471.0 to 30.36)	
IL-1RA (pg/mL), median (IQR)					
Total AD group (*n* = 38)	7.83 (0.07–29.37)	4.77 (0.07–16.79)	0.15	0.0 (−7.91 to 0.0)	0.80
MTX (*n* = 19)	7.05 (0.07–16.79)	5.51 (0.07–13.46)	0.21	−1.13 (−9.74 to 2.50)	
AZA (*n* = 19)	10.21 (0.07–49.03)	1.20 (0.07–30.70)	0.37	0.0 (−2.50 to 0.0)	
IL-9 (pg/mL), median (IQR)					
Total AD group (*n* = 38)	23.91 (3.05–43.13)	18.42 (3.52–30.61)	0.24	0.0 (−12.73 to 3.55)	0.89
MTX (*n* = 19)	23.91 (15.17–40.84)	16.25 (4.75–30.61)	0.50	0.0 (−13.35 to 8.91)	
AZA (*n* = 19)	21.70 (0.92–66.52)	21.70 (0.92–30.61)	0.39	0.0 (−12.52 to 1.10)	
IP-10 (pg/mL), median (IQR)					
Total AD group (*n* = 38)	685.6 (523.7–1042)	636.3 (501.5–898.5)	0.27	−40.21 (−214.0 to 96.59)	0.89
MTX (*n* = 19)	686.3 (613.3–1183)	858.4 (531.3–922.4)	0.62	−33.82 (−266.7 to 187.3)	
AZA (*n* = 19)	680.8 (452.3–863.4)	596.2 (470.7–791.0)	0.23	−42.95 (−134.3 to 71.23)	
VEGF (pg/mL), median (IQR)					
Total AD group (*n* = 38)	32.38 (8.14–60.10)	14.40 (0.95–37.68)	**<0.0001**	−9.54 (−28.64 to 0.0)	0.72
MTX (*n* = 19)	39.11 (15.57–94.06)	29.70 (0.95–66.50)	0.005	−12.84 (−24.07 to 0.0	
AZA (*n* = 19)	29.56 (8.14–48.61)	4.06 (0.95–22.76)	**0.001**	−7.19 (−28.71 to 0.0)	
IL-1β (pg/mL), median (IQR)					
Total AD group (*n* = 38)	0.14 (0.03–0.43)	0.11 (0.03–0.43)	0.44	0.0 (−0.12 to 0.05)	0.67
MTX (*n* = 19)	0.14 (0.08–0.31)	0.14 (0.03–0.43)	0.45	0.0 (−0.17 to 0.06)	
AZA (*n* = 19)	0.08 (0.03–0.66)	0.08 (0.03–0.54)	0.81	0.0 (−0.12 to 0.05)	
IL-6 (pg/mL), median (IQR)					
Total AD group (*n* = 38)	2.01 (0.38–3.45)	1.41 (0.17–2.09)	0.02	−0.26 (−1.71 to 0.09)	0.42
MTX (*n* = 19)	2.09 (0.72–3.45)	1.41 (0.72–2.09)	0.45	−0.34 (−1.71 to 0.69)	
AZA (*n* = 19)	1.92 (0.03–3.79)	1.07 (0.03–1.92)	0.007	0.0 (−1.71 to 0.0)	
IL-7 (pg/mL), median (IQR)					
Total AD group (*n* = 38)	8.40 (7.20–10.40)	8.27 (6.70–10.46)	0.67	−0.13 (−2.14 to 1.59)	0.79
MTX (*n* = 19)	8.27 (7.01–11.27)	8.27 (6.50–9.27)	0.64	0.0 (−1.75 to 0.75)	
AZA (*n* = 19)	8.52 (7.26–10.27)	8.77 (6.76–12.02)	0.95	−0.51 (−2.53 to 2.99)	
IL-13 (pg/mL), median (IQR)					
Total AD group (*n* = 38)	1.81 (0.85–3.17)	1.18 (0.04–2.76)	**0.003**	−0.47 (−1.20 to 0.0)	0.95
MTX (*n* = 19)	1.81 (0.33–3.02)	1.65 (0.15–2.57)	0.10	−0.34 (−1.47 to 0.11)	
AZA (*n* = 19)	1.50 (1.02–3.61)	0.85 (0.04–3.61)	0.008	−0.60 (−0.98 to 0.0)	
MCP1 (pg/mL), median (IQR)					
Total AD group (*n* = 38)	50.67 (38.18–85.12)	53.40 (33.04–68.95)	0.01	−9.46 (−26.26 to 5.94)	0.95
MTX (*n* = 19)	45.65 (39.40–78.55)	50.97 (41.70–69.33)	0.07	−8.92 (25.09–5.35)	
AZA (*n* = 19)	54.21 (31.06–96.61)	55.64 (30.44–67.29)	0.07	−10.24 (−27.92 to 9.57)	
MIP-1b (pg/mL), median (IQR)					
Total AD group (*n* = 38)	114.3 (91.98–152.1)	111.8 (80.28–134.7)	0.004	−12.01 (−25.89 to −1.82)	0.91

© 2019 The Authors. *Journal of the European Academy of Dermatology and Venereology* published by John Wiley & Sons Ltd on behalf of European Academy of Dermatology and Venereology

Table 2 *Continued*

	Week 0	Week 12	*P*-value	Mean difference between week 12 and week 0	*P*-value MTX vs. AZA of the absolute difference between week 12 and week 0
MTX (*n* = 19)	111.1 (91.14–180.3)	109.1 (86.38–142.0)	0.02	−11.31 (−27.36 to −2.65)	
AZA (*n* = 19)	117.5 (92.26–151.8)	112.1 (64.52–131.8)	0.07	−13.29 (−25.73 to 0.87)	
IL-18 (pg/mL), median (IQR)					
Total AD group (*n* = 38)	57.75 (45.64–80.94)	53.61 (36.51–68.93)	0.01	−7.62 (−20.0 to 2.57)	0.37
MTX (*n* = 19)	57.50 (44.92–77.15)	48.76 (36.86–62.42)	**0.001**	−9.23 (−19.70 to −0.97)	
AZA (*n* = 19)	58.98 (45.88–82.46)	54.58 (34.99–99.68)	0.35	−2.89 (−25.85 to 11.14)	
CTACK (pg/mL), median (IQR)					
Total AD group (*n* = 38)	2125 (1420–3108)	1423 (1046–2047)	**<0.0001**	−434.7 (−1381 to −196.7)	>0.99
MTX (*n* = 19)	2195 (1396–3059)	1435 (1049–2004)	**0.0002**	−562.5 (−1340 to −191.1)	
AZA (*n* = 19)	2035 (1428–3258)	1411 (1037–2175)	0.004	−425.8 (−1545 to −245.7)	
MIG (pg/mL), median (IQR)					
Total AD group (*n* = 38)	1091 (765.3–2030)	1077 (643.1–1695)	0.31	−30.99 (−365.4 to 161.5)	0.08
MTX (*n* = 19)	1932 (1004–2175)	1111 (703.1–1915)	0.06	−263.0 (−1112 to 106.5)	
AZA (*n* = 19)	921.6 (672.1–1641)	839.4 (579.4–1621)	0.60	−7.59 (−108.8 to 268.7)	

Differences between baseline and week 12 were considered significant with a *P*-value <0.0031 (Bonferroni correction, 0.05/16). The *P*-value of week 0 vs. week 12 was analysed by the Wilcoxon matched-pairs signed rank test. The *P*-value of Δ absolute value (MTX vs. AZA) was analysed with the Mann–Whitney *U*-test.
AD, atopic dermatitis; AZA, azathioprine; IQR, interquartile range; MTX, methotrexate.

IL-9, IL-10, IL-12, IL-13, IL-18, interferon gamma (IFN-γ), tumour necrosis factor alpha (TNF-α), vascular endothelial growth factor (VEGF), granulocyte-colony stimulating factor (G-CSF), monokine induced by interferon gamma (chemokine (C-X-C motif) ligand 9) (MIG (CXCL-9)), interferon gamma-induced protein 10 (C-X-C motif chemokine Ligand 10) (IP-10 (CXCL-10)), monocyte chemoattractant protein-1 (chemokine (C-C Motif) ligand 2) (MCP-1 (CCL-2)), macrophage inflammatory protein-1 beta (chemokine (C-C motif) ligand 4) (MIP-1β (CCL-4)), regulated on activation, normal T cell expressed and secreted (chemokine (C-C motif) ligand 5) (RANTES (CCL-5)) and Cutaneous T-cell-attracting chemokine (chemokine (C-C motif) ligand 27) (CTACK (CCL-27)).

Levels of thymic stromal lymphopoietin (TSLP) were assessed using a Luminex-based multiplex system (Millipore BV), according to the manufacturer's instructions. The limit of detection was 9.8–10.000 pg/mL. If the Luminex panel measured a value below the detection limit than half of that value was assigned to the cytokine/chemokine.

A patient was excluded for analysis in case of a missing cytokine/chemokine value. Also, only cytokines/chemokines with serum levels that were significantly different ($P < 0.05$) from the healthy volunteer group were analysed, unless stated otherwise. If more than 50% of the values of a cytokine/chemokine were below the lower limit of detection (LLD) evaluated in the total AD group, that cytokine/chemokine was excluded from the analyses. This criterion is based on the article by Domthong *et al.*[17] and in our study related to TSLP (>60% of values below LLD), IL-5 (>62%), IL-1α (>95%) and G-CSF (>95%).

Statistical analysis

Data on patients' characteristics were analysed using SPSS 23.0 for Windows (SPSS, Inc., Chicago, IL, USA) The distribution of dependent data was assessed by D'Agostino & Pearson omnibus normality test. Medians with 1st and 3rd interquartiles (IQR: Q1, Q3) were used for non-normal distributed data, and means with standard deviation were used for normal distributed data.

Biomarker data obtained by ELISA and Luminex data were analysed using Graphpad Prism 6.07 (GraphPad Software, Inc, San Diego, CA, USA). All investigated biomarkers showed a non-normal data distribution. If the natural logarithms (Ln) of the biomarker values showed a normal distribution, then these values were used in the analyses.

Correlations were performed with the Pearson's correlation coefficient on normal distributed data and Spearman's rank test on non-normal distributed data. Comparison analysis of non-normal distributed data was performed with Wilcoxon matched-pairs signed rank tests (paired data) and Mann–Whitney *U*-tests (unpaired data). Comparison analysis of normal distributed data was performed with two-sided paired *t*-tests (paired data) and unpaired *t*-test (unpaired data). Due to multiple testing the P value was adjusted using the Bonferroni correction (α/m) where α was 0.05 and m is the number of selected hypotheses.

© 2019 The Authors. *Journal of the European Academy of Dermatology and Venereology* published by John Wiley & Sons Ltd on behalf of European Academy of Dermatology and Venereology

Results

Patients' characteristics

MTX vs. AZA Of the 43 randomized patients, 38 patients were included in this sub-study (Fig. 1). In the AZA group, one patient did not start on study medication, one did not adhere to the study protocol and two discontinued because of adverse events. In the MTX group, one patient did not complete the study due to an AE. These patients were excluded due to missing blood samples at week 12. Patients' characteristics and baseline values are shown in Table 1.

Response to treatment In the original study, after 12 weeks of treatment, both the MTX group and AZA group showed significant reductions in SCORAD, without a significant difference between treatments.[15]

In this follow-up study, 15 of 38 patients (39%) were responders and 23 (61%) were non-responders. Of the responders, 7 (47%) received MTX and 8 (53%) received AZA.

FLG mutation carriers vs. non-carriers Of the 38 AD patients, 12 (38%) had a *FLG* mutation, 22 had no *FLG* mutation (58%), and of 4 (11%) patients, the mutation could not be determined because, for different reasons, no buccal swap was available as source of DNA for FLG analysis. Of the *FLG* mutation carriers, 6 (47%) received MTX and 6 (53%) received AZA. Of the non-*FLG* mutation carriers, 11 (50%) received MTX and 11 (50%) received AZA. Age was significantly higher in *FLG* mutation carriers (P = 0.001, unpaired

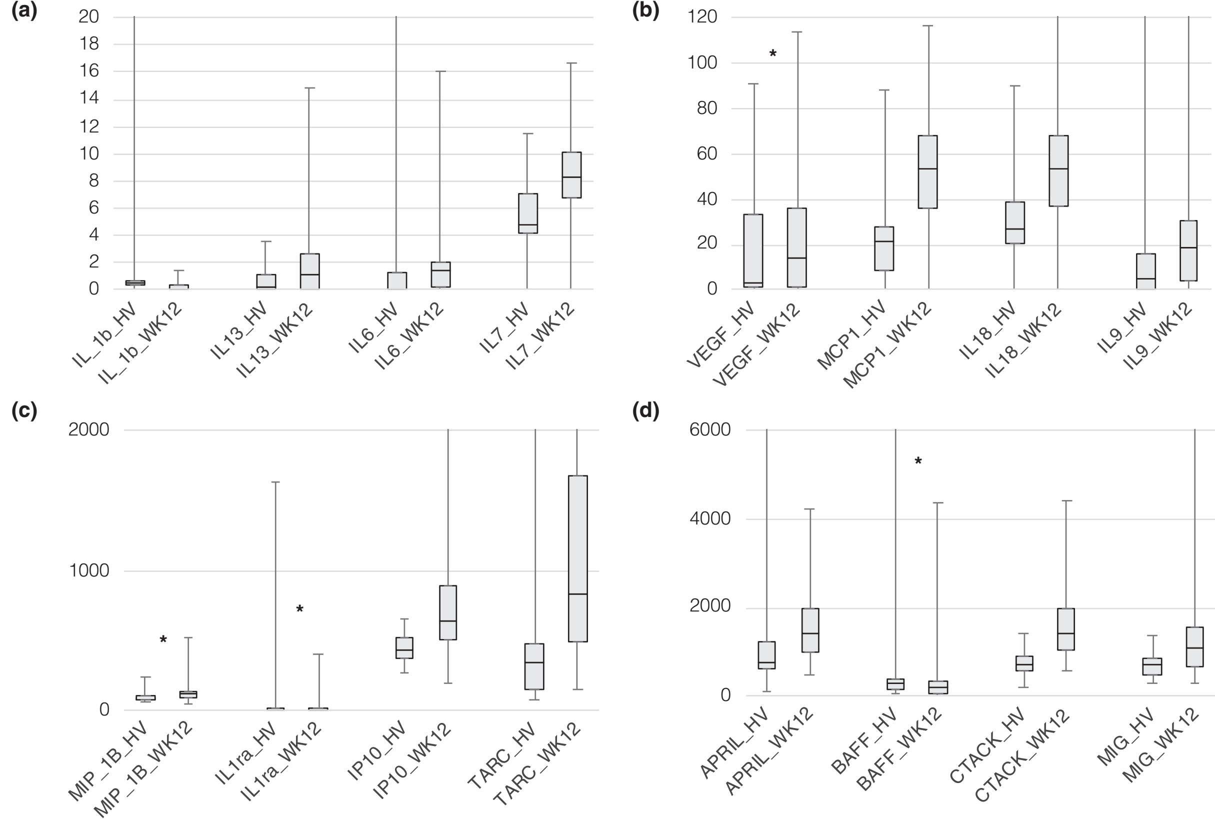

Figure 2 Serum levels of cytokines/chemokines in atopic dermatitis patients after 12 weeks of systemic treatment and healthy volunteers. HV, Healthy Volunteers (n = 18); WK12, week 12 of systemic treatment with methotrexate or azathioprine of AD patients (n = 38, only BAFF had missing values N = 35). At baseline, 16 cytokines/chemokines were significantly different in AD patients compared with 18 healthy volunteers. After 12 weeks of treatment, 4 biomarkers* were not significantly different. Anymore differences were considered significant with a P-value <0.0031 (Bonferroni correction, 0.05/16) analysed with Mann–Whitney U-test. Maximum values a: IL_1b_HV = 38.7; IL6_HV = 56.6. b: IL8_WK12 = 183.9; IL9_HV = 147.3 IL-9_WK12 = 186.6. c: IP_10_WK12 = 3631.9; TARC_HV = 3376.5; TARC_WK12 = 5932.6. d: APRIL_HV = 48 420.0; BAFF_HV = 9948; MIG_WK12 = 6879.5.

© 2019 The Authors. Journal of the European Academy of Dermatology and Venereology published by John Wiley & Sons Ltd on behalf of European Academy of Dermatology and Venereology

t-test) compared with the non-*FLG* mutation carriers with 50.7–34.8 years (means).

TARC, VEGF, IL-13 and CTACK levels decrease significantly during immunosuppressive treatment

At baseline, 16 cytokines/chemokines were significantly different in AD patients compared with 18 healthy volunteers, namely APRIL, BAFF, thymus and activation-regulated chemokine (chemokine (C-C motif) ligand 17) (TARC (CCL-17)), IL-1RA, IL-9, IP-10 (CXCL-10), VEGF, IL-1β, IL-6, IL-7, IL-13, MCP-1 (CCL-2), MIP-1β (CCL-4), IL-18, CTACK (CCL-27) and MIG (CXCL-9). These biomarkers were included in subsequent data analysis. Differences between baseline and week 12 were considered significant if *P*-value <0.0031 [Bonferroni correction, 0.05/16 (number of selected cytokines)].

An overview of the cytokines/chemokines levels at baseline and after 12 weeks of systemic treatment with MTX and AZA are shown in Table 2.

After 12 weeks of treatment, TARC (CCL-17) [mean difference (MD) −403.8 (−734.5 to −53.72), $P < 0.0001$], VEGF [MD −9.54 (−28.64 to 0.0), $P < 0.0001$], IL-13 [MD −0.47 (−1.20 to 0.0), $P = 0.003$] and CTACK [MD −434.7 (−1381 to −196.7), $P < 0.0001$] showed a significant ($P < 0.0031$) decrease in the total AD group. Without Bonferroni correction, IL-6, MCP-1 (CCL-2), MIP-1β (CCL-4) and IL-18 were found significantly decreased as well in this group.

In the MTX treated group, reduction in levels was significant for IL-18 [MD −9.23 (−19.70 to −0.97), $P = 0.001$] and CTACK [MD −562.5 (−1340 to −191.1), $P = 0.0002$] and in the AZA treated group for VEGF [MD −7.19 (−28.71 to 0.0), $P = 0.001$].

The *P*-value ($P < 0.05$) for the absolute difference between the MTX and AZA groups was only for TARC significant ($P = 0.02$), as TARC levels decreased more upon treatment with MTX than with AZA.

Twenty-five per cent of the biomarkers obtain values comparable to the healthy volunteers after 12 weeks of systemic

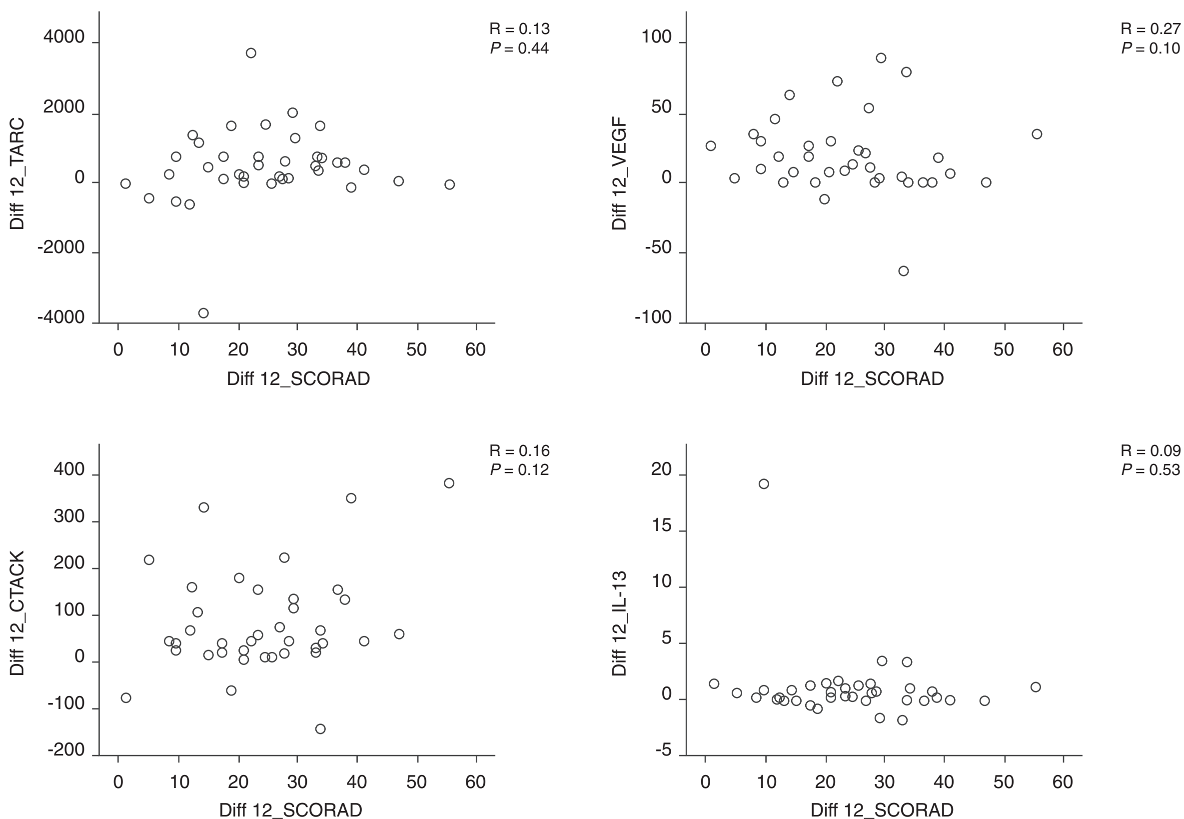

Figure 3 Correlations between ΔTARC (CCL-17), ΔVEGF, ΔCTACK (CCL-27) and ΔIL-13 and change in SCORAD over 12 weeks of treatment with MTX or AZA in patients with AD. AD, atopic dermatitis; AZA, azathioprine; Diff12_XXX, difference in XXX after 12 weeks of treatment; MTX, methotrexate; *P*, *P*-value; *R*, Rho.

© 2019 The Authors. *Journal of the European Academy of Dermatology and Venereology* published by John Wiley & Sons Ltd on behalf of European Academy of Dermatology and Venereology

treatment {Fig. 2, *P*-value <0.0031 [Bonferroni correction, 0.05/16 (number of selected cytokines)]}.

Decreased cytokine levels were not significantly correlated with the change in outcome parameters

Cytokines TARC (CCL-17), VEGF, CTACK (CCL-27) and IL-13 found to be significantly decreased upon systemic treatment were analysed individually to evaluate if this decrease correlated with the change in outcome parameters.

The total SCORAD consists of the oSCORAD, VAS-itch and VAS-sleep loss. For the Bonferroni correction, they were clustered, so two outcome parameters (SCORAD and EASI) and four cytokines/chemokines resulted in 8 correlations leading to a Bonferroni correction with a *P*-value of 0.05/8 = 0.006.

Based on a Bonferroni corrected *P*-value of 0.006, we found that decreased cytokine levels were not significantly correlated with change in primary and secondary outcome parameters (Fig. 3).

None of the biomarkers could identify or discriminate between responders and non-responders

We investigated if cytokine/chemokine profiles differed between responders and non-responders at baseline, to find a predictive biomarker that could differentiate these two groups.

Based on a Bonferroni corrected *P*-value of 0.0031, none of the baseline biomarker levels were significantly different between responders and non-responders, indicating that none of the cytokines/chemokines were able to distinguish between responders and non-responders (Table 3).

FLG and non-*FLG* mutation carriers show similar biomarker profiles

After 12 weeks of treatment, all the outcome parameters showed a significant decrease for both the *FLG* mutation carriers and non-*FLG* carriers (data not shown). No significant differences could be observed in cytokine/chemokine levels at baseline, after 12 weeks of treatment and between the change in cytokine levels after treatment between carriers and non-carriers of *FLG* mutations.

Discussion

Biomarkers may provide tools to predict and monitor therapeutic response.[18] In this study, TARC (CCL-17), VEGF, IL-13 and CTACK showed a significant decrease in the 38 evaluated AD patients who completed 12 weeks of systemic treatment with MTX or AZA, and therefore, these markers showed to be potential severity biomarkers. However, the decreased cytokine levels over 12 weeks of time were not significantly correlated with the change in outcome parameters over 12 weeks of time. In addition, none of the biomarkers could be used to discriminate between responders and non-responders, or *FLG* genotype status, and therefore no potential predictive biomarkers were found.

Thymus and activation-regulated chemokine (chemokine (C-C motif) ligand 17) and IL-13 are mediated by Th-2 cells which play an important role in the pathogenesis of AD in the acute phase of AD cells.

Thymus and activation-regulated chemokine (chemokine (C-C motif) ligand 17) is expressed by keratinocytes in the

Table 3 Baseline serum levels of cytokines/chemokines in responders and non-responders

Cytokines/chemokines	Responders (*n* = 15)	Non-responders (*n* = 23)	*P*-value R vs. NR week 0
APRIL (pg/mL), median (IQR)	1298 (953.0–1609)	1587 (1146–2100)	0.055
BAFF (pg/mL), median (IQR)	74.0 (26.0–240.0)†	109.0 (29.50–508.0)‡	0.57
TARC (pg/mL), median (IQR)	1160 (920.1–2690)	1723 (647.5–2445)	0.75
IL-1RA (pg/mL), median (IQR)	8.61 (0.07–21.91)	7.05 (0.07–41.60)	0.74
IL-9 (pg/mL), median (IQR)	29.48 (17.33–66.52)	21.70 (0.29–40.84)	0.58
IP-10 (pg/mL), median (IQR)	714.1 (670.1–866.3)	618.1 (431.5–1154)	0.31
VEGF (pg/mL), median (IQR)	23.90 (0.95–57.69)	35.19 (8.75–67.34)	0.59
IL-1β (pg/mL), median (IQR)	0.26 (0.08–0.37)	0.08 (0.03–0.60)	0.41
IL-6 (pg/mL), median (IQR)	1.75 (0.72–3.45)	2.09 (0.38–3.45)	0.62
IL-7 (pg/mL), median (IQR)	8.77 (5.74–9.77)	8.27 (7.26–11.77)	0.38
IL-13 (pg/mL), median (IQR)	2.12 (1.02–3.61)	1.50 (0.33–2.72)	0.35
MCP-1 (pg/mL), median (IQR)	44.71 (31.06–76.06)	71.20 (39.40–91.95)	0.41
MIP-1β (pg/mL), median (IQR)	107.6 (90.25–181.2)	117.5 (95.52–145.4)	0.88
IL-18 (pg/mL), median (IQR)	57.50 (45.88–77.15)	57.99 (44.92–90.12)	0.79
CTACK (pg/mL), median (IQR)	2572 (1778–3271)	1812 (1387–3059)	0.13
MIG (pg/mL), median (IQR)	1024 (718.6–2248)	1111 (780.8–2025)	0.74

†*n* = 35. ‡*n* = 20.
Responders to treatment were defined as patients with a SCORAD reduction in 50% or more (SCORAD50) 12 weeks after baseline. Differences at baseline were considered significant with a *P*-value <0.0031 (Bonferroni correction, 0.05/16).
IQR, interquartile range; NR, non-responder; R, responder.

© 2019 The Authors. *Journal of the European Academy of Dermatology and Venereology* published by John Wiley & Sons Ltd on behalf of European Academy of Dermatology and Venereology

epidermis, vascular endothelial cells, T cells and dendritic cells. TARC is described at this moment to be the best single biomarker for assessing disease severity.[2] IL-13 is secreted by Th2 cells and eosinophils, and it is a central regulator in the IgE synthesis. Dupilumab blocks the receptor for IL-4 and IL-13 which results in inhibition of the inflammation. It has shown good efficacy in patients with AD.[14]

Like TARC, VEGF is also derived from endothelial cells, but also from fibroblasts, smooth muscle cells and macrophages, and it takes part in different stages of the angiogenesis, as well as vasodilatation. It promotes survival, migration and proliferation of endothelial cells and keratinocytes and increases the vascular permeability of the skin. Vascular remodelling is known in chronic inflammatory skin diseases.[19] VEGF was also found to play a role in inducing pruritus via epidermal hyper innervation.[20] An active eczema with a high inflammation could therefore be associated with a higher VEGF and possibly more itch, which detracts when it is suppressed by systemic immunosuppressive treatment.

Serum CTACK (CCL27) has previously been put forward as a severity biomarker in adults and showed to have a strong correlation with disease severity.[2] It belongs, like TARC, to the CC family and is thought to play an important role in skin inflammation as it attracts cutaneous lymphocyte antigen-positive memory T cells into the inflammatory sites. CTACK (CCL27) is only expressed in the skin, mostly in the epidermal keratinocytes.[21] It is expected that by suppressing inflammation with systemic treatment, CTACK is reduced.

Besides TARC and CTACK, which we found in this study, also sE-selectin, MDC, LDH and IL-18 were shown to be promising biomarkers.[2] From these biomarkers, only IL-18 was analysed in this study and showed a significant decrease in the 38 evaluated AD patients whom completed 12 weeks of systemic treatment with MTX or AZA but only if it was not corrected for Bonferroni. IL-18 is a member of IL-1 cytokine family, and its levels have been shown to correlate with disease severity in children and adult AD patients.[22,23]

Interestingly, TARC decreased significantly more under MTX than AZA despite of their similar treatment efficacy as assessed by decrease in disease severity.[15] The question is if this reduction is a specific characteristic of the mechanism of action of MTX. Studies with MTX and AZA have been done in patients with rheumatoid arthritis (considered a Th1 dominant condition), but the mechanism of action is still not fully understood.[24,25] It has been shown that serum IL-1β, IL-6 and IL-8 decrease upon MTX treatment.[25] Concerning AZA, one study found that serum levels of IL-6 in rheumatoid arthritis patients did not change upon treatment.[26] The mechanism of actions of these treatments on serum level of cytokines/chemokines is hardly described in the literature and is therewith inconclusive.

Treatment response (SCORAD50) could not be predicted based on the cytokine/chemokine profile of a patient at baseline. Also, the changes in the cytokine/chemokine profiles of responders compared with non-responders were similar for MTX and AZA.

Our study has some limitations. This was an explorative study, and besides looking at the biomarker results in the MTX and AZA group separately, we also pooled the groups because of the small sample sizes. However, MTX and AZA have a different mechanism of action, and it is unknown how these mechanisms have influenced the data. This is an important issue that should be investigated in future studies.

It is a relative small study, and more than 50% of the AD patients had values below the lower limit of detection (LLD) at baseline for TSLP, IL-5 IL-1α and G-CSF. These cytokines were excluded from analysis, and therewith, we could have missed some potential biomarkers.

A combination of biomarkers shows a better correlation with disease severity[27]; however, in this explorative study, multivariable analysis was not sensible as the number of subjects was low.

In conclusion, serum levels of TARC, CTACK, IL-13 and VEGF significantly decreased upon treatment. None of the investigated biomarkers could discriminate between responders and non-responders, and biomarker profiles between *FLG* mutation carriers were similar to those in non-*FLG* mutation carriers.

References

1 McAleer MA, Irvine AD. The multifunctional role of filaggrin in allergic skin disease. *J Allergy Clin Immunol* 2013; **131**: 280–291.
2 Thijs J, Krastev T, Weidinger S *et al.* Biomarkers for atopic dermatitis: a systematic review and meta-analysis. *Curr Opin Allergy Clin Immunol* 2015; **15**: 453–460.
3 Chen Y, Lind Enoksson S, Johansson C *et al.* The expression of BAFF, APRIL and TWEAK is altered in eczema skin but not in the circulation of atopic and seborrheic eczema patients. *PLoS ONE* 2011; **6**: e22202.
4 Dillon SR, Sprecher C, Hammond A *et al.* Interleukin 31, a cytokine produced by activated T cells, induces dermatitis in mice. *Nat Immunol* 2004; **5**: 752–760.
5 Matsushita T, Fujimoto M, Echigo T *et al.* Elevated serum levels of APRIL, but not BAFF, in patients with atopic dermatitis. *Exp Dermatol* 2008; **17**: 197–202.
6 Niyonsaba F, Ushio H, Hara M *et al.* Antimicrobial peptides human beta-defensins and cathelicidin LL-37 induce the secretion of a pruritogenic cytokine IL-31 by human mast cells. *J Immunol* 2010; **184**: 3526–3534.
7 Neis MM, Peters B, Dreuw A *et al.* Enhanced expression levels of IL-31 correlate with IL-4 and IL-13 in atopic and allergic contact dermatitis. *J Allergy Clin Immunol* 2006; **118**: 930–937.
8 Nobbe S, Dziunycz P, Muhleisen B *et al.* IL-31 expression by inflammatory cells is preferentially elevated in atopic dermatitis. *Acta Derm Venereol* 2012; **92**: 24–28.
9 Raap U, Weissmantel S, Gehring M, Eisenberg AM, Kapp A, Folster-Holst R. IL-31 significantly correlates with disease activity and Th2 cytokine levels in children with atopic dermatitis. *Pediatr Allergy Immunol* 2012; **23**: 285–288.
10 Raap U, Wichmann K, Bruder M *et al.* Correlation of IL-31 serum levels with severity of atopic dermatitis. *J Allergy Clin Immunol* 2008; **122**: 421–423.

© 2019 The Authors. *Journal of the European Academy of Dermatology and Venereology* published by John Wiley & Sons Ltd on behalf of European Academy of Dermatology and Venereology

11 Siniewicz-Luzenczyk K, Stanczyk-Przyluska A, Zeman K. Correlation between serum interleukin-31 level and the severity of disease in children with atopic dermatitis. *Postepy Dermatol Alergol* 2013; **30**: 282–285.
12 Szegedi K, Kremer AE, Kezic S *et al.* Increased frequencies of IL-31-producing T cells are found in chronic atopic dermatitis skin. *Exp Dermatol* 2012; **21**: 431–436.
13 Teraki Y, Hotta T, Shiohara T. Increased circulating skin-homing cutaneous lymphocyte-associated antigen (CLA)+ type 2 cytokine-producing cells, and decreased CLA+ type 1 cytokine-producing cells in atopic dermatitis. *Br J Dermatol* 2000; **143**: 373–378.
14 Beck LA, Thaci D, Hamilton JD *et al.* Dupilumab treatment in adults with moderate-to-severe atopic dermatitis. *N Engl J Med* 2014; **371**: 130–139.
15 Schram ME, Roekevisch E, Leeflang MM, Bos JD, Schmitt J, Spuls PI. A randomized trial of methotrexate versus azathioprine for severe atopic eczema. *J Allergy Clin Immunol* 2011; **128**: 353–359.
16 Roekevisch E, Leeflang MMG, Schram ME *et al.* Patients with atopic dermatitis with filaggrin loss-of-function mutations show good but lower responses to immunosuppressive treatment. *Br J Dermatol* 2017; **177**: 1745–1746.
17 Domthong U, Parikh CR, Kimmel PL, Chinchilli VM. Assessing the agreement of biomarker data in the presence of left-censoring. *BMC Nephrol* 2014; **15**: 144.
18 Thijs JL, van Seggelen W, Bruijnzeel-Koomen C, de Bruin-Weller M, Hijnen D. New developments in biomarkers for atopic dermatitis. *J Clin Med* 2015; **4**: 479–487.
19 Samochocki Z, Bogaczewicz J, Sysa-Jedrzejowska A, McCauliffe DP, Kontny E, Wozniacka A. Expression of vascular endothelial growth factor and other cytokines in atopic dermatitis, and correlation with clinical features. *Int J Dermatol* 2016; **55**: e141–e146.
20 Wong LS, Otsuka A, Yamamoto Y *et al.* Vascular endothelial growth factor partially induces pruritus via epidermal hyperinnervation in imiquimod-induced psoriasiform dermatitis in mice. *J Dermatol Sci* 2016; **83**: 148–151.
21 Machura E, Rusek-Zychma M, Jachimowicz M, Wrzask M, Mazur B, Kasperska-Zajac A. Serum TARC and CTACK concentrations in children with atopic dermatitis, allergic asthma, and urticaria. *Pediatr Allergy Immunol* 2012; **23**: 278–284.
22 Sohn MH, Lee KE, Kim KE. Interleukin-18 is associated with increased severity of atopic dermatitis in children. *Allergy Asthma Proc* 2004; **25**: 181–184.
23 Zedan K, Rasheed Z, Farouk Y *et al.* Immunoglobulin e, interleukin-18 and interleukin-12 in patients with atopic dermatitis: correlation with disease activity. *J Clin Diagn Res* 2015; **9**: Wc01–Wc05.
24 Hildner K, Marker-Hermann E, Schlaak JF *et al.* Azathioprine, mycophenolate mofetil, and methotrexate specifically modulate cytokine production by T cells. *Ann NY Acad Sci* 1998; **859**: 204–207.
25 Kremer JM, Lawrence DA, Hamilton R, McInnes IB. Long-term study of the impact of methotrexate on serum cytokines and lymphocyte subsets in patients with active rheumatoid arthritis: correlation with pharmacokinetic measures. *RMD Open* 2016; **2**: e000287.
26 Crilly A, McInnes IB, Capell HA, Madhok R. The effect of azathioprine on serum levels of interleukin 6 and soluble interleukin 2 receptor. *Scand J Rheumatol* 1994; **23**: 87–91.
27 Thijs JL, Nierkens S, Herath A *et al.* A panel of biomarkers for disease severity in atopic dermatitis. *Clin Exp Allergy* 2015; **45**: 698–701.

© 2019 The Authors. *Journal of the European Academy of Dermatology and Venereology* published by John Wiley & Sons Ltd on behalf of European Academy of Dermatology and Venereology