Patient profiling and prediction of response to immune-modulating treatment in moderate-to-severe atopic dermatitis

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Patient profiling and prediction of response to immune-modulating treatment in moderate-to-severe atopic dermatitis

Patiënt profilering en predictie van respons op immuun-modulerende therapie voor matig-tot-ernstig constitutioneel eczeem

(met een samenvatting in het Nederlands)

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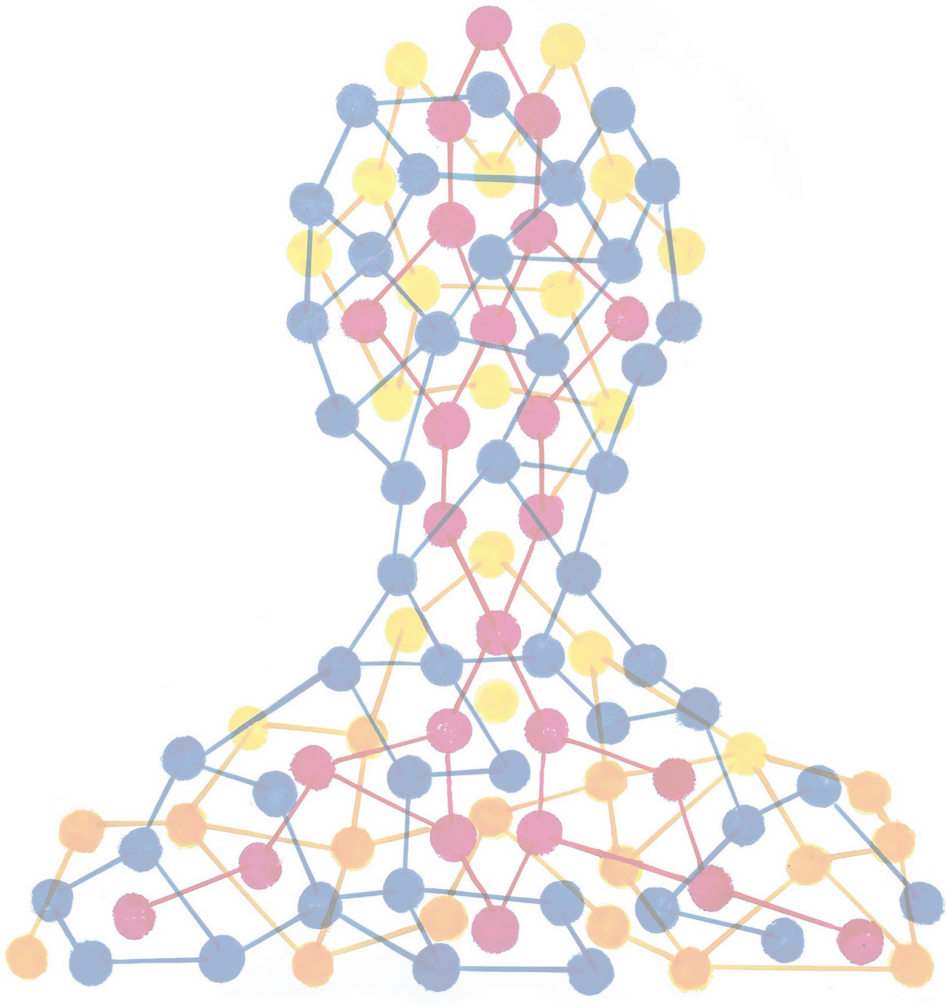
Contents



Chapter 1

General introduction

Atopic dermatitis

Atopic dermatitis (AD) is one of the most common chronic inflammatory skin-diseases worldwide, with an increasing prevalence of 15-30% in children and up to 10% in adults.[1-3] AD can commence at any age, but mostly begins in early childhood. Although it had largely been considered that AD often resolves after childhood, recent studies demonstrate that persistence into adulthood is common, making AD a life-long disease among a part of the patient population.[4] AD is characterized by a chronic relapsing-remitting course with repeated flare ups. Patients suffer from persistent pruritus, pain, sleep disturbance and symptoms of anxiety and depression, resulting in a significantly reduced quality of life.[5] Besides the psychosocial burden, AD also has a substantial economic burden as a result of work absenteeism, lost productivity at work and learning impairments.[6, 7] Patients with AD do not only experience skin-related signs and symptoms, they also have an increased risk for other atopic comorbidities, including asthma, allergic rhinitis, allergic conjunctivitis and food allergy.[8]

Immuno-pathological heterogeneity in atopic dermatitis

AD is a complex and highly heterogeneous disease. AD patients have classically been divided into different phenotypes based on clinical characteristics, such as age, age of onset, disease severity, ethnicity, and the presence of other atopic diseases, such as asthma, allergic rhinitis and food allergy.[9, 10] However, the stratification of patients into clinical phenotypes does not seem to adequately reflect the pathophysiologic diversity among AD patients. Contributing to the complexity of the disease, the pathogenesis of AD is clearly multifactorial. Both genetic and environmental factors are known to be risk factors for the development of AD, but the exact etiology has not been fully understood yet. The two main factors contributing to the pathogenesis of AD are epithelial barrier disruption and immune dysregulation.[11] However, it is still debated whether AD is caused by an intrinsic defect in the immune system triggering barrier dysfunction (the "inside-out" hypothesis), or whether epidermal barrier disruption precedes AD and triggers subsequent immune activation (the "outside-in" hypothesis).[12, 13] In recent years, different genomics, transcriptomics and proteomics profiling studies have revolutionized the understanding of AD pathogenesis. Skin and blood profiling has allowed us to further define the pathogenic pathways of the disease and its subtypes, as well as disease and treatment response biomarkers. [14-17]

AD is considered to be a primarily CD4+ T helper 2 (Th2) cell-driven disease, characterized by an overexpression of type 2 (T2) related cytokines, such as interleukin (IL)-4, IL-5, IL-13, IL-31, and (TARC/CCL17) in both skin and blood.[11] The T2 cytokines in AD skin specifically affect the epidermis by suppressing keratinocyte differentiation, antimicrobial peptide production and filaggrin expression, contributing to epithelial barrier impairment.[18] The defective barrier allows the penetration of allergens and microbes, leading to type 2 inflammation.[19] Besides the T2 pathway, other immune pathways responsible for the development of AD, including T1, T22 and T17, have recently been elucidated. While acute AD lesions are mainly T2/T22 centered, with some T17 overexpression, chronic lesions are characterized by significant increases in T1-related products in addition to the T2 response.[11, 20] The vast majority of infiltrating T-cells present in AD lesions express skin-homing receptors, including cutaneous lymphocyte antigen (CLA).[21, 22] Due to recirculation, peripheral CLA+ T-cells reflect the cutaneous inflammatory response, creating an opportunity for less-invasive translational approaches.[23-25] The role of immune and inflammatory cells in AD is depicted in Figure 1.

Overall, many different biological processes related to both the adaptive and the innate immune system, as well as tissue-related factors play a role in the pathogenesis of AD. Similarly to the clinical heterogeneity that is observed in AD, the different biological processes contribute to the pathogenesis in varying degrees in different patient subpopulations. Despite the highly heterogeneous character of the disease, AD is currently still treated according the "one-size-fits-all" approach.[26] It is likely that the multiple subtypes involving AD differ in their response to a particular treatment. Therefore, the discovery, validation and use of objective biomarkers are important to achieve more personalized clinical and treatment approaches.

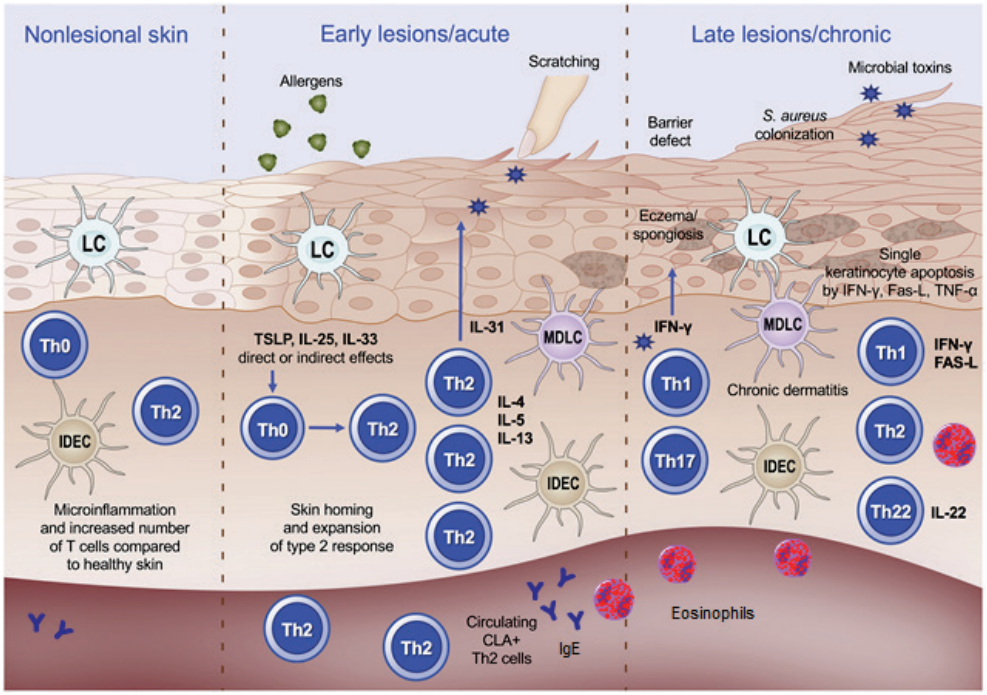


Figure 1. Pathogenesis of atopic dermatitis. In the dermis of nonlesional AD skin, T-cells are slightly increased. Skin-homing CLA+ T-cells exhibit a Th2-dominant profile in acute lesional skin and in the circulation. The systemic T2 response is often accompanied by elevated IgE and eosinophilia. By release of the cytokines thymic stromal lymphopoietin (TSLP), IL-25, and IL-33, keratinocytes directly or indirectly support the type 2 response. IL-31 produced by activated CD4+ T-cells is mainly responsible for pruritus, but not induction of local skin inflammation. In chronic lesional AD skin increased populations of T1-, and sometimes T17- and T22-dominant cells are detected. Other cell types, such as eosinophils, mast cells, innate lymphoid cells, and antigen-presenting cell populations including Langerhans cells (LCs), inflammatory dendritic epidermal cells (IDECs), and monocyte-derived LC-like cells (MDLC) can be detected in AD skin. Figure adapted from "Recent developments and advances in atopic dermatitis and food allergy" by Sugita et al. 2020, Allergology International. 69(2): 204-214.[19]

Current and new therapeutic options

Most AD patients have mild-to-moderate disease and can be adequately treated with topical anti-inflammatory therapy with corticosteroids, calcineurin inhibitors and/or UV light therapy.[27] However, a significant minority of AD patients cannot be controlled by daily use of potent topical steroids with adequate training/instructions, or cannot reduce the frequency/potency of topical steroids to acceptable levels. These patients can be defined as 'difficult to treat' AD, and may require treatment

with systemic immunosuppressive drugs.[28] Traditionally, the systemic treatment of AD relied on broad immunosuppressants, including cyclosporine A, methotrexate, azathioprine and mycophenolate mofetil, which may be limited by ineffectiveness, multiple adverse effects or contraindications.[29, 30] As a result, there has been a large unmet need for effective treatment of moderate-to-severe AD patients. Over the past decade, our improved understanding about the underlying immune dysregulations of AD have led to the development of several novel targeted therapeutics. Due to the central role of type 2 inflammation, the first fully human monoclonal antibody that have become available for moderate-to-severe AD, dupilumab, targets the T2-related cytokines IL-4 and IL-13.[31-34] However, because of the complex and heterogeneous pathophysiology with diverse (endo)phenotypes, other monoclonal antibodies targeting T2-, T22- and T17-related cytokines and more broad-acting small molecule drugs, including janus kinase (JAK)-inhibitors are currently under investigation for the treatment of moderate-to-severe AD.

Dupilumab is currently registered in the United States, Europe, and Japan for adults and children aged >6 years with 'difficult-to-treat' AD, meaning moderate-to-severe AD patients, in whom topical treatment is not sufficient or not advisable.[29, 35, 36] European guidelines additionally recommend to use dupilumab for adult patients in whom other systemic treatment is not advisable, for children dupilumab is the only systemic medicine approved.[29, 36] Dupilumab has shown significantly improved clinical and patient-reported outcomes in moderate-to-severe AD patients in phase III clinical trials and, recently, also in daily practice.[33, 34, 37-41] Its high clinical efficacy has affirmed the importance of the T2 pathway in the pathogenesis of AD. However, only approximately one-third of the dupilumab-treated AD patients achieved complete clinical remission[33, 42, 43], confirming the heterogeneous character of the disease. Dupilumab is the only registered targeted therapy for AD at the moment, but IL-13 antagonists (tralokinumab and lebrikizumab), the IL-31 antagonist nemolizumab, and multiple JAK-inhibitors are expected to follow soon. The registration of these new therapeutics will highly change the current treatment algorithm for AD patients, as proposed in Figure 2. Due to the emergence of numerous therapies targeting different immunological pathways, there is a growing need for patient profiling. Given the heterogeneity of the disease, it is unlikely that every patient will respond equally to different therapies.

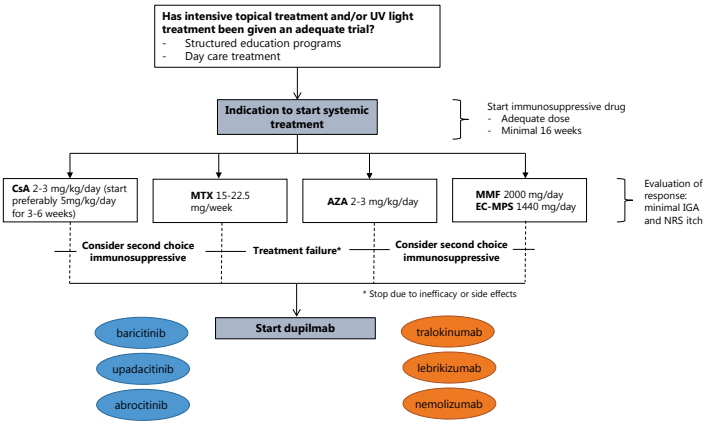


Figure 2. Proposed treatment algorithm for currently available systemic treatment of atopic dermatitis (AD) in Europe. Upcoming therapies displayed in blue and orange will highly change the current treatment algorithm and should in future find their place in more personalized treatment guidelines. Figure adapted from "Dupilumab in atopic dermatitis: rationale, latest evidence and place in therapy" by Lieneke Ariëns et al. 2018, Ther Adv Chronic Dis. 9(9): 159-170. AZA, azathioprine; CsA, cyclosporine A; EC-MPS, enteric-coated mycophenolate sodium; IGA, Investigator Global Assessment; MMF, mycophenolate mofetil; MTX, methotrexate; NRS, Numeric Rating Scale.

The safety profile of dupilumab is favorable, with mostly mild side effects being observed. However, higher rates of conjunctivitis have been reported in dupilumab treated patients (5% to 28%) compared to patients treated with placebo (1% to 11%) in clinical trials, whereas daily practice studies have shown even higher prevalences.[31, 33, 34, 37, 38, 41, 44] The pathomechanism of conjunctivitis development during dupilumab treatment is yet unknown. Remarkably, increased rates of conjunctivitis were not reported in clinical trials evaluating dupilumab for the treatment of asthma[45, 46] or chronic rhinosinusitis with nasal polyposis[47], indicating an AD specific underlying mechanism. Several hypotheses for mechanisms driving conjunctivitis occurring during dupilumab treatment have been proposed, including T17-driven increase of *Demodex* mites leading to rosacea-like conjunctivitis, increased activity of specific ligands involved in atopic keratoconjunctivitis due to IL-4/IL-13 inhibition, a relative ocular under treatment due to lower tissue distribution of dupilumab in the eyes,

and a possible role for the observed early increases in total eosinophil counts.[48-50] Since conjunctivitis seems to be a frequently occurring side effect during dupilumab treatment with potentially relevant treatment consequences[51], a better understanding of the underlying pathomechanism and risk factors is necessary to allow optimal treatment and risk management in clinical practice.

Besides the importance of studies investigating the underlying pathomechanism of the most frequently reported side effects of dupilumab treatment, long-term safety data will be needed to identify any increased risk for infection, malignancy or cardiovascular events. Additionally, long-term evaluations of cellular markers are warranted to investigate whether long-term blockade of IL-4Rα may lead to skewed T-cell responses, since several recent case-reports have reported T1/T17 mediated adverse effects newly developing in AD patients during dupilumab treatment, including psoriasis[52-54], alopecia areata[55], and rosacea[56-58]. Given the recent developments and pipeline for future targeted treatments for AD, it will be most essential to be able to provide the right drug to the right patient, and to better predict and monitor both clinical efficacy as well as (potential) side effects.

Outline of this thesis

The first aim of this these is to further identify AD patient subtypes based on biomarker profiles, which can be helpful to provide the most optimal treatment for the individual patient in future. In a large cohort of severe AD patients that is described in **chapter 2**, we investigated if a predictive biomarker signature could be constructed to identify the subgroup op difficult-to-treat AD patients who require systemic treatment. In **chapter 3**, we focused on the stratification of severe adult AD patients into distinct biomarker driven patient clusters. The heterogeneity of the disease and identification of biomarker based endotypes was further explored in a large cohort of pediatric AD patients in **chapter 4**.

The second aim of this thesis is to clarify the immunological effects of dupilumab on T- and B-cell dynamics and polarization. **Chapter 5** focusses on the short- and long-term effects of dupilumab treatment on IL-4Rα expression and T-cell cytokine production within total and skin-homing subpopulations in moderate-to-severe AD patients. The performance of a biomarker signature predicting disease severity was explored in a longitudinal study including 25 AD patients treated with dupilumab in

chapter 6. In **chapter 7** we investigated the local and central effects of dupilumab treatment on markers of eosinophilic inflammation and activation.

The third aim of this thesis is to elucidate the pathomechanisms underlying different side effects occurring during dupilumab treatment. In a prospective daily practice study that is described in **chapter 8**, we evaluated ophthalmological characteristics and treatment outcomes in moderate-to-severe AD patients who developed conjunctivitis during dupilumab treatment. In **chapter 9**, we described the histopathological characteristics of conjunctivitis occurring during dupilumab treatment, by investigating conjunctival biopsies of six AD patients. Immune cell infiltrates in conjunctival biopsies of six AD patients were further characterized in **chapter 10** by using imaging mass cytometry, an emerging imaging technology that enables high-resolution imaging of multiple markers simultaneously at a subcellular level. In a case report describing two AD patients with dupilumab facial redness in **chapter 11**, we speculated the role of hypersensitivity to *Malassezia* species in the development of this side effect of dupilumab treatment.
The implications and future perspectives of our findings are discussed in **chapter 12**.

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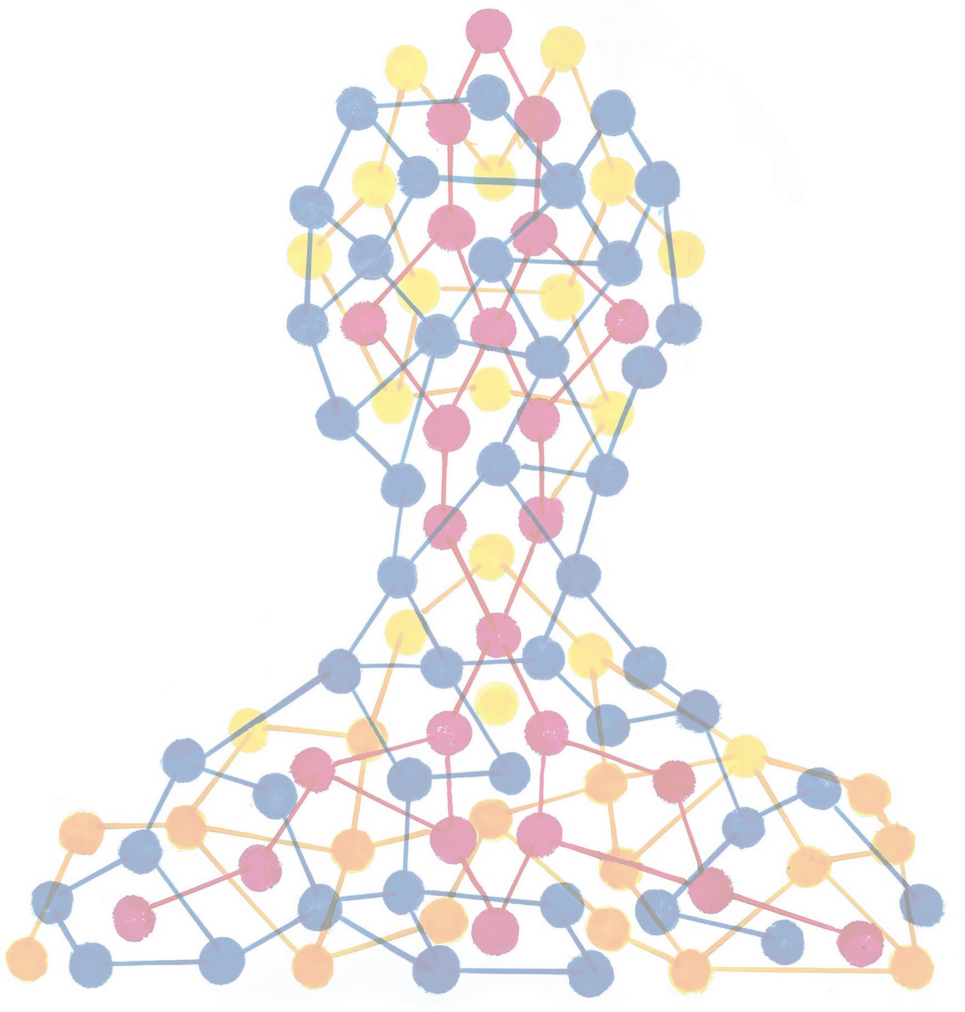

Chapter 2

Early identification of atopic dermatitis patients in need of systemic immunosuppressive treatment

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To the editor,

Atopic dermatitis (AD) is one of the most common chronic inflammatory skin diseases that is known to have profoundly negative effects on patient's quality of life.[1] The majority of AD patients can be controlled with topical corticosteroids, but those with insufficient responses or who cannot reduce the potency/frequency of topical steroids to acceptable levels, will require treatment with systemic immunosuppressive/immunomodulating drugs. This group of patients can be defined as 'difficult-to-treat' AD.

In daily practice, the decision whether or not to start systemic therapy should be based on several factors, like disease severity, quality of life and comorbidities.[2] A single severity measurement can, however, easily over- or underestimate the long-term disease severity of a patient, since AD is characterized by exacerbations and remissions. Difficult-to-treat AD patients often experience a significant delay before optimal treatment is started. Early identification of this group might prevent unnecessary treatment delay. Therefore, the aim of this study was to construct a predictive serum biomarker signature, measured on a single time point, contributing to the separation between difficult-to-treat AD patients requiring systemic treatment and those who can be controlled with only topical therapy.

We retrospectively included 152 severe AD patients (median EASI score 28.8, IQR 25.3–35.4; median age 32.0 years, IQR 22.0–50.8; all Caucasian) from the National Expertise Center for AD in the Netherlands, who were initially inadequately treated with topical corticosteroids. Subsequently, all patients started with intensive topical treatment, defined as the use of at least six weeks of daily treatment with high amounts of potent topical corticosteroids after adequate training and instructions in self-management. Patients with physician reported doubts on treatment compliance were excluded.

During this treatment period 74 severe AD patients (EASI >21 before start of treatment) could be controlled with topical steroids ("controlled disease" group), and 78 severe AD patients (EASI >21 before start of treatment) eventually required treatment with systemic immunosuppressive drugs ("difficult-to-treat" group)(Table 1). Serum was collected before start of intensive topical treatment and 129 serum

biomarkers (Table S1), measured using Luminex-based multiplex immunoassays, were included for analysis.

To construct the prognostic biomarker signature, we used a statistical algorithm previously developed by Mamtani et al.[3] (detailed methods related to patient and sample selection, serum biomarker measurements and statistical analysis are

Table 1. Patient characteristics

Clinical characteristics	Group 1: Controlled disease (n= 74)	Group 2: Difficult to treat (n=78)	p-value differences
Age (years)[1], median [IQR]	29.0 [22.0 – 48.3]	37.0 [22.0 – 52.3]	0.522[a]
Male, n (%)	38 (51%)	47 (60%)	0.269[b]
EASI score, median [IQR]	27.8 [24.7 – 31.5]	29.8 [25.3 – 39.0]	**0.038[a]**
Atopic diseases, n (%)			
- **allergic asthma**	40 (54%)	43 (51%)	0.756[b]
- **allergic rhinitis**	47 (64%)	51 (65%)	0.611[b]
- **food allergy**	26 (35%)	35 (45%)	0.230[b]
- **no other atopic disease besides AD**	15 (20%)	13 (17%)	0.592[b]
- **missing data**	0	3 (4%)	
Age of onset, n (%)			0.370[b]
- **0 – 1 years**	30 (41%)	29 (37%)	
- **2 – 11 years**	30 (41%)	38 (49%)	
- **12 – 18 years**	3 (4%)	1 (1%)	
- **>18 years**	4 (5%)	7 (9%)	
- **missing data**	7 (10%)	3 (4%)	
Hospitalization for AD (after study inclusion and sampling), n (%)	27 (36.5%)	44 (56%)	**0.036[b]**

Categorical variables are presented as counts and percentages; continuous variables are presented as median [IQR]. EASI Eczema Area Severity Intensity; IQR Inter Quartile Range; VAS Visual Analogue Scale [1] age at time of sample collection, [a] Wilcoxon rank sum test, [b] Chi-square test, p-value <0.05 was considered statistically significant

available in the article's Supplemental Information).

Stepwise multiple regression analysis resulted in the selection of eight serum biomarkers, including interleukin (IL)-1b, platelet factor 4 (PF4/CXCL4), cutaneous T-cell attracting chemokine (CTACK/CCL27), Trappin-2, Sclerostin (SOST), gamma-tubulin complex protein 2 (GCP-2), soluble programmed death-1 (sPD-1) and leukocyte associated immunoglobulin like receptor-1 (LAIR-1), which were combined

by using a linear discriminant function analysis to construct the final prediction model for classification of patients into "controlled disease" or "difficult-to-treat". The final model had an R^2 of 0.70, a Wilk's λ of 0.51 and predicted the classification correctly in 125 (82%) out of the 152 patients. Sixteen patients were misclassified as controlled disease and eleven patients were misclassified as difficult-to-treat, resulting in a sensitivity of 78%, a specificity of 86%, a positive predictive value (PPV) of 84% and a negative predictive value (NPV) of 81%. CTACK was the best individual predicting biomarker (AUC value of 0.73). After combining the eight biomarkers, the AUC of the final prediction model raised to 0.89 (Figure 1A and 1B).

Serum levels of IL-1b, PF4/CXCL4, Trappin-2 and SOST were significantly higher in the difficult-to-treat group compared to the controlled disease patients (Figure 1C). Serum levels of CTACK were significantly higher in the controlled disease patients. No differences were found in levels of GCP-2, sPD-1 and LAIR-1. However, these three biomarkers significantly improved the prediction capacity of the final model.

Of the eight identified biomarkers, four have previously been shown to contribute to chronic skin inflammation or AD pathogenesis. PF4/CXCL4 and CTACK/CCL27 are higher expressed in serum of AD patients compared to healthy controls, and correlate with AD severity.[4, 5]Levels of PF4 were, correspondingly, significantly higher, whereas levels of CTACK were significantly lower in our difficult-to-treat group, in which median EASI score was significantly higher (29.8, IQR 25.3–39.0 versus 27.8, IQR 24.7-31.5 in the controlled disease group). However, this small absolute difference in disease severity is not considered to be clinically relevant.[6] Despite PF4 and CTACK have been found to correlate with AD disease severity , both markers are considered not to be the optimal markers to pick up a small difference in disease severity. Serum levels of thymus and activation-regulated chemokine (TARC/CCL17), currently the best performing biomarker for assessing disease severity in AD[5], did not significantly differ between the two groups and was not included in the final model, indicating that the current model is not solely based on differences in disease severity based on a single EASI score, but may reflect the more long-term disease severity and treatment response. IL-1b is a pro-inflammatory cytokine, which can induce IL-20 production and thereby keratinocyte differentiation.[7, 8] Gamma-tubulin complex protein 2 (GCP-2) is a chemoattractant for neutrophilic granulocytes and has shown to be up-regulated by IL-4, one of the main contributors to the pathogenesis of AD.[9]

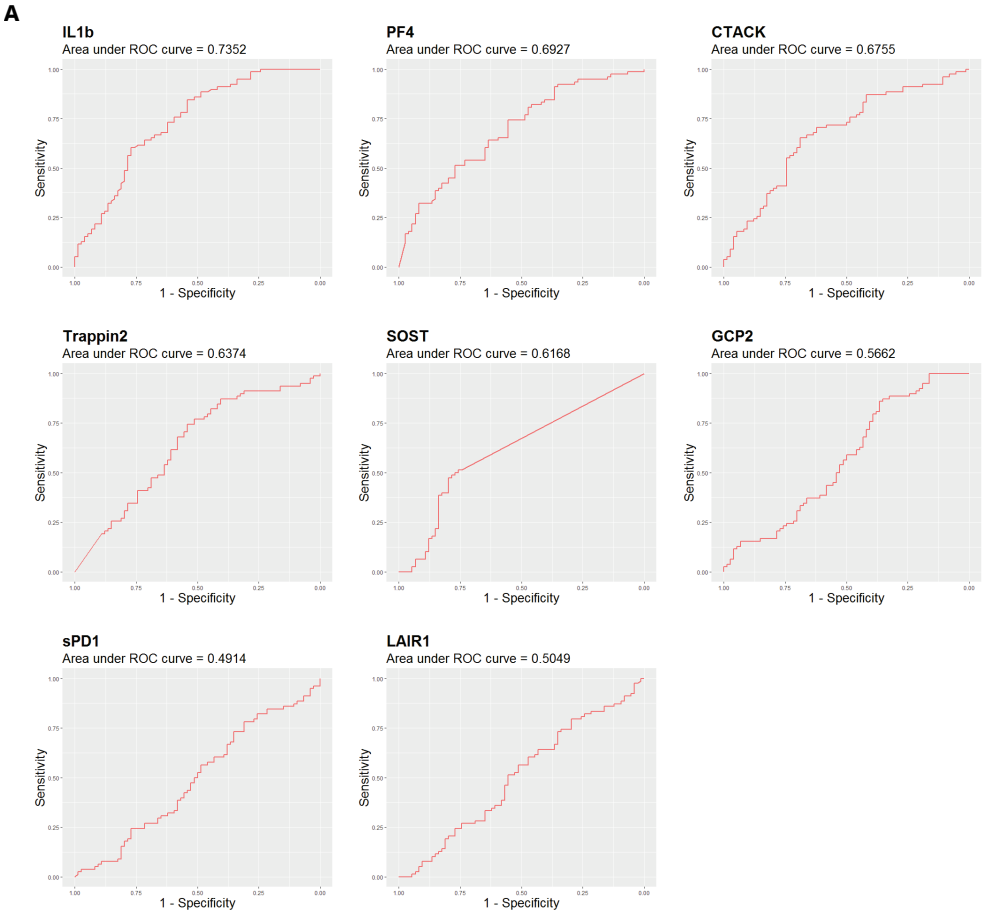
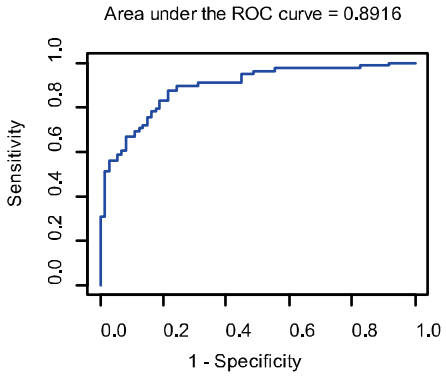


Figure 1. Receiver-operating characteristics and serum levels for individual biomarkers and the final model predicting treatment response in severe AD patients. A. Individual ROC curves for interleukin 1 beta (IL-1b), platelet factor 4 (PF4), cutaneous T-cell-attracting chemokine (CTACK), transglutaminase substrate and WAP domain containing protein/Elafin (Trappin-2), Sclerostin (SOST), gamma-tubulin complex protein 2 (GCP-2), soluble programmed death protein 1 (sPD-1) and leukocyte associated immunoglobulin like receptor 1 (LAIR-1), which were retained in step 2 of the statistical algorithm.

B



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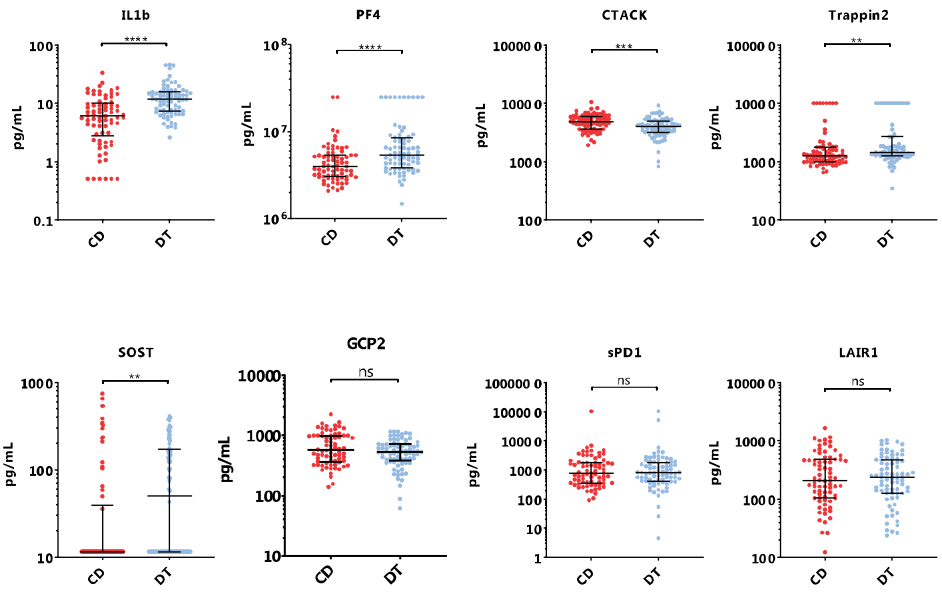

Figure 1. B. Receiver-operating characteristic (ROC) curve for final model which included IL-1b, PF4, CTACK, Trappin-2, SOST, GCP-2, sPD-1 and LAIR-1. Combining these eight biomarkers in a biomarker signature increased the capacity to predict treatment responses in severe AD patients. **C.** Differences in serum biomarker levels between severe AD patients who can be controlled with topical corticosteroids ("controlled disease", CD) and patient who require treatment with systemic immunosuppressive drugs ("difficult to treat", DT) were compared using Mann-Whitney U tests. Horizontal bars represent median biomarker levels with InterQuartile Range. *p<0.05; ** p<0.01; *** p<0.001; ns = non-significant.

The role of the four remaining biomarkers in the pathogenesis of AD has not been explored yet. This is the first study investigating these markers in a large cohort of AD patients. Trappin-2, SOST, sPD-1 and LAIR-1 have all been associated with immune regulation, and might thus play a role in AD pathogenesis. Our results imply that pathophysiological heterogeneity in immunological pathways might underlie differences in treatment responses, and may be used to distinguish a specific subpopulation of difficult-to-treat AD patients in need of systemic treatment from patients who can be controlled with topical therapy.

In the current study, patients were stratified based on treatment history necessary to control the AD. The decision whether or not to start systemic therapy in AD patients is not always easy; several factors need to be considered.[2] The lack of response to adequately applied topical treatment or long-term need of large amounts of topical steroids is a very important indicator for systemic treatment, taken into consideration that much effort should be made to optimize topical treatment. In all included patients, much attention was paid to adherence to topical treatment and evaluation of self-management. However, treatment compliance to topical therapy cannot be fully guaranteed.

With the correlation coefficient, NPV and PPV of the final model appearing to be sub-optimal, a potential danger of using this predictive signature in clinical practice might be unnecessary treatment with systemic immunosuppressive drugs due to incorrectly assigning a patient as 'difficult-to-treat'. Hence, we do not aim to replace clinical decision making by our biomarker signature. Instead, this signature might serve as a valuable addition to the decision whether or not to start systemic therapy in individual AD patients and might accelerate the initiation of optimal therapy. Validation of our biomarker signature in a prospective patient population is necessary to evaluate its applicability and predictive capacity.

In conclusion, this study shows that a constructed predictive signature of eight serum biomarkers is able to identify a subgroup of severe, difficult-to-treat AD patients with a sensitivity of 78% and a specificity of 86%, which might contribute to earlier identification. This signature might serve as a valuable addition to the decision whether to start systemic therapy or not in individual AD patients, and the statistical algorithm used in this study may also be applied to construct biomarker signatures predicting treatment response to systemic immunosuppressive drugs, dupilumab or other therapies in the future. Since more targeted therapies will play an increasingly

important role in AD treatment, prediction of treatment response can significantly contribute to selecting the right treatment for the right patient.

Funding

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SUPPLEMENTAL INFORMATION

METHODS

Patients and samples

Two groups of severe AD patients (EASI > 21), as defined by the criteria of Hanifin and Rajka[1], were included retrospectively from our AD database, including all AD patients treated at our center, who have given written informed consent for the use of the data recorded in their electronic medical records. Electronic medical records were manually screened and patients were stratified based on their treatment history necessary to control the eczema. Multiple severity measurements over time were used to define these groups. Following our local treatment protocols, all patients were initially treated with intensive topical treatment, defined as the use of at least six weeks of daily treatment with high amounts of potent topical corticosteroids after adequate training and instructions. Patients with physician reported doubts on treatment compliance were excluded.

Group 1 consisted of patients with severe AD (EASI >21), who could be "controlled" with topical corticosteroids (controlled disease). Controlled disease was defined as an EASI score of 7 or less. Group 2 consisted of patients with severe AD (EASI >21) who needed systemic treatment to control their AD (difficult to treat). Group 2 included (a) patients with uncontrolled severe AD despite intensive topical treatment, or (b) patients with severe disease experiencing exacerbations upon tapering of topical treatment to safe maintenance schedules, who are therefore in need of systemic (immunosuppressive) therapy. Patients who were treated with oral immunosuppressive drugs or UV-light therapy within three months before sampling were excluded.

The following data were retrospectively retrieved from the patient's electronic medical files: sex, age, EASI score at moment of sampling, history of asthma, allergic rhinitis, allergic conjunctivitis and food allergy, age of onset of AD and history of hospitalization for AD. Serum samples were routinely collected before start of treatment during uncontrolled disease (EASI>21) and stored at -80 degrees Celsius in a biobank until analysis.

The protocol used in this study was approved by the Institutional Review Board of the University Medical Center Utrecht, adhering to the Declaration of Helsinki Principles.

Power calculation

The sample size (n) needed for this study was determined using a frequentist approach. The disease status aimed to predict in this study applies to a binominal test; having "difficult to treat" AD or not. This will give a sequence of 0's and 1's which are called 'x'. The entries of 'x' will have a Bernoulli distribution with success probability 'p'. The estimate of 'p' is given by $p = \sum x/n$.

In order to answer the question how big 'n' should be, additional information on biomarkers as disease predictors in AD is needed. The best known biomarker for disease severity in AD is serum thymus and activation-regulated chemokine (TARC). Serum TARC levels have been determined in relatively large numbers of patients from different studies, showing pooled correlation coefficients of 0.60 (95% CI 0.48–0.70) and 0.64 (95% CI 0.57–0.70) in longitudinal and cross-sectional studies, respectively.[2] To calculate the sample size we used a sensitivity cut-off value of 0.60 and a power of 0.80, so there is an 80% chance to detect a sensitivity of $p=0.60$, with a significance level controlled of $\alpha=0.05$. By using the "pwr" package in R.[3] we calculated a sample size of 152 AD patients (effect size=0.21) needed to match our required sensitivity.

Serum protein biomarker analysis

A panel of 143 serum biomarkers (all markers currently available in our center) were measured using Luminex technology at the Multiplex Core Facility of the Laboratory for Translational Immunology (UMC Utrecht, The Netherlands), using an in-house validated panel of analytes, listed in Table S1. Uniquely color-coded magnetic beads (MagPlex Microsperes, Luminex, Austin, Texas) were conjugated to antibodies specific for the reported analytes and incubated with 50 µL of standard dilutions per sample for 1 hour (continuous shaking in the dark). Samples were diluted in High Performance Elisa buffer (HPE; Sanquin, The Netherlands). Pre-treatment of samples included filtration and incubation with HeteroBlock to prevent interference by binding of heterophilic antibodies. Plates were washed (Bio-Plex Pro II Wash Station; Bio-Rad, Hercules, California, USA) and a corresponding cocktail of biotinylated detection antibodies was added for 1 hour. Repeated washings were followed by a

10 minute streptavidin-phycoerythrin (PE) incubation. Fluorescence intensity of PE was measured using a Flexmap 3D system (Luminex) and analyzed by using BioPlex Manager software version 6.1; (Bio-Rad) using 5-parameter curve fitting.[4] Serum biomarkers with signals above or below the assay detection limit in >60% of the samples were excluded for further analyses, resulting in 129 unique serum biomarkers selected for further analysis.

Statistical analysis

Data were analyzed using R version 3.4.1 (R Foundation for Statistical Computing, Vienna, Austria) and the SPSS (Statistical Package for the Social Science) software for Windows version 21.0 (SPSS Inc., Chicago, IL, USA). Differences in clinical characteristics between the two patient groups were compared using the Wilcoxon rank sum test for continuous variables, and with the chi-square test for categorical variables. P-values lower than 0.05 were considered statistically significant.

Serum samples that were above or below the assay limits of detection were given values equivalent to the lower limit divided by two or the upper limit multiplied by two. Actual concentration data were normalized by a log-transformation.

Prognostic biomarker signature

To construct the prognostic biomarker signature, we used the method that has been previously developed by Mamtani et al[5]. This method consists of three steps: 1. screening the biomarkers individually based on the Performance Index (Pi) which is a function of the estimated area under the receiver characteristic curve (AUC); 2. using stepwise multiple regression analysis to select the top ranked n-1 biomarkers; 3. combining the selected biomarkers using a linear discriminant function (Figure 1). In the stepwise multiple regression analysis, a retention criterion of 0.01 was used to define if a biomarker should be kept in the multivariate model. The best model was assessed by its R^2 and its complement Wilks' λ. The Wilks' λ is a measure of how well the model separates the cases into groups. Smaller values of Wilks' λ indicate greater discriminatory ability of the model.

Posterior probabilities from the linear discriminant function analysis were used to define a predicted classification (group 1: "controlled disease" or group 2: "difficult to treat") for each individual. If the posterior probability for a given patient was higher for group 1 than for group 2 the predicted classification was defined as group 1, and vice versa. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the final prediction model were calculated based on predicted and observed classifications.

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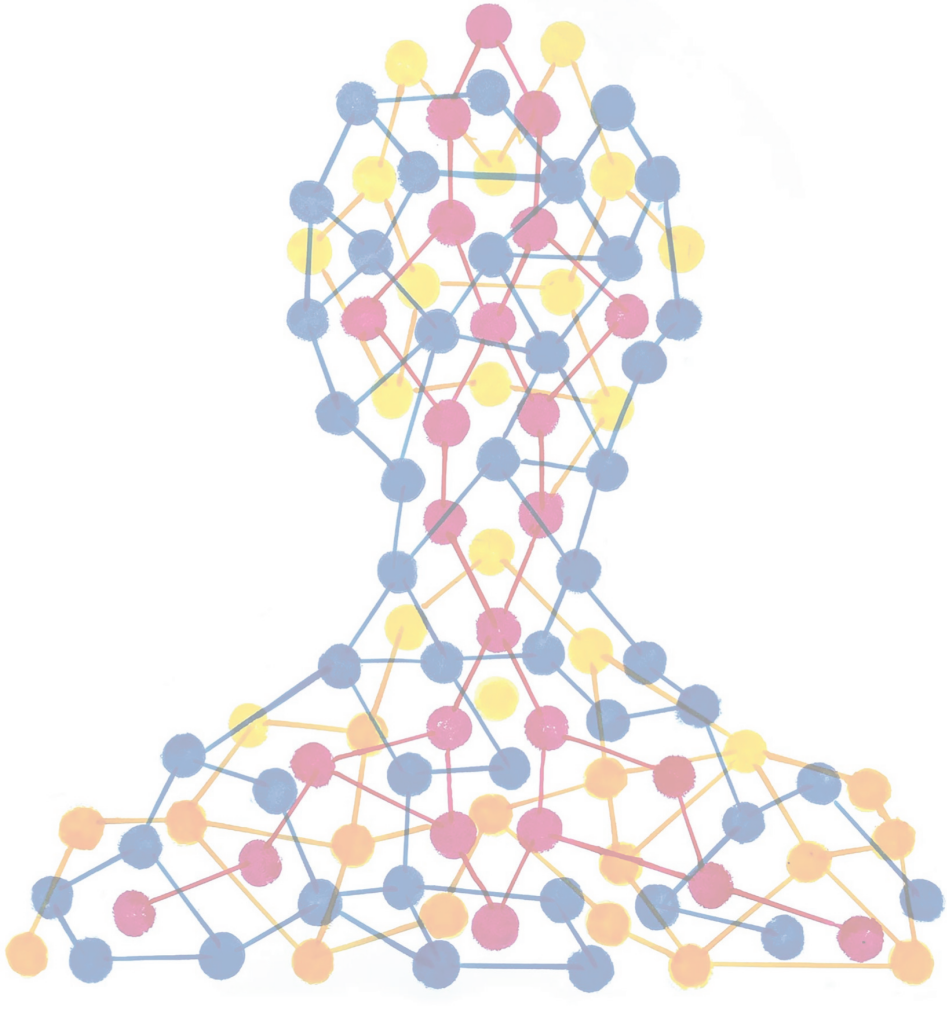

Chapter 3

Confirmation of multiple endotypes in atopic dermatitis based on serum biomarkers

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ABSTRACT

Background: Atopic dermatitis (AD) is a highly heterogeneous disease, both clinically and biologically, whereas patients are still treated according the "one-size-fits-all" approach. Stratification of patients into biomarker-based endotypes is important for future development of personalized therapies.

Objective: To confirm previously defined serum biomarker-based patient clusters in a new cohort of AD patients.

Methods: A panel of 143 biomarkers was measured using Luminex technology in serum samples of 146 severe AD patients (median EASI 28.3, IQR 25.2-35.3). Principal components analysis followed by unsupervised k-means cluster analysis of the biomarker data was used to identify patient clusters. A prediction model was built based on a previous cohort to predict in which of the four previously identified clusters the patients of our new cohort would belong.

Results: Cluster analysis identified four serum biomarker-based clusters of which three (cluster B, C and D) were comparable to the previously identified clusters. Cluster A (33.6%) could be distinguished from other clusters as being "skin-homing chemokines/IL-1R1 dominant" cluster, cluster B (18.5%) as "Th1/Th2/Th17 dominant" cluster C (18.5%) as "Th2/Th22/PARC dominant" and cluster D (29.5%) as "Th2/eosinophil inferior" cluster. Additionally, using a prediction model based on our previous cohort we accurately assigned the new cohort to the four previously identified clusters by including only 10 selected serum biomarkers.

Conclusion: We confirmed that AD is heterogeneous on the immuno-pathological level and identified four distinct biomarker-based clusters of which three were comparable with previously identified clusters. Cluster membership could be predicted with a model including 10 serum biomarkers.

INTRODUCTION

Atopic dermatitis (AD) is one of the most common chronic inflammatory skin diseases worldwide and is characterized by a diverse clinical manifestation and a highly heterogeneous pathophysiology.[1] Classically, AD is stratified into different disease phenotypes according to clinical characteristics, including age, age of onset, ethnicity, and the presence of other atopic diseases such as allergic rhinitis and asthma.[2, 3] Despite the highly heterogeneous character of the disease, there is currently no endotype-specific published data for any licensed drug for the treatment of AD in Europe or the United States. Therefore, the current treatment guidelines for AD could not consider disease subtypes, resulting in a high unmet need in individualized treatment options.

In the past decade more and more advances have been made in characterizing the immunologic differences underlying AD. Although AD is considered to be a primarily T helper (Th)2 cell-driven disease, it has now become clear that Th1, Th17, and Th22 cytokine pathways are likely to contribute to AD pathogenesis as well.[4 5, 6] Due to the heterogeneity of the disease, it is unlikely that novel molecular therapies targeting specific immunological pathways will be equally effective in all AD patients, which makes the stratification of subtypes of AD patients of increasing importance. The identification of patients by relevant and validated biomarkers is a prerequisite for a more personalized therapeutic approach.[7] Nevertheless, the distinct molecular mechanisms driving different disease subtypes of AD, previously defined as endotypes[8], are yet inadequately described.

In a recently published study, we identified four clearly differentiated clusters of patients using a data driven approach on 147 biomarkers measured in 193 moderate-to-severe AD patients[9]. Each cluster was characterized by a specific serum biomarker profile, implying that distinct underlying immuno-pathological pathway drives each cluster. Two of these clusters where characterized by a Th2-dominated biomarker profile[9], suggesting that the patients in these clusters would be ideal candidates for Th2 inhibiting therapies, such as the recently developed anti-interleukin (IL)-4Rα monoclonal antibody dupilumab or the anti-IL-13 antibodies tralokinumab and lebrikizumab. Stratification of patients into distinct endotypes might contribute to the development of personalized medicine approaches and precision based care in the future. However, the previously defined patient clusters still need to be replicated in an independent patient population.

The aim of the present study is to confirm the previously identified AD patient clusters based on distinct serum biomarker profiles, by using the same data driven approach on a new cohort of severe AD patients. Additionally, we aim to build a prediction model enabling the stratification of patients into one of the four previously defined clusters by using a small set of selected markers, which might be incorporated in clinical trials or standard practice as a convenient tool to identify endotypes in the future.

METHODS

Patients and samples

To confirm the endotypes on a clinically well-defined large cohort of severe AD patients, who are most eligible for systemic/biological treatments, we used data of a previously reported cohort including AD patients, who were selected based on AD severity (Eczema Area Severity Intensity score, EASI >21) and only treated with topical corticosteroids, which original aim was to predict the need of systemic therapy.[10] Out of the 152 patients of this cohort, six patients were also included in the cohort from the study of Thijs et al.[9]; in order to lower the risk of bias, these six patients were excluded for the current study, resulting in 146 patients included in the current study. Clinical characteristics were retrospectively extracted from the patients' electronic medical records. For the current study, AD severity was assessed by using EASI score, according to the Harmonizing Outcomes Measures in Eczema (HOME) recommendations.[11] Severity scores from the previous cohort[9] were measured before the availability of these recommendations, and were assessed by using Six Area, Six Sign Atopic Dermatitis (SASSAD) severity score, which was the standard severity score in our center at that time. Both severity scores incorporate grading of AD signs and assessment of body region involvement. All patients were diagnosed with AD according to the criteria of Hanifin and Rajka.[12] The protocols of this study were approved by the Institutional Review Board of the University Medical Center Utrecht (the Netherlands) adhering to the Declaration of Helsinki Principles.

Serum protein biomarkers

A panel of 143 serum biomarkers (all markers currently available in our center) were measured using Luminex technology[9, 13] at the Multiplex Core Facility of the Center for Translational Immunology (UMC Utrecht, The Netherlands), using an in-house

validated panel of analytes, listed in Supplemental Table E1 (see this article's Online Supporting Information at https://drive.google.com/drive/folders/1X7pdu-VLbwzBWvzLuPbbufrkRktA9n3o?usp=sharing). The same biomarker measurements as previously published[10] were used in the current study.

Uniquely color-coded magnetic beads (MagPlex Microsperes, Luminex, Austin, Texas) were conjugated to antibodies specific for the reported analytes and incubated with 50 µL of standard dilutions per sample for 1 hour (continuous shaking in the dark). Samples were diluted in High Performance Elisa buffer (HPE; Sanquin, The Netherlands). Pre-treatment of samples included filtration and incubation with HeteroBlock to prevent interference by binding of heterophilic antibodies. Plates were washed (Bio-Plex Pro II Wash Station; Bio-Rad, Hercules, California, USA) and a corresponding cocktail of biotinylated detection antibodies was added for 1 hour. Repeated washings were followed by a 10 minute streptavidin-phycoerythrin (PE) incubation. Fluorescence intensity of PE was measured using a Flexmap 3D system (Luminex) and analyzed by using BioPlex Manager software version 6.1; (Bio-Rad) using 5-parameter curve fitting.[9]

Baseline thymus and activation-regulated chemokine (TARC)/C-C motif chemokine (CCL)17 levels, currently the best performing and accepted biomarker for disease severity[14], were measured in routine care using Quantikine® ELISA immunoassays (R&D systems, Inc., Minneapolis, MN, USA).

Statistical methods

Replication of the four distinct patient clusters

Principal components analysis (PCA) followed by unsupervised k-means cluster analysis of the serum biomarker data was used to identify patient clusters. Additionally, PCA followed by k-means cluster analysis was repeated on the pooled serum biomarker data of the current cohort and the original cohort from the study of Thijs et al[9]. Clinical characteristics and averages of serum biomarkers were analyzed between the clusters using one-way ANOVA for normally-distributed variables, Kruskal-Wallis test for non normally-distributed variables, and χ^2 test for percentages.

Prediction model based on previously defined clusters

We built a prediction model based on the biomarker data used in the study of Thijs et al.[9] to predict in which of the four previously identified clusters the patients in our cohort would belong. The prediction model was built using a supervised random

forest approach (package randomForest[15] in R) . The importance of each biomarker in the classification of patients was estimated using the mean decrease in accuracy. The prediction model accuracy, defined as the fraction of correctly predicted cases (1 – model error rate), was studied for several prediction models using all the 140 markers common between the two cohorts, as well as the top 70, top 20 and top 10 biomarkers. A flowchart presenting all the steps of the prediction model can be found as Supplemental Figure 1.

Statistical analyses were performed using R Project software version 3.4.1 (R Foundation for Statistical Computing, Vienna, Austria)[16], and the SPSS (Statistical Package for the Social Science) software for Windows version 21.0 (SPSS Inc., Chicago, IL, USA). Serum biomarker data were normalized by Box-Cox transformation before analyses. Before replication of the cluster analysis and building the prediction model, we merged the serum biomarker data of both datasets and corrected them for batch effects concerning two different batches (package sva[17] in R). P-values lower than 0.05 were considered statistically significant.

RESULTS

Patient characteristics and cluster replication

Among the 146 patients, 56% were male, median (IQR) age was 30.5 years (22.0 – 50.0), median EASI score 28.3 (IQR 25.2 – 35.3), median TARC/CCL17 level 4192 pg/mL (IQR 2088 - 8496) and all were treated with topical corticosteroids at the moment of sampling. Patient characteristics from the current and original study[9] are summarized in Table 1, disease severity in the current cohort differed from the original cohort[9], in which patients with moderate-to-severe AD (median TARC/CCL17 level 1766 pg/mL, IQR 874 – 5215, ($p<.001$)) were included. Besides, the current cohort had a significantly higher percentage of patients who were treated with any systemic immunosuppressive drug (not including systemic corticosteroids) within one year before sampling (22.6% vs 11.4%, respectively, $p=0.010$). Age, sex, atopic comorbidities, and age of onset did not significantly differ between the two cohorts. A total of 143 serum biomarkers were determined via multiplex immunoassay. After principal components analysis on the Box-Cox transformed serum biomarker dataset, the cumulative percentage of variance showed that 90% of the dataset's variance was described by the first 48 principal components (Figure 1). The first 48

Table 1. Baseline characteristics

Clinical characteristics	Current cohort (n=146)	Original cohort[9] (n=193)
Age (years)[1], median [IQR]	30.5 [22.0 – 50.0]	30.0 [21.0 – 42.0]
Male, n (%)	81 (56)	81 (42)
EASI score, median [IQR]	28.3 [25.2 – 35.3]	NA
SASSAD score, median [IQR]	NA	31.6 (23.0 – 37.5)
Serum TARC/CCL17 levels, median [IQR]	4191.5 [2087.5 – 8495.5]	NA
Atopic diseases, n (%)		
- **allergic asthma**	80 (55)	88 (46)
- **allergic rhinitis**	95 (65)	124 (64)
- **food allergy**	59 (40)	NA
- **no atopic disease**	25 (17)	50 (26)
- **missing data**	1 (1)	3 (2)
Age of onset, n (%)		
- **0 -1 yrs**	58 (38)	88 (46)
- **2 – 11 yrs**	64 (44)	74 (38)
- **12 – 18 yrs**	4 (3)	8 (4)
- **>18 yrs**	10 (7)	15 (8)
- **missing data**	10 (7)	8 (4)
Hospitalization for AD, n (%)	64 (44)	NA
History of immunosuppressive drug[2] use <1 year before sampling, n (%)	22 (11)	33 (23)

Categorical variables are presented as counts and percentages; continuous variables are presented as median [InterQuartileRange]. EASI Eczema Area Severity Intensity; IQR Inter Quartile Range; SASSAD Six Area, Six Sign Atopic Dermatitis; VAS Visual Analogue Scale; [1] age at time of sample collection; [2] including azathioprine, cyclosporine A, methotrexate, enteric-coated mycophenolate sodium, mycophenolate mofetil and tacrolimus.

principal components were included in the unsupervised k-means cluster analysis, resulting in the identification of four distinct patient clusters (cluster A, B, C and D, which are displayed in terms of the first three principal components in Figure 2A). The cluster membership per patient was added back to the complete dataset and clinical characteristics were compared between the four clusters (Table 2). Averages of serum biomarker levels were calculated per cluster to characterize the biomarker profiles driving the four clusters (Figure 2B, supplemental Table E2 in this article's Online Supporting Information) and were also compared with the previously identified clusters[9] (reported as cluster 1, 2, 3 and 4; Figure 2B).

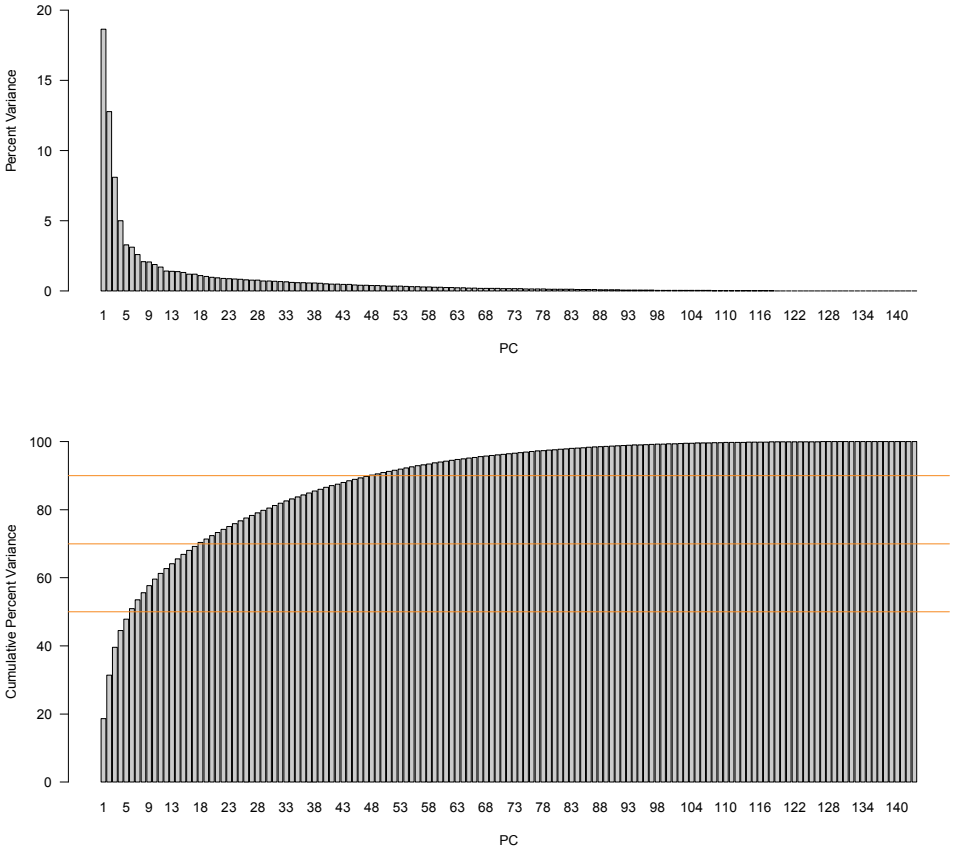

Figure 1. Variance described by principal components. The first six principal components (PCs) describe 50% of the variance and the first 48 PCs describe 90% of the variance in the Box-Cox normalized serum biomarker dataset.

A

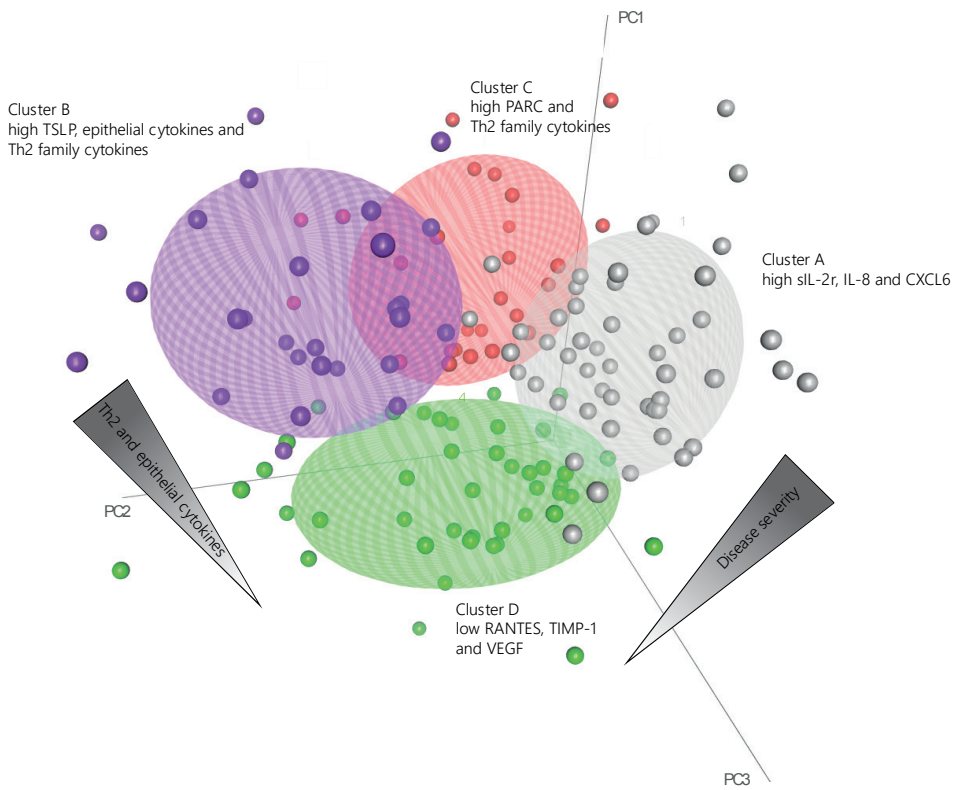

Figure 2. Comparison of clusters identified in original and replication cohort. A. Using unsupervised k-means clustering of the first 48 principal components (PCs) resulted in the identification of four patient clusters (A, B, C and D). All 146 patients are presented in a three-dimensional plot in terms of the first three PCs. Colors and colored ellipses represent clusters. PC1 explained 18.7% of the variance, PC2 explained 12.8% of the variance and PC3 explained 8.1% of the variance. Clinical characteristics were analyzed between the four clusters (Table 2). A difference between the clusters was found only in disease severity. Averages of serum analytes were calculated and compared per clusters to characterize cluster's unique biomarker profiles.

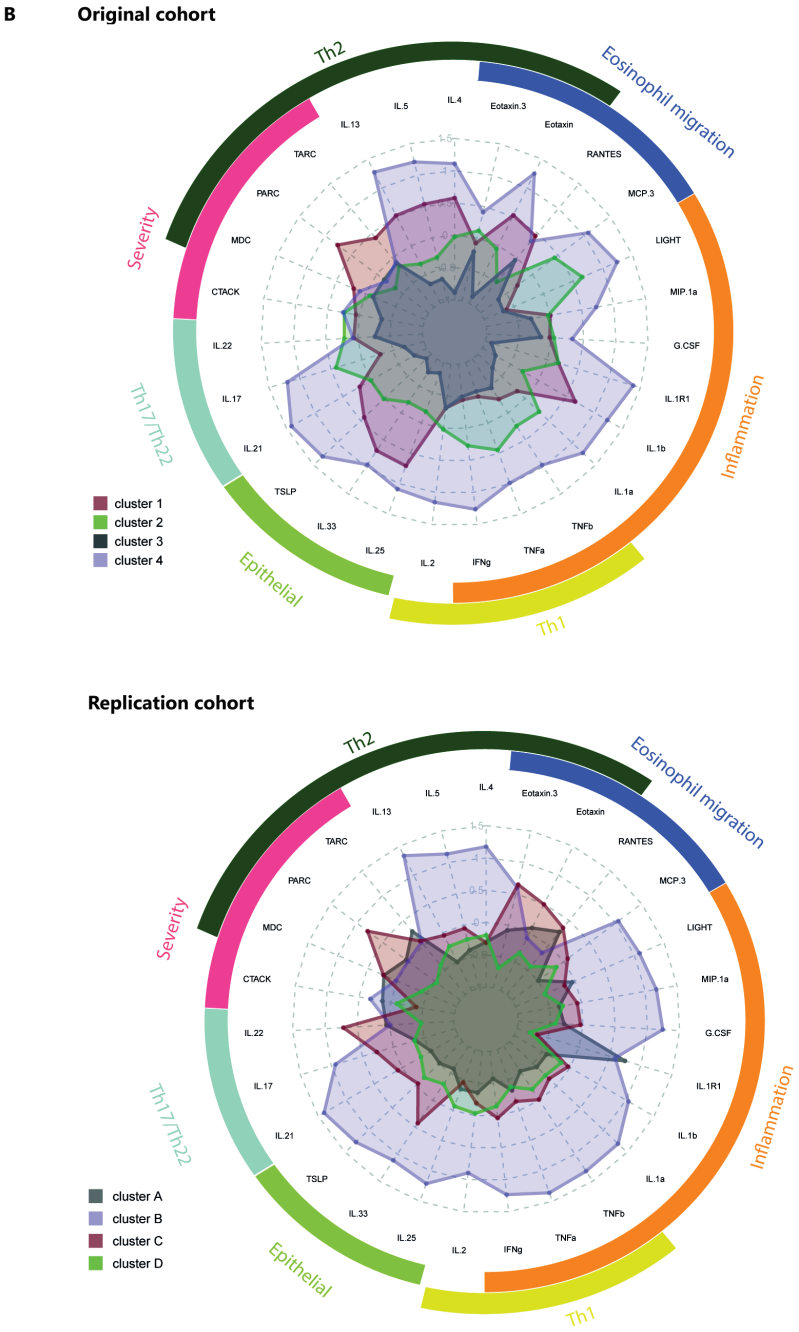

Figure 2B. Averages of Box-Cox transformed serum biomarker data per cluster of the replication cohort were compared with the previously defined clusters 1, 2, 3 and 4. Radar plots show selected markers that were significantly higher or lower expressed in one of the clusters compared to the other clusters. The biomarker profile of cluster B resembled the profile of the previously identified cluster 4, cluster C resembled cluster 1, and cluster D resembled cluster 2.

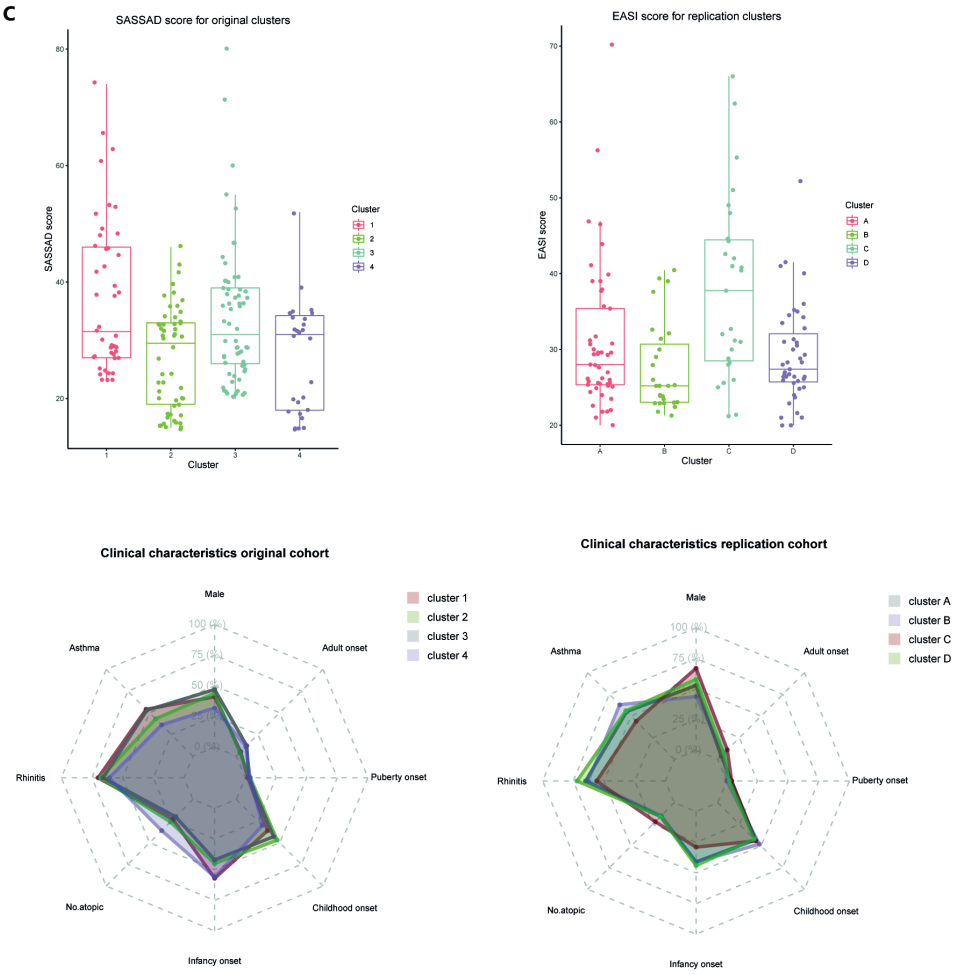

Figure 2C. Clinical characteristics per cluster of the replication cohort were compared with the previously defined clusters 1, 2, 3 and 4. Box plots represent median with IQR; the upper whisker extends to the largest value no further than 1.5*IQR from the third quartile; the lower whisker extends to the smallest value at 1.5*IQR to the first quartile. Disease severity was assessed by Six Area, Six Sign Atopic Dermatitis (SASSAD) severity score in the original cohort and by Eczema Area Severity Intensity (EASI) score in the current cohort. Both severity scores include only gradation of AD signs and body region involvement, and do not take patient reported outcomes into account. Maximum score for SASSAD is 108, maximum score for EASI is 72. Disease severity was relatively higher in clusters 1 and 3, and A and B, compared to the other clusters. Radar plots show that no differences were found in other clinical characteristics between the patient clusters in both cohorts.

Cluster A represented 33.6% of the AD patients. Median age in this cluster was 35.0 years (IQR 22.5 – 51.0), and median EASI score was 28.0 (IQR 25.3 – 35.6). Cluster A was distinct from cluster C and D by having higher levels of C-C chemokines (CTACK/CCL27, TARC/CCL17, MDC/CCL22 and RANTES/CCL5) and IL1R1 ("skin-homing chemokines/IL-1R1 dominant" cluster). Regarding clinical characteristics, the percentage of patients who were treated with any systemic immunosuppressive drug (not including systemic corticosteroids) within one year before sampling, was significantly higher in cluster A compared to the other clusters (37 vs 18, 11 and 16% respectively, p=0.043). Cluster A was the only cluster that did not correspond to any of the previously defined clusters.[9]

Cluster B represented 18.5% of the AD patients, with a median age of 25.0 years (IQR 20.0 – 50.0) and median EASI score of 25.2 (IQR 23.0 – 31.4). It was characterized by a high inflammatory state, distinctive from the other clusters by the highest levels of Th2-(IL-4, IL-5, IL-13), Th1-(IFNγ, TNFα, TNFß), Th17-(IL-17, IL-21) and epithelial-related (IL-25, IL-33, TSLP) cytokines ("Th1/Th2/Th17 dominant" cluster) as shown in Figure 2B. Cluster B was comparable with the biomarker profile of the previously defined cluster 4.

Cluster C represented 18.5% of the AD patients and had a median age of 32.0 years (IQR 22.0 – 55.0). Patients in this cluster had significantly higher severity scores compared to cluster A, B and D (median EASI 37.8 IQR 28.2 – 44.6, p=0.001). Cluster C was uniquely defined by high levels of Th2 related cytokines (PARC, IL-13, IL-5, eotaxin and eotaxin-3), IL-22 and IL-33 ("Th2/Th22/PARC dominant" cluster). The biomarker profile of cluster C resembled the profile of the previously identified cluster 1.

Cluster D represented 29.5% of the AD patients, had a median age of 32.0 years (IQR 23.0 – 48.0) and median EASI score of 27.4 (IQR 25.6 – 32.8). It was characterized by a relatively low inflammatory state, particularly distinctive from the other clusters by low serum levels of Th2/severity- (MDC, PARC, TARC) and eosinophil-related markers (RANTES, eotaxin and eotaxin-3) ("Th2/eosinophil inferior" cluster). The biomarker profile of cluster D resembled the profile of the previously identified cluster 2.
Other clinical characteristics including age, sex, atopic comorbidities, age of onset, and hospitalization rate did not significantly differ between the four clusters (Table 2 and Figure 2C).

Table 2. Clinical characteristics for the four AD patient clusters

Clinical characteristics	Cluster A (n=49)	Cluster B (n=27)	Cluster C (n=27)	Cluster D (n=43)	p-value
(% AD patients)	33.6	18.5	18.5	29.5	
Age (years)[1], median [IQR]	35.0 [22.5 – 51.0]	25.0 [20.0 – 50.0]	29.0 [22.0 – 55.0]	32.0 [23.0 – 48.0]	0.535
Male, n (%)	26 (53)	12 (44)	18 (67)	25 (58)	0.401
EASI score, median [IQR]	28.0 [25.3 – 35.6]	25.2 [23.0 – 31.4]	37.8 [28.2 – 44.6]	27.4 [25.6 – 32.8]	0.001
TARC baseline (pg/mL), median [IQR]	5024 [2816 – 11750]	3501 [1388 – 11500]	4142 [2068 – 16000]	3278 [1787 – 5430]	0.037
Atopic diseases, n (%)					
- **allergic asthma**	27 (55)	17 (63)	12 (44)	24 (56)	0.582
- **allergic rhinitis**	32 (65)	17 (63)	15 (56)	31 (72)	0.285
- **foodallergy**	22 (45)	12 (44)	6 (22.2)	19 (44)	0.238
- **no atopic disease**	8 (16)	4 (15)	6 (22)	7 (16)	0.852
Age of onset, n (%)					
- **0 -1 yrs**	20 (41)	11 (41)	8 (29)	19 (44)	0.955
- **2 – 11 yrs**	21 (43)	13 (48)	12 (44)	18 (42)	
- **12 – 18 yrs**	2 (4)	0	1 (4)	1 (2)	
- **>18 yrs**	2 (4)	2 (7)	3 (11)	3 (7)	
- **missing data**	4 (8)	1 (4)	3 (11)	2 (5)	
Hospitalization for AD, n (%)	22 (45)	13 (48)	12 (44)	17 (40)	0.522
History of immunosuppressive drug use[2] <1 year before sampling, n (%)	18 (37)	5 (18)	3 (11)	7 (16)	0.043

Categorical variables are presented as counts and percentages; continuous variables are presented as median [InterQuartileRange]. EASI Eczema Area Severity Intensity; IQR Inter Quartile Range; VAS Visual Analogue Scale; [1] age at time of sample collection; [2] including azathioprine, cyclosporine A, methotrexate, enteric-coated mycophenolate sodium, mycophenolate mofetil and tacrolimus

In addition, we analyzed the merged datasets (previously published and the present one) using a PCA and k-means cluster analysis, and here again we identified four patient clusters. Biomarker profiles of the merged clusters were largely comparable with the original patient clusters[9]. "Merged-cluster" 1 was characterized by the lowest levels of epithelial cytokines and IL-1 related cytokines, "merged-cluster" 2 by the highest levels of Th2 family cytokines, IL-1 related cytokines and TSLP, "merged-

cluster" 3 by the highest levels of Th2 family cytokines and pulmonary and activation-regulated chemokine (PARC/CCL18), and "merged-cluster" 4 by the lowest levels of IFNα and apelin. Of the patients who clustered together in the original clusters, 88.3% clustered again together in one of the "merged-clusters" (Supplemental Table E3 in this article's Online Supporting Information). For the replication cohort, 68.5% of the patients clustered together again in one of the merged clusters.

Cluster prediction

As we could (re)confirm three of the four previously defined patient clusters, we next used a supervised random forest approach using the biomarker data of Thijs et al.[9] to build a prediction model of cluster membership (cluster 1, 2, 3 ,or 4) for the patients of the current cohort. Only biomarkers determined in both datasets were used for this analysis, resulting in a total of 140 serum biomarkers. Biomarkers were sorted by prediction importance based on the mean decrease in accuracy. The different steps of this analysis are described in Supplemental Figure 1.

The top 10 biomarkers were IL-37, IL-1rα, XCL-1, eotaxin/CCL11, IL-1ß, IL-26, LIGHT/tumor necrosis factor superfamily (TNFSF)14, IL-1r1, epidermal growth factor (EGF) and TSLP (Supplemental Table E4 in this article's Online Supporting Information). The accuracy of the prediction model, on the original study[9], including all 140 biomarkers was 94.1% (Figure 3 and Supplemental Figure 2) and the out of bag (OOB) estimate of error rate (i.e. number of incorrect classifications) was 5.3% (Table 3). When including only the top 10, top 20 and top 70 biomarkers, the accuracy was 86.7%, 90.4% and 93.6% respectively. The OOB estimate of error rate was 13.8%, 9.6%, and 5.3% respectively (Supplemental Tables E5 – 7 in this article's Online Supporting Information).

We then applied the models on the current dataset and used as reference the cluster membership of the model including all 140 markers. We compared this membership with the ones predicted by including only the top 10, the top 20 and top 70 markers. The model accuracy including the top 70 biomarkers was 87.0% and 73.3% and 81.5% using the model including the top 10 and the top 20 respectively.

Table 3. Model accuracy for predictive model including all 140 biomarkers

		Original cluster				
		1	**2**	**3**	**4**	**Class error**
Predicted cluster	**1**	**41**	0	3	0	6.8%
	2	2	**51**	1	0	5.6%
	3	2	0	**60**	0	3.2%
	4	0	2	0	**26**	7.1%

Confusion matrix showing the accuracy of the model built on the original dataset of 140 overlapping serum biomarkers measured in 193 moderate-to-severe AD patients, used to predict cluster membership in the current cohort of 146 severe AD patients. The OOB estimate rate of accuracy for the model was 5.3%.

DISCUSSION

Due to the development of new therapies for AD targeting different cytokine pathways, the stratification of patients into endotypes driven by distinct molecular pathways is of increasing importance in order to move towards more personalized medicine. In a recent study we classified AD patients into four serum biomarker based clusters that could represent endotypes.[9] By using the same data driven approach, the current study once more identified four patient clusters with a distinct profile of serum biomarkers in a different cohort of severe AD patients. Regarding their biomarker profiles, three of the four clusters (representing 66.4% of the patients) were similar to the previously identified clusters. Endotyping of AD patients may contribute to precision medicine by allowing treatment to be tailored for individual patients and may be important to better inform which patient is most likely to benefit from specific targeted therapies.[18]

Since three of the four clusters were confirmed in the current study, we were able to further characterize and name the clusters by their discriminating biomarker profiles. Patients stratified into cluster B were characterized by a relatively high inflammatory state and could be distinguished from the other clusters as being the "Th1/Th2/Th17 dominant" cluster. Cluster C shared relatively high Th2-related cytokine levels with

the "Th1/Th2/Th17 dominant" cluster, although separated itself from the other clusters by showing high levels of IL-22 and PARC. Cluster D was characterized by the lowest levels of eotaxins, RANTES, PARC, MDC, TARC and IFNα and was thereby defined as the "Th2/eosinophil inferior" cluster. Patients identified in cluster A showed higher levels of the C-C chemokines CTACK/CCL27, TARC/CCL17, MDC/CCL22 and RANTES/CCL5. CTACK/CCL27, TARC/CCL17, and MDC/CCL22 are known to bind the C-C chemokine receptors CCR10, and CCR4[19, 20] respectively, thereby enabling skin-specific homing of CD4+ T-cells.[21] RANTES/CCL5 is a ligand for CCR3 and CCR5 and is considered to maintain eosinophilic infiltration in chronic inflammation of AD skin.[22] Furthermore, patients in cluster A showed higher expression of serum IL-1R1 levels. Previous studies have shown that serum IL-1R1 levels are significantly increased in AD patients compared to healthy controls[23], and that the upregulation of IL-1R1 is associated with frequent exacerbations in asthma patients.[24] Based on the underlying biomarker profile, cluster A could be defined as the "skin-homing chemokines/IL-1R1 dominant" cluster.
Although cluster D showed the lowest expression of several severity related markers (TARC, PARC and MDC), this was not reflected by the lowest EASI score. Similar to the previous cohort, the four identified AD patient clusters in the present study were clinically distinguished by disease severity, with the "Th2/Th22/PARC dominant" cluster showing a significantly higher EASI score. However, since all patients had high lesions/signs scores, we consider this difference as not clinically relevant. Furthermore, we were unable to identify an association between the four clusters and other clinical characteristics including age of onset or atopic comorbidities. This result highlights the challenges in identifying patient subgroups based only on clinical features and might indicate that individualized treatment options should not be based on clinical phenotypes of AD, but in fact, on biomarker based endotypes.

One of the four clusters (the "skin-homing chemokines/IL-1R1 dominant" cluster A) identified in the current study could not be matched with one of the previous clusters of the study by Thijs et al.[9] An explanation for the different fourth cluster could be the difference in the percentage of patients who used immunosuppressive drugs within one year before sampling, as this percentage was higher in the current cohort compared to the original one and, moreover, significantly higher in the non-corresponding "skin-homing chemokines/IL-1R1 dominant" cluster A. The majority of these patients were treated with cyclosporine A (CsA) in the year before sampling. CsA is a calcineurin inhibitor that selectively acts on T-cells through inhibiting signal transduction mediated by T-cell receptor activation.[25] It has been shown previously

that CsA treatment in AD patients suppresses the levels of IL-2-, IFNγ- and IL-4/IL-5/IL-13-producing T-cells and T-cell products including TARC/CCL17.[26-28] However, data on the long-term effects of CsA treatment on serum biomarkers after discontinuation are lacking. In agreement with previous findings in CsA treated AD patients, patients in the "skin-homing chemokines/IL-1R1 dominant" cluster A showed the lowest serum levels of IFNγ, IL-4, IL-5 and IL-13.

The ability to endotype patients based on serum biomarkers has already been demonstrated in asthma, where anti-IL-13, anti-IL-4/IL-13 and anti-IL-5 therapies appeared to be more effective in "Th2-dominant" patients groups. Clinical trials investigating the efficacy of treatment with the anti-IL-4/IL-13 receptor monoclonal antibody dupilumab in AD patients showed that only 35%-40% of the patients achieved clear or almost clear skin [29-31], which corresponds to the percentages of patients in the "Th1/Th2/Th17 dominant" and "Th2/Th22/PARC dominant" clusters. On the other hand, the numbers of non-responders to dupilumab treatment in AD patients are very low[30, 32], indicating that Th2 cytokines might not be the most relevant markers to discriminate AD patients in this overall Th2-high population. This might also explain why the top 10 biomarkers based on the mean decrease in accuracy of our prediction model that were found to be distinctive for the four clusters did not include any Th2-related markers. Although two clusters shared a relatively Th2-low cytokine profile compared to the other two clusters, these patients still express higher levels of Th2-related cytokines compared to levels that have previously been reported for healthy controls.[9, 23] Prediction of treatment response by serum biomarker profiles in AD patients is scarce. A single phase 2b study investigating treatment with tralokinumab (anti-IL-13) suggested that baseline serum DPP-4 levels, reflecting IL-13 activity, might serve as a predictive biomarker for AD patients who may benefit from tralokinumab treatment.[33] Furthermore, a phase 2a study of IL-22 blockade with fezakinumab showed that patients with higher baseline expression of IL-22 had greater disease improvement during fezakinumab treatment[34], although IL-22 expression was measured in skin biopsies, which is hard to implement in daily practice. Theoretically, patients in our "Th2/Th22/PARC dominant" cluster might be optimal candidates for anti-IL-22 treatment.

Both the original and the current study made use of a panel of more than 140 serum biomarkers to confirm the presence of four endotypes within AD patients. Although, for the implementation of serum biomarker based endotypes in clinical trials and daily practice, a smaller set of markers is desired. In the current study we built a

prediction model, based on the biomarker data used in the study of Thijs et al.[9], that could be used to classify our patients into one of the four original clusters with a good accuracy, even when using only the top 10 biomarkers (IL-37, IL-1rα, XCL-1, eotaxin/CCL11, IL-1ß, IL-26, LIGHT/TNFSF14, IL-1r1, EGF and TSLP). Surprisingly, none of those markers are Th2-related cytokines but they consisted of IL-1-, IL-10- and epithelium-related markers. We, hence, hypothesize that markers related to other cytokine pathways might be (more) important to define endotypes in an overall Th2-dominant disease such as AD. In the future, the application of such prediction model, resulting in a small panel of biomarkers, might enhance tailored decision making in the management of moderate-to-severe AD patients and might contribute to more personalized medicine. However, the use of the prediction model should be tested in longitudinal studies and randomized controlled trials with drugs targeting specific pathways first.

A limitation of the study is that the current cohort was not completely independent from the original one, since it included patients from the same center with comparable demographic characteristics. The previous study by Thijs et al. [9] included patients with moderate-to-severe AD with a broad range of severity scores, whereas our cohort included patients with only severe AD, which makes it more difficult to generalize the results for the complete spectrum of severity. However, severe AD patients are the most eligible for systemic/biological therapies, and may therefore be the most appropriate group to use endotypes to target specific therapies in future trials and daily practice. A strength of our study, was the confirmation of three of the four previously identified clusters within a cohort that was not originally designed as a validation cohort.

While we aimed to divide AD patients into distinct endotypes based on blood molecular profiles, previous studies have proposed to integrate models of lesional and non-lesional skin with blood measures, as well as clinical parameters, to reflect disease outcomes in AD. Wen et al.[35] demonstrated a Th2/Th22 profile in blood of Asian AD patients, with lower Th1-related cytokine levels compared with European American patients, which was attributed to reciprocal downregulation of this axis by the increased Th17 pathway in the skin[36]. Zhou et al. [6] compared AD endotypes among different age groups by evaluating lesional and non-lesional skin, as well as serum biomarkers. This study found decreases in Th2/Th22 activation and increases in Th1/Th17 axes with age in AD patients combined with a normalization of epithelial abnormalities. Although, integrated blood-skin biomarker models might be a more

holistic way to build a disease profile, we believe that only serum biomarker endotyping can advance personalized therapeutics and may be more patient friendly. Establishing blood biomarker profiles is particularly crucial in children, in whom skin biopsies are challenging. To confirm our findings in different clinical subgroups of AD patients, it would be very interesting to perform a separate evaluation of endotypes in a cohort of Asian and/or African American and paediatric and/or elderly AD patients. Furthermore, biomarker based cluster analysis in patients with other inflammatory skin diseases, including psoriasis, lichen planus or contact dermatitis might be useful as control for our results in future.

The present study provided the first step in the confirmation of our previously reported serum biomarker based patient clusters[9], by replicating three of the four clusters in a different retrospective cohort. Additionally, we constructed a prediction model which was able to stratify patients into one of the four clusters by using only 10 serum biomarkers. The use of a small set of biomarkers to predict patients' cluster status may easily be incorporated in clinical trials and standard practice. Given the introduction of new targeted therapies for AD, the use of endotypes may be helpful, since patient with different endotypes might respond differently to the same treatment. Future longitudinal clinical studies are needed to investigate whether the defined endotypes are stable over time and whether patients might switch between clusters over clinical course (after treatment with systemic immunosuppressive or immunomodulating drugs for instance). Subsequently, confirmation of the endotypes and prediction model in clinical trials including AD patients treated with drugs targeting specific pathways will be the final step in the confirmation of endotypes in AD.

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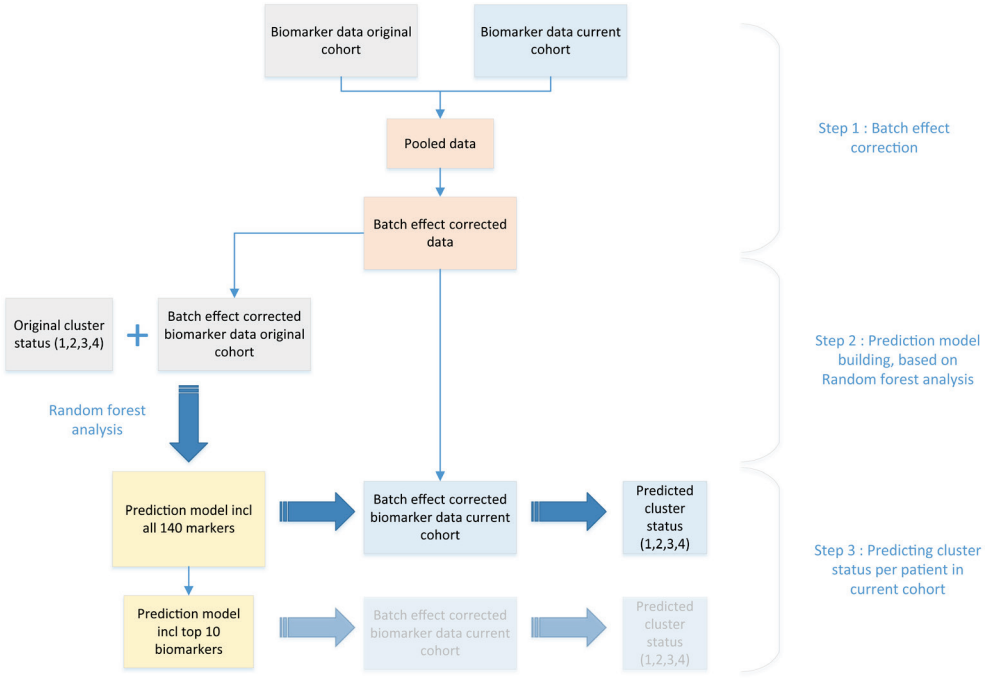
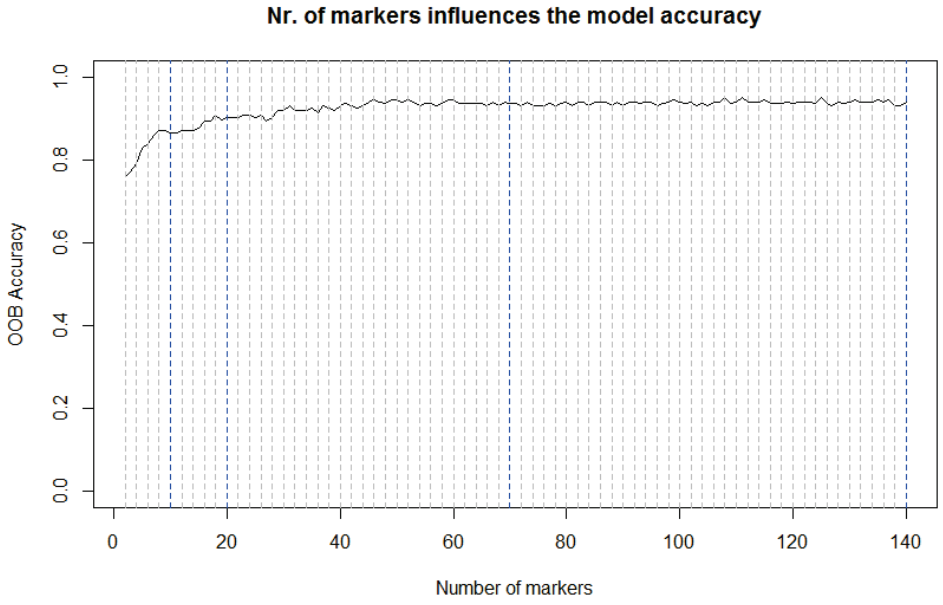
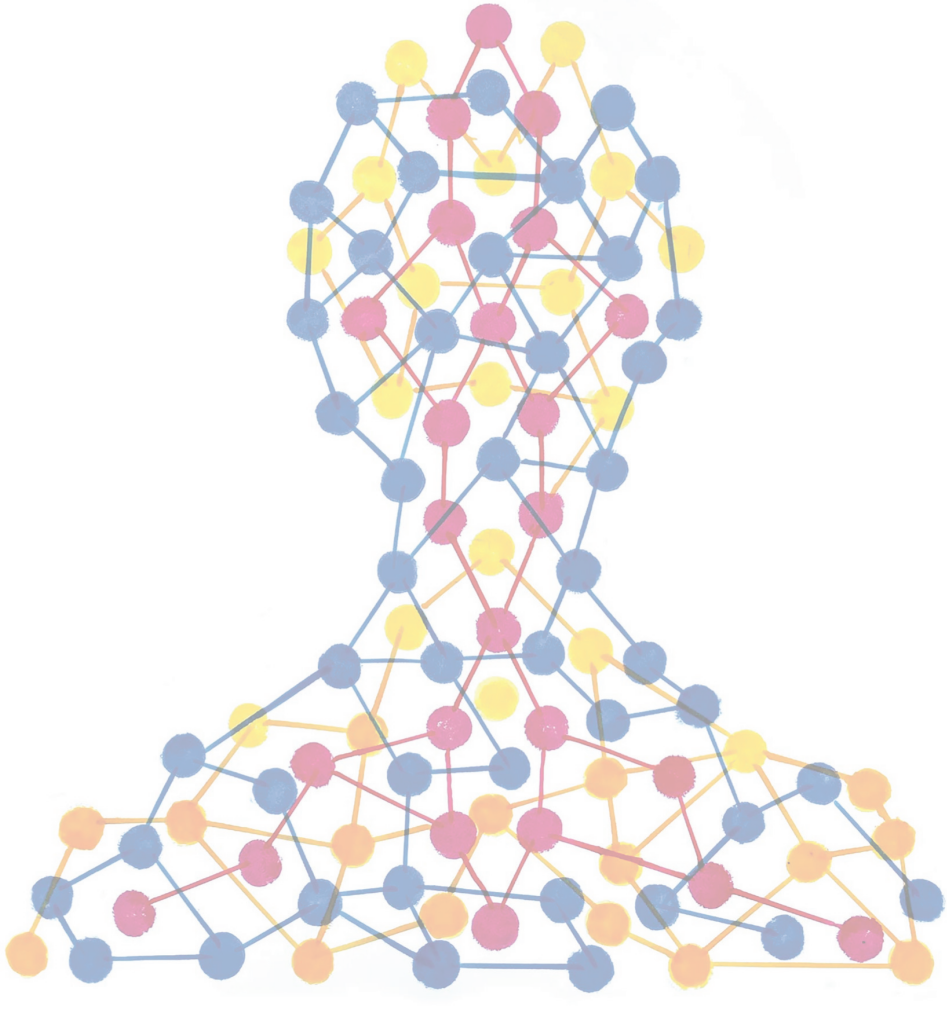
SUPPLEMENTAL FIGURES



Supplemental Figure 1. Cluster status prediction model. Flowchart presenting all steps of the prediction model based on the biomarker data used in the study of Thijs et al.[2] to predict in which of the four previously identified clusters the patients in the current cohort would belong.



Supplemental Figure 2. Prediction model's accuracy. The prediction model build on the original cohort including all 140 overlapping biomarkers showed an OOB accuracy (defined as the number of correct classifications divided by the number of patients) of 94.1% for classification of cluster status per patient in the current cohort. The model including the top 70 markers showed an OOB accuracy of 93.6%, the model including the 20 markers showed an OOB accuracy of 90.4% and the model including the top 10 markers showed an OOB accuracy of 86.7%.



Chapter 4

Unraveling heterogeneity in pediatric atopic dermatitis: identification of serum biomarker based patient clusters

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ABSTRACT

Background: Increasing evidence shows that pediatric AD differs from adult AD on a biological level. Broad biomarker profiling in all age ranges of pediatric AD patients is however lacking.

Objective: To identify serum biomarker profiles in AD children aged 0–17 years and compare these with previously found profiles in adult AD.

Methods: Luminex multiplex immunoassays were used to measure 145 biomarkers in serum from 240 children with AD (aged 0–17 years). Principal components analysis followed by unsupervised k-means cluster analysis were performed to identify patient clusters. Patients were stratified into age groups (0-4 years, 5-11 years, and 12-17 years) to assess association between age and cluster membership.

Results: Children aged 0-4 years had highest levels of Th1- and Th17-related markers. Th2-related markers did not significantly differ between age groups. Similarly to adults, cluster analysis identified four distinct pediatric patient clusters ("Th2/retinol dominant", "skin-homing dominant", "Th1/Th2/Th17/IL-1 dominant", and "Th1/IL-1/eosinophil inferior" cluster). Only the "Th1/Th2/Th17/IL-1 dominant" cluster resembled one of the previously identified adult clusters (representing 18% of children and 18.5% adults respectively). While AD severity was associated with cluster memberships, no association with age or age of onset was found.

Conclusion: Four distinct patient clusters based on serum biomarker profiles could be identified in a large pediatric AD cohort, of which one was similar to previously identified adult clusters. The identification of endotypes driven by distinct underlying immunopathological pathways might be useful to define pediatric AD patients at risk of persistent disease and may necessitate different targeted treatment approaches.

INTRODUCTION

Atopic dermatitis (AD) is the most common chronic inflammatory skin disease, affecting up to 20% of children and up to 10% of adults.[1-3] AD can present at all ages, but mostly begins in early childhood. Although the general consensus is that most pediatric AD patients will eventually 'outgrow' the disease, recent studies suggest that persistence into adulthood is more common than previously recognized.[4] The clinical presentation and distribution of AD in childhood and adults is clearly different[5-7], and atopic comorbidities, including food allergy, asthma, and allergic rhinitis, develop over the course of infancy and childhood, described as the 'atopic march'.[8, 9] Besides the well-known differences in clinical presentation, increasing insights into blood and skin profiles show substantial differences between pediatric and adult AD.[10-13] Although both populations show significant Th2 activation in skin and blood, early-onset pediatric AD also shows Th17/Th22 skewing, but lacks the Th1 upregulation that is seen in adults.[10, 12, 13]

In the past decade it has become increasingly clear that adult AD is not only heterogeneous based on clinical characteristics, but that different pathophysiological mechanisms can be defined in different subgroups of patients. In recent studies, we identified four clearly differentiated adult AD patient clusters, each characterized by a unique serum biomarker profile.[14, 15] These clusters might represent endotypes in which the disease is driven by a distinct underlying mechanism. However, heterogeneity on a biological level has not yet been confirmed in pediatric AD. While most pediatric biomarker data are based on studies in infants and young children with recent-onset AD, broad blood profiling in all age ranges of pediatric AD patients is limited.

The use of endotypes may be helpful to identify AD patients who respond well to specific treatments. The increasing understanding of molecular pathways involved in chronic AD has accelerated the development of more targeted systemic therapies for adult and adolescent AD patients, and will eventually move to children.[16] This necessitates even more the identification of pediatric AD endotypes to optimize safe and effective personalized treatment approaches. Early treatment and AD control in pediatric patients may impact the natural history of the disease.

In the present study, we investigated biomarker profiles in children with AD aged between 0 and 17 years and compared these profiles with the previously found adult

AD endotypes. We expect that by defining biomarker profiles in pediatric AD, we will eventually be able to predict the course of the disease, and optimize personalized medicine approaches.

METHODS

Study design and data collection

Patients and samples

Serum samples from 240 children aged 0 – 17 years, diagnosed with AD as defined by the criteria of Hanifin and Raijka[17], were retrospectively selected. Sera were collected at the Wilhelmina Children's Hospital (University Medical Center Utrecht) between 2014 – 2017, and stored at -80 degrees Celsius in a biobank until analysis. Exclusion criteria for this study were use of systemic immunosuppressive drugs within four weeks before blood sampling. Disease severity was assessed by the Eczema Area and Severity Index (EASI) score. Clinical characteristics were retrospectively extracted from the patients' electronic medical records. Before study inclusion, parents signed institutional review board-approved written consent, according to the Declaration of Helsinki principles. Protocols of this study were approved by the Institutional Review Board of the University Medical Center Utrecht (Utrecht, The Netherlands).

Serum biomarkers

To characterize disease heterogeneity and to identify specific pediatric AD patient clusters, we quantified 145 analytes by a multiplex immunoassay based on Luminex technology[18] as previously described[15], using an in-house validated panel of analytes listed in Supplemental Table E1 (see this article's Online Supporting Information at https://drive.google.com/drive/folders/1X7pdu-VLbwzBWvzLuPbbufrkRktA9n3o?usp=sharing). The panel was selected based on our previous studies in adult AD.[14, 15] Serum samples that were above or below the assay limits of detection were given values equivalent to the lower limit of quantification divided by two or the upper limit of quantification multiplied by two.

Statistical analyses

Serum biomarker data was cleansed of patients with any missing data (1 patient was removed), and subjected to Box-Cox transformation before analyses. To identify

differences in serum biomarker levels within different age groups, patients were stratified into three groups: 0 – 4 years, 5 – 11 years, and 12 – 17 years.

The normalized biomarker data were analyzed using a principal components analysis (PCA), followed by unsupervised k-means cluster analysis, as previously described.[14, 15] The optimal number of clusters was determined by using the elbow method, which looks at the total within-cluster sum of square (WSS) as a function of the number of clusters.[19] The optimal number of clusters was selected to be such that adding another cluster does not significantly reduce the total WSS. To investigate the differences between the age groups, we looked *a posterori* if the defined clusters were associated with age groups. Additionally, we compared the biomarker profiles found in our pediatric AD cohort with the previously described biomarker profiles in adults AD patients.[14, 15]

Clinical characteristics and serum biomarker levels between the age groups and patient clusters were compared using chi-square tests for categorical variables or one-way ANOVA for continuous variables, followed by pairwise t-tests or chi-square tests when appropriate, with Benjamini-Hochberg correction for multiple comparisons, controlling False Discovery Rate (FDR). FDR adjusted P-values <0.05 were considered statistically significant. The association of serum biomarkers with disease severity was evaluated by using Spearman correlation coefficients. All statistical analyses were performed using R Project software (version 3.4.1)[20].

RESULTS

Patient characteristics and age groups

A total of 240 pediatric AD patients (mean age 8.2 years, SD= 5.5) were included. AD disease severity at the moment of sampling ranged from clear to severe, with a mean EASI score of 14.6 (SD=10.7). Clinical characteristics are summarized in Table 1. Disease severity was not significantly different between children aged 0 – 4 years old (n=77, mean EASI 13.3, SD=10.1), 5 – 11 years (n=84, mean EASI 14.3, SD=11.3), and 12 – 17 years (n=79, mean EASI 16.2, SD=10.5)(Table 2). The presence of other atopic comorbidities, including asthma (p=0.001), allergic rhinitis (p=<0.001) and food allergy (p=0.050) was significantly higher in the oldest age group (12 – 17 years). The

youngest age group (0 – 4 years) included significantly more males compared to the other two groups (55.8%, compared to 31.0% and 39.2%, p=0.005).

Table 1. Baseline characteristics total cohort of pediatric AD patients

Clinical characteristics	Total group (n=240)
Age (years)†, mean (SD)	8.2 (5.5)
Male, n (%)	100 (41.7)
EASI score, mean (SD)	14.6 (10.7)
Atopic comorbidities, n (%)	
- allergic asthma	84 (35.0)
- allergic rhinitis	108 (45.0)
- food allergy	87 (36.3)
- no atopic comorbidities	77 (32.1)
Age of onset, n (%)	
- 0 -1 years	180 (75.0)
- 2 – 11 years	48 (20.0)
- 12 – 18 years	2 (0.8)
- missing	10 (4.2)

Categorical variables are presented as counts and percentages; continuous variables are presented as mean with Standard Deviation (SD). EASI Eczema Area Severity Intensity; † age at moment of sample collection.

Table 2. Clinical comparison of different age groups

Clinical characteristics	0 – 4 years (n=77)	5 – 11 years (n=84)	12 – 17 years (n=79)	p-value
Age (years)†, mean (SD)	2.0 (1.4)	7.6 (1.9)	14.9 (1.7)	<0.001
Male, n (%)	43 (55.8)	26 (31.0)	31 (39.2)	0.005
EASI score, mean (SD)	13.3 (10.1)	14.3 (11.3)	16.2 (10.5)	0.271
Atopic comorbidities, n (%)				
Allergic asthma	15 (19.5)	30 (35.7)	39 (49.4)	0.001
Allergic rhinitis	15 (19.5)	40 (47.6)	53 (67.1)	<0.001
Food allergy	21 (27.3)	29 (34.5)	37 (46.8)	0.050
No atopic comorbidities	37 (48.1)	27 (32.1)	13 (16.5)	<0.001
Age of onset, n (%)				0.084
0 -1 years	63 (81.8)	64 (76.2)	53 (67.1)	
2 – 11 years	11 (14.3)	16 (19.0)	21 (26.6)	
12 – 18 years	NA	NA	2 (2.5)	
missing	3 (3.9)	4 (4.8)	3 (3.8)	

Categorical variables are presented as counts and percentages; continuous variables are presented as mean with Standard Deviation (SD). Clinical characteristics between the age groups were compared using a one-way ANOVA or chi-square test when appropriate. P values <0.05 were considered statistically significant. EASI Eczema Area Severity Intensity; † age at moment of sample collection.

Serum biomarker levels were first compared between the three age groups (Figure 1 and supplementary Table E2). By applying this supervised approach, the youngest children, aged 0 – 4 years, were characterized by the highest levels of innate, mostly Th1-skewing markers (IL-18, MCP1/CCL2, TNF receptor 2), epithelial proliferation and differentiation (epidermal growth factor [EGF]), B-cell homing (BLC/CXCL13), adhesion molecules (P-selectin, sICAM), the adipokine adiponectin, and the proinflammatory cytokine macrophage migration inhibitory factor (MIF). Children aged 0 – 4 years were also characterized by the lowest levels of the Th17-related marker trappin2/elafin. Children aged 5 – 11 years were distinguished from the other age groups by the highest levels of the TNF superfamily members TWEAK/TNFSF12 and TACI/TNFRSF13B. The oldest children, aged 12 – 17 years, were characterized by the highest serum levels of markers related to tissue remodeling (MMP-1, MMP-3, MMP-9), and the lowest levels of the adhesion molecule sICAM and the multifunctional glycoprotein osteopontin (OPN). Th2-related (IL-5, IL-13, TARC/CCL17, MDC/CCL22, MCP-4/CCL13) and Th22-related (IL-22) markers were higher expressed in children aged 0 – 4 years compared to the other age groups, however, not statistically significant.

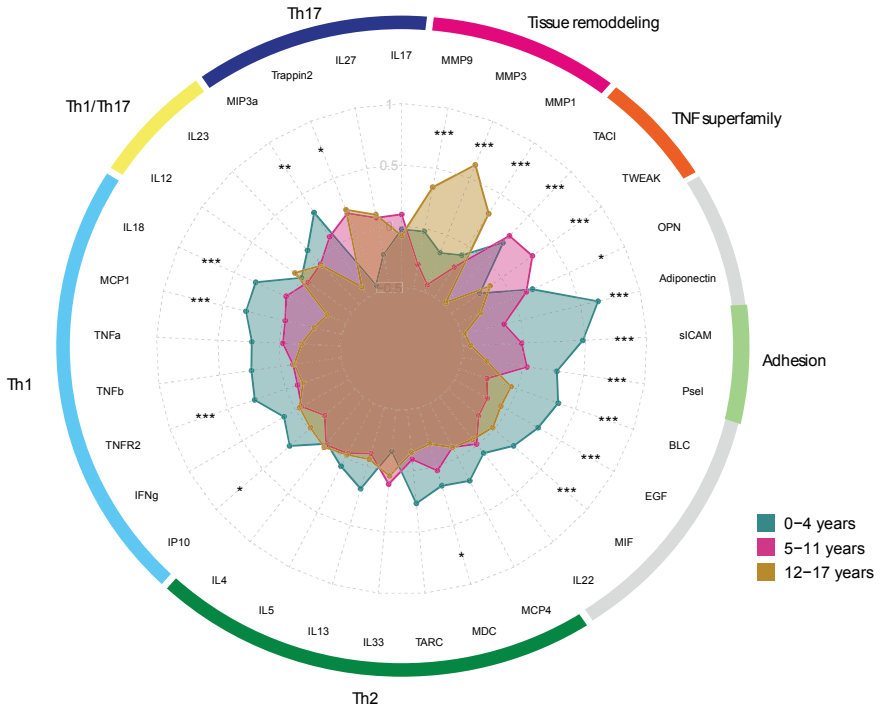

Figure 1. Biomarker profiles in pediatric AD children divided in three different age groups. Averages of Box-Cox transformed serum biomarker levels were compared between AD children aged 0 – 4 years, 5 – 11 years, and 12 – 17 years at the moment of sampling. Radar plot shows biomarker profiles per age group for selected markers based on significance and AD-related pathways. Spoke lengths represent means of Box-Cox transformed data per variable. Significance levels for one-way ANOVA results are presented with asterisks. P-values lower than 0.05 were considered statistically significant Significance levels correspond to the following P-values: *P < 0.05, **P < 0.01, and ***P < 0.001.

Correlation of biomarkers with disease severity

We next investigated which serum biomarkers were associated with AD disease severity by determining correlation of each measured serum biomarker with EASI scores in all 240 pediatric patients (Figure 2). Significant positive correlations between disease severity were found for PARC/CCL18 (r=0.63), apelin (r=0.53), IL-1R2 (r=0.49), TARC/CCL17 (r=0.48), MMP-1 (r=0.48), CTACK (r=0.41), elastase (r=0.40), I309 (r=0.40), MDC (r=0.38), sVCAM (r=0.36), E-selectin (r=0.36), IL-22 (r=0.31), and S100A8 (r=0.31). EASI score was significantly negatively correlated with RBP4 (r=-0.68), CatS (r=-0.66), ACE (r=-0.48), IL-25 (r=-0.36), IL-26 (r=-0.36), NAP2 (r=-0.35), and MMP-8 (r=-0.30). Overall, correlation coefficients from these markers with disease severity were comparable between the three age groups and the total cohort (Supplementary Table E3).

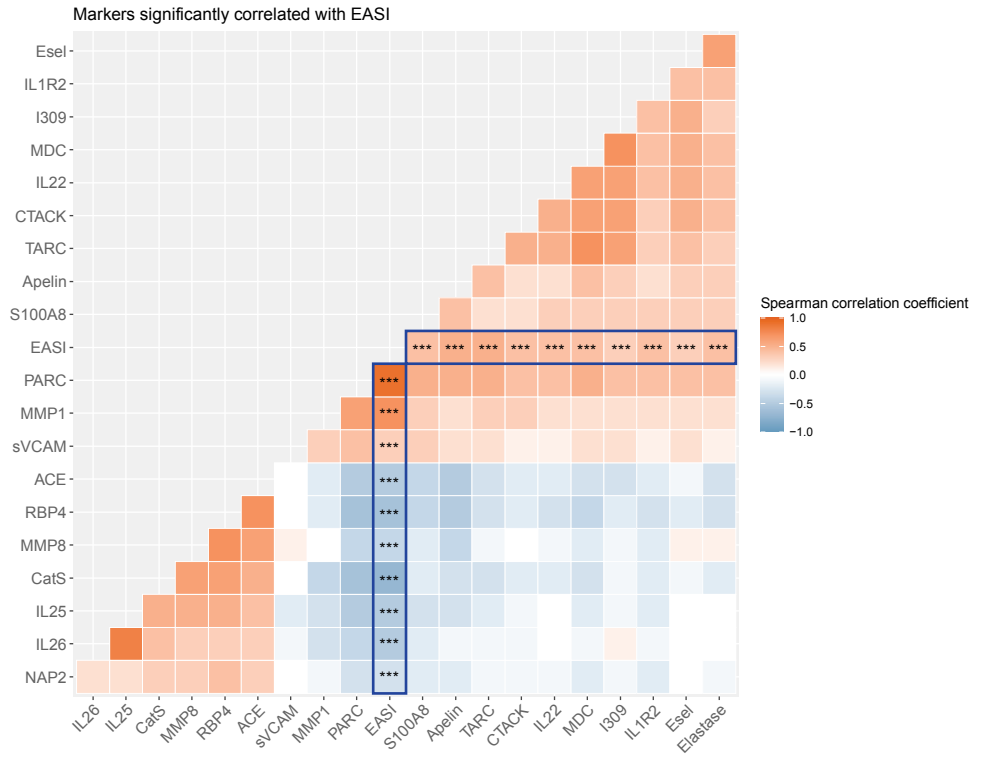

Figure 2. Correlation of disease severity with serum biomarkers in pediatric AD patients. Heatmap of Spearman correlations between serum biomarkers and AD disease severity measured by Eczema Area and Severity Index (EASI). Heatmap includes only serum biomarkers that significantly correlate with EASI score and have Spearman correlation coefficients >0.30 or <-0.30. Red denotes positive and blue denotes negative correlations. Blue boxes marks correlations between serum biomarkers and EASI, *** represents P-value <0.001.

Characterization of pediatric AD clusters

In the next step, unsupervised analyses was performed on the Box-Cox transformed serum biomarker data of all 240 pediatric AD patients to identify distinct patient clusters based on serum biomarker profiles. After PCA, the cumulative percentage of variance showed that the first 50 principal components described at least 90% of the dataset's variance (Figure 3A), and were, hence, included in the unsupervised k-means cluster analysis. By applying the elbow method on the k-means clustering, four clusters were indicated as the appropriate number of clusters (Figure 3B – C). Clinical characteristics were compared between the four clusters (Table 3). Pediatric AD patient clusters were not influenced by age, as age did not significantly differ between the four clusters (Table 3, p=0.11), and patients from the three age groups were equally divided over the four clusters (Figure 3D, p=0.074).

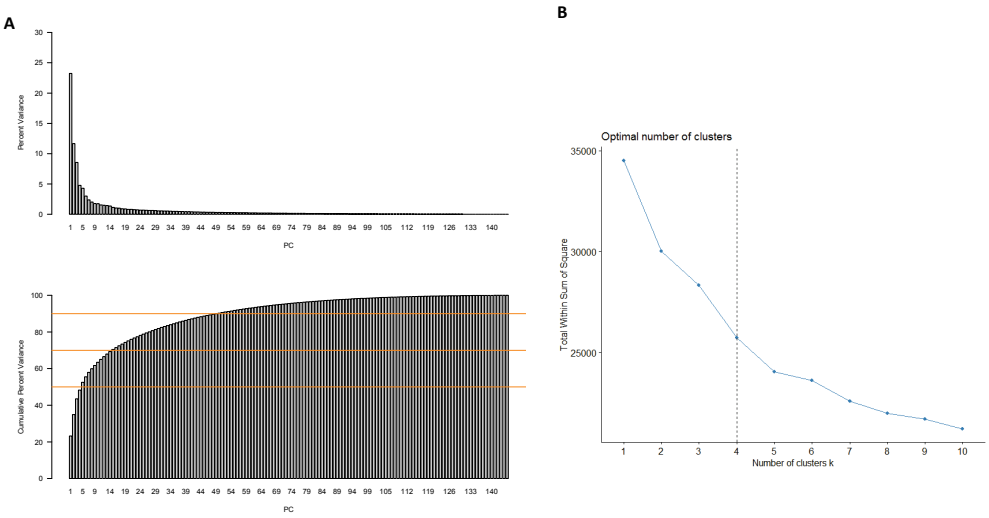

Figure 3. Principal component and cluster analyses of pediatric AD patients. A. Variance described by principal components. The first five principal components (PCs) describe 50% of the variance and the first 50 PCs describe 90% of the variance in the Box-Cox normalized serum biomarker. **B**. The optimal number of clusters was determined by using the elbow method, which looks at the total within-cluster sum of square (WSS) as a function of the number of clusters. The optimal number of clusters was defined as four.

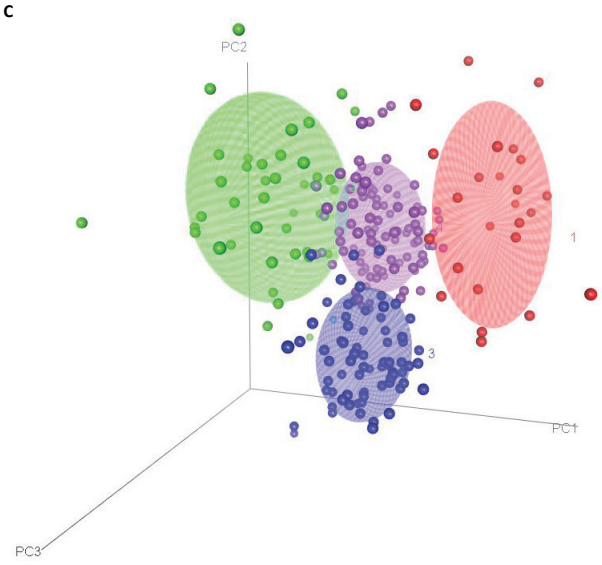

Figure 3C. Using unsupervised k-means clustering of the first 50 PCs resulted in the identification of four pediatric AD patient clusters (1, 2, 3, and 4). 239 patients are presented in a three-dimensional plot in terms of the first three PCs. Colors and colored ellipses represent clusters. PC1 explained 23.3% of the variance, PC2 explained 11.7% of the variance and PC3 explained 8.6% of the variance.

Figure 3D. Two-dimensional plot of 239 patients in terms of the first two PCs. Colors and colored ellipses represent clusters. Symbols represent the three age groups. Patients from all three different age groups were equally divided over the three clusters.

Averages of serum biomarker levels were calculated per cluster to characterize the biomarker profiles driving the four clusters (Figure 4; supplementary Table E4). Cluster 1 was the largest cluster, representing 41% of the pediatric AD population. Cluster 1 patients had a mean age of 8.7 years (SD=5.7 years) and had the lowest mean EASI score (mean 9.2 years, SD=5.4). This cluster was distinct from the other three by having the highest levels of the acute phase protein retinol binding protein 4 (RBP4). Besides, the Th2 cytokines IL-4, IL-5, IL-13, and TSLP, the Th17-related cytokines IL-23 and IL-26 were higher in cluster 1 compared to cluster 2 and 4, but lower compared to cluster 3. Cluster 1 could be defined as the "Th2/retinol dominant" cluster.

Cluster 2 represented 31% of the patients and had a mean age of 8.8 years (SD=5.3 years). Patients in this cluster had a significantly more severe AD compared to the

other clusters ($p<0.001$), with a mean EASI score of 27.8 (SD=7.5). This cluster also had the highest incidence of food allergy (53.4%). Its biomarker profile was characterized by the highest levels of apelin, and markers related to skin-homing (PARC/CCL18, TARC/CCL17, and CTACK/CCL27), and the lowest levels of markers related to tissue remodeling and angiogenesis (adiponectin, MMP-8, TIMP1). Cluster 2 was defined as the "skin homing dominant" cluster.

Cluster 3 represented 18% of the patients, had a mean age of 6.9 years (SD=5.4 years) and mean EASI score of 10.5 (SD=9.1). Cluster 3 was uniquely defined by having the highest levels of biomarkers related to the Th1 pathway (IL-2, IL-12, IFNα, IFNγ, TNFα, TNFß, MIG/CXCL9, ITAC/CXCL11), the Th2 pathway (IL-4, IL-5, IL-13, eotaxin-3/CCL26, TSLP, MCP-4/CCL13), the Th17 pathway (IL-23, IL-26, MIP3a/CCL20, GM-CSF), the IL-1 family pathway (IL-1α, IL-1Rα, IL-1R1, IL-18BPa, IL-37), the TNF superfamily pathway (TNFR1, TNFR2, TWEAK/TNFSF12, LIGHT/TNFSF14), and T-cell activation (sIL2Rα). Cluster 3 could be described as the "Th1/Th2/Th17/IL-1 dominant" cluster.

Cluster 4 represented 10% of the patients, mean age in this cluster was 6.6 years (SD= 4.9 years) and mean EASI score 12.3 (SD=9.1). Patients from this cluster had the lowest incidence of food allergy (24.0%). Regarding the serum biomarker profile, cluster 4 was distinct from the other three clusters by having the highest levels of the chemokines RANTES/CCL5 and PF4/CXCL4, and the monocyte activation marker soluble CD14. Besides, cluster 4 showed the lowest levels of biomarkers related to the Th1 pathway (MIG/CXCL9, ITAC/CXCL11, and MIP1b/CCL2), eosinophil trafficking (eotaxin-1/CCL11 and eotaxin-3/CCL26), the IL-1 family pathway (IL1R1, IL-18BPa), the TNF superfamily pathway (TNFR1, TNFR2, TWEAK/TNFSF12), neutrophil activation and trafficking (elastase, GCP2), and T-cell activation and skin-homing (sIL2Rα , CTACK). This cluster was defined as the "Th1/IL-1/eosinophil inferior" cluster.

In summary, we could identify four distinct pediatric AD patient clusters. Two of the four clusters showed skewing towards the Th2 pathway (cluster 1 and 3), of which cluster 3 was further characterized by a strong immune activation state, related to both innate as well as T-cell immunity. Cluster 2 was clinically defined by the highest EASI score and was characterized by a biomarker profile skewed towards skin-homing-related markers. Besides elevation of few innate immunity-related markers, cluster 4 was overall distinguished by a relatively low inflammatory state.

Table 3. Clinical comparison four clusters

Clinical characteristics	Cluster 1 (n=98)	Cluster 2 (n=73)	Cluster 3 (n=43)	Cluster 4 (n=25)	p-value
Age (years)[1], mean (SD)	8.7 (5.7)	8.8 (5.3)	6.9 (5.4)	6.6 (4.9)	0.109
min – max	0 – 17	0 – 17	0 – 17	0 – 17	
Male, n (%)	52 (53.1)	23 (31.5)	20 (46.5)	5 (20.0)	0.004
EASI score, mean (SD)	9.2 (5.4)	27.8 (7.5)	10.5 (9.1)	12.3 (9.1)	<0.001
Atopic comorbidities, n (%)					
Allergic asthma	39 (39.8)	27 (37.0)	12 (27.9)	6 (24.0)	0.272
Allergic rhinitis	43 (43.9)	38 (52.0)	16 (37.2)	11 (44.0)	0.755
Food allergy	29 (29.6)	39 (53.4)	13 (30.2)	6 (24.0)	0.017
No atopic comorbidities	33 (33.7)	17 (23.3)	16 (37.2)	10 (40.0)	0.267
Age of onset, n (%)					0.384
0 -1 yrs	77 (78.6)	55 (75.3)	26 (60.5)	21 (84.0)	
2 – 11 yrs	17 (17.3)	15 (20.5)	13 (30.2)	3 (12.0)	
12 – 18 yrs	2 (2.0)	0 (0)	0 (0)	0 (0)	
missing	2 (2.0)	3 (4.1)	4 (9.3)	1 (4.0)	

Categorical variables are presented as counts and percentages; continuous variables are presented as mean with Standard Deviation (SD). Clinical characteristics between the patient clusters were compared using a one-way ANOVA or chi-square test when appropriate. P values <0.05 were considered statistically significant. EASI, Eczema Area Severity Intensity; [†] age at moment of sample collection.

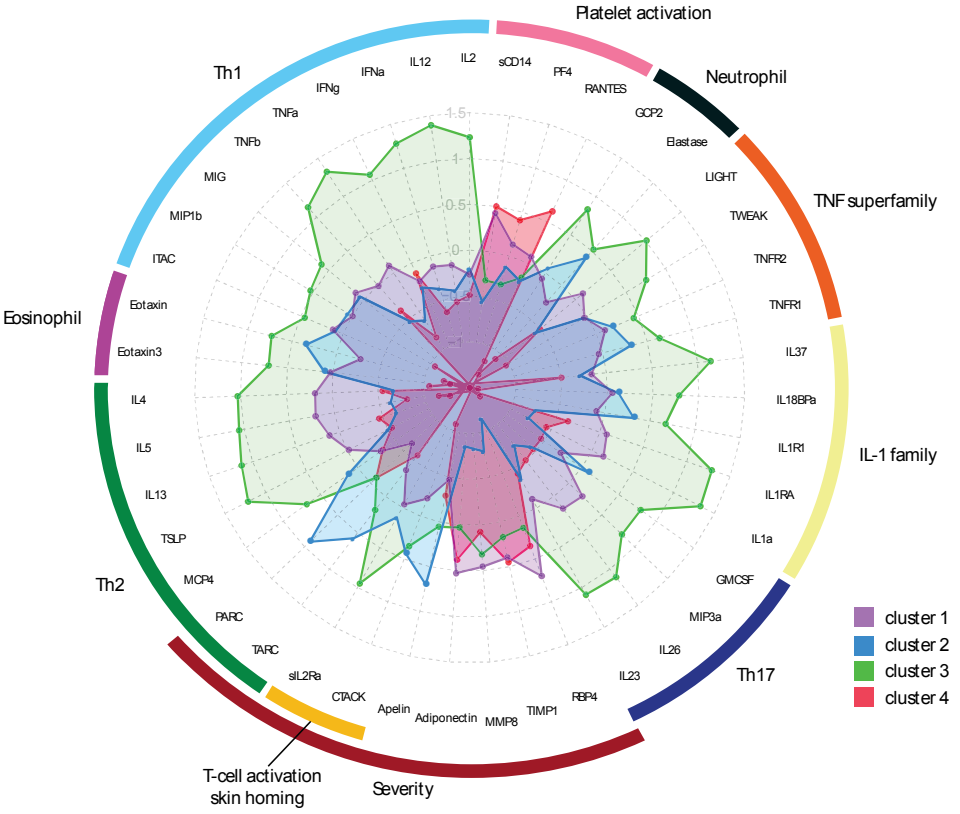

Figure 4. Biomarker profiles of four distinct pediatric AD patient clusters. Averages of Box-Cox transformed serum biomarker levels were compared between the four identified pediatric AD clusters (cluster 1, 2, 3, and 4). Radar plot shows biomarker profiles per cluster for selected markers that were significantly higher or lower expressed in one of the clusters compared to the other clusters. Spoke lengths represent means of Box-Cox transformed data per variable.

DISCUSSION

This is the first study to broadly characterize serum biomarker profiles in a large cohort of pediatric AD patients aged 0 - 17 years. We confirmed heterogeneity at the level of serum biomarkers in pediatric AD patients and identified four patient clusters based on their unique systemic immune profiles, by using an unsupervised clustering approach. Our results suggest unique endotypes in pediatric AD patients, possibly arguing for personalized, endotype-driven therapeutic approaches, rather than the currently used “one-size-fits-all” concept.

Blood biomarker profiles of early-onset pediatric AD have previously been characterized by an upregulation of Th2, Th17 and tissue remodeling markers, and lacked the Th1 upregulation that is seen in adult AD.[10, 11, 13, 21] In contrast to these findings, our pediatric AD patients aged 0 – 4 years, corresponding in age to the previously studied early pediatric AD cohorts[10-13, 22], was characterized by higher expression of innate activation markers, mostly related to Th1, and decreased levels of the Th17 marker trappin/elafin, compared to the older children. The prior studies characterized blood profiles of pediatric AD patients within 6 months after disease onset compared to age-matched healthy controls, which might explain the different findings. Th1-related markers have been identified as marker for disease chronicity and immune development, but in the view of our findings, they might also represent other immune-related mechanisms distinguishing infant and toddler AD from older children and adolescents.[23] The innate activation markers significantly upregulated in the youngest group (IL-18, MCP-1/CCL2, and TNFR2) have been proven to contribute to both Th1 and Th2 cytokine-mediated inflammation. Besides, IL-18 and MCP-1/CCL2 are associated with severity of pediatric AD.[24-28] Pediatric AD is supposed to be an even more Th2-dominant disease than adult AD. Although not significant, other Th2-related markers including IL-5, IL-13, TARC/CCL17, MDC, and MCP-4 were higher expressed in the youngest children, compared to the 5 – 17 years old children. As AD is a primarily Th2-driven disease, it could be that Th2 cytokines are upregulated in all pediatric AD patients, and therefore not different within the three age groups. Serum samples from age-matched healthy controls are needed to further investigate this.

The previously described positive correlations of pediatric AD severity with TARC/CCL17, PARC/CCL18, CTACK/CCL27, MDC/CCL22, E-selectin, and the IL-1 decoy receptor IL-1R2 were also present in our study.[10, 29-31] MMP-1, an inflammatory marker related to tissue remodeling, and previously described to be negatively associated with skin scores in early-onset AD patients (mean age 1.8 , SD= 1.6 years)[10], showed positive correlation with EASI in our cohort. In a previous study by Thijs et al.[32] MMP-1 also showed a significant positive correlation with disease severity in adult AD. The difference in the direction of the correlation of MMP-1 with disease severity may therefore reflect age and chronicity of the disease. Retinol binding protein (RBP)4 showed a strong negative correlation with EASI scores in our cohort. Both lower retinol levels and RBP4 expression have been detected in skin samples of adult AD, and a negative association of serum retinol with AD severity was reported in children.[33, 34] Retinol has important immunomodulatory effects, and

decreased levels of RBP and vitamin A are associated with infection and inflammation.[35-37] These data might support the negative correlation with EASI in our pediatric cohort as an effect of excessive skin inflammation. In our large pediatric AD cohort, EASI was scored by several different physicians. The subsequent higher inter-rater variability might have resulted in relatively lower correlation coefficients in our study.

By using an unsupervised clustering approach, we could identify four pediatric AD patient clusters, characterized by certain serum biomarkers that were significantly differentially expressed compared to the other clusters. Patients stratified in cluster 1 had the lowest disease severity and were characterized by the highest levels of RBP4, which showed the strongest negative correlation with EASI. Additionally, cluster 1 showed higher levels of IL-4, IL-5, IL-13 and TSLP, and could be defined as the "Th2/retinol dominant" cluster. Patients in cluster 1 and 3, representing 59% of the patients, shared a Th2-cytokine high profile, corresponding to the percentage of Th2-dominant patients as previously reported in adults.[14, 15] These patients would hypothetically be the most ideal candidates for Th2 targeting drugs.

In contrast to the other three clusters, cluster 4 showed a relatively low inflammatory state, with no clear immune skewing, and could be distinguished from the other clusters as being the "Th1/IL-1/eosinophil inferior" cluster. Cluster 4 was defined by elevation of the monocyte activation markers RANTES/CCL5, PF4/CXCL4, and sCD14. Elevated platelet activation, as shown by higher levels of PF4/CXCL4, has been suggested to play a role in the pathomechanism of chronic skin inflammation in AD, by inducing leukocyte recruitment and through direct activation of local capillary endothelial cells and attraction of effector T-cells to the skin.[38] Both RANTES/CCL5, a potent eosinophil, monocyte, basophil and lymphocyte chemo-attractant, and the monocyte activation marker sCD14 have shown evidence of association with AD as well.[39-41]

By focusing on the driving pathways per patient cluster, only one out of the four pediatric AD clusters was comparable to one of the previously defined endotypes in adult AD patients.[14, 15] The biomarker profile of pediatric "Th1/Th2/Th17/IL-1 dominant" cluster 2 corresponds to the profile of the "Th1/Th2/Th17 dominant" cluster identified in adult AD patients. These results strengthen the previous findings that blood profiles in pediatric AD differ from adult AD patients. However, the identified biomarker-based pediatric patient clusters in the current study were not influenced by age or age of onset. Added to this, the absolute differences in

biomarker profiles between the four unsupervised identified clusters were more pronounced than, and might overrule the differences in serum biomarker levels between the three age groups that were found by supervised analysis. Although three of the pediatric patient clusters differ from the previously identified adult AD clusters, our results might indicate that the distinct pathophysiologic mechanisms driving the heterogeneity of pediatric AD cannot be solely assigned to the difference in age or duration of the disease, and argue for endotype-specific, rather than uniform or age-specific therapeutic strategies.

One of the most important questions regarding disease heterogeneity in pediatric AD is in which patient the disease will resolve and in whom it will persist into adulthood. One could speculate that patients with resolving childhood AD and persisting disease may represent separate endotypes. Early identification and targeted treatment of the non-resolving endotype might theoretically prevent the atopic march and persistence of AD into adulthood. Previous birth cohort studies have shown that one of the strongest risk factor for non-resolving AD is disease severity, and that the presence of asthma and allergic rhinitis did not affect the course of AD.[42-44] In contrast to the previous studies in adults[14, 15], our current study showed that cluster membership of pediatric AD patients was influenced by disease severity. Patients in cluster 3 had significantly higher EASI scores compared to the other three clusters. Their driving biomarker profile was characterized by the highest levels of the Th2 cytokine PARC/CCL18 and apelin, and the lowest levels of RBP4, MMP-8, and ACE, all biomarkers that were significantly correlated with EASI scores. Patients in cluster 3 represented 31% of the total cohort, which is consistent with data from large birth cohorts showing that up to one third of the children diagnosed with AD had persistent disease.[42-44] On the other hand, patients in cluster 2 showed a comparable biomarker profile to the adult AD patients previously stratified into the "Th1/Th2/Th17 dominant" cluster[15], and might thus be considered to have a higher risk for non-resolving AD. Longitudinal follow-up studies are needed to confirm the endotype of each cluster, and to investigate whether the persistence of AD is related to one of the four endotypes and whether endotypes are stable over time or might change after treatment with systemic immunosuppressive of immunomodulatory drugs. Comparing the profile of cleared versus persistent pediatric AD, will better define biomarker-specific characteristics that predict AD clearance.

Despite inclusion of different age groups, this study was not longitudinal and thus did not follow the same cohort over time. Another limitation is the lack of age-

matched healthy controls, which makes it difficult to distinguish disease-specific from age-specific differences in biomarker profiles, although the patient clusters were not influenced by age.

By using an unbiased and unsupervised profiling approach, the findings of this study indicate that pediatric AD is a biologically heterogeneous disease. We could identify four distinct patient clusters based on serum biomarker profiles in a large cohort of pediatric AD patients aged 0-17 years. Cluster membership was not influenced by age or age of onset, but disease severity seems to be associated with patient clustering. The identification of endotypes driven by distinct underlying immunopathological pathways might be useful to define pediatric AD patients at risk of persistent disease and may necessitate different targeted treatment approaches. Future longitudinal studies will be needed to further validate the endotypes and may provide additional insights into the stability of endotypes in pediatric AD patients over time.

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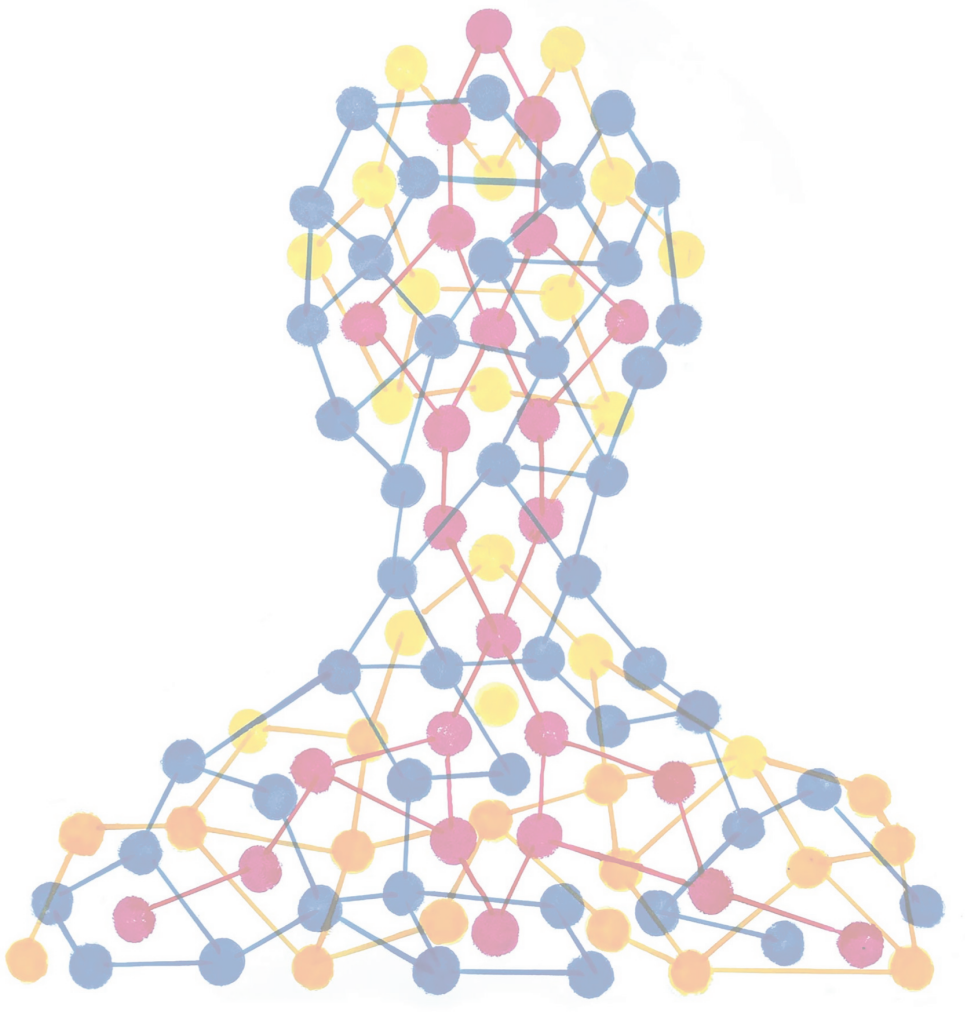

Chapter 5

Early and long-term effects of dupilumab treatment on circulating T-cell functions in moderate-to-severe atopic dermatitis patients

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ABSTRACT

Dupilumab, a monoclonal antibody targeting the interleukin-4 receptor alpha (IL-4Rα), markedly improves disease severity in atopic dermatitis (AD) patients. However, the effect of IL-4Rα blockade on dynamics of circulating skin-homing T-cells, which are crucial players in the pathologic mechanism of AD, has not been studied yet. In addition, it remains unknown whether dupilumab treatment induces long-lasting T- and B-cell polarization. Therefore, we studied the short- and long-term effects of dupilumab treatment on IL-4Rα expression and T-cell cytokine production within total and skin-homing (CLA+/CCR4+) subpopulations in moderate-to-severe AD patients. Dupilumab treatment completely blocked IL-4Rα expression and STAT-6 phosphorylation in CD19+ B-cells and CD4+ T-cells already within two hours of administration and through week 52. Although no change in the proportion of skin-homing T-cell subsets was found, dupilumab treatment significantly decreased the percentage of proliferating (Ki67+) and Th2/Th22 cytokine-producing skin-homing CD4+ T-cells already at week 4. No evidence of general Th-cell skewing following a year of dupilumab treatment was found. In summary, dupilumab treatment rapidly and stably inhibited IL-4Rα, which was accompanied by a strong early functional immunological effect specifically on skin-homing T-cells, without effecting overall Th-cell skewing on the long-term.

INTRODUCTION

The pathogenesis of atopic dermatitis (AD), a common chronic-inflammatory skin disease, is mainly driven by CD4+ T helper 2 (Th2) cell-mediated responses. The type 2 inflammation in AD is characterized by profound overexpression of type 2-related cytokines, such as interleukin (IL)-4, IL-5, IL-13, IL-31, and thymus and activation-regulated chemokine (TARC/CCL17), in both skin and blood.[1] Recently, activation of other T-cell axes, such as Th1, Th17/IL-23 and Th22 has also been reported in AD patients.[2]

During the past decades more insight into the pathogenesis of AD has led to the development of novel targeted therapies. Dupilumab (Dupixent®) is the first targeted antibody-based treatment for moderate-to-severe AD that has been approved in the EU, US, Japan, and other countries.[3] It is a fully human monoclonal IgG4 antibody, targeting the IL-4-receptor alpha, best known for the regulation of IgE production by B-cells, and in a lesser amount also expressed on T-cells, where it promotes differentiation of Th2 cells.[4, 5] By dually inhibiting the signaling of IL-4 and IL-13, dupilumab has demonstrated significantly improved clinical and patient-reported outcomes in moderate-to-severe AD patients.[6-12] Previous studies have shown that dupilumab significantly reduces circulating serum levels of type 2 biomarkers and suppresses Th2, Th17, and Th22 inflammatory pathways in lesional skin as early as 4 weeks after treatment initiation.[7, 13] However, the effect of IL-4Rα blockade on circulating, skin-homing T cells, which are crucial players in the pathologic mechanism of AD, has not been studied yet.

Peripheral tissue-homing receptors enable T-cell subsets to traffic through distinct domains of non-lymphoid peripheral tissues.[14] The cutaneous lymphocyte antigen (CLA) and the chemokine receptors (CCRs) CCR4 and CCR10 have been proposed as critical mediators of skin-specific Th-homing, by binding to E-selectin, TARC/CCL17 and CTACK/CCL27, respectively.[15, 16] CLA+ T cells recirculate between skin and peripheral blood, where they might reflect effector T-cells in AD skin lesions.[17] Therefore, circulating skin-homing T-cells might serve as cellular peripheral biomarkers and as a source of translational knowledge in T-cell mediated skin diseases as AD.[18] In addition, an important unanswered question is whether long-term blockade of IL-4Ra may lead to skewed T-cell responses with increasing Th1/Th17 polarization. Several recent case-reports have reported Th1/Th17-

mediated adverse effects developing newly in AD patients during dupilumab treatment, including psoriasis [19-21], alopecia areata [22], and rosacea [23-25].

In this study, we investigated the effects of dupilumab on the peripheral total and skin-homing T-cell functional dynamics and polarization in moderate-to-severe AD patients after 4, 16, and 52 weeks of treatment. In addition, we measured IL-4Rα expression on T- and B-cells, and responsiveness of these cells to recombinant human IL-4.

RESULTS

Dupilumab treatment rapidly blocks IL-4Rα on B- and T-cells and downstream signaling reflecting clinical response

To longitudinally study short- and long-term effects of dupilumab treatment on IL-4Rα expression and T-cell cytokine production, we included ten moderate-to-severe AD patients (6 male; median age 50.0, IQR 46.0 – 54.5, median EASI score 16.8, IQR 13.8 – 21.1) treated with 300mg dupilumab every other week for at least 52 weeks (experiment 1, Supplemental Table S1 in this article's Online Supporting Information at https://drive.google.com/drive/folders/1X7pdu-VLbwzBWvzLuPbbufrkRktA9n3o?usp=sharing). Comparable to previous studies [6-8, 26], dupilumab treatment significantly improved measures of disease severity from week 4 through week 52, including Eczema Area Severity Index (EASI) scores and serum TARC/CCL17 levels, with the most robust decrease during the first four weeks of treatment (p=0.002; see Supplemental Figure S1a-1b in this article's Online Supporting Information).

The rapid clinical response in our patients was reflected by a complete blockade of IL-4Rα on total and naïve (CD27-IgD+) CD19+ B-cells at week 4, which remained stable through week 52 (Figure 1A, Supplemental Figure S2 in this article's Online Supporting Information). Although T-cells expressed much lower levels of IL-4Rα than (naïve) B-cells at baseline and in HC samples, also T-cells (especially CD4+ T-cells) showed a significant reduction in measurable IL-4Rα levels from baseline through weeks 4-52 (Figure 1B).

No differences in peripheral blood (naïve) CD19+ B-cell and CD3+ T-cell numbers (data not shown) and CD4+/CD8+ T-cell ratio were observed over time

(Supplemental Figure S3 in this article's Online Supporting Information). Reduced measurable IL-4Rα levels were accompanied by clear binding of dupilumab antibodies to the surface of both T- and B-cells after 4 and 16 weeks of treatment, with no differences between these two time points (Figure 1C-D).

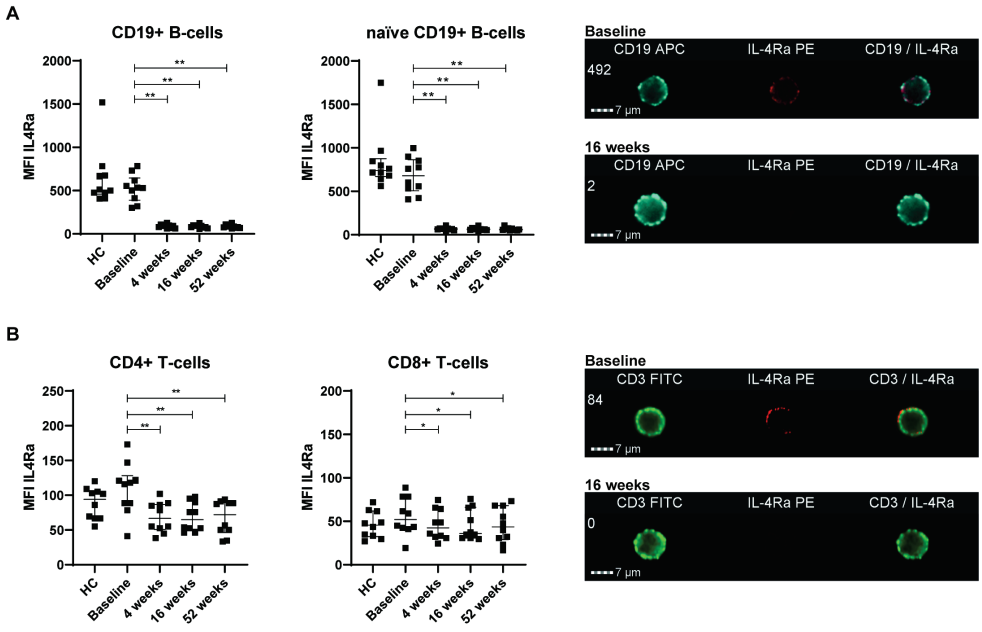

Figure 1. Effect of dupilumab binding on measurable IL-4Rα levels. A. The median fluorescence intensity (MFI) of IL-4Rα on total and naïve (CD27-IgD+) CD19+ B-cells after 4, 16 and 52 weeks of treatment, compared to healthy controls (HC) (left and middle panel), median with interquartile range are presented. Representative ImageStream visualization of measurable IL-4Rα levels (red) on a CD19+ (blue) B-cell at baseline and after 16 weeks of treatment (right panel). **B**. MFI of IL-4Rα on CD4+ and CD8+ T-cells before initiation of dupilumab treatment (baseline) and after 4, 16 and 52 weeks of treatment, compared to HC (left and middle panel), median with interquartile range are presented. Representative ImageStream visualization of measurable IL-4Rα levels (red) on a CD3+ (green) T-cell at baseline and after 16 weeks of treatment (right panel).

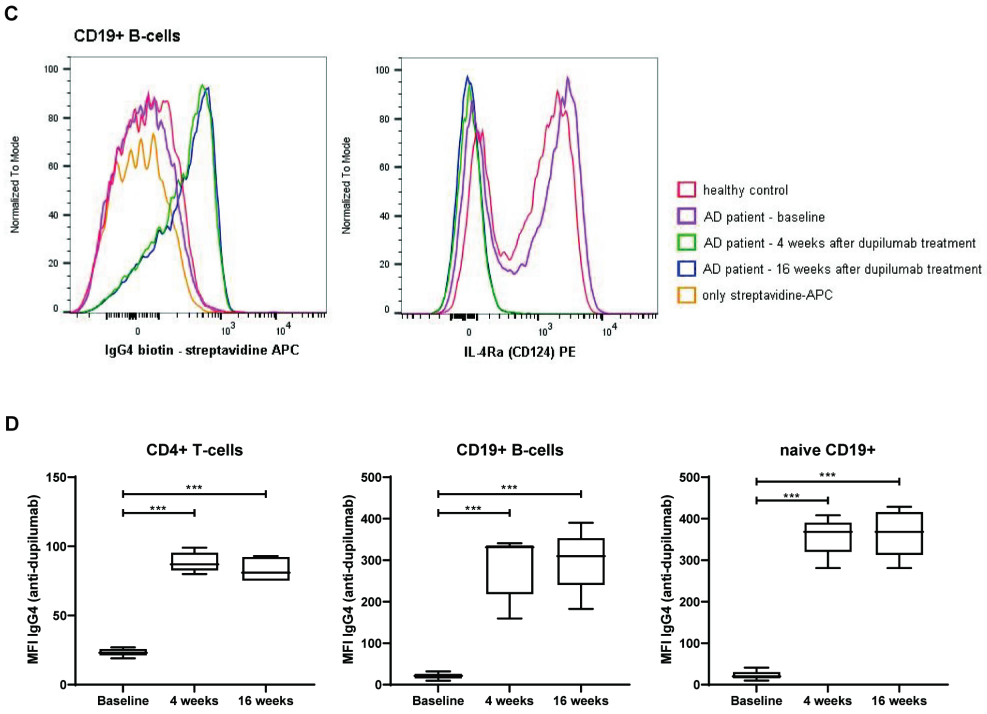

Figure 1. C. Representative histogram of flow cytometry analysis of IgG4 (dupilumab) (left) and IL-4Rα (right) on CD19+ B-cells from an AD patient before initiation of dupilumab (purple), and after 4 weeks (green) and 16 weeks of dupilumab treatment (blue), an healthy control (red), and the control staining including only the secondary antibody streptavidine-APC (yellow). **D**. MFI of IgG4 (dupilumab) on CD4+ T-cells, CD19+ B-cells and naïve CD19+ B-cells from 5 AD patients before dupilumab treatment (baseline) and after 4 and 16 weeks of treatment. MFI IgG4 was corrected for MFI only secondary antibody streptavidin-APC. Boxes represent median with interquartile range, whiskers indicate minimum and maximum. Significance levels correspond to the following P values: *P < .05, **P < .01 and ***P < .005

Next, we investigated the very early effects of dupilumab on T- and B-cell IL-4Rα occupancy, and on their reactivity to IL-4. No appreciable differences were found between patients at baseline and healthy controls in respect to receptor expression and functional response. However, already within two hours after the first dupilumab administration, IL-4Rα detection was abolished, and reciprocal dupilumab binding was detected on the surface on all cell types studied (Figure 2A-B). On a functional level, this finding indicated a weaker signalling of intracellular phosphorylated signal transducer and activator of transcription 6 (pSTAT6) in response to stimulation with recombinant human IL-4 *in vitro* (Figure 2C, Supplemental Figure S4 in this article's Online Supporting Information).

Correspondingly, TARC/CCL17 levels in supernatants of lesional and non-lesional skin biopsies decreased (although not significantly) from baseline through week 4 in a clinically comparable cohort of eight AD patients (experiment 5, Supplemental Table S1 in this article's Online Supporting Information) treated with dupilumab (Supplementary Figure S1C in this article's Online Supporting Information). Thereby, TARC/CCL17 levels in supernatants of lesional skin biopsies were significantly higher ($p=0.028$) compared to supernatants of non-lesional skin biopsies at baseline as well as at 4 weeks. Total serum IgE levels were significantly higher at baseline compared to HC samples and steadily decreased from baseline through week 52 in all patients (Supplemental Figure S1D in this article's Online Supporting Information). In summary, dupilumab showed a rapid and stable blockade of IL-4Rα accompanied by a decrease in clinical and severity-related molecular markers, both locally and systemically.

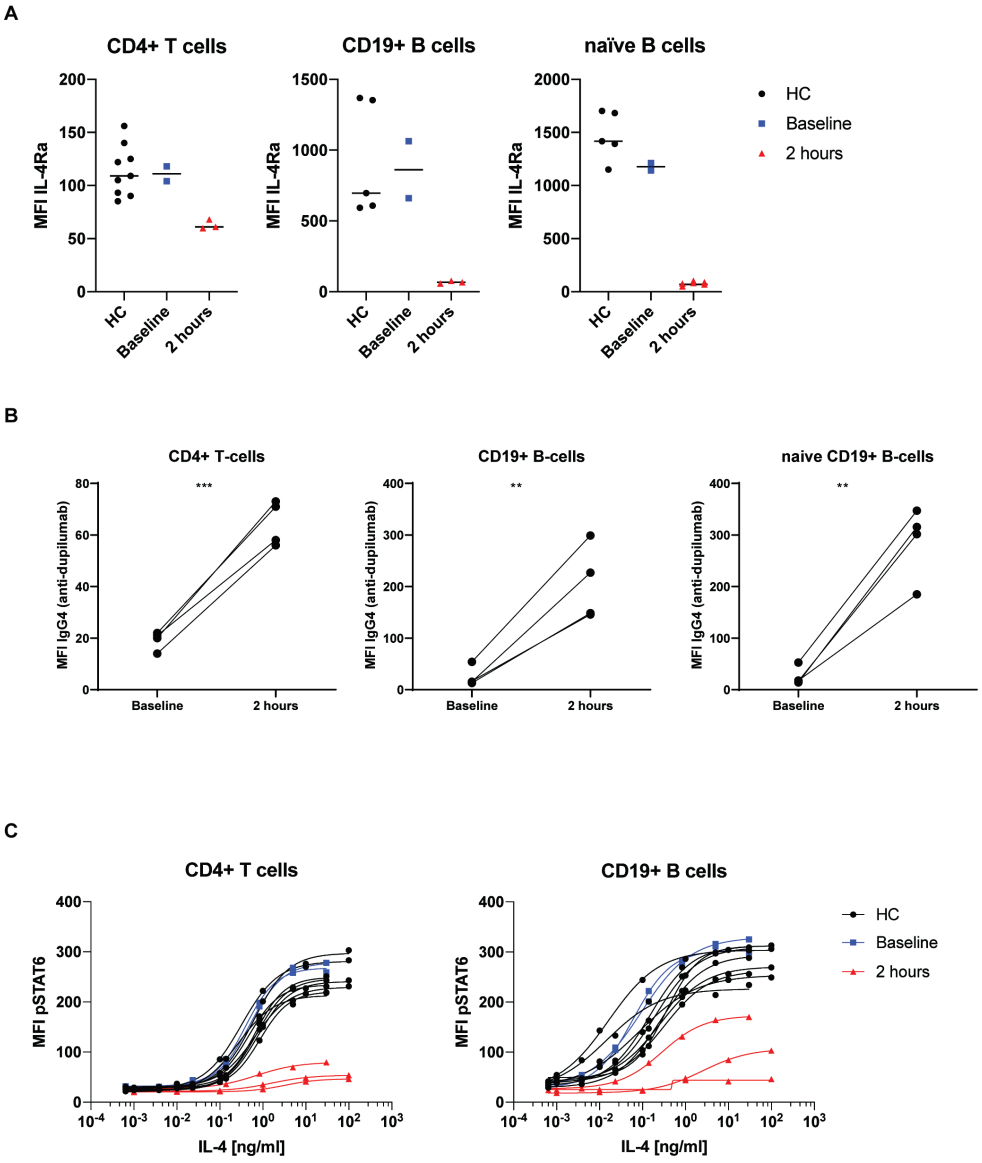

Figure 2. Very early effects of dupilumab binding on measurable IL-4Rα levels and signaling. **A.** The median fluorescence intensity (MFI) of IL-4Rα on CD4+ T-cells, total and naïve (CD27-IgD+) CD19+ B-cells before (in 2 patients) and within 2 hours after first dupilumab administration (in 3 patients) compared to healthy controls (HC). Horizontal lines represent median values. **B.** MFI of IgG4 (anti-dupilumab) on CD4+ T-cells, CD19+ B-cells and naïve CD19+ B-cells from four AD patients before and within 2 hours after the first dupilumab administration. Significance levels correspond to the following P values: *P < .05, **P < .01 and ***P < .005. **C.** Dose-response curves of pSTAT6 response to IL-4 of CD4+ T-cells as well as total CD19+ B-cells.

Dupilumab treatment modulates proliferation of skin-homing T-cells without affecting the proportion of total and skin-homing T-cell subsets

Next, we analyzed the effect of dupilumab treatment on the proportion and proliferation of different T-cell populations. Surface staining was used to examine changes in the total (CD3+), helper (CD4+), cytotoxic (CD8+), skin-homing T-cells (CLA+), and the skin-homing subpopulations (CCR4+/CLA+, CCR10+/CLA+ and CRTH2+/CLA+)during dupilumab treatment (Figure 3a). Absolute lymphocyte counts measured at the time PBMCs were isolated did not change during dupilumab treatment, besides a slight increase from baseline through week 16 (data not shown). At baseline, the proportion of total skin-homing (CLA+) CD4+ T-cells and specific CCR10+CLA+CD4+ T-cells in PBMCs from AD patients was slightly lower compared with HC samples (Figure 3A). The vast majority of CLA+CD4+ T-cells consisted of CD45RA- memory T-cells (Supplemental Figure S5 in this article's Online Supporting Information), with no difference between HC and patients. CCR4+ and CRTH2+ CLA+CD4+ T-cells in patients at baseline did not significantly differ from HC. There were no differences in CD8+ T-cell (skin-homing) subsets between patients and controls (data not shown).

Although the percentage of skin-homing CD4+ and CD8+ T-cells in PBMCs from AD patients remained relatively stable during dupilumab treatment (Figure 3A – B), a striking difference in the proliferation of skin-homing T-cells before and during dupilumab treatment was noted. First of all, the frequency of proliferating (Ki67+) T-cells was highly increased in the CLA+ population compared to the CLA- population, especially in the CD4+ T-cells (Figure 3C), which might be explained by the (effector) memory phenotype of the skin-homing T-cells. Furthermore, the proportion of proliferating (Ki67+) CLA+CD4+ and CLA+CD8+ T-cells was significantly higher in PBMCs of AD patients at baseline compared to HCs and significantly decreased from baseline through week 16 and 52 of dupilumab treatment (Figure 3C). Similar effects during treatment were observed in the CLA- Ki67+ CD8+ and CD4+ T-cells. Taken together, these results might indicate that dupilumab treatment suppresses proliferation of skin-homing T-cells, while their relative proportion remains unaffected.

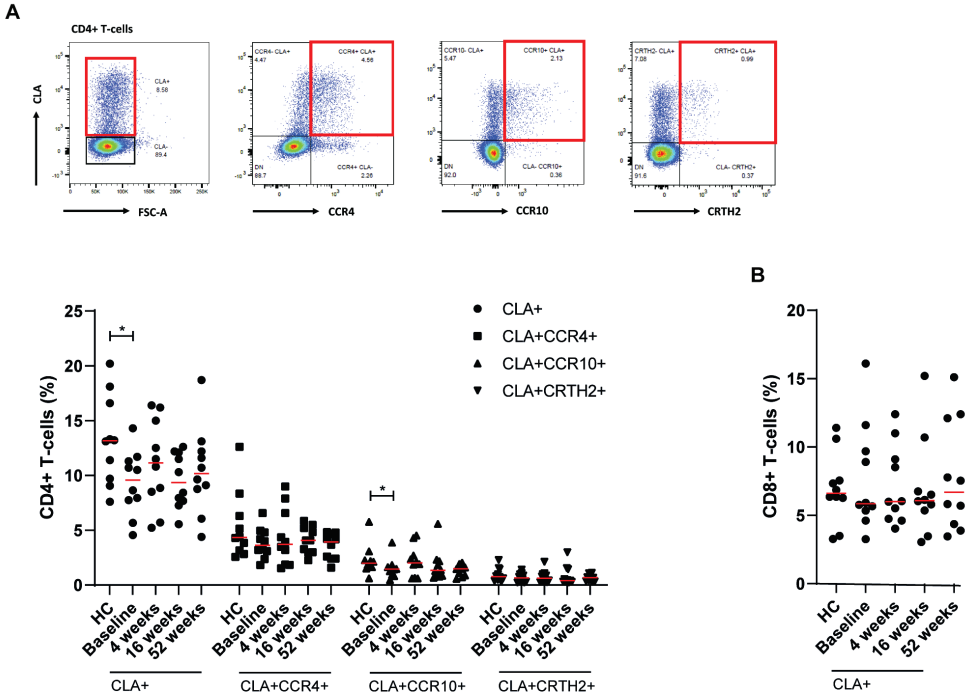

Figure 3. Effect of dupilumab treatment on skin-homing CD4+ and CD8+ T-cell subtypes. Representative flow cytometry gating strategy for and percentages of several skin-homing subpopulations within total CD4+ T-cells (**A**), within total CD8+ T-cells (**B**) in PBMCs from ten AD patients during dupilumab treatment, compared to healthy controls (HC). CLA, cutaneous lymphocyte antigen; CCR4, C-C chemokine receptor type 4; CCR10, C-C chemokine receptor type 10; CRTH2, chemoattractant receptor-homologous molecule expressed on Th2 cells; Significance levels correspond to the following P values: *P < .05, **P < .01 and ***P < .005

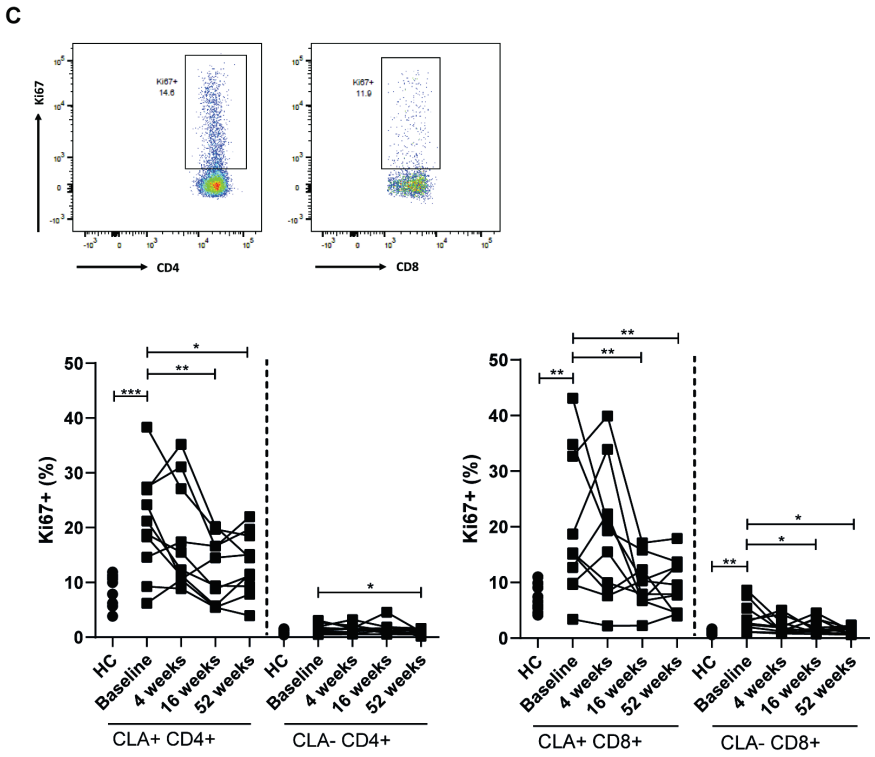

Figure 3. C. Representative flow cytometry gating strategy for and percentages of proliferating (Ki67+) skin-homing (CLA+) and non skin-homing (CLA-) CD4+ and CD8+ T-cells in PBMCs from ten AD patients during dupilumab treatment, compared to healthy controls (HC). CLA, cutaneous lymphocyte antigen; CCR4, C-C chemokine receptor type 4; CCR10, C-C chemokine receptor type 10; CRTH2, chemoattractant receptor-homologous molecule expressed on Th2 cells; Significance levels correspond to the following P values: *P < .05, **P < .01 and ***P < .005

Dupilumab treatment downregulates Th2/Th22 cytokine-producing skin-homing T-cells

To study the early functional effect of dupilumab treatment on the T-cell cytokine production, PBMCs were stimulated with PMA/Ionomycin for four hours and intracellularly stained for Th subset-related cytokines (Supplemental Figure S6 in this article's Online Supporting Information). At baseline, a significantly higher production of Th2- and Th22-related cytokines by CLA+CD4+ T-cells was observed in PBMCs from AD patients compared to HCs (Figure 4A). Already after four weeks of dupilumab treatment, a significant reduction of IL-4 (p=0.013), IL-5 (p=0.007), IL-13 (p=0.005), and IL-22 (p=0.007) production in CLA+CD4+ T-cells was found.

Similar effects were observed in CCR4+ (Supplemental Figure S7 in this article's Online Supporting Information) and CCR10+ CLA+CD4+ T-cells (data not shown). No differences were observed in the cytokine production within CLA-CD4+ T-cells after 4 weeks of treatment, suggesting a selective effect on skin-homing T-cells.

Production of Th1-related cytokines was significantly higher for IFNγ and significantly lower for TNFα in CLA+CD4+ T-cells from AD patients at baseline compared to HC (Figure 4B). This effect was not visible in the CLA-CD4+ T-cell compartment and this did not change during the first 4 weeks of treatment in CD4+ CLA+ and CLA- T-cell populations. A decline in IL-17 production by CLA+CD4+ T-cells was detected in 7 out of 10 patients at week 4 (Figure 4C). Additionally, cytokine analyses by Luminex immunoassays of the PBMC culture supernatant 72h after anti-CD3 stimulation at baseline and 4, 16, and 52 weeks after initiation of dupilumab treatment were performed to evaluate the effect on total cytokine producing capability of all PBMCs. In line with the previous results, no changes in total IL-5, IL-13, IL-17, IL-22, TNFα, or IFNγ total cytokine production were observed over time, except for a transient decrease in total IL-4 production (Supplementary Figure S8 in this article's Online Supporting Information).

Overall, rapid effects of dupilumab treatment on Th2/Th22 cytokine production were selectively observed in the skin-homing CD4+ T-cell population.

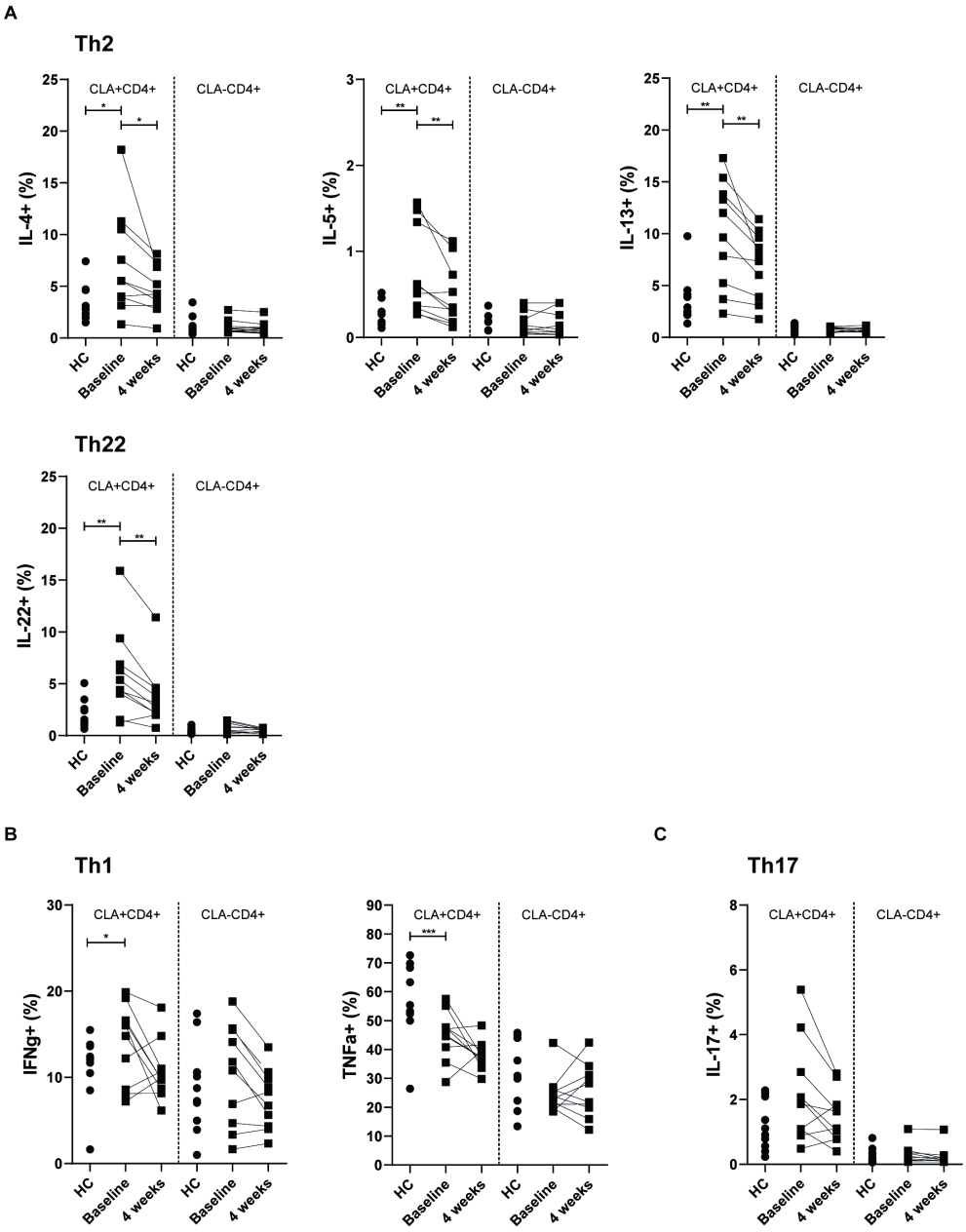

Figure 4. Short-term effect of dupilumab treatment on cytokine producing (skin-homing) CD4+ T-cells. Percentages of Th2-(IL-4, IL-5, IL-13) and Th22-related (IL-22) (**A**), Th1-related (IFNγ, TNFα) (**B**) and Th17-related (IL-17) (**C**) cytokine producing skin-homing (CLA+) and non skin-homing (CLA-) CD4+ T-cells in PBMCs from ten AD patients at baseline and after 4 weeks of dupilumab treatment, compared to healthy controls (HC). Significance levels correspond to the following P values: *P < .05, **P < .01 and ***P < .005

No evidence of long-term polarization towards Th1/Th17/Th22 cytokines is observed during dupilumab treatment

In the long term, the overall effect of dupilumab treatment on the Th2-cytokine-producing CLA+CD4+ and CLA-CD4+ T-cells remained relatively stable (Figure 5A). However, after a decrease in the first four weeks of treatment, the production of Th2-related cytokines by CCR4+CLA+CD4+ T-cells significantly increased from week 4 through week 52, not exceeding baseline levels (Figure 5B). Interestingly, the percentage of CD4+ regulatory T-cells (CD25+FOXP3+) significantly and stably increased in AD patients during dupilumab treatment (Figure 6A). The increase could be attributed almost completely to the CLA+CD4+ T-cell compartment, again pointing to a specific effect on skin-homing cells.

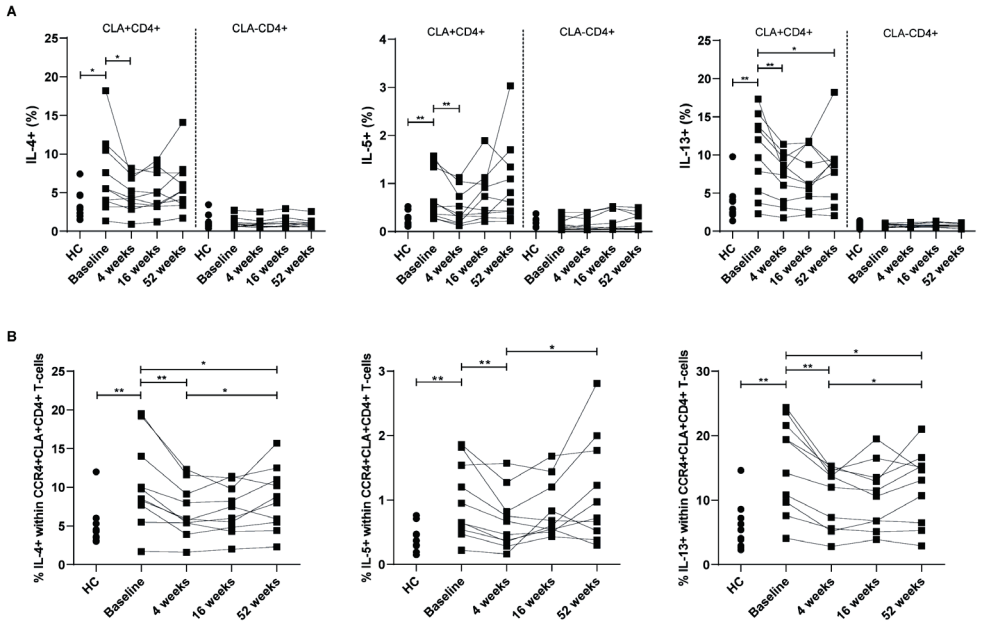

Figure 5. Long-term effect of dupilumab treatment on Th2-related cytokine producing (skin-homing) CD4+ T-cells. Percentages of IL-4, IL-5 and IL-13 producing total skin-homing (CLA+) and non skin-homing (CLA-) (**A**) and specific CLA+CCR4+ (**B**) CD4+ T-cell in PBMCs from AD patients during 52 weeks of dupilumab treatment, compared to healthy controls (HC). Significance levels correspond to the following P values: *P < .05, **P < .01 and ***P < .005

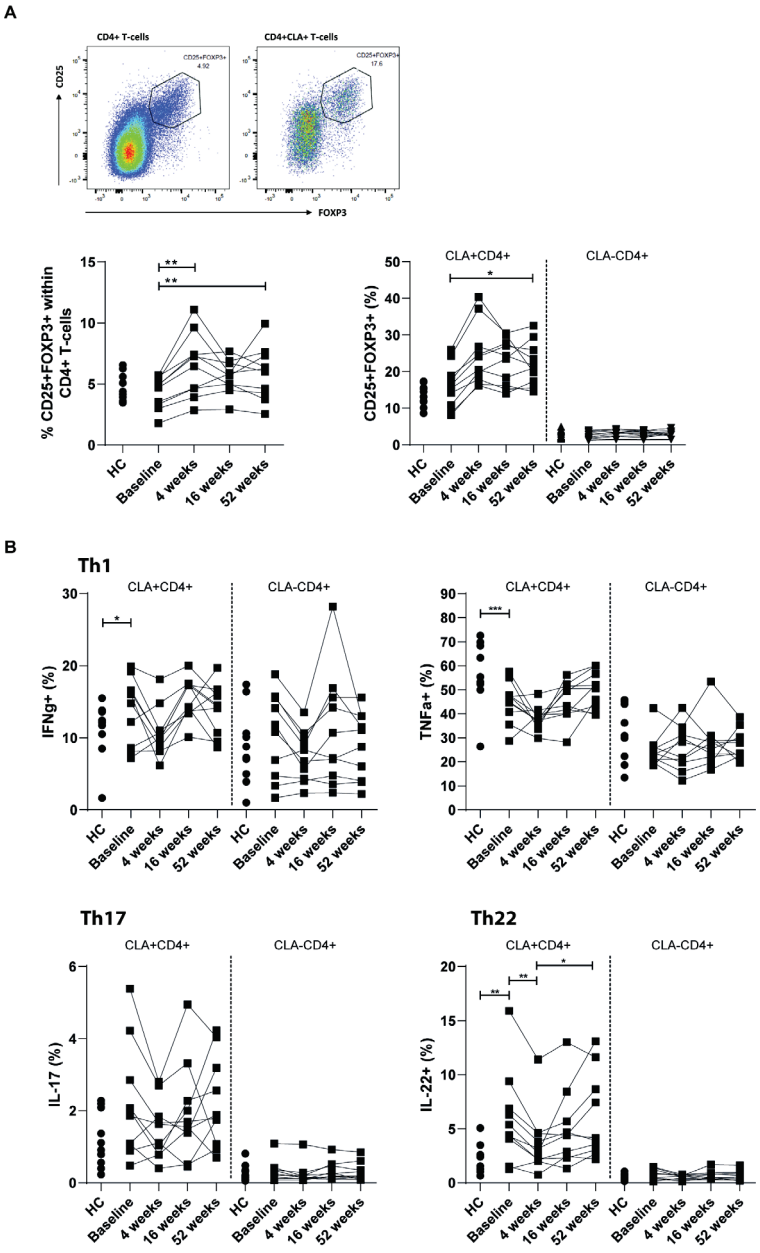

Figure 6. No signs of long-term Th1/Th17/Th22 T-cell skewing, but significantly increasing Treg proportions. A. Representative flow cytometry gating strategy for and percentages of regulatory (CD25+FOXP3+) within CD4+ T-cells (lower left panel) and within CLA+ and CLA-CD4+T-cells (lower right panel) in PBMCs from AD patients during 52 weeks of dupilumab treatment, compared to healthy controls (HC). **B.** Percentages of Th1-(IFNγ, TNFα), Th17-(IL-17) and Th22-related (IL-22) cytokine producing skin-homing (CLA+) and non skin-homing (CLA-) CD4+ T-cells in PBMCs from AD patients during 52 weeks of dupilumab treatment, compared to HC. Significance levels correspond to the following P values: *P < .05, **P < .01 and ***P < .005

Concerning the long-term possible polarization of T-cells towards the production of Th1-, Th17-, and Th22-related cytokines, a significant increase of IL-22 producing CLA+ CD4+ T-cells from week 4 through week 52 was noted (Figure 6B). Although not significant, the mean percentages of IFNγ-, TNFα- and IL-17-producing CLA+CD4+ T-cells also increased from week 4-52 during dupilumab treatment. In four out of ten individual patients IL-17 production by CCR4+CLA+CD4+ T-cells exceeded baseline levels after 52 weeks of treatment (Supplementary Figure S9 in this article's Online Supporting Information). One of these patients developed severe rosacea after 40 weeks.

In sum, after a rapid downregulation of the Th2/Th22 cytokine producing skin-homing CD4+ T-cell population, a slight increase was observed after 52 weeks. Overall, dupilumab does not seem to have strong long-term polarizing effects on the peripheral Th composition. However, in some individuals the balance may be tipped towards a more Th1/Th17 phenotype, especially within the skin-homing T-cell population.

DISCUSSION

The clinical efficacy of dupilumab treatment supports the hypothesis that type 2 cytokines IL-4 and IL-13 are critical mediators of AD pathogenesis. Elucidating the mechanisms of action of this targeted drug is of great importance for a better understanding of the pathogenesis of the disease. This is the first evaluation of the mechanistic effects of IL-4Rα blockade on the peripheral T- and B-cell compartment in moderate-to-severe AD patients.

The current study confirmed the mechanism of action of dupilumab by demonstrating a substantial functional blockade of the IL-4Rα on B- and T-cells already within two hours after the first dosage. The very rapid initial response was followed by a stable blockade of IL-4Rα on CD19+ B-cells and CD4+ T-cells from week 4 through week 52, which was concomitant with clinical efficacy. The significant decrease of IL-4Rα MFI observed is most likely reflecting full occupancy of IL-4Rα by dupilumab monoclonal antibodies, as confirmed by IgG4 antibody detection on T- and B-cells during treatment. Additionally, we confirmed that the IL-4Rα blockade by dupilumab is of functional relevance, by showing weaker pSTAT6 responsiveness

to IL-4 already within 2 hours after the first administration. Although no signs of receptor internalization were observed until 16 weeks of treatment, we cannot rule out a change in IL-4Rα expression levels due to shedding or changes in gene expression in the long term. Pharmacokinetic studies have shown that, following the first subcutaneous dose of 600mg, the maximum serum concentration of dupilumab is achieved after approximately one week.[27] However, actual functional blockade of the IL-4Rα on effector cells has not been confirmed before.

Previous studies have shown that the number of circulating type 2 cytokine-producing skin-homing T-cells is increased in the peripheral blood of AD patients.[28, 29] Our study confirmed this by showing a higher production of type 2 cytokines by CLA+CD4+ T-cells in AD patients at baseline compared to healthy controls. Data regarding mechanistic changes during dupilumab treatment are scarce. Recently, dupilumab treatment was observed to downregulate the expression of genes related to type 2 inflammation in lesional skin after 4 and 16 weeks of treatment as well as to decrease Th2-related serum biomarkers.[13, 30] Our study showed that, during the first four weeks of treatment, dupilumab significantly suppressed the type 2 cytokine production in CLA+, but not in CLA-CD4+ peripheral blood T-cells. Similar effects were observed in specific subtypes (CCR4+, CCR10+, CRTH2+) of skin-homing CLA+CD4+ T-cells. In accordance with our findings, a recent *in vitro* study including 12 AD patients and 6 HCs showed that IL-4Rα blockade could reduce the production of the type 2 cytokines IL-4, IL-5, IL-13 ,and IL-31 by proliferating CD4+ T-cells.[31] However, this study found an increase of IFNy production in CD4+ T-cells, whereas in our study no increase in the production of Th1- and Th17-related cytokines was observed. The skin-homing peripheral T-cell population in our study might have been affected by (re-)migrating T-cells from the skin compartment due to reduced TARC/CCL17 expression in skin. In contrast to the study of Brøgger et al., in which PBMCs were stimulated *in vitro* with and without a neutralizing monoclonal antibody against IL-4Rα, our study analyzed actual *in vivo/ex vivo* effects of dupilumab treatment in AD patients, and our results suggest that dupilumab treatment selectively affects only the skin-homing T-cell population. No difference in expression of IL-4Rα on skin-homing T-cell subsets was observed between patients at baseline and HCs. Another reason for increased sensitivity to dupilumab treatment may be the relatively high turnover of the CLA+ T-cell compartment we observed at baseline, which decreased upon treatment. Finally, priming of naïve T-cells may also be affected by dupilumab, possibly preventing induction and/or differentiation of skin-homing T-cells.

The IL-4Rα plays an important role in inducing B-cell proliferation and isotype switching, resulting in high levels of circulating IgE.[5] Our study showed that after blocking the IL-4Rα, dupilumab treatment steadily decreased IgE levels from baseline through week 52 in all ten patients, even including the patients who showed normal IgE (<100 IU/ml) levels at baseline. Our findings are in accordance with previous studies, showing significant reduction in total serum IgE levels from baseline through week 16 in AD patients[11, 30, 32] and a gradual reduction throughout 52 weeks of dupilumab treatment in asthma patients[33]. The long-term IgE suppression found in our study suggests that dupilumab treatment adequately suppresses IL-4 and IL-13 activity and might indicate long-term effects on the atopic phenotype.

The functional immunological effect of dupilumab treatment was mainly observed in the first four weeks of treatment. After the significant decrease from baseline through week 4, the production of type 2 cytokines by CCR4+CLA+CD4+ T-cells gradually increased again. A similar trend in the production of IL-4, IL-5 and IL-13 in total skin-homing T-cell population was observed. This effect may be explained by a small decrease in biological efficacy after the initial high pulse dosing, or compensatory mechanisms on a biological level, e.g. upregulation of the IL-4Rα. At the same time, our results suggest that IL-4Rα occupancy remains stable over time. Despite the gradual increase in type 2 cytokine producing skin-homing T-cells on the long term, lasting effects of dupilumab treatment were observed on clinical efficacy and total IgE levels, which both continued to decrease until one year of treatment. These results show the differential dynamics of immune-modulating effects on T- and B-cells by dupilumab treatment.

Recent case reports describing new onset or worsening of rosacea[22], alopecia areata[23-25, 34] and psoriasis[19-21] during dupilumab treatment, suggest possible skewing of the helper T-cell profile towards a Th1/Th17 phenotype as a results of IL-4R signaling antagonism. Since it has been shown that IL-4 can act as a negative regulator of the Th1 and Th17 pathways, suppression of the IL-4/IL-13 signaling pathway could result in alteration of the Th1/Th2/Th17 balance and may predispose patients to Th1/Th17-mediated diseases.[35] However, our study showed no signs of general immune skewing towards Th1 or Th17 pathways on the total CD4+ T-cell (data not shown) and specific skin-homing T-cell levels during the first year of dupilumab treatment. This might be the effect of increased control and suppression of T-cell responses, as especially the proportion of skin-homing regulatory CD4+CD25+FOXP3 T-cells (Treg) significantly increased in PBMCs from AD patients

during dupilumab treatment. Tregs are known to suppress immune responses by suppressing effector T-cells and play a major role in controlling asthma and allergy.[36] Accordingly, the expansion of Tregs might also contribute to the improvement of clinical signs and symptoms of AD in our patients. The occurrence of Th1/Th17-mediated diseases as adverse effects during dupilumab treatment might be explained by T-cell skewing on the level of individual patients, since our results show that in four out of ten patients IL-17 production by CCR4+CLA+CD4+ T-cells exceeded baseline levels after 52 weeks of treatment. Remarkably, one of these patients developed a severe rosacea after 40 weeks of dupilumab treatment. It remains to be shown whether dupilumab induced IL-17 production, is able to induce neutrophil activation in AD patients, since it has been shown that IL-4Ra-mediated signaling can inhibit neutrophil migration and function.[37, 38]

The strength of this is study is the inclusion of both AD patients and healthy controls, as well as the long-term follow up until 52 weeks of dupilumab treatment. Additionally, the evaluation of skin-homing CLA+ T-cells in the peripheral blood, which might reflect the cutaneous immune responses, creates an opportunity for less invasive, translational approaches and might eliminate the need for skin biopsies in future studies. Although results were very consistent between the different cohorts included, the small number of included patients for the assessment of very early treatment effects was a limitation of this study.

Overall, this study confirms the mechanism of action of dupilumab treatment by demonstrating a (very) rapid and stable blockade of the IL-4Rα on B-cells and T-cells accompanied by a strong early functional immunological effect (after 4 weeks), specifically in skin-homing T-cells of AD patients treated with dupilumab. Although there were no signs of general immune skewing on the T-cell level following a year of dupilumab treatment, the continuous decrease in total IgE levels and increase in IL-17 production by skin-homing T-cells in a selection of patients may indicate long-term effects on the atopic phenotype. For the future, monitoring peripheral (skin-homing) T-cell responses might be a useful tool to predict/monitor treatment efficacy, potential side effects, and guide tapering strategies in AD patients.

MATERIALS & METHODS

Patient characteristics, blood and biopsy collection

This study included adult patients with moderate-to-severe AD from a larger prospective, observational cohort study in which patients who were treated with dupilumab in daily practice, and were enrolled in the Dutch Bioday Registry at the National Expertise Center for Atopic Dermatitis from the University Medical Center Utrecht (ClinicalTrials.gov Identifier: NCT03549416, retrospectively registered June 8, 2018). At baseline, patients received a loading dose of 600mg dupilumab subcutaneously, followed by 300mg dupilumab subcutaneously every other week. Concomitant treatment with topical corticosteroids was allowed. Patients using oral immunosuppressive drugs within 2- (fast acting drugs including systemic steroids or cyclosporine A) or 4- (slow acting drugs including azathioprine, methotrexate and mychophenolate mofetil) weeks before screening were excluded. Blood samples were collected before initiation of dupilumab treatment (baseline) and after 4, 16 and 52 weeks of treatment from ten moderate-to-severe AD patients (experiment 1). Blood samples from ten adult healthy controls, who have not experienced AD or any other atopic disease were obtained from the Mini Donor Service at University Medical Center Utrecht. To study actual binding of the dupilumab antibodies to the IL-4Rα after 4 and 16 weeks of dupilumab treatment (experiment 2), a clinically comparable subgroup of five adult AD patients were included. For the study of very early effects, blood samples of four (experiment 3) and three (experiment 4) patients, respectively, were taken within two hours after first dupilumab administration. Skin biopsies (3mm) of lesional and non-lesional skin were collected from a clinically comparable cohort of eight adult moderate-to-severe AD patients at baseline and week 4 of dupilumab treatment (experiment 5). Posttreatment biopsies were taken from the same location as pretreatment biopsies, close to prior biopsy scars. Clinical data were extracted from an online Good Clinical Practice database (BioDay registry). All patients signed Institutional Review Board-approved written consent, adhering to the Declaration of Helsinki Principles.

The Eczema Area and Severity Index (EASI) score was used to evaluate disease severity. Additionally, TARC/CCL17 levels, currently the best performing and accepted biomarker for disease severity[39], were measured in routine care using Quantikine® ELISA immunoassays (R&D systems, Inc, Minneapolis, MN).

Assessments

Cell isolation

Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Paque (GE Healthcare, Eindhoven, the Netherlands) density gradient centrifugation. PBMC were frozen in RPMI1640 medium supplemented with 2mM L-glutamine, 100 IU/ml penicillin-streptomycin, 20% Fetal Bovine Serum (FBS) and 10% DMSO (Sigma Aldrich, Saint Louis, MO), and stored at -170°C until use.

Flow cytometry

PBMC were thawed in a 37°C water bath, washed, and re-suspended in RPMI 1640 medium (Gibco, Grand Island, NY) containing 10% Fetal Bovine Serum (FBS) with the addition of L-glutamine and penicillin-streptomycin.

Experiment 1

250.000 – 500.000 PBMC were plated in round-bottom 96-well plates. To determine cell death, eBioscience Fixable Viability Dye eFluor® 506 (Invitrogen, Carlsbad, CA) in PBS was used. Surface staining of multiple T- and B-cell markers (see panel 1 - 3 in Supplemental Table S2 in this article's Online Supporting Information) was performed for 25 min at 4 ºC. Surface staining of IL-4Rα (CD124) PE was performed for 25 min at 37°C, using an optimization protocol after testing different temperatures. For intracellular and -nuclear staining, cells were fixed and permeabilized by using eBioscience Fixation and Permeabilization buffers (Invitrogen, Carlsbad, CA), and stained with Granzyme B FITC, Ki67 AF647 and FOXP3 PE.

For intracellular cytokine production, cells were first stimulated with phorbol 12-myristate 13-acetate (PMA) (20 ng/ml; Sigma-Aldrich, Saint Louis, MO) and ionomycin (1.0 µg/ml; Sigma-Aldrich, Saint Louis, MO) for a total of 4 hours. Golgistop (1/1500; BD Biosciences, San Jose, CA) was added for the last 3.5 hours of cell culture. Afterwards, cells were incubated with the fixable viability dye and surface antibodies (see panel 4-6 in Supplemental Table S2 in this article's Online Supporting Information), and then fixed, permeabilized, and intracellularly stained with IFNy PE-Cy7, IL-4 BV711 IL-5 PE, TNF-alpha APC, IL-13 PerCP-Cy5.5, IL-17a PE, IL-22 APC.

Experiment 2-3

0,5 – 1,0 x 10^6 PBMC were plated to study actual binding of the dupilumab antibodies to the IL-4Rα. Surface staining (see panel 7 Supplemental Table S2 in this article's

Online Supporting Information) was performed for 25 min at 37 ºC, followed by a 25 min incubation of 2nd antibody: streptavidin-APC at 4 ºC.

Experiment 4
For determination of reactivity to IL-4, freshly isolated PBMCs were incubated with titrated amounts of recombinant human IL-4 (Peprotech, Cranbury, NJ) in RPMI + 1% FBS for 15 min at 37 ºC, followed by immediate fixation for 10 min at 37 ºC using BD Phosflow Fix Buffer I (BD Biosciences, San Jose, CA) and staining of surface antigens (see panel 8A + 8B in Supplemental Table S2 in this article's Online Supporting Information) for 30 min at 4 ºC. Subsequently, cells were permeabilized using BD Phosflow Perm Buffer III (BD Biosciences, San Jose, CA) at 4 ºC for 1 hour, followed by intracellular staining of phosphorylated signal transducer and activator of transcription 6 (pSTAT6, Y641) for 30 min at room temperature.

Stained cells from all experiments were resuspended in PBS containing 2% FBS and 0.1% sodium-azide (Sigma-Aldrich, Saint Louis, MO). Data acquisition was performed on a FACS LSR Fortessa (BD Biosciences, San Jose, CA) and data was analyzed using FlowJo Software (Tree Star Inc.).

ImageStream
PBMC were thawed and resuspended in PBS (Sigma-Aldrich, Saint Louis, MO) (0,5 – 1,0 x 10^6 living cells/well). Surface staining of CD3 FITC, CD19 APC and IL-4Rα PE (panel 9 Supplemental Table S2 in this article's Online Supporting Information) was performed for 25 min at 37°C. Data acquisition was performed on the Amnis ImageStream (Millipore Sigma, Billerica, MA, USA) and on-focus cells were analyzed using Amnis IDEAS software (Millipore Sigma).

Cell cultures
100.000 PBMC were plated in a round bottom 96-well plate in RPMI1640 medium containing 2mM L-glutamine, 1% penicillin-streptomycin and 10% FBS. PBMC were stimulated by 0.1 µg/ml coated anti-CD3 (Invitrogen, Carlsbad, CA) and incubated for 72 hours at 37°C. Supernatants were collected and stored at -80°C until use.

Skin biopsies in culture
Full-thickness biopsies (3mm) of lesional and non-lesional skin were collected and incubated in DMEM (1x) + Glutamax (Gibco, Grand Island, NY) containing 10% FBS and 100 U/ml penicillin-streptomycin (Invitrogen, Carlsbad, CA). Biopsies were

weighted before they were placed in a 48-wells plate (Corning, Glendale, AZ) at 37°C. Supernatants were collected after 24 hours and immediately cryopreserved at -80°C until use, as previously prescribed in psoriasis patients.[40]

Multiplex immunoassay

Concentrations of 16 cytokines and chemokines were measured in supernatants by Luminex multiplex immunoassay[41] at the Multiplex Core Facility of the Center for Translational Immunology (UMC Utrecht, The Netherlands), using an in-house validated panel of analytes, listed in Table S1. Uniquely color-coded magnetic beads (MagPlex Microsperes, Luminex, Austin, TX) were conjugated to antibodies specific for the reported analytes and incubated with 50 µL of standard dilutions per sample for 1 hour (continuous shaking in the dark). Samples were diluted in High Performance Elisa buffer (HPE; Sanquin, The Netherlands). Pre-treatment of samples included filtration 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) and a corresponding cocktail of biotinylated detection antibodies was added for 1 hour. Repeated washings were followed by a 10 minutes streptavidin-phycoerythrin (PE) incubation. Fluorescence intensity of PE was measured using a Flexmap 3D system (Luminex) and analyzed by using BioPlex Manager software version 6.1; (Bio-Rad) using 5-parameter curve fitting.[42] Supernatant samples that were above or below the assay limits of detection were given values equivalent to the lower limit divided by two or the upper limit multiplied by two.

Total IgE ELISA

Maxisorp 96-wells nunc plates (ThermoFisher Scientific, Rochester, NY) were coated with 4 µg/ml goat anti-human IgE (catalog number A80-108A, Bethyl Laboratories, Montgomery, TX) for 60 min at RT. Three washes were performed with PBS containing 0.05% TWEEN20. The plates were incubated with PBS containing 5% FBS for 30 min at RT to block nonspecific interactions. After washing, patient samples were added in different dilutions (1:10, 1:100 and 1:1000) and incubated for 60min at RT. Serum from healthy controls was taken along as negative control. Afterwards, the detection antibody mouse anti-human IgE biotin (0.1 µg/ml) (BD Biosciences Pharmingen, San Diego, CA) was added and incubated for 60 min at RT. Streptavidin-HRP (100 ng/ml) (Sanquin, the Netherlands) was used for the detection of biotins and was incubated for 30 min at RT. TMB substrate (Biolegend, San Diego, CA) was added to the wells and the reaction was stopped by 1M H_2SO_4 when wells turn into

blue. The read-out was performed within 30 min on the ELISA-reader, 450nm (BMG Labtech, Ortenberg, Germany). 570nm was taken along to correct for the background of every well. Data was analyzed using Clario Star (BMG Labtech).

Statistical analysis

Statistical analyses were performed using SPSS (for Windows, version 25.0, SPSS Inc.) and Prism (version 7.4; Graphpad). Flow cytometric data were presented with medians. The Wilcoxon Signed Rank Test was used to compare two continuous variables in the same patients. Differences between baseline and healthy control flow cytometric data were assessed by the Wilcoxon Rank Sum Test. P-values lower than 0.05 were considered statistically significant.

Acknowledgements

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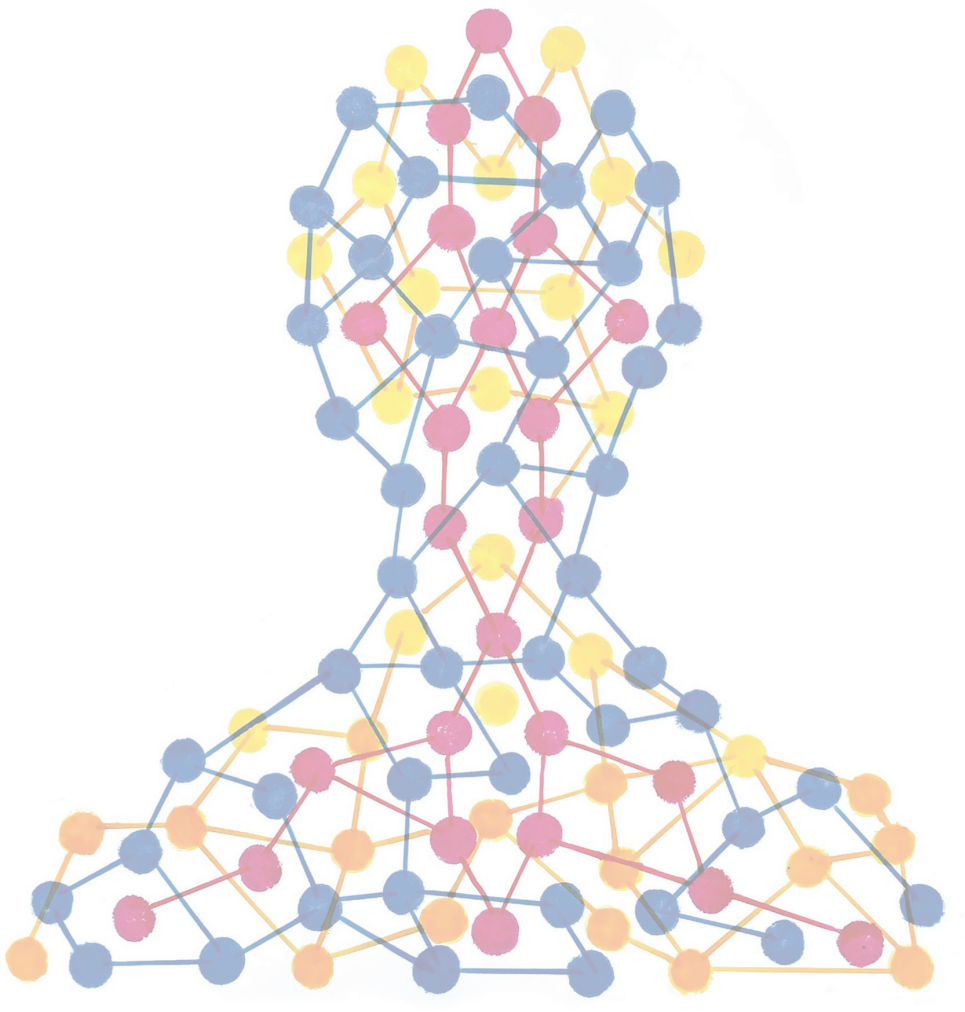

Chapter 6

EASI p-EASI: predicting disease severity in atopic dermatitis patients treated with dupilumab using a combination of serum biomarkers

Allergy. 2020; 75(12); 3287-3289

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To the editor,

During the past decade, new more targeted therapies for the treatment of atopic dermatitis (AD) have been developed and are currently under investigation in clinical trials. However, the comparison of results from different clinical studies remains challenging, given the substantial variation in clinician-rated outcome measures that are currently used, and high inter- and intra-observer dissimilarities.[1] An objective and consistent outcome measurement is hence needed to successfully compare different treatment options. Serum thymus and activation-regulated chemokine (TARC/CCL17) is currently the best performing objective biomarker for disease severity in AD.[2] Nevertheless, the correlation with disease severity is not strong enough to replace clinical outcome measures. Therefore, we previously proposed to use a biomarker combination reflecting different underlying pathways and showed that a biomarker signature including TARC, soluble interleukin(IL)-2-receptor (sIL-2R) and IL-22 was a significantly better predictor of disease severity than a single biomarker.[3] All three biomarkers have been reported to contribute to AD pathogenesis. TARC is a T-cell attracting chemokine, involved in T-cell recruitment to the skin[3], sIL-2R is a proven marker of T-cell activation in vivo[4], and IL-22 induces keratinocyte proliferation and inhibits terminal differentiation, thereby promoting epidermal hyperplasia and barrier defects.[5] Since the model was developed to predict Eczema Area and Severity Index (EASI) scores, this objective outcome measure was named "the predicted-EASI" (p-EASI). The p-EASI was developed using data of AD patients treated with topical corticosteroids (TCS), and recently validated in cyclosporin A (CsA) treated AD patients.[6] In the near future, novel immune-modulating drugs will transform the management of moderate-to-severe AD. Therefore, we aim to validate the p-EASI in patients treated with dupilumab, the first human monoclonal antibody-based treatment approved for adults with moderate-to-severe AD.[7]

We included 25 adult patients with moderate-to-severe AD from a previously published prospective cohort[8] (median age 32, IQR 27-49, 15 male, Supplemental Table 1). Patients using oral immunosuppressive drugs within two (fast-acting) or four (slow-acting) weeks before baseline were excluded. Patients were treated with dupilumab 600mg at initiation followed by 300mg every other week for 16 weeks. Disease severity was assessed by EASI score and serum was collected before initiation of dupilumab treatment (t_0) and after eight (t_1), twelve (t_2) and sixteen (t_3) weeks of treatment. Serum TARC, sIL-2R, and IL-22 levels were measured via Luminex-based

multiplex immunoassays using an in-house validated panel of analytes, as previously described.[3, 6] Differences between time points were evaluated by Wilcoxon matched-pairs signed-rank test. Patients signed Institutional Review Board-approved written consent, adhering to the Declaration of Helsinki Principles.

Dupilumab treatment significantly decreased median EASI scores from baseline (19.3, IQR 14.2 – 24.5) through week 8 (median EASI 5.2, IQR 2.4 – 8.0, $p < 0.0001$), week 12 (3.8, IQR 2.1 – 6.6, $p<0.0001$), and week 16 (median EASI 3.9, IQR 2.4 – 7.6, $p<0.0001$). Median serum TARC and IL-22 levels significantly decreased from t_0 to t_1 and remained stable onwards until t_3 (Figure 1). No significant change in median serum sIL2r levels was observed during the 16 weeks of dupilumab treatment.

Serum biomarker levels were used to calculate the p-EASI scores at the different time points using the following previously published signature[3]: *(-36.12+18.49*logTARC+0.009*IL-22-0.009*sIL-2R)*(1-treatment)+ (-5.82+4.04*logTARC+0.003*IL-22-0.003*sIL-2R)*treatment*, in which treatment refers to dupilumab treatment, and can be either *No=0,* or *Yes=1*. The observed and predicted EASI scores showed a high correlation (Spearman correlation $r=0.67$, $p<0.0001$). Median EASI and p-EASI were 19.3 (IQR 14.2 – 24.5) and 17.3 (IQR 8.5 – 20.2), respectively, before treatment, 5.2 (IQR 2.4 – 8.0) and 2.8 (IQR 1.6 – 3.9), respectively, after eight weeks, 3.8 (IQR 2.1 – 6.6) and 2.7 (IQR 1.7 – 4.0), respectively, after twelve weeks, and 3.9 (IQR 2.4 – 7.6) and 3.1 (IQR 1.9 – 4.0), respectively, after 16 weeks of dupilumab treatment (Figure 2). Additionally measured biomarkers (Supplemental Table 2) were not considered to have added value over the current signature, based on correlation with disease severity.

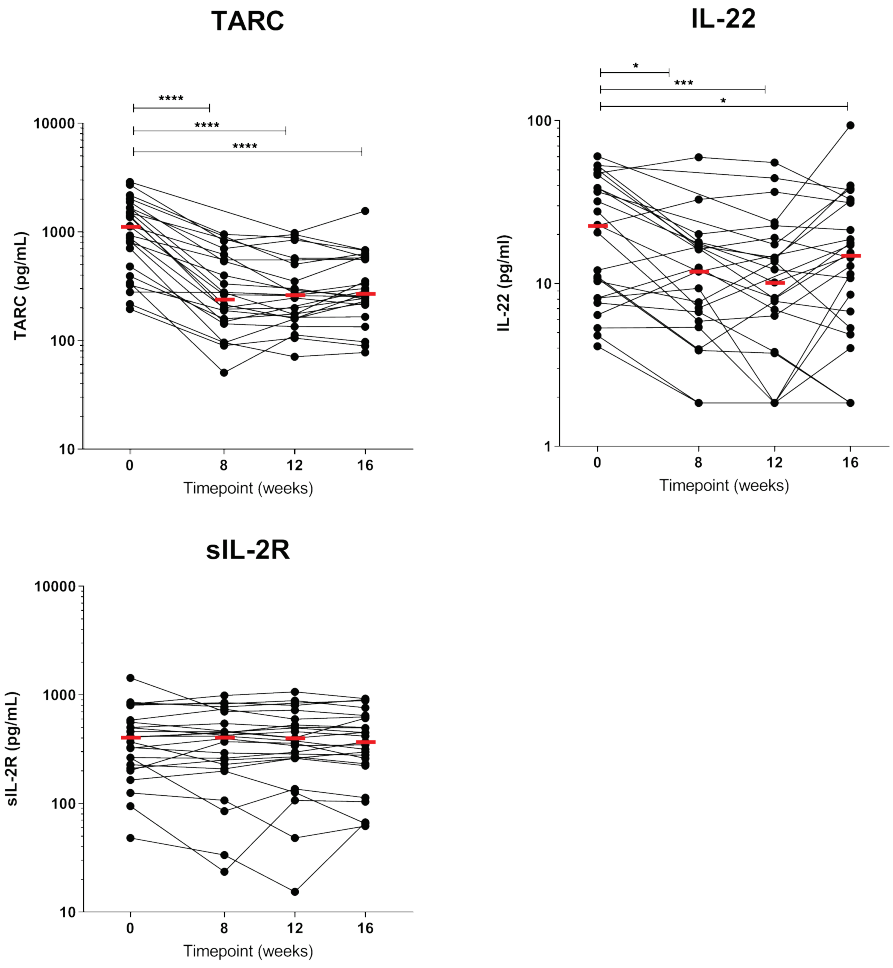

Figure 1. Serum TARC, IL-22 and sIL-2R levels from 25 AD patients before initiation of dupilumab treatment (week 0) and after eight, twelve, and sixteen weeks of treatment. Red lines represent median. Significance levels correspond to the following P values: *P < .05, **P < .01 and ***P < .005.

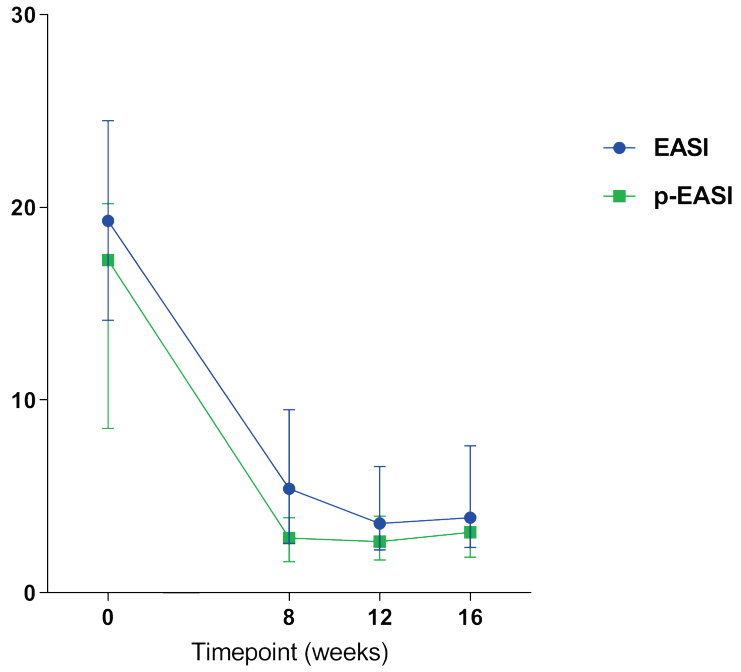

Figure 2. Median EASI and predicted EASI (p-EASI) scores in 25 AD patients before initiation of dupilumab treatment (week 0) and after eight, twelve, and sixteen weeks of treatment. Error bars represent inter quartile range.

The current study demonstrates that the p-EASI corresponds closely to disease severity in AD patients before and after 8–16 weeks of dupilumab treatment. In comparison with our previous TCS and CsA treated AD cohorts, the correlation between EASI and p-EASI was slightly lower in dupilumab treated AD patients. This may be explained by the sIL-2R levels remaining stable during dupilumab treatment, which was not observed in the other two cohorts. By targeting the IL-4Rα, dupilumab specifically inhibits the T helper (Th)2-related cytokines IL-4 and IL-13. Although sIL-2R is known to reflect T-cell activation and correlate to AD disease severity[4, 9], our results suggest that it is not influenced by dupilumab treatment. This might reflect that this biological only targets T-cell phenotypes directly involved in AD pathogenesis (Th2 cells). In comparison, CsA and, to a lesser amount TCS, have a broad systemic immunosuppressive effect, targeting multiple T-cell phenotypes and related cytokines. As few patients did show a quick drop in sIL-2R, extending the current model to a larger patient population including different phenotypes of AD might identify subtypes of AD patients in whom sIL-2R is an important marker.

The difference between EASI and p-EASI was larger at baseline and after eight weeks of treatment, compared to the later time points. The difference at baseline might be explained by an overestimation of the EASI score due to a more severe disease at the moment of dupilumab initiation compared to the other time points. The EASI is a subjective score reflecting the visible skin lesions, while the p-EASI objectively reflects the extent and intensity of AD lesions, and might be ahead of clinical signs. Since dupilumab is a systemic immunomodulating drug, changes in serum biomarkers might occur before clinical signs improve. This is supported by our finding that the lowest median serum TARC/CCL17 level was observed at week 8, while lowest median EASI score was observed at week 12. Similar results were reported in the previous study of Guttman-Yassky et al.[10], investigating 54 moderate-to-severe AD patients treated with dupilumab for 16 weeks, where the mean percentage change from baseline in serum TARC levels was the highest at week 4. In future, the change in p-EASI during the first weeks of treatment might potentially be used to predict response to dupilumab in AD patients.

The current study demonstrates that a biomarker signature (p-EASI) consisting of serum biomarkers TARC, IL-22 and sIL-2R, adequately predicts disease severity in AD patients treated with dupilumab, in addition to previously published cohorts of TCS and CsA treated AD patients.[3, 6] The use of p-EASI measured via a standardized assay will help to improve comparability of study outcomes in future clinical trials on new more targeted therapies for AD, but may also be helpful as an objective measure for treatment effects in daily practice.

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SUPPLEMENTAL TABLES

Supplemental Table S1. Baseline characteristics

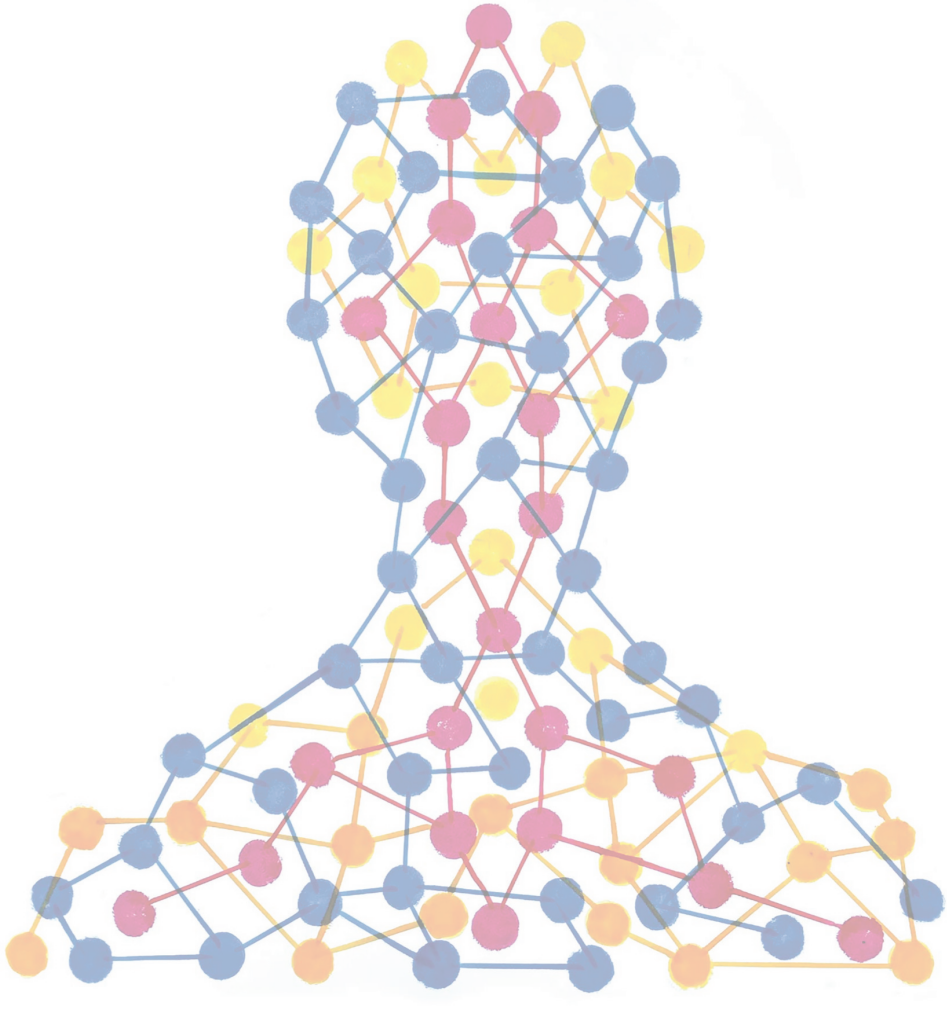
Clinical characteristics	Total group (n=25)
Age (years)[1], median (IQR)	32.0 (26.5 – 49.0)
Male, n (%)	15 (60)
Atopic comorbidities, n (%)	
Allergic asthma	17 (68)
Allergic rhinitis	21 (84)
Food allergy	18 (72)
Allergic conjunctivitis	20 (80)
Previous use of systemic immunosuppressants for atopic dermatitis*, n (%)	25 (100)
History of ≥2 oral immunosuppressants, n (%)	15 (60)
Previous use of cyclosporine A, n (%)	24 (94)
Previous use of methotrexate, n (%)	8 (32)
Previous use of azathioprine, n (%)	7 (28)
Previous use of mycophenolate mofetil, n (%)	8 (32)
EASI score, median (IQR)	19.3 (14.2 – 24.5)
IGA score, median (IQR)	3.0 (3.0 – 4.0)
Weekly average pruritus NRS, median (IQR)	6.0 (4.5 – 8.0)

EASI, Eczema Area Severity Index; IGA, Investigator Global Assessment; IQR, interquartile range; NRS, numeric rating scale; [1] Age at the moment of sampling. *Patients using oral immunosuppressive drugs at any time point or within two (fast-acting drugs including cyclosporine A and systemic corticosteroids) or four weeks (slow-acting drugs including azathioprine, methotrexate, mycophenolate mofetil and tacrolimus) before baseline were excluded

Supplemental Table S2. Additional biomarker panel: correlation with disease severity

Biomarker	Correlation coefficient	p-value
IL-4	**-0,353**	**0**
IL-5	-0,198	0,056
IL-6	-0,083	0,428
IL-12	0,148	0,154
IL-13	-0,196	0,059
IL-17	-0,035	0,738
IL-20	-0,118	0,258
IL-21	**-0,255**	**0,013**
IL-23	-0,156	0,134
IL-26	-0,091	0,385
IL-31	-0,168	0,106
TNFa	-0,088	0,396
TSLP	-0,009	0,931
Eotaxin-1	0,146	0,161
Eotaxin-3	**0,296**	**0,004**
IL-8	-0,133	0,201
OSF2	0,164	0,113
Elastase	0,086	0,407
PARC	**0,568**	**0**

Spearman correlation coefficients were calculation for correlation between nineteen additionally measured serum biomarkers with disease severity (EASI score). Biomarkers that showed significant correlation with disease severity are marked in bold. Of these markers, only PARC showed a correlation coefficient higher than 0.40. From our previous study[3], we know that PARC levels highly correlate with disease severity, but that it showed no added value over TARC in a panel of biomarkers for disease severity, probably due to a high correlation between PARC and TARC (r=0.510 in the current study). Therefore, PARC was not added to the signature.



Chapter 7

The effect of dupilumab treatment on eosinophil homing and activation in patients with atopic dermatitis

Submitted

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ABSTRACT

Background: Treatment with dupilumab, a monoclonal antibody against interleukin(IL)-4Rα, has shown high efficacy in atopic dermatitis (AD) patients. Dupilumab treatment is, however, clearly associated with eosinophilia in the peripheral blood. The mechanism behind this phenomenon has not been elucidated.

Objective: To evaluate local effects of dupilumab treatment on markers of eosinophilic inflammation in skin biopsies, and characterize the activation state of peripheral blood eosinophils before and after dupilumab treatment of AD patients.

Methods: The activation state of peripheral blood eosinophils was analyzed in 16 AD patients before and after 16 weeks of dupilumab treatment by measuring multiple surface markers using flow cytometry, and this was compared with healthy controls. Skin biopsies from 10 AD patients before and after 16 weeks of dupilumab were evaluated for eosinophil counts and eotaxin expression.

Results: An increase of CCR3 and CD44 expression on eosinophils was observed during dupilumab treatment. Eosinophil counts and eotaxin expression in lesional skin significantly decreased during treatment. Eosinophils in peripheral blood from AD patients showed an increased activation state compared to healthy controls, which did not change after dupilumab treatment.

Conclusion & Clinical Relevance: Our results support the concept that treatment with dupilumab decreases eosinophil trafficking to the skin, possibly leading to peripheral blood eosinophilia. Peripheral blood eosinophils of AD patients exhibited an activated phenotype, which was not altered by 16 weeks of dupilumab treatment suggesting that IL-4Rα is not involved in the production of eosinophil activating mediators in active AD. It may be prudent to monitor eosinophil counts and markers of eosinophil (pre-)activation during dupilumab treatment of AD and other diseases.

INTRODUCTION

Atopic dermatitis (AD) is one of the most common chronic inflammatory skin diseases, with a prevalence of up to 10% in adults.[1, 2] AD is classically characterized by type 2 inflammation and associated with increased production of interleukin(IL)-4, IL-5 and IL-13.[3] IL-4 and IL-13 induce T helper (Th)2 cell differentiation, promote IgE class switching, and stimulate eosinophil recruitment.[4, 5] IL-5 is the primary cytokine in eosinophil differentiation and proliferation, and also stimulates trafficking of eosinophils into tissues.[6, 7] Upon terminal differentiation, eosinophils are released into the circulation and recruited into tissues in response to chemokines, particularly those of the eotaxin family. Eosinophils can produce IL-4 and IL-13, further promoting Th2 responses.[8] However, the role of eosinophils in AD has been a subject of debate for years.[9, 10] Even though enough evidence has been provided that eosinophil granule proteins are prominently deposited in AD lesions[11] and serum levels correlate with AD disease activity[12], short term treatment with anti-IL-5 (mepolizumab) did not result in significant improvement of symptoms of patients with AD, despite a decrease of circulatory eosinophils.[13]

Due to the evolving understanding of AD pathogenesis in the past decade, several novel targeted therapeutics are being developed for AD. In contrast to anti-IL-5 targeting alone, treatment with dupilumab, the first monoclonal antibody blocking the shared receptor subunit for IL-4 and IL-13, has demonstrated efficacy and safety in moderate-to-severe AD patients.[14-16] However, previous studies have shown that dupilumab treatment is associated with the occurrence of eosinophilia.[16-18] Increased peripheral blood eosinophil numbers observed during dupilumab treatment in AD patients were mostly transient and not associated with clinical symptoms.[17, 18] Although, in dupilumab-treated asthma patients, few cases of eosinophilia were accompanied by clinical symptoms.[19] In the study of Ariens et al.[18] serum eotaxin-1 and eotaxin-3 levels decreased during dupilumab treatment in AD patients, without significant changes in serum IL-5 levels. Furthermore, dupilumab treatment locally reduced eosinophil-related markers in nasal secretions and polyp tissues in a cohort of chronic rhinosinusitis with nasal polyposis patients.[20] These findings suggest that eosinophilia observed during dupilumab treatment might be a result of reduced homing of eosinophils to the tissues, rather than an increase in eosinophilopoiesis. However, the exact mechanism remains unknown. Part of the AD patients do not exhibit eosinophilia before or during dupilumab treatment, which is possibly related to the individual's Th2 status and atopic comorbidities.[21-23] In

addition to the increase in the number of eosinophils, it is therefore also important to investigate whether eosinophils are activated during dupilumab treatment, which might potentially lead to tissue damage. In the current study, we evaluated the local effect of dupilumab treatment on markers of eosinophilic inflammation in skin biopsies, and characterized the activation state of peripheral blood eosinophils in AD patients before and after dupilumab treatment.

METHODS

Subjects and study design

This study included patients from the Dutch BioDay Registry at the National Expertise Center for Atopic Dermatitis from the University Medical Center Utrecht (ClinicalTrials.gov Identifier: NCT03549416), a large prospective, observational cohort study in which patients with moderate-to-severe AD are enrolled. Clinical data, including peripheral blood eosinophil levels over time, were extracted from an online Good Clinical Practice database (BioDay registry). Blood eosinophilia was defined as eosinophil counts $\geq 0.45 \times 10^9$ /L, according to the local laboratory reference limits. Patients were treated with a subcutaneous dose of 600 mg dupilumab at start of treatment, followed by 300 mg every other week for at least 16 weeks. For the majority of patients systemic immunosuppressive treatment was discontinued before starting dupilumab treatment, but concomitant treatment with topical corticosteroids was allowed. This study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice. Study protocols were approved by the local Medical Research Ethics Committee (METC 18/239), and each patient provided written informed consent.

Sample collections

Fresh blood samples were collected from 16 AD patients at three different time points: at baseline prior to treatment with dupilumab, after 4 weeks and after 16 weeks of dupilumab treatment, and from nine healthy controls. Skin biopsies (3mm) of lesional and non-lesional skin were collected from a clinically comparable cohort of ten adult patients with moderate-to-severe AD at baseline and after 16 weeks of dupilumab treatment. Posttreatment biopsies were taken from the same location as pretreatment biopsies, close to prior biopsy scars.

Blood sample processing and stimulation

Blood samples were collected in sodium heparin tubes (Vacuette® Greiner bio-one, Kremsmünster, Austria). Directly after collection, whole blood samples were either stimulated with 1µM-formylmethionine-leucyl-phenylalaline (fMLF) (Sigma-Aldrich, St. Louis, USA) in a 37°C water bath for 10 minutes or left on the bench for 10 minutes. Thereafter, erythrocytes were lysed using an ice cold lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM NA_2EDTA). After this, the remaining leukocytes were washed and resuspended in a staining buffer consisting of PBS supplemented with 0.32% w/v trisodium citrate (prepared by the pharmacy of the University Medical Centre Utrecht) and 10% w/v human pasteurized plasma solution (GPO, Sanquin, Amsterdam, the Netherlands).

Flow cytometry

One million cells at a concentration of 40 million cells per mL were stained with antibodies for 30 minutes on ice, and washed twice before analysis on the LSR-Fortessa flow cytometer (BD, Mountain View CA, USA). The following antibodies were used for staining: from BD: CD44-FITC (clone L178), CD66b-PerCP-Cy 5.5 (clone G10F5), CD35-AF647 (clone E11) and CD11b-AF700 (clone ICRF44); from Biolegend: CD193-BV605 (clone 5E8) and CD62L-APC-Cy7 (clone DREG-56); from Sony Biotechnology: CD16-BV785 (clone 3G8) and CD14-PB (clone HCD14).

Granulocytes were identified on the basis of FSC/SSC (Supplementary Figure E1C). After that monocytes were excluded using CD14 (Supplementary Figure E1D). Finally eosinophils were identified as $CD16^{neg}$ and $CD193^{high}$ cells (Supplementary Figure E1E).

Immunohistochemistry

Skin biopsies were fixed in 10% buffered formalin and embedded in paraffin. Immunohistochemistry (IHC) was performed on paraffin-embedded tissue sections by using purified mouse anti-human mAbs for major basic protein (MBP) (Thermo Scientific, clone BMK13) and eotaxin (Abcam, clone EPR5825). Random fields of equal size were selected to assess superficial dermal/epidermal eosinophil counts on skin sections stained with major basic protein (MBP). MBP+ eosinophils were counted manually by one investigator and one experienced dermato-pathologist. Only positively stained cells with a nucleus were included in the counting. Mean cell densities were calculated and expressed as cells/mm2. Eotaxin expression and

extracellular MBP deposition, indicating degranulation of eosinophils, were rated on a four-point grading scale (0 – 3) by the dermato-pathologist.

Data analysis

FlowJo v10 (LLC, Ashland, OR, USA) was used to analyze the flow cytometry data. Statistical analysis was performed using GraphPad Prism 7.04 (Graphpad Software, La Jolla, CA, USA). Data are presented as median ± interquartile range (IQR). A Wilcoxon matched-pairs signed rank test was performed to compare two paired groups and a Mann-Whitney test was used for non-paired analysis. For comparisons between more than two groups, a Friedman tests or a Kruskal-Wallis test was used. The association of eosinophil counts in lesional skin with disease severity was evaluated by using Spearman correlation coefficients. A p-value ≤ 0.05 was considered statistically significant. Graphs and figures were modified using Adobe Illustrator CS6 v16.0.0 (Adobe Systems, San Jose, CA, USA).

RESULTS

Patient characteristics

A total of 26 patients were enrolled for this study (Table 1); sixteen patients for blood sample analysis (median Eczema Area Severity Index [EASI] at baseline 14.9, IQR 10.6 – 18.5) and ten patients for skin biopsy evaluation (median EASI at baseline 19.2, IQR 11.4 – 29.9). Baseline characteristics did not significantly differ between both groups (Table 1). One patient in the biopsy group and one patient in the blood sample group still used cyclosporine A at baseline, which was tapered and stopped in the first four weeks of treatment. Disease severity significantly decreased in both groups from baseline through week 4 (p=0.000 and p=0.005, respectively) and week 16 (p=0.000 and p=0.005, respectively) (Figure 1A). The proportion of patients with eosinophilia, defined as eosinophil number >0.45 $x10^9$ /L, increased or remained stable from baseline (5 patients [31.3%] in the blood sample group and 6 patients [60%] in the biopsy group) to 4 – 16 weeks after dupilumab treatment (11 patients [68.8%] in the blood sample group and 6 patients [60%] in the biopsy group)(Figure 1B). Blood eosinophil levels >3.0 $x10^9$ in two patients at week 16 could not be explained by AD severity, other immunosuppressive drugs or comorbidities. Increased blood eosinophil levels were not associated with symptoms and did not result in dose adjustment or treatment discontinuation of dupilumab.

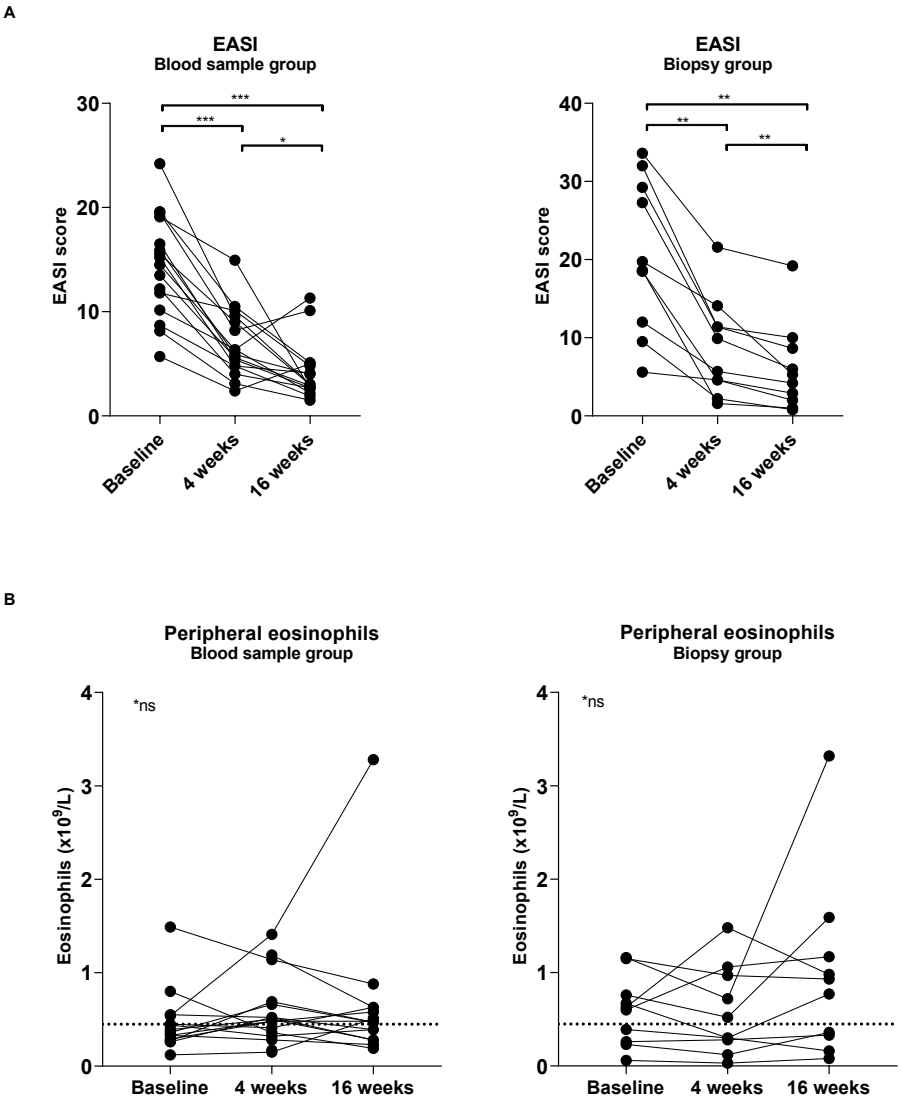

Figure 1. The effect of dupilumab treatment on disease severity and peripheral blood eosinophil counts. Disease severity measured by Eczema Area Severity Index (EASI) scores (**A**) and peripheral blood eosinophil counts (**B**) during 16 weeks of dupilumab treatment in a total of 26 patients (n=16 in the blood sample group and n=10 in the biopsy group). * P≤ 0.05, ** P≤ 0.01, and *** P≤ 0.001.

Dupilumab treatment decreased lesional skin eosinophils and eotaxin expression

Compared to non-lesional AD skin, increased numbers of eosinophils and increased eotaxin expression was observed in lesional skin from AD patients before start of dupilumab treatment (Figure 2A-B). Eotaxin was predominantly expressed on the epidermis and endothelial cells of the dermis, but not in eosinophils. During dupilumab treatment, the number of eosinophils/mm^2 in lesional skin significantly decreased (p=0.0234, Figure 2B). Eosinophil counts in lesional skin positively correlated with EASI scores (*r*=0.548; p=0.017)(Figure 2C). Extracellular deposition of MBP in lesional skin specimens, indicating eosinophil degranulation, decreased in 6 patients (data not shown). Eotaxin expression on dermal endothelial cells of lesional skin decreased from baseline through week 16 of dupilumab treatment in 6 patients (data not shown). Overall these data indicate that dupilumab treatment leads to a decrease of eosinophil numbers in the skin of AD patients, possibly by the inhibition of local eotaxin expression.

Increased expression of trafficking markers CCR3 and CD44 on peripheral blood eosinophils after treatment with dupilumab

To test whether eosinophilia after dupilumab is caused by reduced homing of eosinophils to the skin, we tested the expression of two surface markers related to trafficking on eosinophils in the peripheral blood. The expression of CD193 (or C-C chemokine receptor 3/CCR3) and CD44 on eosinophils in peripheral blood from AD patients at baseline was not different from HCs (Figure 3). However, after 4 weeks of dupilumab treatment, the expression of both receptors was significantly increased in AD patients. From week 4 through week 16, CD193/CCR3 expression decreased again to near-baseline levels (Figure 3A). In contrast to CD193/CCR3, the increase of CD44 was persistent until 16 weeks of treatment (Figure 3B). Summarizing, dupilumab treatment induces (temporarily) elevated expression of the eosinophil trafficking markers CD193/CCR3 and CD44 on peripheral blood eosinophils.

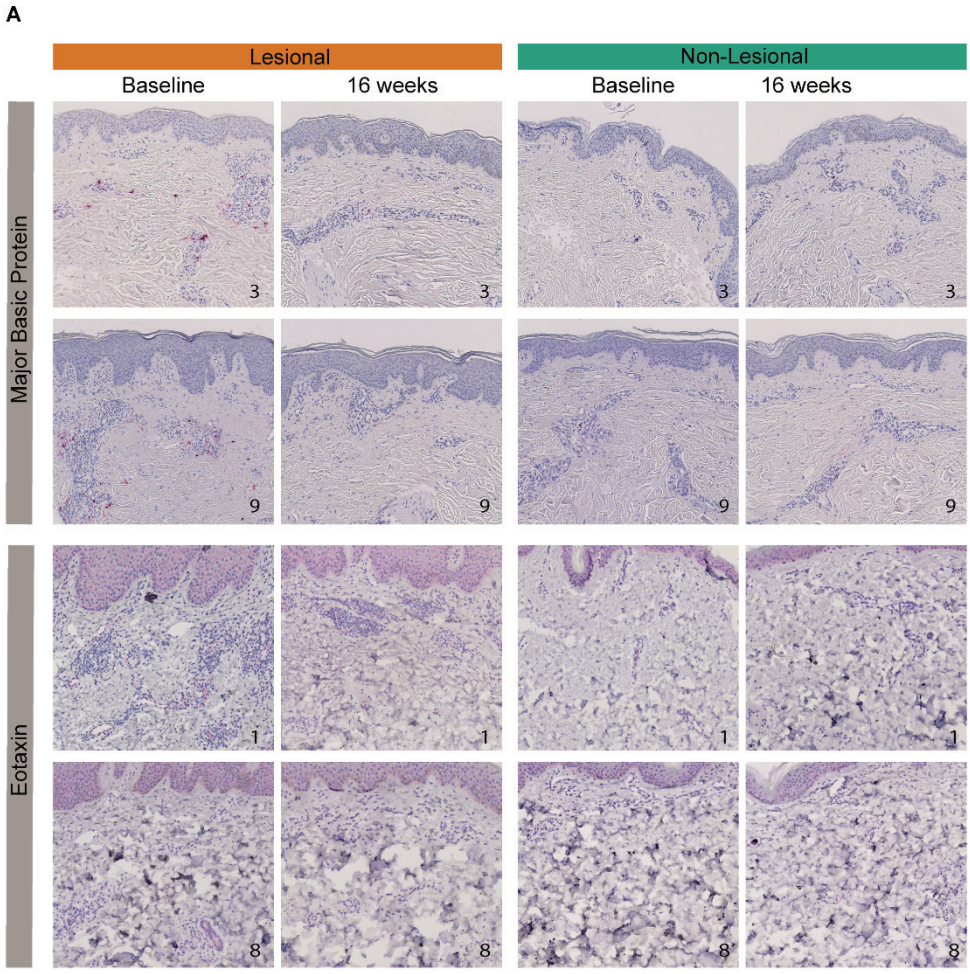

Figure 2. Eosinophil counts and eotaxin expression in skin biopsies from atopic dermatitis patients treated with dupilumab. **A**. Representative histologic images from lesional and non-lesional skin specimens of AD patients before and after 16 weeks of dupilumab treatment stained with major basic protein (MBP) and eotaxin.

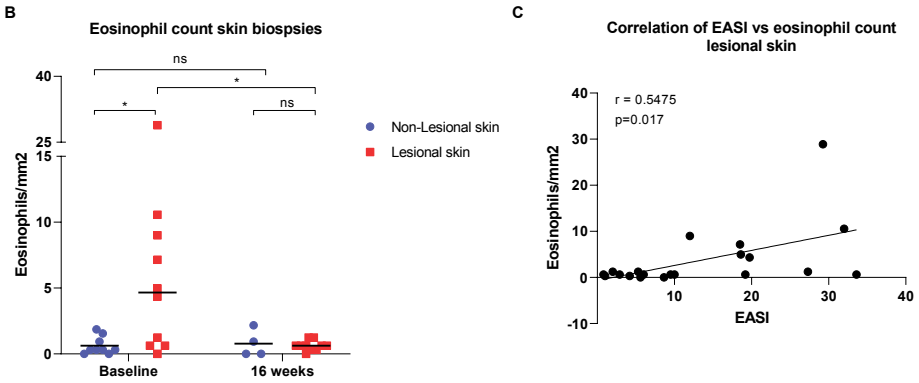

Figure 2. B. MBP+ eosinophil counts per mm^2 in lesional and non-lesional skin specimens of AD patients before and after 16 weeks of dupilumab treatment. **C**. Correlation of Eczema Area Severity Index (EASI) scores and MBP+ eosinophil counts in lesional skin specimens of AD patients before and after 16 weeks of dupilumab treatment. * P≤ 0.05.

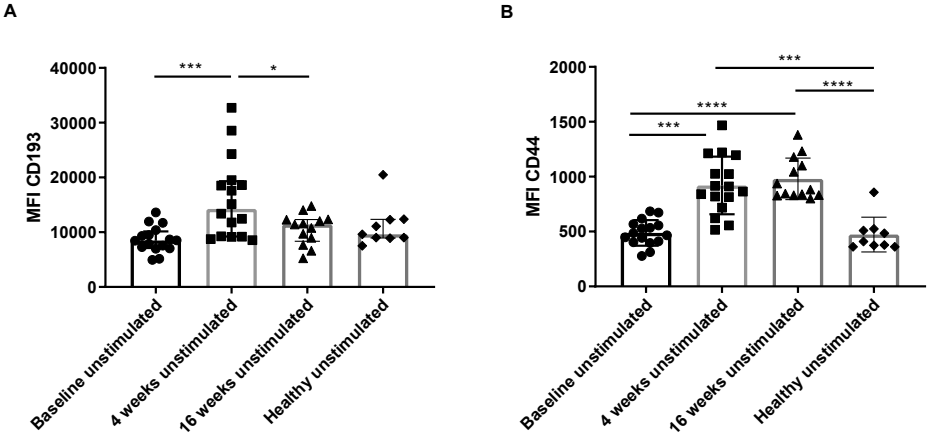

Figure 3. Comparison of surface level of trafficking markers CD193/CCR3 and CD44 on peripheral blood eosinophils. The median fluorescence intensity (MFI) of CD193/CCR3 (**A**) and CD44 (**B**) is plotted for AD patients at baseline (n=16, circles), after 4 weeks of treatment (n=16, squares) and after 16 weeks of treatment (n=13, triangles) with dupilumab. The MFI is compared to surface markers on eosinophils from healthy volunteers (n=9, diamonds). The individual data points with the median and interquartile range are shown. A Friedman test (to compare the different treatment phases) or a Kruskal-Wallis test (to compare healthy volunteers to AD patients) was performed for statistical analysis. All groups were compared but only statistical significant results are indicated. * P≤ 0.05, *** P≤ 0.001 and **** P≤0.0001.

Eosinophil activation in peripheral blood of AD patients was not affected by treatment with dupilumab

It is unknown whether eosinophils in dupilumab treated AD patients are activated. Therefore, we tested whether circulatory eosinophils in AD patients treated with dupilumab were characterized by signs of increased priming and/or degranulation. Activation of eosinophils can be characterized by increased expression of several eosinophil surface proteins including CD11b, CD35, and CD66b, or decreased expression of, for example, CD62L/L-selectin.[24, 25] At baseline, the eosinophilic expression of CD66b, CD35, and CD11b was significantly higher, and the expression of CD62L was significantly lower in peripheral blood from AD patients compared to healthy controls in the current study (Figure 4). No significant change in CD66b, CD35, CD11b or CD62L was observed after 4 and 16 weeks of dupilumab treatment. Eosinophil responsiveness (change of activation markers after stimulation), as shown by the ratio between the mean fluorescence intensity (MFI) of fMLF stimulated/unstimulated eosinophils for CD66b, CD35 and CD11b, was lower in eosinophils from AD patients compared to HCs, and did not change during dupilumab treatment (Supplementary Figure E2). The ratio for CD62L was significantly higher in AD patients at baseline compared to HCs, and increased from baseline through week 16. In conclusion, peripheral blood eosinophils in AD patients show an increased activation status compared to healthy controls. This enhanced activation status is not altered after treatment with dupilumab.

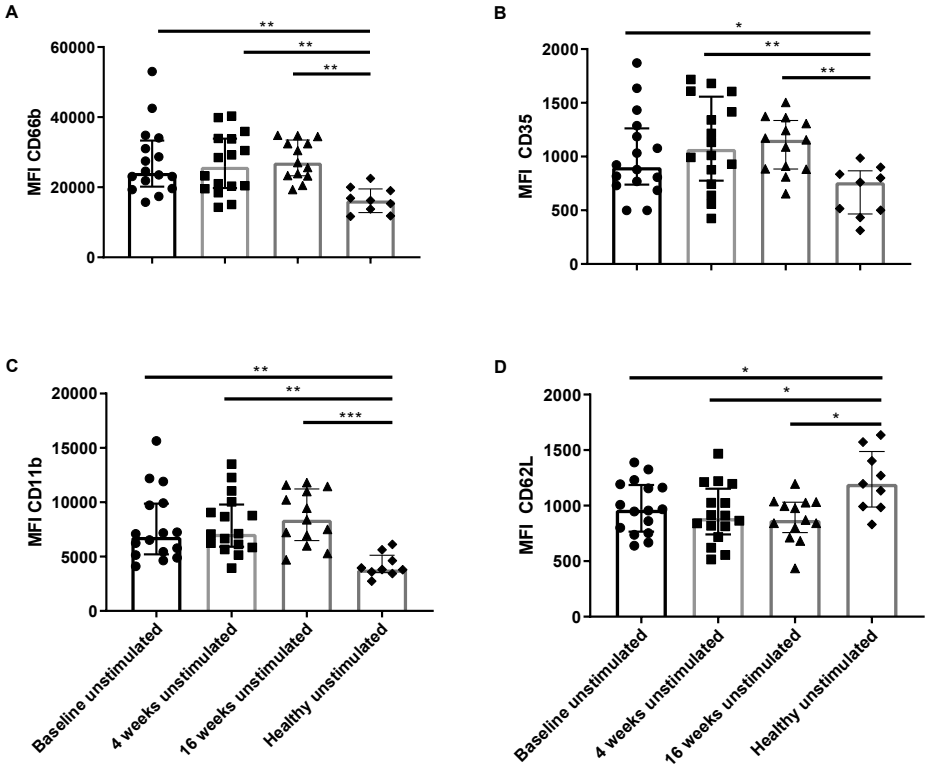

Figure 4. Comparison of activation markers on peripheral blood eosinophils. The median fluorescence intensity (MFI) of CD66b (**A**), CD35 (**B**), CD11b (**C**) and CD62L (**D**) is plotted for AD patients at baseline (n=16, circles), after 4 weeks of treatment (n=16, squares) and after 16 weeks of treatment (n=13, triangles) with dupilumab. The MFI is compared to surface markers on eosinophils from healthy volunteers (n=9, diamonds). The individual data points with the median and interquartile range are shown. A Friedman test (to compare the different treatment phases) or a Kruskal-Wallis test (to compare healthy volunteers to AD patients) was performed for statistical analysis. All groups were compared but only statistical significant results are indicated. * P≤ 0.05, ** P≤ 0.01 and *** P≤0.001.

DISCUSSION

The clinical efficacy of dupilumab treatment in moderate-to-severe AD patients has proven the central role of IL-4 and IL-13 cytokine pathways in the pathogenesis of the disease. The current study aimed to increase the understanding of the role of eosinophilic inflammation in AD and the mechanism underlying eosinophilia

occurring during dupilumab treatment. Our findings of a decrease in lesional skin eosinophils and eotaxin expression during the first weeks of dupilumab treatment, supports the concept that dupilumab treatment inhibits eosinophil trafficking to the skin. We additionally demonstrated that peripheral blood eosinophils from AD patients show an activated phenotype, which is not influenced by 16 weeks of dupilumab treatment. The increased expression of CD193/CCR3, CD44 and several activation markers indicate that eosinophils still have increased potency to migrate into the skin, but apparent decreased skin chemokines and potentially endothelial adhesion molecule expression limit eosinophil skin migration.

The expression of CD193/CCR3 on eosinophils in AD patients at baseline did not differ from healthy controls. However, after 4 weeks of treatment with dupilumab, the expression of this receptor was significantly increased in AD patients, which decreased again after 16 weeks of treatment with dupilumab to levels comparable to baseline. CD193/CCR3 is the principal receptor involved in eosinophil attraction by binding to its ligands eotaxin-1, eotaxin-2, eotaxin-3, monocyte chemoattractant protein (MCP)-2, MCP-3, MCP-4, and RANTES/CCL5.[26, 27] It has previously been demonstrated that CD193/CCR3 and eotaxin levels are markedly increased in lesional skin from AD patients compared to non-atopic controls.[28] The upregulation of CD193/CCR3 on peripheral blood eosinophils found in our patients might be a compensatory response to the reduced local and systemic availability of eotaxin during dupilumab treatment that was shown in the current and previous studies.[18, 20] The production of eotaxins by endothelial cells is orchestrated via IL-13 and to a lesser extent via IL-4.[29] Besides, IL-4 and IL-13 selectively induce vascular cell adhesion molecule-1 (VCAM-1) expression on endothelial cells that is involved in eosinophil migration to tissues via the counter-receptor VLA-4.[30, 31] Blockade of IL-4 and IL-13 signaling by dupilumab treatment might lead to a decrease in both the local and systemic eotaxin production as well as VCAM-1 expression on endothelial cells, resulting in reduced eosinophil recruitment to the skin. This is in line with our findings that both eotaxin expression and eosinophil counts decreased in the lesional skin of AD patients during 16 weeks of dupilumab treatment. The (transient) eosinophilia observed in a subset of dupilumab treated patients could therefore be the result of dupilumab treatment preventing eosinophils from entering the skin, and possibly other tissues, and thus eosinophils accumulating in the bloodstream.[4, 32] However, since eosinophil survival in the blood is short (hours)[33], there must be a continuing stimulus for eosinophil production. Major growth factors for eosinophils are IL-5, granulocyte/macrophage colony-stimulating factor (GM-CSF), and IL-3.

Eotaxin and IL-5 predominantly regulate the mobilization of eosinophils from the bone marrow to the peripheral blood.[7] While eotaxin levels are demonstrated to decrease during dupilumab treatment, levels of IL-5, the most critical marker for eosinophil proliferation and differentiation, remain stable.[18, 34, 35] Whether eosinophils are actively involved in the pathogenesis of AD, or whether they are only bystander cells, remains to be determined. MBP+ eosinophil counts in lesional skin highly correlated with disease severity in our study, and serum eosinophil cationic protein (ECP) levels have previously been correlated to AD severity[12, 36], which supports at least a role for eosinophils as markers for disease activity and response to treatment.

As earlier described in vitro, downregulation of CD193/CCR3 expression might be the result of eotaxin-1 induced desensitization.[37] Therefore, it is conceivable that a reduction in serum eotaxin levels during dupilumab treatment[18] might lead to an increase of CD193/CCR3 expression as shown in our study. Alternatively, it is possible that peripheral blood eosinophils are overall more mature after treatment with dupilumab. We have previously shown that in homeostasis, CD193/CCR3 is a maturation marker on eosinophils.[38] So, a lack of homing of circulatory eosinophils might lead to aging of eosinophils in the circulation which will lead to an increase of CD193/CCR3 expression. An additional hypothesis may be that in AD patients in general, blood eosinophils with the highest CCR3 expression levels are the ones that migrate to the skin, and thus during dupilumab treatment stay in the circulation, causing an increase in mean CCR3 levels on blood eosinophils.

CD44 expression on eosinophils also increased in our patients during treatment with dupilumab. In contrast to CD193/CCR3, the increase of CD44 was persistent during 16 weeks of treatment. CD44 is a proteoglycan expressed on all leukocytes and is possibly involved in cell trafficking by mediating rolling and adhesion to hyaluronic acid.[39] For eosinophils, CD44 is known as an activation marker.[40] In asthma, CD44 is mainly expressed on sputum eosinophils, supporting the concept that CD44 facilitates homing of eosinophils to the tissue. Interestingly, the expression of CD44 on peripheral blood eosinophils is higher in asthma patients that are well-controlled opposed to poorly controlled patients.[40] This again suggests that $CD44^{high}$ eosinophils are more likely to transmigrate to the tissue. One might expect that inhibition of transmigration to the tissue as a result of dupilumab will lead to an increase of the median CD44 expression on eosinophils.[25] CD44 expression remained high after longer treatment with dupilumab (16 weeks), which suggests that homing

of eosinophils to the skin is still reduced. This is in line with the reduced numbers of eosinophils in lesional skin after 16 weeks of dupilumab treatment observed in our study. A previous study of Guttman-Jassky et al.[41] found similar results in lesional skin biopsies from AD patients after 16 weeks of dupilumab treatment. However, the reduction of major basic protein-positive eosinophils did not reach statistical significance when compared with placebo treated patients in this study.[41]

The expression of several activation markers on eosinophils of our AD patients was significantly higher (CD66b, CD35 and CD11b) or lower (CD62L) at baseline compared to healthy controls, which points at an increased activation state of eosinophils.[24, 25] This is in concordance with the previous finding that primed eosinophils are also found in the peripheral blood of patients with other Th2-mediated diseases.[25, 42] Eosinophil activation was not affected by 4 or 16 weeks of treatment with dupilumab in our study, which suggests that IL-4 and/or IL-13 are not involved in the (pre)activated state of eosinophils in the peripheral blood of AD patients. Conversely, anti-IL-5 treatment with mepolizumab have been demonstrated to decrease expression or activation state of some eosinophil surface proteins, presumably by inhibiting IL-5-mediated activation.[43] The enhanced activation status before and during dupilumab treatment is supported by our finding that eosinophils from AD patients are less reactive to fMLF compared to healthy controls. This partial refractoriness of eosinophils has also been found in other type 2-related diseases, such as asthma.[25, 44]

Although our study provides evidence for the role of dupilumab treatment in inhibiting IL-4 and IL-13-mediated migration of eosinophils from the peripheral blood to the skin, no significant change in peripheral blood eosinophil counts was found in our patients, which is a limitation of this study. However, our data are in concordance with those published in to the previous study by Ariëns et al.[18], by showing that the proportion of patients presenting with eosinophilia increased during dupilumab treatment. The occurrence of eosinophilia in a subset of patients suggests that the increase in eosinophil counts depends on the degree of chemokine expression and the rate of eosinophil production in an individual patient.[45] To the best of our knowledge, no adverse events have yet been attributed to eosinophilia in dupilumab treated AD patients.[18, 45] In a large phase III trial investigating dupilumab in moderate-to-severe asthma, 0.2% of the patients developed eosinophilia accompanied by clinical symptoms, including eosinophilic pneumonia and myositis.[19] Furthermore, one case of eosinophilic pneumonia associated with

dupilumab has been reported in a daily practice setting.[46] Our findings of increased activation of peripheral eosinophils before and during treatment, raises awareness of the potential eosinophil mediated collateral damage to healthy tissues. In a recent study we demonstrated the presence of numerous eosinophils in conjunctival tissue of dupilumab-treated AD patients who developed conjunctivitis.[47] However, their potential role in the etiology of this type of conjunctivitis still needs to be elucidated. Increase in the number of activated eosinophils has been associated with elevation of serum ECP, indicating that the eosinophil compartment exhibited a primed state.[48] A recent study including patients with chronic rhinosinusitis with nasal polyposis observed that dupilumab treatment significantly reduced ECP concentrations in nasal polyp tissue after 16 weeks, just as a local reduction in eotaxin-2 and eotaxin-3.[20] No data concerning serum ECP levels during dupilumab treatment are available yet, but Doran et al.[49] showed that serum ECP and eosinophil-derived neurotoxin (EDN) levels remained unchanged during lebrikizumab (anti-IL-13) treatment of asthma patients, while blood eosinophil numbers increased. However, serum ECP and EDN levels significantly decreased in the placebo group, indicating that anti-IL-13 treatment may lead to higher degree of eosinophil granule proteins in serum than placebo. Therefore, it might be prudent to monitor eosinophil counts, eosinophil (pre)activation and ECP levels during dupilumab treatment in patients with AD and other Th2-related diseases.

In conclusion, our study showed that dupilumab treatment significantly decreased local presence of eosinophils and production of eotaxin in lesional skin, combined with an increased expression of CD193/CCR3 and CD44 on eosinophils in peripheral blood in AD patients treated with dupilumab. These results support the concept that treatment with dupilumab decreases eosinophil trafficking to the skin. Furthermore, peripheral blood eosinophils of AD patients show an elevated activation state compared to healthy controls, which is not altered after 16 weeks of treatment with dupilumab. Further studies are needed to elucidate the potential risks of (transient) increases of activated eosinophils in dupilumab treated patients.

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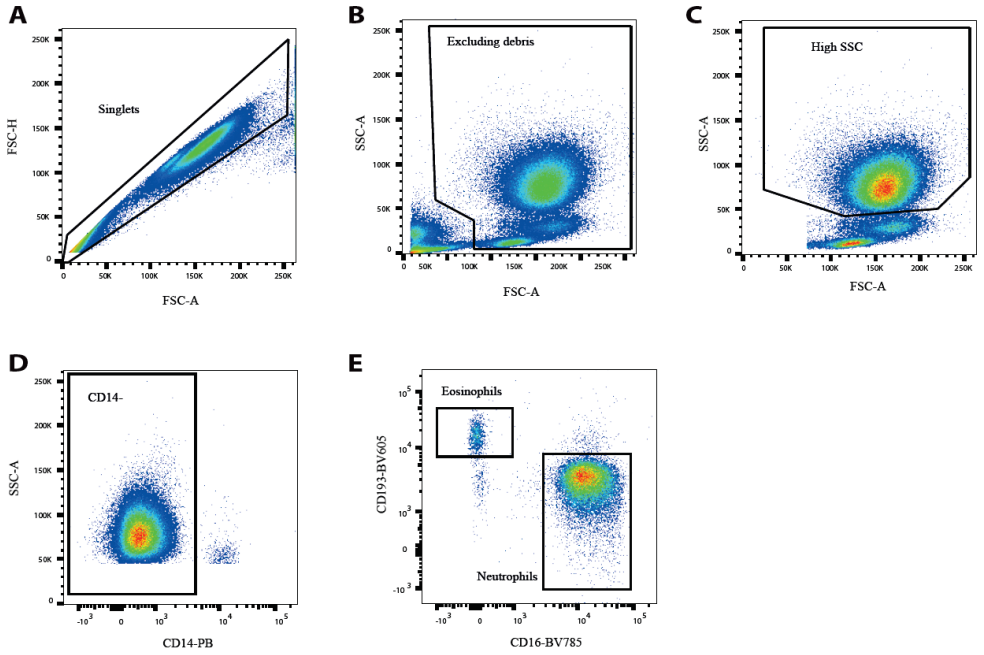
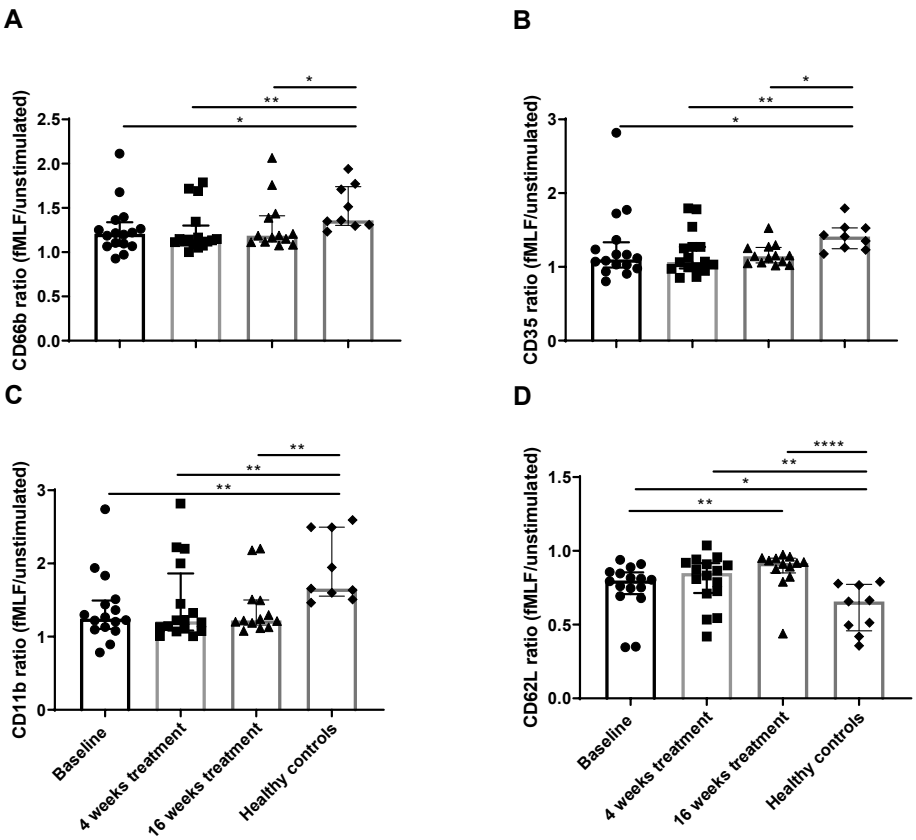
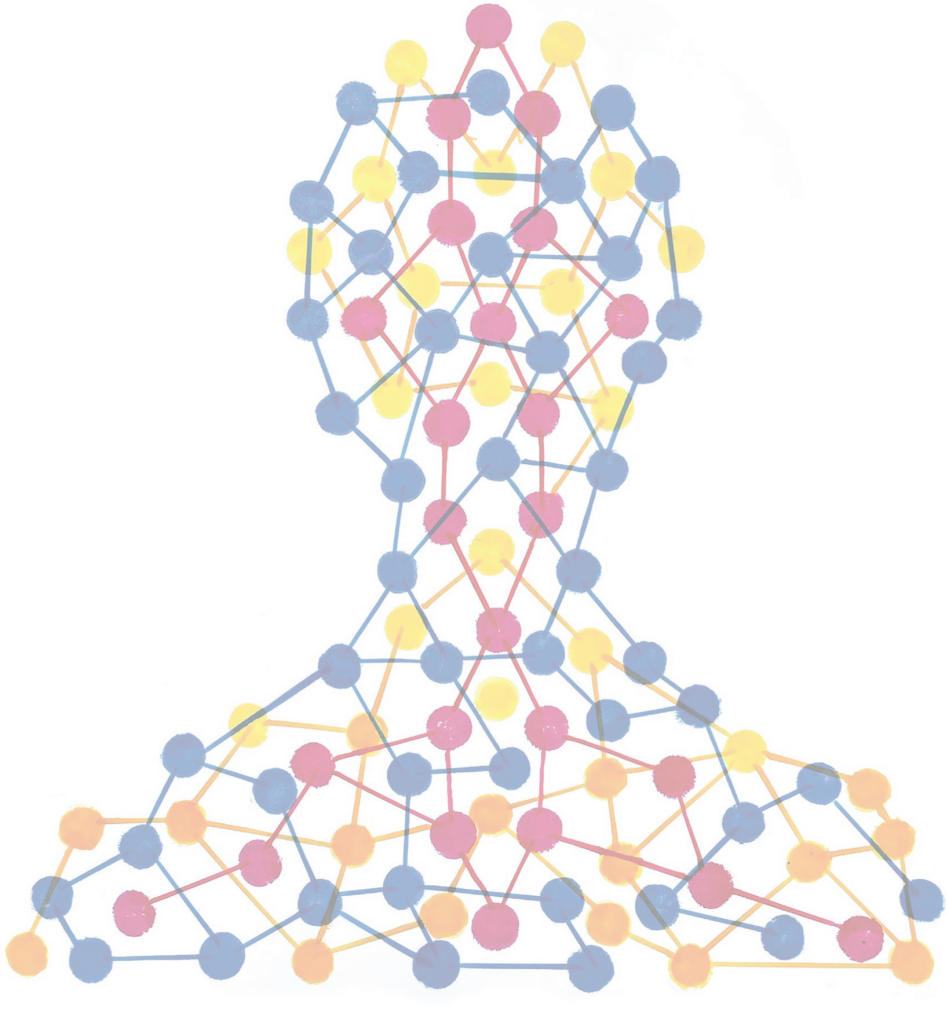
SUPPLEMENTAL FIGURES



Supplemental Figure E1. The different steps to gate eosinophils are depicted. Cells were gated for singlets (**A**), debris was excluded (**B**), SSC^{high} (**C**), exclusion of CD14^{high} (**D**) and finally eosinophils were gated as CD16^{neg} and CD193^{high} (**E**).



Supplemental Figure E2. The ratio between the MFI of 1µM fMLF stimulated and unstimulated eosinophils was calculated for CD66b (**A**), CD35 (**B**), CD11b (**C**) and CD62L (**D**). This is plotted for AD patients at baseline (n=16, circles), after 4 weeks of treatment (n=16, squares) and after 16 weeks of treatment (n=13, triangles) with dupilumab. This ratio is also compared to samples taken from healthy volunteers (n=9, diamonds). The individual data points with the median and interquartile range are shown. A Friedman test (to compare the different treatment phases) or a Kruskal-Wallis test (to compare healthy volunteers to AD patients) was performed for statistical analysis. All groups were compared but only statistical significant results are indicated. * P≤ 0.05, ** P≤ 0.01 and **** P≤0.0001.



Chapter 8

Long-term follow-up and treatment outcomes of conjunctivitis during dupilumab treatment in patients with moderate-to-severe atopic dermatitis

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Dupilumab is the first biologic treatment for atopic dermatitis (AD) and its effectiveness and safety are proven.[1] Although conjunctivitis is the most frequently reported side effect during dupilumab treatment in both clinical trials and daily practice, data on the clinical course of conjunctivitis during long-term use of dupilumab are lacking.[1-3] This prospective daily practice study evaluates ophthalmological characteristics and long-term treatment outcomes of ophthalmologist-confirmed conjunctivitis during dupilumab treatment in moderate-to-severe AD patients. During a 12-month evaluation period, 167 moderate-to-severe AD patients were treated with dupilumab 300 mg every 2 weeks at the University Medical Center Utrecht, the Netherlands. Patients reporting ophthalmological symptoms who could not be controlled with lubricant drops and/or tacrolimus skin ointment (1mg/g) for the external eyelids were referred to an ophthalmologist. Further (anti-inflammatory) ophthalmological treatment was prescribed by the ophthalmologist, and individually chosen per patient.

Conjunctivitis was reported in 66/167 (39.5%) patients, of whom 33 were referred to an ophthalmologist. Ophthalmologist-confirmed conjunctivitis was reported in 33/167 (19.8%) patients (17 female; mean age 45.7 years, standard deviation (SD) 14.3; mean Eczema Area Severity Index (EASI) at baseline 21.7 (SD 9.5), Supplemental Table E1). History of (allergic) conjunctivitis was present in 24/33 (72.7%) patients. None of the 33 patients reported conjunctivitis symptoms at start of dupilumab. In the 33 referred patients, patient-reported eye symptoms, such as redness, tearing and itching, developed within a median of 33 days (interquartile range (IQR) 28.0-61.0) after starting dupilumab. Ophthalmological characteristics were examined and graded in terms of severity by an experienced ophthalmologist following the UTrecht OPhthalmic Inflammatory and Allergic disease (UTOPIA) ocular surface score (Table 1). Overall conjunctivitis severity was based on grading of different ophthalmological characteristics (Figure 1A-B).

Ophthalmological characteristics	Severity*			
	None	**Mild**	**Moderate**	**Severe**
Blepharitis	No blepharitis	Bubbles, mild hyperemia of the eyelid	Hyperemia and mild swelling of the eyelid	Severe hyperemia, thickening, keratinization, scarring of the eyelid
Meibomian gland dysfunction	No Meibomian gland dysfunction	Bubbles, after pressing an oily substance is formed	Plugs or bubbles, after pressing an thicker substance is formed	Plugs or scarring, after pressing no substance is formed
Tarsal conjunctivitis	No tarsal conjunctivitis	Mild swelling and hyperemia, mild papillae	Larger papillae, moderate swelling and hyperemia	Moderate characteristics and/or keratinization, ulceration, sclerosis
Bulbar conjunctivitis	No bulbar conjunctivitis	Mild swelling and hyperemia	Moderate swelling and hyperemia in all quadrants	Severe swelling and hyperemia, mucus and excessive tearing, photophobia.
Limbitis	No limbitis	Mild swelling and hyperemia	Evident swelling/hyperemia over >3 clock hours	Severe swelling/hyperemia, conjunctival vascularization extending the normal limbus edge
Limbal vascularization	No abnormal limbal vascularization	Fine vascularization along the limbus	Moderate vascularization to the limbus >3 clock hours, or fine vascularization extending the normal limbus barrier	Strong vascularization extending the normal limbus barrier in >3 clock hours
Corneal punctate	No corneal punctate	Some punctate limited to the interpalpebral region	Fiddled punctate, extending the interpalpebral region or strongly present in the interpalpebral region	Significantly diffuse punctate and/or confluent
Hurricane pattern	No hurricane pattern	Elongated small and narrow punctate along the limbus to <0.25 radius and <1 clock hour	Long and thin punctate in hurricane pattern, up to <0.5 radius and <3 clock hours	Evident hurricane pattern >3 clock hours and/or 0.5 radius (cross pupil)
Overall severity of the conjunctivitis	None / mild / moderate / severe conjunctivitis**			

Table 1. UTrecht OPhthalmic Inflammatory and Allergic disease (UTOPIA) ocular surface score. * None = 0 points; mild = 1 point; moderate = 2 points; severe = 3 points. ** 0 = no conjunctivitis; 1-4 = mild conjunctivitis (unless the score consists of only Meibomian gland dysfunction and punctate, then the total score is 0); 5-8 = moderate conjunctivitis; ≥9 = severe conjunctivitis.

During the first ophthalmological consultation, mild, moderate, and severe conjunctivitis were diagnosed in 22 (66.7%), 7 (21.2%), and 4 (12.1%) of the 33 referred patients, respectively (Fig. 1B). Most frequently reported ophthalmological characteristics were tarsal and bulbar conjunctivitis, and blepharitis (in 28 (84.8%), 25 (75.8%), and 22 (66.7%) patients, respectively). Six (18.2%) patients presented with limbitis (Fig. 1A).

The most frequently prescribed ophthalmological treatments during follow up included corticosteroid eye drops, tacrolimus skin ointment for the external eyelids, and lubricant drops (in 24 (72.7%), 25 (75.8%), and 26 (78.8%) patients, respectively, Supplemental Table E2). During follow-up (mean 17.5 (SD +/- 3.4) months, dosing interval of dupilumab was prolonged to 300 mg every three to five weeks in 10/33 (30%) patients because of conjunctivitis, resulting in improvement of eye symptoms in six patients and remission in one patient. Discontinuation of dupilumab due to ocular pathology was necessary in 3/33 (9.1%) patients, showing improvement or remission in all cases (Fig 1C). Ineffectiveness of dupilumab led to discontinuation in 2/33 (6.1%) patients.

After follow-up, 24/28 (86%) patients who continued dupilumab treatment were still suffering from conjunctivitis (Fig. 1B). New-onset limbitis during follow-up was seen in eight more patients (8/27, 29.6%) patients; in six cases despite ophthalmic anti-inflammatory treatment.

The conjunctivitis outcome during a follow-up of 17.6 months (SD +/-3.5), was evaluated for 28/33 (84.8%) patients who continued dupilumab, by comparing the first conjunctivitis severity category with the latest follow-up category (Fig. 1D). Outcomes were categorized into worsened (worsening with ≥1 category), stable (unchanged category), improved (improvement with ≥1 category) or complete remission (no conjunctivitis). Complete remission was seen in 4/28 (14%) patients; of these, two were still using anti-inflammatory eye drops or tacrolimus ointment for the external eyelids. Improvement of conjunctivitis occurred in 7/28 (25%) patients, of which six were still using anti-inflammatory eye drops. Uncontrolled conjunctivitis, meaning stable or worsened conjunctivitis, was seen in 17/28 (61%) patients. Ophthalmic anti-inflammatory therapy was prescribed for all of these 17 patients; however, 2/17 patients reported being non-compliant.

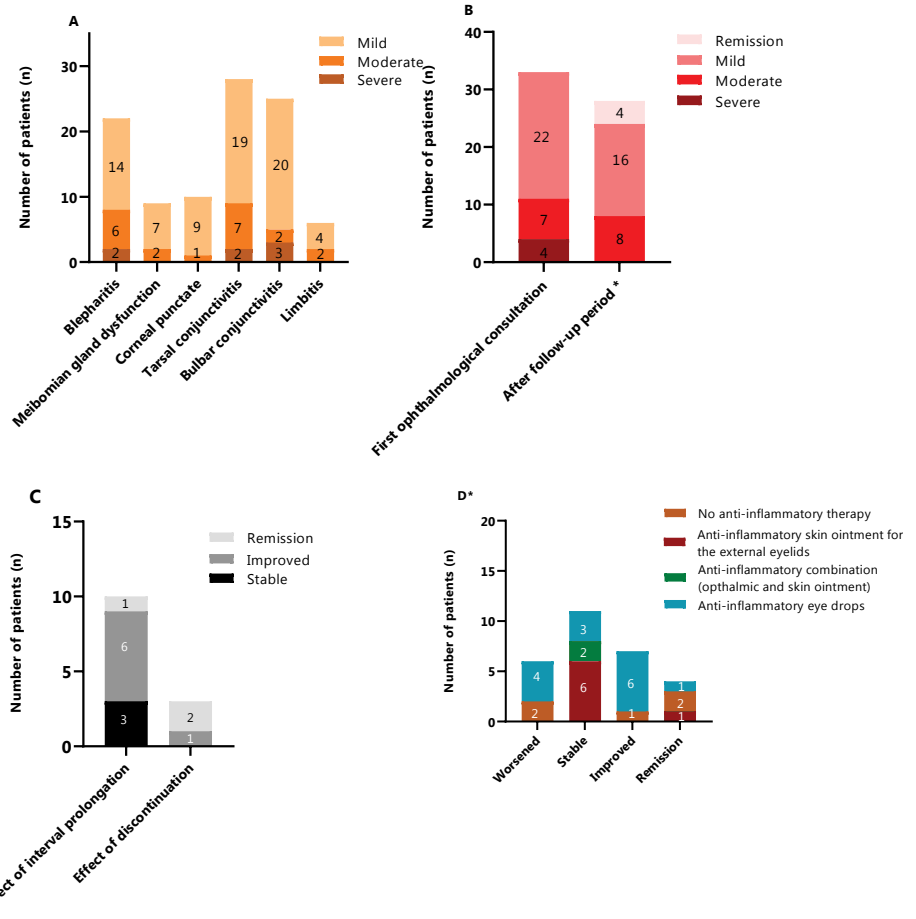

Figure 1. Results of 33 atopic dermatitis patients diagnosed with conjunctivitis during dupilumab treatment. **A.** Ophthalmic characteristics at the first ophthalmological consultation (n=33). **B.** Severity of conjunctivitis at the first consultation (n=33) and after follow-up (n=28). **C.** Effect of dose adjustment of dupilumab due to ocular pathology. **D.** Outcome and treatment of conjunctivitis after follow-up (n= 28)*. *Discontinued patients (n=5) were excluded.

Literature regarding conjunctivitis during dupilumab is limited by small sample sizes, short follow-up duration, and lack of thorough and standardized ophthalmological investigation. In contrast, all 33 patients of our study underwent standardized examination by an ophthalmologist followed by long-term follow up. Several pathomechanisms have been suggested to be responsible for the development or worsening of conjunctivitis during dupilumab treatment in AD patients, such as rosacea-like conjunctivitis, focal scarcity of intra-epithelial goblet cells, and relative

ocular under treatment due to lower tissue distribution of dupilumab in the eyes.[2, 4, 5] The last hypothesis seems in contradiction with our finding that interval prolongation or discontinuation of dupilumab resulted in improvement of the conjunctivitis. The management of conjunctivitis during dupilumab treatment is challenging. Previous case series and case reports have described several therapeutic options, including tacrolimus eye ointment, fluorometholone eye drops, cyclosporine eye drops, and lifitegrast eye drops, leading to improvement in most cases.[6-8] The majority of our patients received combination therapy and most patients remained dependent on ophthalmic medication. Anti-inflammatory eye drops and/or tacrolimus ointment for the external eyelids were prescribed most often.

In contrast to clinical trial data, reporting that most conjunctivitis cases recovered or resolved while continuing dupilumab treatment, our results show more persistent ophthalmological signs and symptoms despite adequate ophthalmic treatment. Remarkably, 8/33 (24.2%) patients developed limbitis during follow-up; in six cases despite adequate ophthalmic anti-inflammatory treatment. Limbal stem cells are vital for corneal healing and the barrier function of the limbus. Chronic limbitis may lead to irreversible limbal stem cell deficiency, which could lead to irreversible long-term visual loss, making adequate monitoring of conjunctivitis necessary.[9]

This study has some limitations. Firstly, since all patients were seen in an AD expertise center, the population consisted of more severe AD patients. As severity of AD may be related with the development of conjunctivitis during dupilumab treatment, this may have affected the results.[2] Secondly, not all patients may have been compliant with ophthalmic treatment, which might have resulted in under treatment of the conjunctivitis. Lastly, ophthalmological examination by an ophthalmologist was not performed before starting dupilumab; therefore, pre-existing ophthalmological pathology cannot be excluded.

In conclusion, this study shows ophthalmologist-confirmed conjunctivitis in 33/167 (19.8%) AD patients treated with dupilumab in a one-year period. During long-term ophthalmological follow-up, the majority of these patients still suffered from mild-to-moderate conjunctivitis despite treatment. Dose adjustment or discontinuation of dupilumab due to ocular pathology was needed in 10/33 and 3/33 of the patients, respectively.

Acknowledgements

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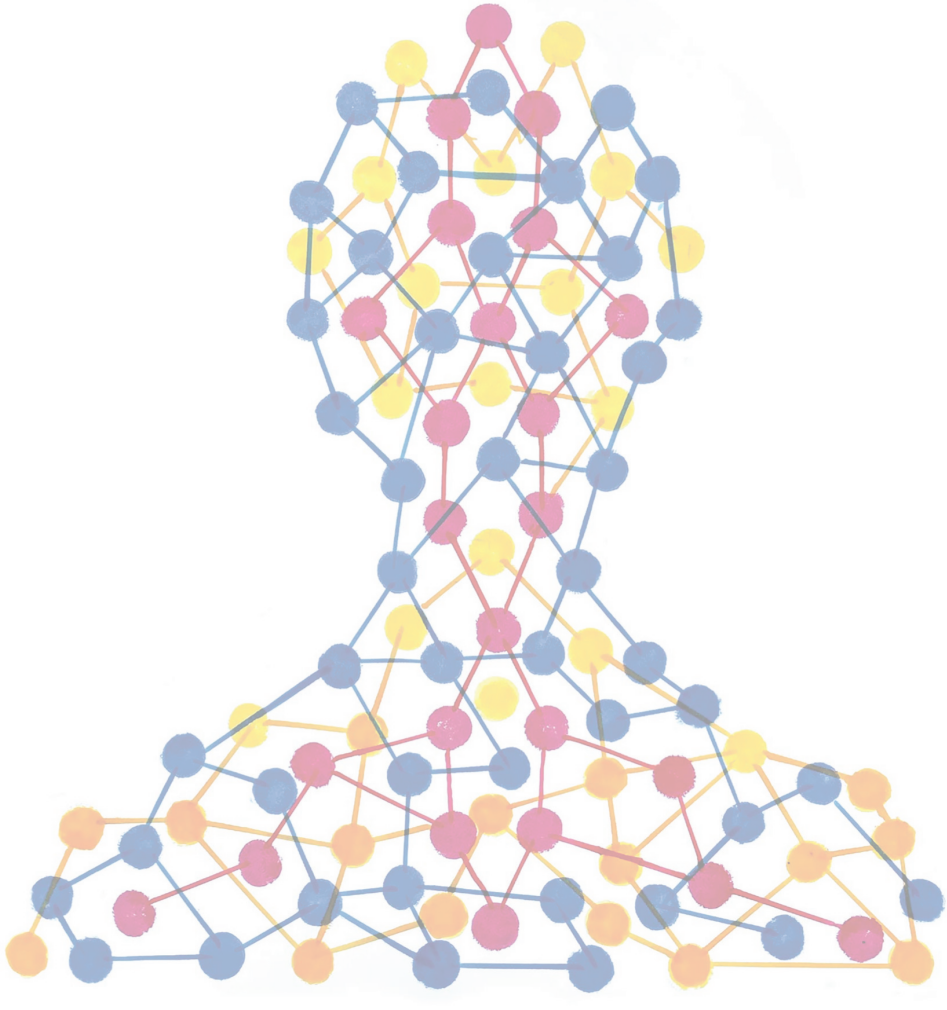
SUPPLEMENTAL TABLES

Characteristics	Total group (n=33)
Sex, female, n (%)	17 (51.5)
Age (years) at start of dupilumab, mean (SD)	45.7 (14.3)
Age of primary onset AD	
Childhood, n (%)	28 (84.8)
Adolescence, n (%)	4 (12.1)
Adult, n (%)	1 (3.0)
Number of prior immunosuppressive systemic treatments for AD (used for at least 3 months), median (IQR)	2.0 (1.0 – 4.0)
Hospitalized for AD ever, n (%)	27 (81.8)
Atopic comorbidities	29 (87.9)
Allergic asthma, n (%)	23 (69.7)
Allergic rhinitis, n (%)	23 (69.7)
Allergic conjunctivitis, n (%)	24 (72.7)
Food allergy, n (%)	21 (63.6)
AD related parameters at start dupilumab	
EASI score baseline, mean (SD)	21.7 (9.5)
TARC (pg/ml), median, (IQR)	2856 (1271 – 8000)
Eosinophils ($x10^9$/L), median (IQR)	0.38 (0.26 – 0.72)
AD related parameters at referral to the ophthalmologist	
EASI score, mean (SD)	8.0 (5.8)
TARC (pg/ml), median (IQR)	625 (413 – 938)
Eosinophils ($x10^9$/L), median (IQR)	0.62 (0.30 – 1.30)
Number of days between start dupilumab and development of eye symptoms, median (IQR)	33.0 (28.0 – 61.0)
Number of days between start dupilumab and referral to the ophthalmologist, median (IQR)	94.0 (54.5 – 147.5)
Number of ophthalmological consultations, median, (IQR)	4.0 (2.5 – 8.0)
Total follow-up period (both dermatological and ophthalmological) (months), median (IQR)	22.0 (18.0 – 24.0)
Follow-up period since ophthalmological baseline(months), mean, (SD)	17.5 (3.4)
History of ocular disease (excluding allergic conjunctivitis)	11 (33.3)
History of atopic keratoconjunctivitis, n (%)	5 (45.5)
Active conjunctivitis at start dupilumab, n (%)	0 (0.0)
Rosacea	
History of rosacea, n (%)	4 (12.1)
Rosacea flare during follow-up, n (%)	6 (18.2)
Development of head-neck dermatitis during follow-up, n (%)	2 (6.1)

Supplemental Table E1. Baseline table. Data are n (%) unless otherwise indicated. Childhood is <12 years, adolescence is 12-17 years old and adult is >18 years old. AD = atopic dermatitis; SD = standard deviation; IQR = interquartile range; EASI = Eczema Area Severity Index; TARC = Thymus- and Activation-Regulated Chemokine

Prescribed therapies as treatment for conjunctivitis during follow-up	n=33
Lubricant drops	26 (78.8)
Anti-inflammatory therapy for the external eyelids	25 (75.8)
Antihistamine eye drops	14 (42.4)
Corticosteroid eye drops	24 (72.7)
Other anti-inflammatory therapy (eye drops/eye ointment)	12 (36.4)
Combined anti-inflammatory and antimicrobial therapy (eye drops/ eye ointment)	10 (30.3)
Other therapy	3 (9.1)

Supplemental Table E2. Treatment for conjunctivitis, number of total prescribed treatments during follow-up. Data are n (%) unless otherwise indicated. Multiple therapies per patient. Anti-inflammatory treatment for the external eyelids included tacrolimus skin ointment; corticosteroid eye drops included fluormetholone, dexamethasone, hydrocortisone, softacor, prednisolone; antihistamine eye drops included ketotifen; other anti-inflammatory therapy (eye drops/ eye ointment) included tacrolimus eye ointment, cyclosporine A eye drops; combined anti-inflammatory and antimicrobial treatment (eye drops/ eye ointment) are terracortril, tobradex; other therapies are cross-linking, bandage lens with chloramphenicol.



Chapter 9

Goblet cell scarcity and conjunctival inflammation during treatment with dupilumab in patients with atopic dermatitis

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Dear editor,

Higher rates of conjunctivitis have been reported in atopic dermatitis (AD) patients treated with dupilumab, a human monoclonal antibody that inhibits the signaling of interleukin (IL)-4 and IL-13, versus patients treated with placebo in phase III clinical trials.[1] However, the exact pathomechanism of this potential treatment-limiting side effect has not been clarified. Given the necessity of optimal treatment and risk management in clinical practice, the aim of this study was to describe the histopathological characteristics of conjunctivitis during dupilumab treatment in AD patients.

Participants, selected from the Bioday registry, consisted of 74 moderate-to-severe AD patients treated with dupilumab for at least 16 weeks. Of these, 23% developed ophthalmologist-confirmed conjunctivitis requiring anti-inflammatory treatment. We sequentially included six patients (three male; median age 39 years, interquartile range [IQR] 29-54) in whom a diagnostic conjunctival biopsy of the inferior fornix was performed by the ophthalmologist before initiation of ocular anti-inflammatory treatment. Biopsies were fixed, paraffin-embedded and stained with haematoxylin and eosin (HE) for histological assessment, and additionally with CD3/CD4 (T helper [Th] cells) and Alcian Blue (mucus-containing goblet cells [GCs]). Conjunctival biopsies of two healthy controls were included from the local pathology database and stained with Alcian Blue. Biopsies were assessed by two independent experienced pathologists. This study did not fall under the scope of the Medical Research Involving Human Subjects Act which was confirmed by the local Medical Research Ethics Committee (METC 18/537).

The most prominent histopathological feature in conjunctival biopsies from patients with AD developing conjunctivitis during dupilumab treatment was scarcity of intraepithelial GCs. Median GC density was 3.3 cells/mm (IQR 1.1 – 4.9)(Figure 1A-B) in patients with AD with conjunctivitis vs. 28.3 and 36.3 cells/mm in the two control samples. Five patients showed a multicellular immune-cell stromal infiltrate, consisting mainly of T cells (CD3+) and eosinophils (Figure 1C). Epithelial migration of eosinophils and lymphocytes was seen in respectively four and five out of six patients.

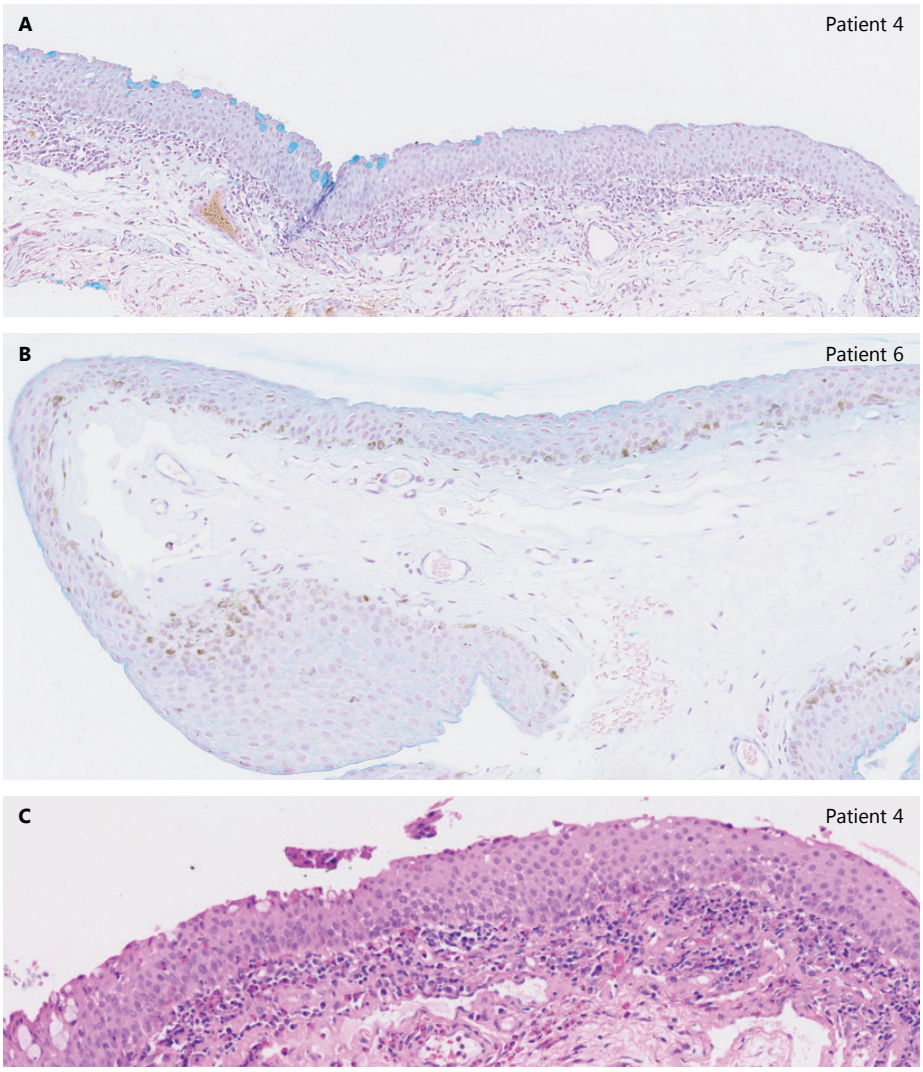

Figure 1. Alcian blue-stained histological sections of the inferior bulbar conjunctiva under light microscopy shows the presence of decreased goblet-cell density in patients with AD treated with dupilumab (original magnification × 40). **A.** Regions with no goblet cells (GCs) interspersed with smaller regions of normal GC density. **B.** In patient 6 no GC was found in the conjunctival biopsy. **C.** Haematoxylin and eosin stained histological sections of the inferior bulbar conjunctiva under light microscopy show the presence of a superficial inflammatory multicellular infiltrate in the conjunctival stroma consisting of mainly T cells and eosinophils, partially migrating into the conjunctival epithelium.

Conjunctival GCs are specialized mucus-secreting cells, vital for ocular surface function.[2] GC density varies between different conjunctival regions, with higher numbers in the normally covered locations of the open eye. [3] In healthy individuals lower forniceal GC counts vary between 8.8 and 30 cells/mm.[4] All patients included in our study had a marked decreased amount of GCs (median of 3.3 cells/mm) vs. controls (median 32.3 cells/mm).

Mice studies have demonstrated that ocular IL-13 expression normally stimulates GC proliferation and mucus secretion.[5] By blocking IL-13, dupilumab treatment may lead to GC hypoplasia, as IL-4Rα is expressed on conjunctival epithelium. This might result in decreased mucin production, subsequent tear film instability and mucosal epithelial barrier dysfunction, leading to conjunctival inflammation in a subpopulation of (predisposed) patients with AD. Clinically, the loss of GC-produced factors may result in dry eyes, as was reported by all patients, and subsequently irritative conjunctivitis. As in this study biopsies were performed after initiation of dupilumab, GC scarcity might already be present before dupilumab treatment, although patients did not experience ocular symptoms at start of treatment.

Our histopathological findings do not correspond with the histopathology of atopic keratoconjunctivitis and allergic conjunctivitis, which is associated with an increased GC density and increased mucus production, probably due to IL-13 overexpression.[6, 7] Dupilumab treatment might theoretically be beneficial in these typical Th2-mediated ocular surface diseases.

It has been proposed that dupilumab treatment could increase *Demodex* numbers in hair follicles, causing ocular rosacea-like disease.[8] Ocular rosacea is a Th17-driven disease characterized by an inflammatory cell infiltrate, mainly consisting of CD4+ T cells, but not eosinophils.[9] The unique combination of low conjunctival GC numbers accompanied by numerous lymphocytes and eosinophils found in this study may imply a new entity of conjunctivitis in dupilumab-treated patients with AD.

Only patients with new onset of conjunctivitis symptoms or worsened symptoms in cases of pre-existing conjunctivitis were included in this study; these probably do not represent all conjunctivitis cases during dupilumab treatment. In daily practice, we experience some patients reporting improvement of conjunctivitis symptoms during dupilumab treatment, underlining the heterogeneity of the conjunctivitis.

Limitations of this study are small sample size, and collection of conjunctival biopsies at one single time point. Therefore, dynamic differences in histopathological features before and during dupilumab treatment could not be studied. Nevertheless, the histopathological features and findings were very consistent, and constitute a first clue in the underlying pathomechanism of dupilumab-associated conjunctivitis. However, the exact pathomechanism of this new entity of conjunctivitis could not be fully elucidated.

In conclusion, this study found a remarkable scarcity of conjunctival GCs accompanied by an inflammatory T-cell- and eosinophilic infiltrate in patients with AD with conjunctivitis during dupilumab treatment. We hypothesize that the IL-13 blocking effect of dupilumab might lead to reduction of GCs and mucin production in a subpopulation of patients with AD, which may potentially result in irritative conjunctivitis. A prospective study further characterizing conjunctivitis in patients with AD before and during dupilumab treatment will start soon.

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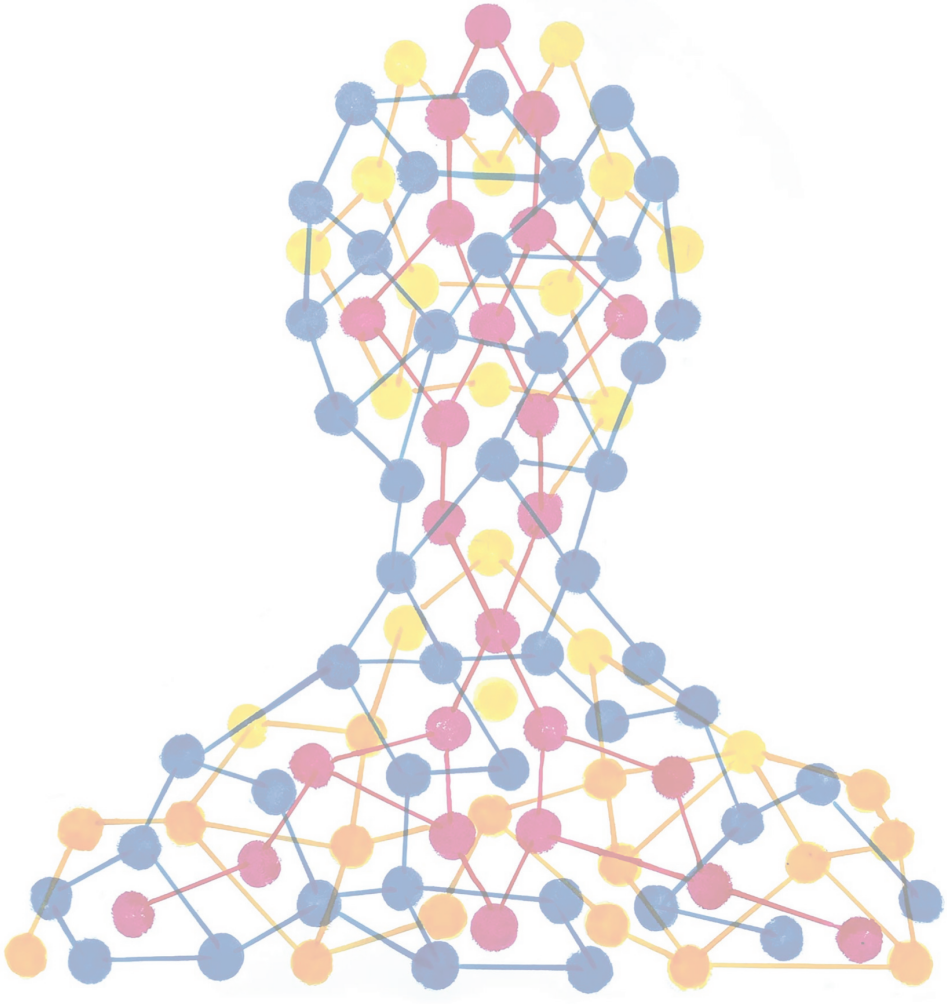

Chapter 10

Conjunctival inflammation in dupilumab-treated atopic dermatitis comprises a multicellular infiltrate with elevated T1/T17 cytokines: a case series study

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ABSTRACT

Background: Conjunctivitis is one of the most commonly reported adverse events during dupilumab treatment, the first biological for atopic dermatitis (AD) targeting (interleukin)IL-4 and IL-13. However, the exact pathogenesis of conjunctivitis has not been clarified. We sought to characterize infiltrating immune cells in conjunctival tissue from dupilumab-treated AD patients, in order to improve the understanding of this new entity of conjunctivitis.

Methods: This case-series study included six AD patients who developed conjunctivitis during dupilumab treatment. Paraffin-embedded conjunctival biopsies were histologically assessed. Additionally, biopsies were stained with a selected panel of 27 antibodies for imaging mass cytometry, an emerging imaging technology that enables in situ protein expression analysis of multiple markers simultaneously at a subcellular resolution.

Results: In addition to a scarcity of intraepithelial goblet cells (GCs), the subepithelial cellular infiltrate in inflamed conjunctival tissue of dupilumab-treated AD patients comprises a diverse panel of infiltrating immune cells, including highly activated and proliferating CD4+ and CD8+ T-cells, but also B-cells, macrophages, monocytes, and dendritic cells. Besides increased cytotoxic activity, elevated T1 (IFNγ, TNFα) and T17 (IL-17) cytokine production was observed within infiltrates.

Conclusion: Our findings indicate that dupilumab-associated IL-4 and IL-13 suppression in combination with increased local T1-related cytokine production may underlie the loss of GCs and their essential immunomodulatory role in the conjunctiva, leading to dry eyes, a highly activated diverse multicellular infiltrate, and tissue damage. In future studies, evaluation of conjunctival GC numbers and tear IFNγ measurements might identify AD patients at risk of developing conjunctivitis who might benefit from early, preventive anti-inflammatory ocular treatment.

INTRODUCTION

Dupilumab (Dupixent®), a fully human monoclonal antibody directed against the interleukin (IL)-4 receptor-alpha-subunit, is the first antibody-based treatment for atopic dermatitis (AD).[1] By inhibiting the signaling of the type 2 cytokines IL-4 and IL-13, which are key drivers in the pathogenesis of AD, dupilumab has demonstrated efficacy and safety in moderate-to-severe AD.[2, 3] Conjunctivitis is a frequently reported adverse event in dupilumab-treated AD patients[4], which in some cases led to discontinuation of treatment.[3, 5] Surprisingly, higher rates of conjunctivitis during dupilumab treatment were not reported in asthma[6, 7] or chronic rhinosinusitis with nasal polyposis trials[8], indicating an AD specific underlying mechanism. To date, the exact pathogenesis of conjunctivitis observed during dupilumab treatment has not been clarified. In a recent case-series study describing histopathology of conjunctival biopsies, we demonstrated a scarcity of intraepithelial goblet cells (GCs), accompanied by a mixed immune-cell infiltrate, consisting of numerous T-cells and eosinophils.[9] In the current case-series study we aimed to further specify these infiltrating cells, in order to improve the understanding of this new entity of conjunctivitis, and optimize treatment and risk management in clinical practice in future.

MATERIALS AND METHODS

Patients

AD patients who developed conjunctivitis during treatment with dupilumab 300mg every other week at the National Expertise Center for Atopic Dermatitis (department of Dermatology and Allergology, University Medical Center Utrecht, the Netherlands) were screened for inclusion. Patients with new onset or worsened conjunctivitis confirmed by an ophthalmologist after starting dupilumab and in whom a diagnostic conjunctival biopsy was performed were eligible for inclusion. Patients were not treated with any ocular anti-inflammatory medication at the moment of biopsy. Ophthalmological characteristics were examined and graded in terms of severity by an experienced ophthalmologist following the Utrecht Ophthalmic Inflammatory and Allergic disease ocular surface score.[10] Clinical data were extracted from an online Good Clinical Practice database called BioDay registry, including a prospective cohort

of adult patients with moderate-to-severe AD treated with dupilumab in daily practice. ClinicalTrials.gov identifier: NCT03549416, retrospectively registered June 8, 2018. All patients signed Institutional Review Board-approved written consent, adhering to the Declaration of Helsinki Principles.

Biopsy collection

Topical local anesthetic with 0.4% oxybuprocaine hydrochloride and 0.5% tetracaine was applied to the conjunctival surfaces. After topical anesthetic, the lower eyelid was retracted and the peripheral inferior bulbar conjunctiva was grasped with forceps. A strip of approximately 3mm in diameter from the bulbar conjunctiva near the inferior fornix was excised using Westcott scissors. Hemostasis was achieved by applying pressure for two to three minutes and patients were treated witch prophylactic antibiotic eye ointment for three days. Bulbar conjunctival biopsies of two healthy controls (HCs), obtained during corneal transplantation due to endothelial dysfunction were included as comparison.

Sample slides

Conjunctival biopsies were fixed in 10% neutral buffered formalin, paraffin-embedded, and three slides containing consecutive 5 μm-thick sections of all samples were prepared. One slide was stained with haematoxylin and eosin (HE) for histological assessment and another with Alcian blue for identification of goblet cells (GCs). A third slide was stained for imaging mass cytometry (IMC)(Figure 1a). IMC is an emerging imaging technology that combines immunohistochemistry with high-resolution laser ablation of stained tissue sections followed by CyTOF mass cytometry. This approach enables sharp-contrast imaging of up to 37 proteins simultaneously at a subcellular resolution.[11] In addition to conjunctival biopsy sections of patients and controls the sample slide for IMC analysis contained a Tissue Micro Array (TMA) section. The TMA consisted of cores from ten different tissues (tonsil, breast, placenta, skin, liver, lung, small intestine, ovarian, spleen, colon). In this way, each antibody could be positively validated on tissues known to express the target antigen and negatively validated on tissues known to be negative for that antigen.

Imaging Mass Cytometry

Antibodies and metal conjugation

All antibodies in the panel were initially tested by immunofluorescent staining on *Formalin-Fixed Paraffin-Embedded* (FFPE) sections of tonsil or spleen (depending on anticipated epitope abundance) and colon. Next, the purified carrier-free IgG or polyclonal antibodies were conjugated to lanthanide metals (Fluidigm, San Fransisco, CA, USA) (Supplemental Table E1 in this article's Online Supporting Information at https://drive.google.com/drive/folders/1X7pdu-VLbwzBWvzLuPbbufrkRktA9n3o?usp=sharing) using the MaxPar antibody labeling kit and protocol (Fluidigm). After conjugation, all antibodies were eluted in antibody stabilization buffer (Candor Bioscience) to obtain a final concentration of 0.5 mg/ml and stored at 4°C. Before sample slide hybridization, all antibodies were tested by IMC analysis of tonsil or spleen and colon to ensure that antibody specificity was not affected by conjugation and to determine the optimal antibody concentration.

Staining

The slide was baked for 1.5 hours at 60°C, deparaffinized with fresh xylene for 20 min and subsequently rehydrated in descending grades of ethanol (100% (10 min), 95%, 80%, 70% (5 min each). After washing for 5 min in milliQ and 10 min in PBST (PBS containing 0.1% Tween-20), heat-induced epitope retrieval was conducted in Tris/EDTA (10 mM/1 mM, pH 9.5) for 30 min in a 95°C water bath. The slide was allowed to cool to 70°C before washing in PBST for 10 min. To decrease non-specific antibody binding, tissue sections were blocked with 3% BSA and Human TruStain FcX (1:100, BioLegend) in PBST for 1 hour at RT. The antibody cocktail was prepared by mixing all antibodies at concentrations specific for the assay in PBST+0.5% BSA. After careful removal of the blocking buffer, the slide was incubated overnight at 4°C with the antibody cocktail. Following three 5 min washes in PBST and rinsing in milliQ the tissue was counterstained with 0.1% toluidine blue for 5 min to enable tissue structure visualization under bright field microscopy if desired. Upon washing for 5 min in milliQ, the slide was incubated with Ir-intercalator (1:500 in PBST, Fluidigm) for 60 min at RT. Finally, the slide was washed in milliQ and air dried at least for 20 min at RT.

High-spatial resolution laser ablation of tissue sections

Images were acquired at a resolution of 1 µm using a Hyperion Imaging System (Fluidigm). Regions of interest (ROIs) were selected based on hematoxylin and eosin stains performed on consecutive sections after which areas with approximate size of 1,000 × 1,000 µm for conjunctival samples and 500 x 500 µm for TMA samples were ablated and acquired at 200 Hz.

Image visualization

Pseudo-colored intensity maps were generated of each mass channel. Composite images were created for each sample using Image J (version 1.47), and any changes to the brightness or contrast of a given marker were consistent across all samples.

Cytokine signal intensity

To compare cytokine expression, three types of ROIs were selected within the samples: T-cell infiltrated ROIs from patient samples, non-infiltrated "control" ROIs from patient samples, and control ROIs from HC samples. ROIs including blood vessels were excluded (based on H&E and morphology). A maximum of 10 ROIs of equal size (60 x 90 µm) per type per patient were selected based on composite images including CD4, CD8, CD14 and Ecadherin. The mean signal intensity per μm^2 was calculated using Fiji (Fiji is just ImageJ, version 1.51g).

Statistical analyses

Statistical analyses were performed using R Project software version 3.4.1 (R Foundation for Statistical Computing, Vienna, Austria)[12]. Regarding baseline chacteristics, continous variables were presented as median with interquartile range (IQR) and categorical variables as number (n) with percentages. Cytokines mean signal intensity per μm^2 were compared using the paired t-test for comparison of infiltrated and non-infiltrated ROIs from patients, and unpaired t-test for comparison of ROIs from patients and HCs. P-values lower than 0.05 were considered statistically significant.

RESULTS

Patients and samples

The current study evaluated conjunctival biopsies from six AD patients (4 male; median age 38.5 years, interquartile range [IQR] 29.0 - 56.5, median Eczema Area Severity Index [EASI] score before starting dupilumab 20.5, IQR 15.4 - 27.9)(Table 1a) with active conjunctivitis developed during dupilumab treatment (median time to onset 59.5 days, IQR 51.5 - 80.5), of which for five histopathology was previously reported.[9] Conjunctival biopsies of two healthy controls (HCs) were included as comparison. As graphically depicted in Figure 1A, conjunctival biopsies collected from the inferior fornix were fixed, paraffin-embedded, stained with haematoxylin & eosin or Alcian blue for histological assessment, and additionally stained with a selected panel of 27 metal-conjugated antibodies (Supplemental Table E1 in this article's Online Supporting Information) for IMC.

Table 1. Clinical and ophthalmological characteristics

A. Clinical characteristics

Patient	Age†	Sex	History of conjunctivitis	EASI baseline	Time to onset of conjunctivitis (days)
1	20	Male	No	25.6	56
2	32	Female	Yes	16.4	112
3	32	Male	Yes	16.8	56
4	61	Female	Yes	34.8	38
5	45	Male	No	12.3	63
6	55	Male	No	24.1	70

B. Ophthalmological characteristics

Patient	Bulbar conjunctivitis	Palpebral conjunctivitis	Blepharitis	Limbitis	Cornea punctata	GC density (cells/mm)
1	ODS: mild	ODS: moderate	ODS: mild	No	No	5.9
2	No	ODS: moderate	No	No	No	1.4
3	ODS: severe	ODS: severe	ODS: moderate	ODS: moderate	ODS: moderate	3.3
4	No	No	ODS: mild	No	ODS: mild	3.3
5	OD: Mild	OD: Moderate OS: mild	OD:moderate OS: mild	OD: moderate	No	0
6	ODS: mild	ODS: moderate	ODS: moderate	No	No	1.9

† Age at initiation of dupilumab treatment. Ophthalmological characteristics were examined and graded in terms of severity by an experienced ophthalmologist following the Utrecht Ophthalmic Inflammatory and Allergic disease ocular surface score.10 EASI, Eczema Area Severity Index.

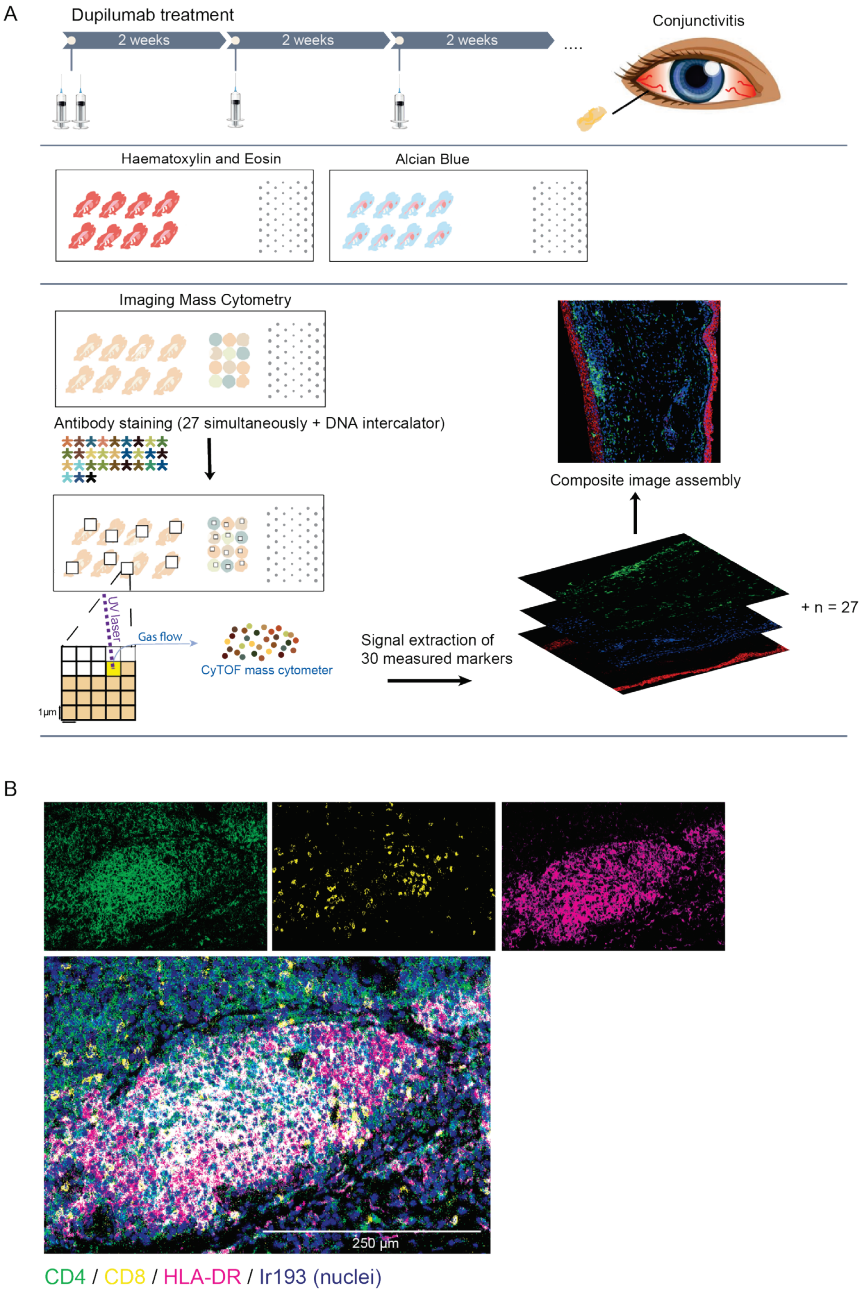
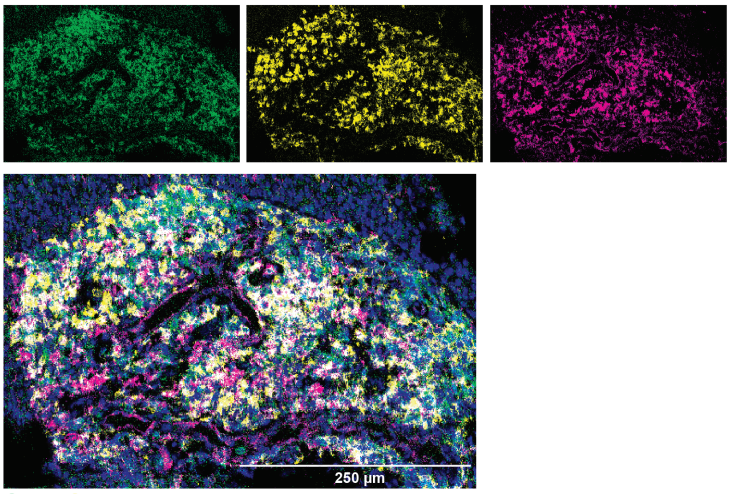


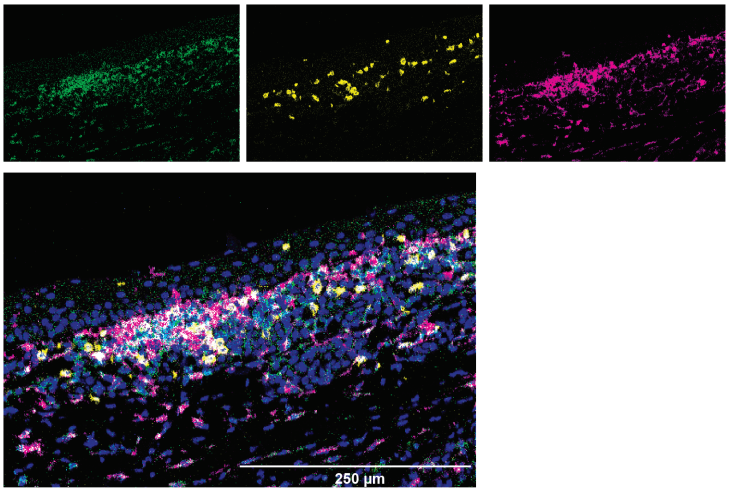
Figure 1. Representative conjunctival tissue imaging mass cytometry images of infiltrating immune cells. A. Graphical workflow of sample collection, section staining, and Imaging Mass Cytometry. **B.** Composite images derived from Imaging Mass Cytometry of conjunctival biopsy samples from AD patients developing conjunctivitis during dupilumab treatment. Representative images of patient 3 showing overlay of CD4 (green), CD8 (yellow), HLA-DR (magenta), and DNA intercalator (Ir) 193 (blue).

C



CD4 / CD8 / HLA-DR / Ir193 (nuclei)

D



CD4 / CD8 / HLA-DR / Ir193 (nuclei)

Figure 1. Composite images derived from Imaging Mass Cytometry of conjunctival biopsy samples from AD patients developing conjunctivitis during dupilumab treatment. Representative images of patient 4 (**C**), and patient 5 (**D**) showing overlay of CD4 (green), CD8 (yellow), HLA-DR (magenta), and DNA intercalator (Ir) 193 (blue).

Clinical features and goblet cell density

History of (allergic) conjunctivitis was present in three of the six (50%) patients (Table 1b). None of the six patients had a history of rosacea. The most prominent symptom reported in all of these patients was redness of the conjunctiva. The majority of patients further reported dryness, burning, tearing, and itching of the eyes. Most frequently reported ophthalmological characteristics were tarsal and bulbar conjunctivitis (83.3% and 66.7%, respectively), and blepharitis (83.3%). Two patients presented with limbitis. Median goblet cell density was 2.6 cells/mm (IQR 1.1 – 4.0) in the six patients compared to goblet cell density of 4.1 cells/mm and 9.8 cells/mm in the HC samples. Of note, biopsies from HCs were taken from the pericorneal conjunctiva, which in healthy individuals has a tendency toward lower GC density compared to normally covered regions of the eye, such as the inferior palpebral conjunctiva, from which patient samples were taken.[13, 14]

Characterization of infiltrating cells

Besides a scarcity of intraepithelial GCs, a subepithelial infiltrate was observed in conjunctival biopsies from all patients, ranging from mild to extensive. Infiltrating cells mainly consisted of CD3+CD4+ T-helper cells and CD3+CD8+ cytotoxic T-cells (Figure 1B-D). Infiltrating T-cells colocalized ICOS/CD278, Ki67 and/or HLA-DR in all patients, indicating an activation state accompanied by local proliferation (Figure 2A). Numerous regulatory T-cells (CD3+FOXP3+) were identified in conjunctival infiltrates of patients 1, 3, 4 and 5 (Figure 2B), while few CD3+FOXP3+ T-cells were observed patient 2 and 6, and both HC samples, mainly located near the epithelium (Supplemental Figure E1 in this article's Online Supporting Information). Besides infiltrating T-cells, several HLA-DR+ cells, including CD11c+ dendritic cells, CD14+ monocytes, and CD68+ macrophages were identified in patients 1, 3, 4, and 5 (Figure 3, Supplemental Figure E1 in this article's Online Supporting Information). Few CD14+ and CD68+ cells were observed in patient 2 and 6. CD20+ B-cells were present in the three patients with the most extensive infiltrate (patient 3, 4 and 5), which were not found in HC samples. Few CD56+ natural killer cells and γδ T-cells were observed in patient and HC samples (Supplemental Figure E1 in this article's Online Supporting Information).

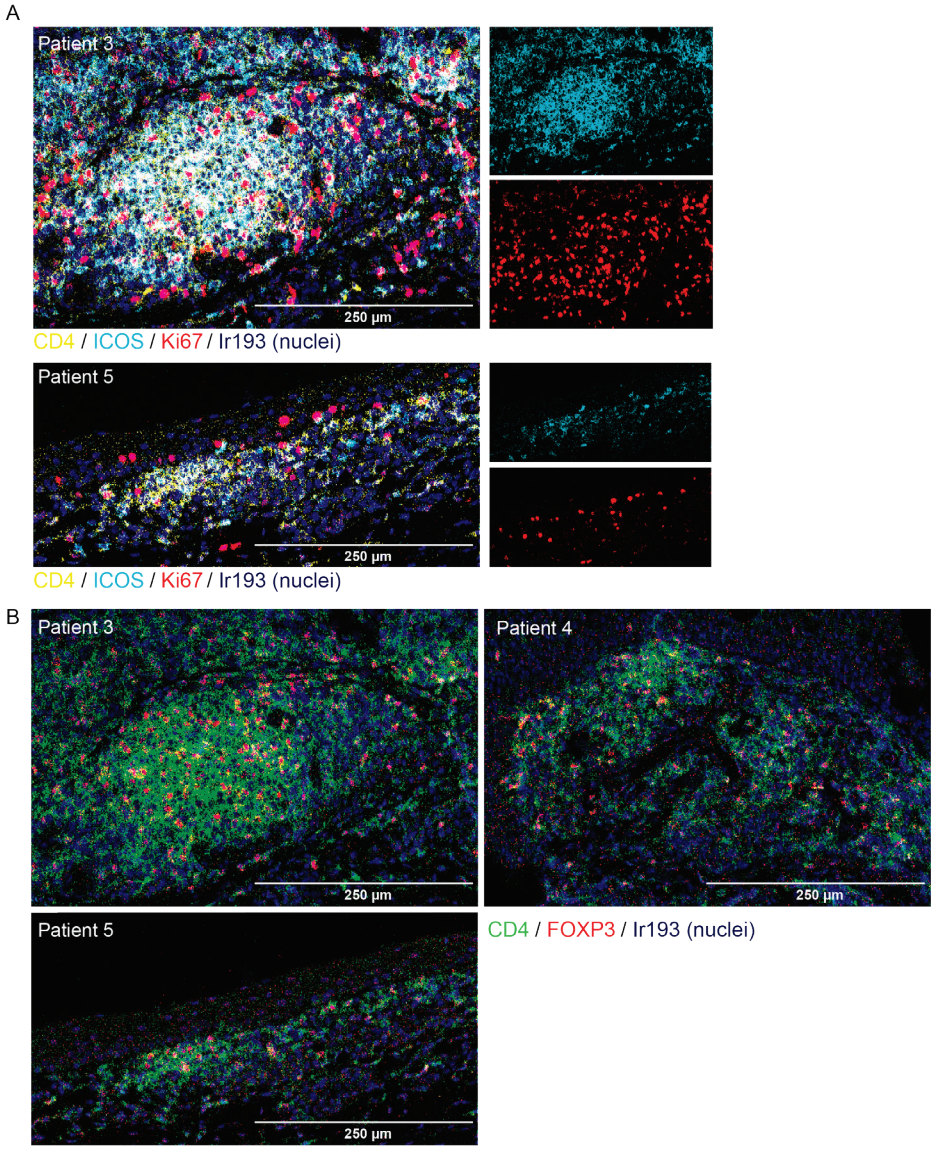


Figure 2. Representative conjunctival tissue imaging mass cytometry images of T-cell activation and proliferation. Composite images derived from Imaging Mass Cytometry of conjunctival biopsy samples from AD patients developing conjunctivitis during dupilumab treatment. **A.** Representative image of patient 3 showing overlay of CD4 (yellow), CD27/Inducible T-cell COStimulator (ICOS) (cyan), Ki67 (red), and intercalator (Ir) 193 (blue). **B.** Representative image of patient 3, 4 and 5 showing overlay of CD4 (green), FOXP3 (red), and Ir 193 (blue), showing numerous regulatory T-cells within subepithelial infiltrates.

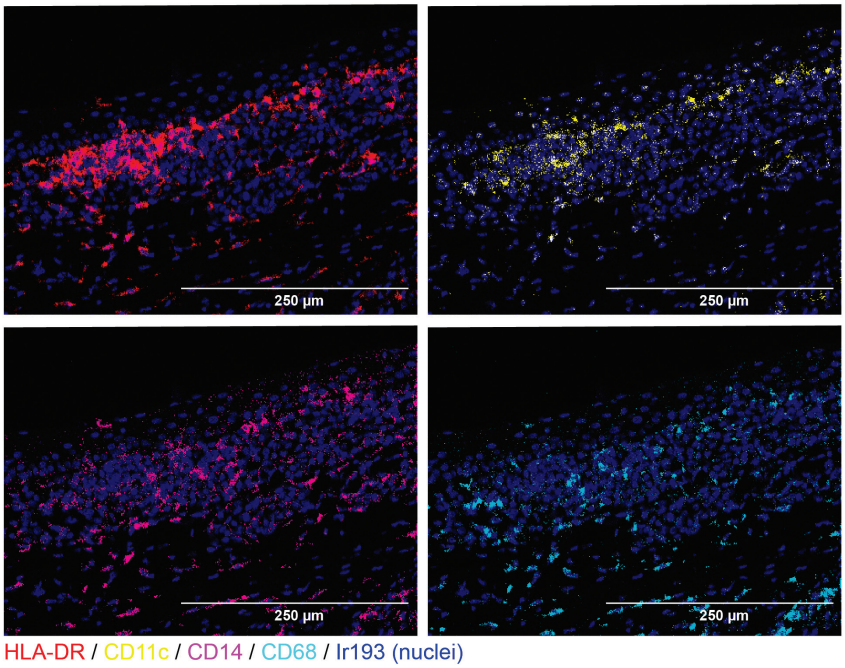


Figure 3. Representative conjunctival tissue imaging mass cytometry images of infiltrating HLA-DR positive cells. Composite images derived from Imaging Mass Cytometry of conjunctival biopsy samples from AD patients developing conjunctivitis during dupilumab treatment. Representative image of patient 5 showing overlay of HLA-DR (red, upper left), CD11c (yellow, upper right), CD14 (magenta, lower left), and CD68 (cyan, lower right) with DNA intercalator (Ir) 193 (blue).

Cytokine expression levels and cytotoxic activity

Cytokine production of IFNγ, TNFα, IL-10, and IL-17 within the immune cell infiltrates were evaluated by calculating the mean signal intensity per μm^2, and compared to non-infiltrated reference regions of interest (ROIs) from the same patient and ROIs from HC samples. Significantly increased signals of IFNγ, TNFα, IL-10, and IL-17 were observed within subepithelial cell infiltrates in all patient samples compared to non-infiltrated reference regions and HCs (Figure 4). Differences were most pronounced for IFNγ and IL-10. In five patients, infiltrating cells, including CD8+ T-cells, co-localized granzyme B, indicating cytotoxic activity (Figure 5).

In summary, we found a diverse immune cell infiltrate with T-cells displaying an activated phenotype, and increased T1 and T17 cytokine production, as well as granzyme B cytotoxic responses within conjunctival tissue of dupilumab treated AD patients who developed conjunctivitis.

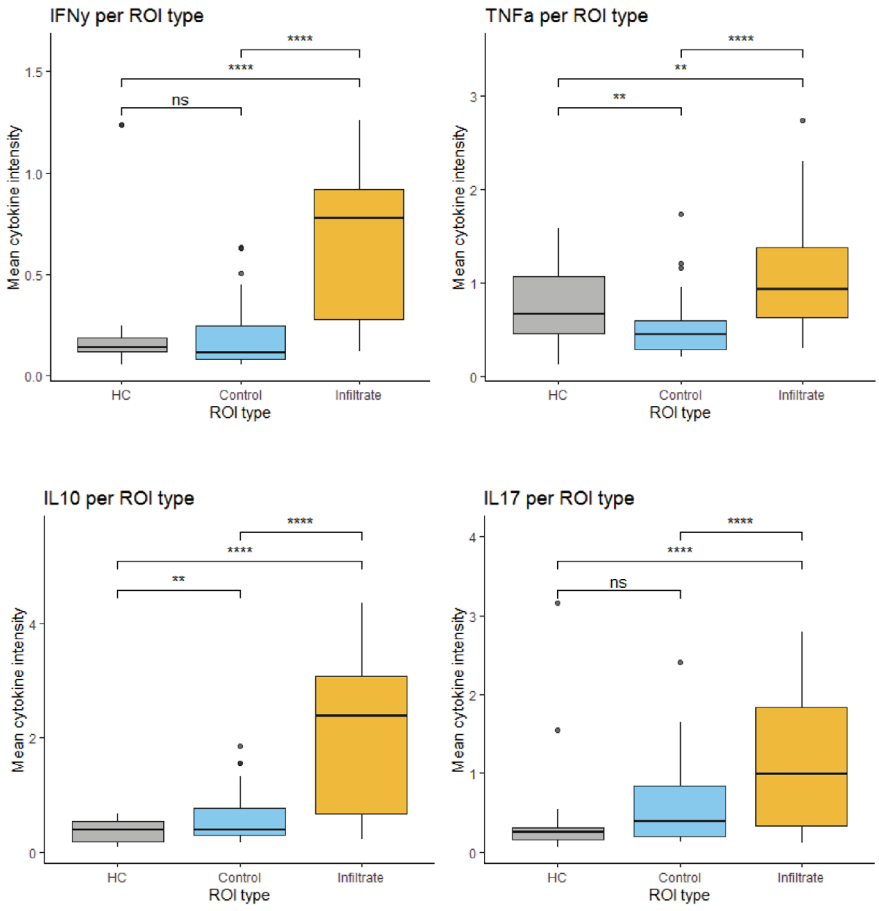


Figure 4. Mean cytokine signal intensity plotted for IFNγ, TNFα, IL-10 and IL17. Mean signal intensities per µm were calculated from three types of region of interest (ROI) within the samples: T-cell infiltrated ROIs from patient samples, non-infiltrated reference ROIs from patient samples, and control ROIs from HC samples. A maximum of 10 ROIs of equal size per type per patient were selected based on composite images including CD4, CD8, CD14 and Ecadherin. Selected ROIs were not including or were not near to any blood vessel. Boxes represent medians with first and third quartiles (lower and upper hinges). The upper and lower whiskers extend from the hinge to the largest and smallest value, respectively, no further than 1.5* interquartile range. Cytokines mean signal intensity per µm were compared using the paired t-test for comparison of infiltrated and non-infiltrated ROIs from patients, and unpaired t-test for comparison of ROIs from patients and HCs. P-values lower than 0.05 were considered statistically significant. Significance levels correspond to the following P values: *P < .05, **P < .01 and ***P < .005

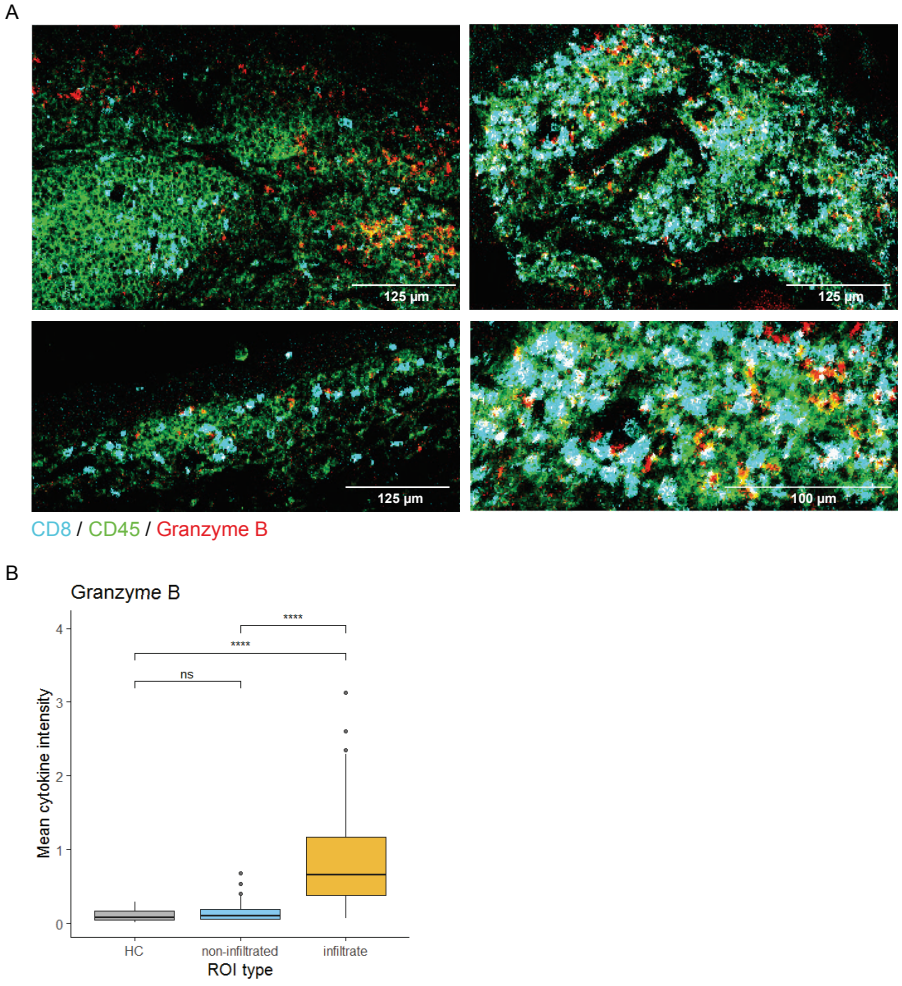


Figure 5. Cytotoxic activity of infiltrating cells within conjunctival tissue is shown by increased granzyme B expression. A. Composite images derived from Imaging Mass Cytometry of conjunctival biopsy samples from patient 3 (upper left), patient 4 (upper and lower right), and patient 5 (lower left), showing overlay of CD8 (cyan), CD45 (green), and granzyme B (red). Magnified image of patient 3 (lower right) shows multiple CD8+ T-cells colocalized granzyme B, represented by white colored areas. **B**. Mean cytokine signal intensity plotted granzyme B. Mean signal intensities per µm were calculated from three types of region of interest (ROI) within the samples: T-cell infiltrated ROIs from patient samples, non-infiltrated reference ROIs from patient samples, and control ROIs from HC samples. A maximum of 10 ROIs of equal size per type per patient were selected based on composite images including CD4, CD8, CD14 and Ecadherin. Selected ROIs were not including or were not near to any blood vessel. Boxes represent medians with first and third quartiles (lower and upper hinges). The upper and lower whiskers extend from the hinge to the largest and smallest value, respectively, no further than 1.5* interquartile range. Cytokines mean signal intensity per µm were compared using the paired t-test for comparison of infiltrated and non-infiltrated ROIs from patients, and unpaired t-test for comparison of ROIs from patients and HCs. P-values lower than 0.05 were considered statistically significant. Significance levels correspond to the following P values: *P < .05, **P < .01 and ***P < .005

DISCUSSION

Conjunctivitis observed in dupilumab treated AD patients is a complex, multifactorial phenomenon, and various hypotheses have been raised about its yet unknown underlying etiology.[4] Ocular surface diseases, such as allergic conjunctivitis and atopic keratoconjunctivitis (AKC) are well-known comorbidities associated with AD.[4, 15] The uncovering of pre-existing ocular disorders, such as AKC, may explain the increased incidence of conjunctivitis in dupilumab-treated AD patients contrary to the low incidence of conjunctivitis in dupilumab trials in other T2 diseases.[4] The infiltrating cells characterized in conjunctival biopsies from AD patients developing conjunctivitis in our study are consistent with previous findings in severe chronic AKC, where conjunctival inflammation was dominated by CD4+ and HLA-DR+ cell densities, combined with increased IFNγ and IL-17 expression, rather than the T2-associated cytokines observed in the acute phase.[16] By suppressing the IL-4/IL-13 pathway, dupilumab might aggravate the shift towards a more T1- and T17-dominant profile, which has been suggested as a potential function of disease chronicity in AKC, and may explain why conjunctivitis during dupilumab treatment occurs more frequently in severe and chronic atopic patients.[16, 17] However, AKC is associated with increased goblet cell numbers, which is inconsistent with our findings.[18]

The patients included in the current study showed a scarcity of intraepithelial GCs, as previously reported.[9] A previous study analyzing conjunctival biopsies from 30 cataract patients without clinical changes of the conjunctiva, showed a mean GC density of 30.21 ± 14.32 cells per mm in the lower forniceal conjunctiva, compared to a median GC density of 2.6 cells/mm in our patients. The conjunctival epithelium normally is a GC rich tissue, and IL-13 is the predominant cytokine promoting GC proliferation and mucus secretion.[19, 20] Various types of dry eye diseases have been associated with GC loss, and GC density has previously been inversely correlated to IFNγ expression and the proportion of HLA-DR+ cells in the bulbar conjunctiva of dry eye patients.[20-22] Additionally, dry eye mouse models lacking conjunctival GCs demonstrated increased numbers of CD45+ inflammatory cells as well as CD11c+ cells in the conjunctiva.[23, 24] CD4+ T-cells from these mice exhibit a higher proliferation state and increased expression of IFNγ and IL-17.[24] These results are in line with our findings in dupilumab-treated AD patients developing conjunctivitis, indicating that conjunctival GCs have an important immunomodulatory function and

might suppress the production of dry eye-inducing cytokines, including IFNγ. By inhibiting IL-13, dupilumab might affect GC development and function, resulting in reduced production of immunoregulatory factors, such as Transforming Growth Factor Beta 2 (TGFß2) and retinoic acid[20], by conjunctival GCs, promoting conjunctival inflammation and ocular surface disease.

Treatment with ocular cyclosporine A (CsA) emulsion has been proven to significantly increase GC density in patients with dry eye syndrome and to reduce T-cell infiltration, activation, and cytokine expression of especially IFNγ in conjunctival biopsy samples of AKC patients.[25, 26] In view of the findings described in the current study, CsA eye drops and/or other calcineurin inhibitors such as tacrolimus eye ointment, might have the potential to suppress the conjunctival inflammation and restore the development and function of GCs, thereby preventing severe persistent ocular complications in dupilumab treated AD patients. Successful treatment with CsA eye drops and tacrolimus 0.03% eye ointment has already been described in cases of conjunctivitis occurred during dupilumab treatment.[4, 27]

Besides T1 and T17-related cytokines, we also found increased expression of the immunoregulatory cytokine IL-10 within subepithelial cell infiltrates compared to non-infiltrated control regions and HCs. Together with the influx of regulatory (FOXP3+) T-cells in some of the patients, these findings suggest increased immunosuppressive activity within the conjunctiva of dupilumab treated AD patients, likely as compensatory mechanism to the extensive inflammatory-cell infiltrates. Although IL-10 has been shown to reduce eosinophil inflammation, mice studies conversely demonstrated that IL-10 might play in important role in the T2 response and skin and long eosinophilia.[28-30] This suggests that elevated levels of IL-10 might also explain the presence of eosinophils as previously reported[9] in the conjunctival biopsies.[31]

A limitation of this study is the relatively small sample size due to the difficulty of recruiting patients who were willing to undergo a conjunctival biopsy, and the lack of baseline samples before initiation of dupilumab. A further limitation is the lack of T2-related cytokines or other type markers examined in the conjunctival biopsies. Nevertheless, we were able to obtain a clear and consistent characterization of the local conjunctival infiltrate.

In conclusion, our study demonstrates that the cellular infiltrate observed in conjunctival tissue of AD patients developing conjunctivitis during dupilumab

treatment encompasses large numbers of highly activated CD4+ and CD8+ T-cells but also B cells, macrophages, monocytes, and dendritic cells. Additionally, increased expression of T1- and T17-related cytokines and increased cytotoxic activity was found compared to healthy controls. The T1 dominance promoted by IL-4/IL-13 suppression, combined with the direct effect of IL-13 suppression on GCs potentially result in the loss of GCs and their essential immunomodulatory role in the conjunctiva, hence leading to dry eyes, a highly activated multicellular infiltrate, and tissue damage. Evaluation of conjunctival GC numbers with less invasive techniques such as conjunctival impression cytology and IFNγ concentrations in tears might identify AD patients who are at risk of developing conjunctivitis and who might benefit from preventive ocular anti-inflammatory treatment.

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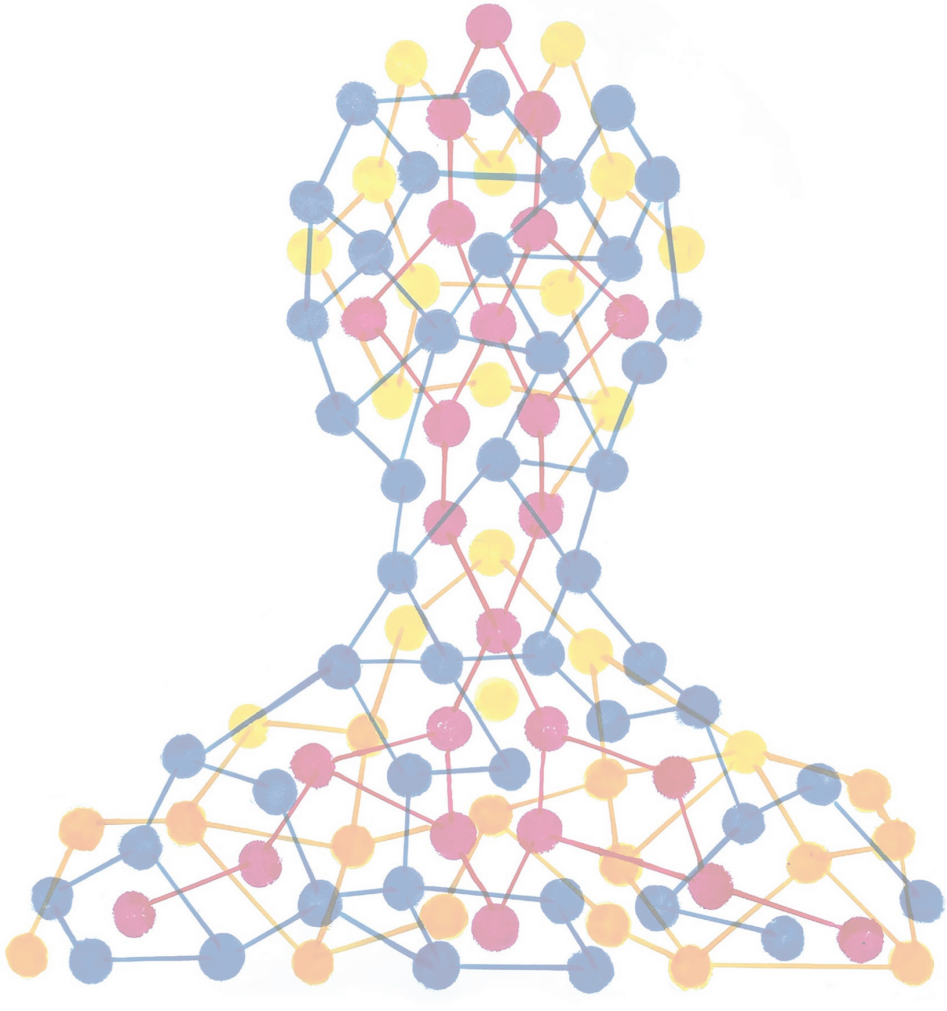

Chapter 11

Dupilumab facial redness: Positive effect of itraconazole

JAAD case reports 2019; Oct 5(10); 888-891.

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INTRODUCTION

Dupilumab, a fully human monoclonal antibody that inhibits interleukin (IL) 4 and IL-13 by blocking the shared IL-4 receptor α and thereby suppressing the T helper type 2–mediated inflammatory response (Th2), is the first biological treatment for moderate to severe atopic dermatitis (AD). We present 2 cases of dupilumab facial redness (DFR), which was not reported in phase 3 clinical trials investigating the efficacy and safety of dupilumab. However, DFR is found to affect approximately 10% of patients treated with dupilumab in daily practice.[1] In both of our cases, DFR was considered to be caused by hypersensitivity to *Malassezia* species. The differential diagnosis included allergic contact dermatitis (ACD) and rosacea.

CASE 1

A 39-year-old man with severe AD since childhood was treated with dupilumab 300 mg every 2 weeks after a loading dose of 600 mg subcutaneously at our outpatient clinic. Initially, significant improvement of AD was observed. However, after 11 weeks of dupilumab treatment, the patient developed worsening of redness and scaling of the face, accompanied by itch and pain, that did not respond to treatment with topical corticosteroids. Physical examination showed erythematous and scaly plaques exclusively affecting the head and neck (Figure 1, *A*), raising clinically suspicion of head-neck dermatitis (HND). Because of the painful appearance, atypical rosacea was also considered. Histopathologic examination showed remarkable parakeratosis with numerous neutrophilic granulocytes, acanthosis, and spongiosis. In the upper dermis, a dense infiltrate of lymphocytes, neutrophils, and eosinophils was observed (Figure 2). The histopathologic findings did not correspond with rosacea. Additionally, an elevated serum level of *Malassezia*-specific immunoglobulin E (48.50 kU/L; reference value, 0.0-0.34 kU/L) was found.

Consequently, treatment with oral itraconazole 200 mg once daily was started, and treatment with dupilumab was continued. After 1 week of itraconazole treatment, the patient reported significant improvement of signs and symptoms during telephonic evaluation. During re-evaluation at our outpatient clinic after 3 weeks of itraconazole treatment, signs and symptoms were completely cleared (Fig 1, *B*). Treatment with oral itraconazole 200 mg once daily was continued for a total period of 1 month.

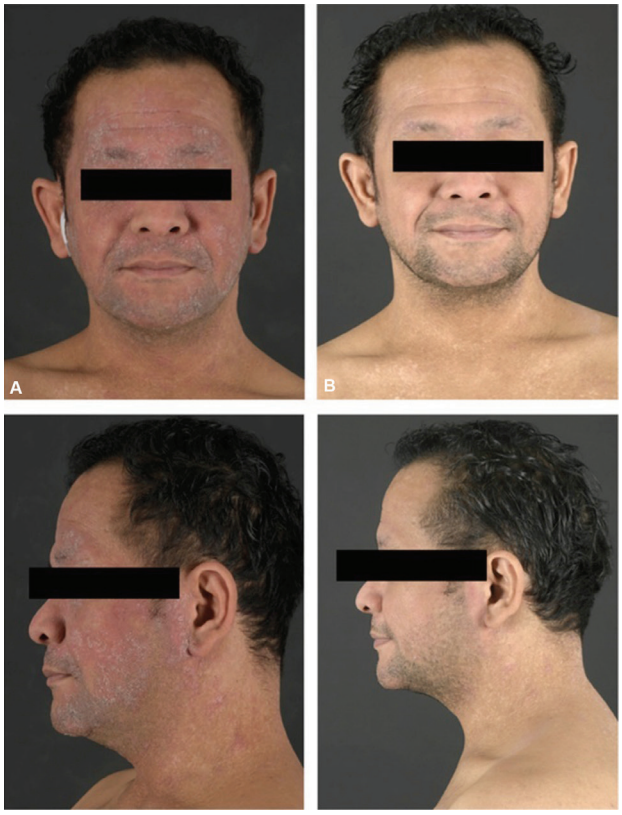

Figure 1. A. Before treatment with oral itraconazole. **B.** During treatment with oral itraconazole.

CASE 2

A 29-year-old man with severe AD since childhood was treated with dupilumab 300 mg every 2 weeks after a loading dose of 600 mg subcutaneously at our outpatient clinic. Initially, significant improvement of AD was observed. However, after 6 months of dupilumab treatment, the patient developed erythematous and scaly plaques in the face, accompanied by itch and pain, that did not respond to treatment with topical corticosteroids. Because of clinical suspicion for rosacea, treatment with topical ivermectin was initiated without success. Patch testing was performed to exclude ACD but did not result in clinically relevant positive reactions. Histopathology showed an identical pattern as presented in case 1. The clinical diagnosis of HND was suspected, and treatment with oral itraconazole 200 mg once daily led to significant improvement of signs and symptoms while dupilumab was continued.

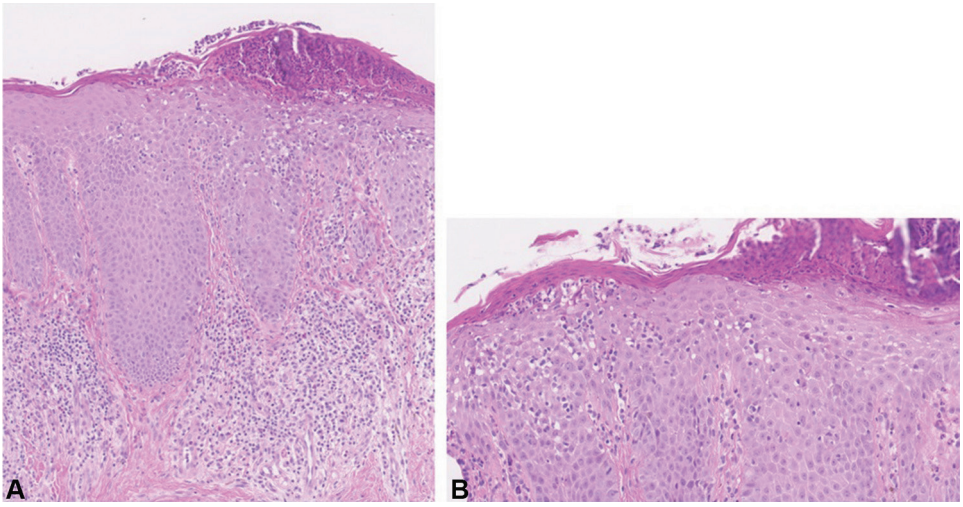

Figure 2. Histopathology of case 1. **A**. Parakeratosis with numerous neutrophilic granulocytes, acanthosis, and spongiosis. In the upper dermis, there is a dense infiltrate of lymphocytes, neutrophils, and eosinophils. **B.** Neutrophilic granulocyte migration through the epithelium.

DISCUSSION

To our knowledge, this is the first case report suggesting *Malassezia* hypersensitivity as a possible cause for DFR. An elevated *Malassezia*-specific immunoglobulin E level was found in 1 patient, and in both cases, HND was clinically suspected and improved after itraconazole treatment.

HND is a clinical diagnosis that can be observed in patients with AD from adolescence. The skin disease is characterized by erythematous and scaling plaques affecting the head and neck, accompanied by itch, and mostly inadequately responding to topical corticosteroids. *Malassezia furfur*, a yeast belonging to normal skin flora and mostly located on skin rich in sebaceous glands (head and neck), probably plays a role in the pathophysiology. Hypothetically, because of disturbed skin barrier function in patients with AD, *Malassezia furfur* can easily penetrate the skin and locally impair and activate keratinocytes, consequently enhancing inflammation. In response to *Malassezia* antigen load, T cells further activate B cells to produce *Malassezia*-specific immunoglobulin E.[2]

In the first case, a high serum level of *Malassezia*-specific immunoglobulin E was found, which strengthens the *Malassezia* hypersensitivity theory. Elevated serum levels of *Malassezia*-specific immunoglobulin E have been previously described in AD and HND patients.[2-4] In a Dutch study investigating *Malassezia*-specific immunoglobulin E in patients with AD with and without HND, all patients with AD and HND had elevated serum levels of *Malassezia*-specific immunoglobulin E (100%), in contrast to patients with AD but without HND (13.6%).[3] Elevated serum levels of *Malassezia*-specific immunoglobulin E have been shown to be specific for patients with HND in contrast to patients with seborrheic dermatitis or pityriasis versicolor.[4]

To our knowledge, this is the first publication to include evaluation of skin biopsy samples in DFR. The histopathologic characteristics of both DFR and HND have not been clarified yet. In both of our cases, a heterogeneous histology was observed with characteristics of eczema underlying a (reactive) neutrophilic dermatosis, probably resulting from the *Malassezia* yeast. There was no histopathologic evidence of rosacea, such as dilated capillaries in the upper dermis and perivascular and/or perifollicular mononuclear cell infiltrates. Seborrheic dermatitis was not likely due to numerous neutrophilic granulocytes.

The positive response to oral itraconazole in our patients supports the *Malassezia* hypersensitivity theory. This finding is in line with randomized, placebo-controlled trials describing significant clinical improvement after treatment with systemic antimycotics in patients with AD with suspected HND.[2, 5-7] Both daily use of 200 mg itraconazole and 200 mg ketoconazole are recommended for a treatment duration of 1 to 2 months, followed by long-term twice weekly treatment if necessary. Itraconazole is preferred because of the smaller risk of hepatotoxicity.

In patients presenting with DFR and not responding to oral itraconazole, patch testing is reasonable; some previous published case reports described DFR as a result of paradoxical worsening of ACD.[1, 8, 9]

Different hypotheses have been suggested for the development of DFR, including triggering of Th1-mediated skin diseases such as psoriasis, ACD, and rosacea by blocking the Th2 pathway.[8-10] DFR due to *Malassezia* hypersensitivity, a more Th2-driven condition, cannot be explained by this theory.

In conclusion, for patients with AD presenting with DFR, *Malassezia* hypersensitivity should be considered, with rosacea and ACD as differential diagnoses. *Malassezia*-specific immunoglobulin E and histologic examination may further clarify the diagnosis. In addition, positive treatment response to itraconazole supports the diagnosis. In the case of significant clinical improvement, treatment with oral itraconazole once daily should be continued for 1 to 2 months, followed by long-term twice-weekly treatment if necessary.

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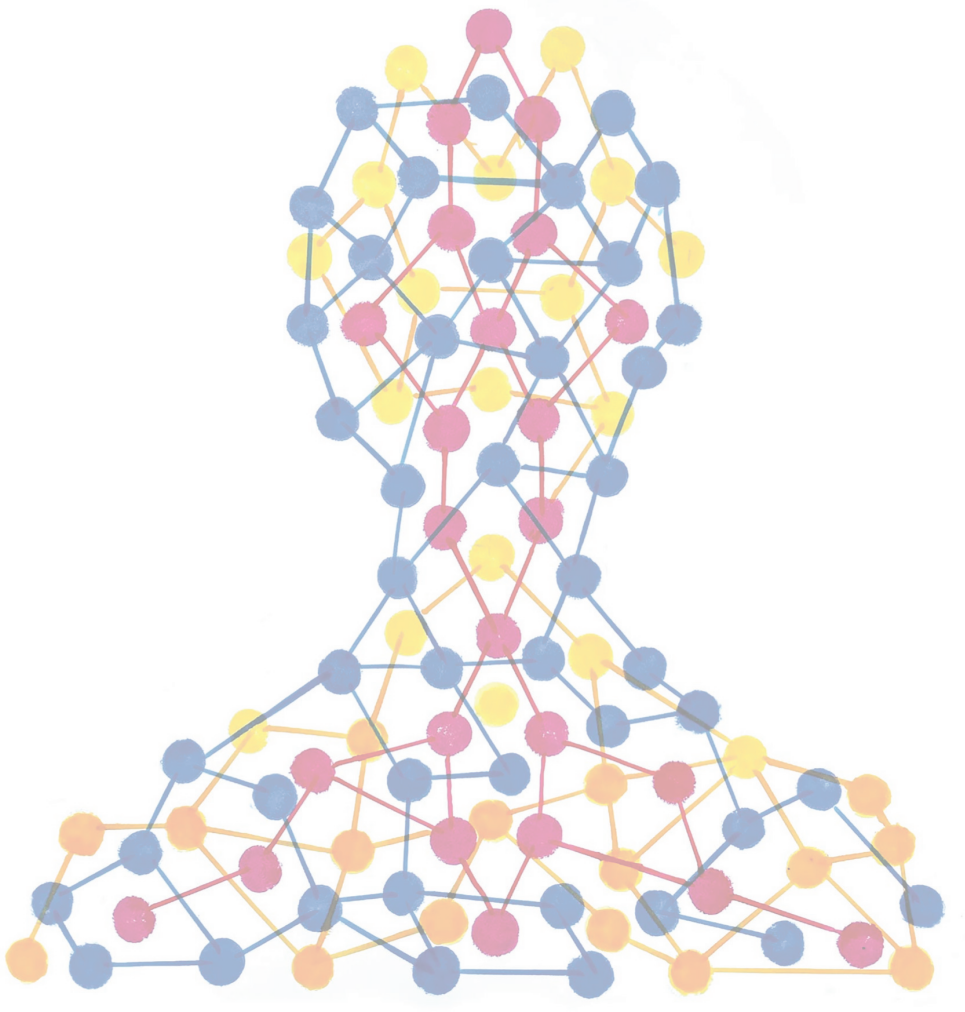


Chapter 12

General discussion

Atopic dermatitis (AD) is a complex and highly heterogeneous disease, but is currently still treated according the "one-size-fits-all" approach. The research presented in this thesis aimed to: 1) further identify AD patient subtypes based on biomarker profiles, which can be helpful to find the most optimal treatment for the individual patient ; 2) clarify the early and long term immunological effects of dupilumab, the first biologic treatment for AD; 3) elucidate the pathomechanisms underlying different side effects occurring during dupilumab treatment, mainly focusing on conjunctivitis.

The implications, clinical recommendations and suggestions for future research regarding the main findings of this thesis will be discussed in this chapter.

MAIN FINDINGS OF THIS THESIS

Biomarker based patient profiling

- A predictive signature consisting of eight serum biomarkers is able to identify the subgroup of difficult-to-treat AD patients who are in need of systemic treatment with a sensitivity of 78% and a specificity of 86%. - **Chapter 2**
- Four biomarker based patient clusters are identified in a large cohort 142 severe adult AD patients. The biomarker profiles of three clusters are consistent with previously identified clusters. Additionally, a constructed prediction model is able to stratify patients into one of the four clusters by using only 10 serum biomarkers. - **Chapter 3**
- Four distinct patient clusters based on serum biomarker profiles are identified in a large cohort of 240 pediatric AD patients, of which one is similar to previous adult endotypes. - **Chapter 4**

Effects of dupilumab treatment on molecular parameters and dynamics

- Dupilumab treatment rapidly and stably inhibits IL-4Rα in AD patients, which is accompanied by a strong early functional immunological effect specifically on skin-homing T-cells. Although there are no signs of general T-cell skewing following a year of dupilumab treatment, the continuous decrease in total IgE levels may indicate long- term effects on the atopic phenotype. - **Chapter 5**
- A biomarker signature (p-EASI) consisting of serum biomarkers TARC, IL-22, and sIL-2R, adequately predicts disease severity in AD patients treated with dupilumab. - **Chapter 6**
- Dupilumab treatment significantly decreases local presence of eosinophils and production of eotaxins in the skin, combined with an increased expression of CD193/CCR3 and CD44 in the peripheral blood from AD patients treated with dupilumab, which supports the concept that treatment with dupilumab decreases eosinophil trafficking to the skin. - **Chapter 7**

Side effects of dupilumab treatment and underlying pathophysiology

- In a retrospective cohort of dupilumab-treated AD patients, ophthalmologist-confirmed conjunctivitis is observed in 33 (19.8%) patients. During long-term ophthalmological follow-up, the majority of these patients still suffer from mild-to-moderate conjunctivitis despite treatment. Dose adjustment or discontinuation of dupilumab, due to ocular pathology, is needed in respectively 30% and 9% of the patients. **- Chapter 8**
- A remarkable scarcity of conjunctival GCs accompanied by an inflammatory T-cell- and eosinophilic infiltrate is found in conjunctival biopsies in six AD patients who developed conjunctivitis during dupilumab treatment. **- Chapter 9**
- The cellular infiltrate observed in conjunctival tissue of AD patients developing conjunctivitis during dupilumab treatment encompasses large numbers of highly activated CD4+ and CD8+ T-cells but also B cells, macrophages and monocytes. In addition, increased expression of T1- and T17-related cytokines and increased cytotoxic activity is found compared to healthy controls. **- Chapter 10**
- Malassezia hypersensitivity might be a possible cause for dupilumab facial redness, which is supported by elevated Malassezia-specific immunoglobulin E level and positive treatment response to itraconazol. **– Chapter 11**

BIOMARKER-BASED PATIENT PROFILING

The current treatment guidelines for AD mainly focus on the severity of skin lesions measured using clinical scores, and do not take the individual pathogenesis of the disease into account. Given the highly heterogeneous character of AD, it is unlikely that every patient will respond equally to different therapies. The recent introduction of novel targeted therapies for AD has driven the need for patient stratification based on immunological biomarkers. Unraveling different underlying biomarker pathways will not only provide us a better understanding of the pathogenesis of AD, but will also increase treatment efficacy and safety, further enabling more personalized clinical care.

Who needs systemic therapy: predicting the need in severe atopic dermatitis patients

The first step towards a personalized treatment approach would be to divide patients into a subgroup of patients who can be controlled with topical therapy only, and a subgroup in need of systemic therapy. The subgroup of AD patients who cannot be controlled by daily use of potent topical steroids, or cannot reduce the frequency/potency of topical steroids to acceptable levels can be defined as difficult-to-treat AD and require systemic therapy. The decision whether or not to start with systemic therapy in AD patients is not always easy. As AD is characterized by exacerbations and remissions, a single disease severity measurement can easily over- or underestimate the long-term disease severity of a patient. Several factors should be considered before deciding to start systemic therapy, such as the number and severity of disease flares over time, the amount and potency of topical steroids needed to control the eczema, the number of courses with oral corticosteroids, and the patient-reported itch scores, which sometimes remain very high despite relatively low physician-reported severity scores, and can be very disabling for the patient. Additionally, the impact on the quality of life at several time points and treatment burden, including time spent on treatment and treatment costs, should be taken into account.[1] As a result of the multifactorial decision-making, many difficult-to-treat AD patients are subjected to a significant delay before they can start systemic therapy.

In **chapter 2** we constructed a predictive signature, including the serum biomarkers interleukin (IL)-1β, platelet factor 4 (PF4/CXCL4), cutaneous T-cell attracting chemokine (CTACK/CCL27), Trappin-2, Sclerostin (SOST), gamma-tubulin complex

protein 2 (GCP-2), soluble programmed death-1 (sPD-1) and leukocyte associated immunoglobulin like receptor-1 (LAIR-1), which was able to identify the subgroup of difficult-to-treat AD patients with a sensitivity of 78% and a specificity of 86%. The use of this signature might in future contribute to earlier identification of AD patients indicated for systemic treatment. The predictive biomarker signature was constructed in order to prevent unnecessary treatment delay in difficult-to-treat AD patients. Hence, we do not aim to replace clinical decision making by our biomarker signature. For example, persistent noncompliance to topical therapy despite adequate education, might be a reason to switch to systemic therapy, but cannot be considered in a biomarker signature. Our predictive signature might rather serve as a valuable addition to the decision whether or not to start systemic therapy in individual AD patients and might accelerate the initiation of optimal therapy. Validation of our signature in different prospective cohorts is needed to confirm its applicability in daily practice.

Defining biomarker-based patient clusters

After the identification of AD patients who require systemic therapy, the next step is to find the most optimal drug for the individual patient. Given the variety of (upcoming) treatments targeting specific cytokine pathways we believe that it is important to stratify patients based on the most important immunological drivers of their AD, rather than subgrouping based on clinical phenotypes. This is strengthened by a recent study of Tavecchio et al.[2] comparing the efficacy of dupilumab treatment in patients representing six different AD phenotypes based on their clinical presentation. Clinical improvement and improvement in quality of life was comparable in patients representing different AD phenotypes.[2] The patients with prurigo and nummular eczema phenotypes responded more slowly than the patients with other phenotypes, but reached EASI-75 scores (at least 75% reduction from baseline in EASI) similar to the other ones after 16 and 52 weeks of treatment.[2] Additionally, previous studies evaluating the efficacy of dupilumab treatment were also not able to identify good clinical predictors for response, such as disease severity, age of onset and intrinsic versus extrinsic AD.[3-5] This indicates that clinical phenotypes might be less suitable compared to molecular based endotypes to define sub-populations of AD patients who are the best candidates for various (targeted) treatments. It is therefore important to develop useful (combinations of) biomarkers to predict treatment efficacy, especially considering the economic burden of such targeted therapies. Current clinical trial inclusions are primarily based

on disease severity, rather than endotypical characteristics. This raises the possibility that an investigative drug might be highly effective for a subset of affected subjects with a specific endotype, but that the number of these subjects within the test cohort is too small to detect significant changes at group level.

It is expected that a combination of different biomarkers is more suitable than an individual biomarker for the stratification of a complex disease such as AD. Endotypes are made of a collection of biomarkers, and describe distinct pathophysiologic mechanisms at a cellular and molecular level driving the disease.[6] Several ways of endotyping AD patients have already been described. AD endotypes have immunologically been characterized based on specific clinical, ethnic or demographic patient groups, including pediatric versus adult AD[7, 8], younger versus older adults[9], and African versus Asian and European patients[10, 11]. Thijs et al.[12] were the first to encompass endotyping based on unsupervised molecular profiling across a broad spectrum of AD patients. By using an unsupervised data driven approach, they could classify adult AD patients into four distinct patient clusters, based on serum biomarker profiles. These clusters could represent subgroups of AD patients that have unique pathophysiologic mechanisms driving the disease, which can be defined as endotypes. To be able to apply endotyping in future clinical studies and daily practice, the validation of these findings is very important. In **chapter 3** we used the same data driven approach on a separate cohort of 146 adult patients with severe AD. By measuring the same panel of serum biomarkers, we again identified four AD patient clusters with a distinct serum biomarker profile. Three out of the four clusters were comparable to the previously identified clusters. Based on the serum biomarker levels, patients could be stratified into the "Th1/Th2/Th17 dominant" cluster, the "Th2/Th22/PARC dominant" cluster, the "Th2/eosinophil inferior" cluster, or the "skin-homing chemokines/IL-1R1 dominant" cluster. The latter cluster, representing 33.6% of the patients, was not comparable to one of the previous clusters. The difference in their biomarker profiles might be explained by the high proportion of patients who used systemic immunosuppressive drugs within one year before sampling, which was significantly higher in the cohort described in **chapter 3**, compared to the previous study, and also in the "skin-homing chemokines/IL-1R1 dominant" cluster compared to the other three clusters. Ciclosporin A (CsA), a calcineurin inhibitor selectively acting on T-cells[13], was the most commonly used immunosuppressive drug in these patients. Besides the highest levels of IL-1R1 and the skin-homing C-C chemokines CTACK/CCL27, TARC/CCL17, MDC/CCL22 and RANTES/CCL5, patients in the "skin-homing chemokines/IL-1R1 dominant" cluster

showed the lowest serum levels of IFNγ, IL-4, IL-5 and IL-13. This is in accordance with previous studies showing that CsA treatment in AD patients suppresses the levels of T-cells producing IL-2, IL-4, IL-5, IL-13, and IFNγ.[14-16] Although , oral immunosuppressive drugs were discontinued within at least two (fast-acting drugs) or four (slow-acting drugs) weeks before blood collection in all patients, a persistent immunological effect of the oral immunosuppressive drugs cannot be ruled out. Future longitudinal studies including large patient populations are needed to investigate whether patients belong to one cluster or whether they switch during the course of the disease or after immunosuppressive or immunomodulatory treatment.

Who needs which therapy: defining the most optimal treatment for the individual patient

The specific biomarker pathways distinguishing the different patient clusters may be particularly meaningful for the application of molecularly targeted drugs, and defining the most optimal treatment for the individual patient, since different endotypes might respond differently to the particular treatments. Previously, few single biomarkers have been proposed for the prediction of response to targeted therapies in AD. Serum total IgE was one of the first biomarkers used to divide AD patients into a subgroup of non-IgE-associated "intrinsic" and IgE-associated "extrinsic" AD.[17] Although overall anti-IgE treatment with omalizumab has shown inconclusive results in AD patients, a meta-analysis of 13 studies reported that lower IgE serum levels were associated with a significantly better response compared to higher IgE concentrations.[18] Other attempts that have been made to predict response to targeted treatments in AD based on single biomarkers include the presence of high IL-22 skin expression for anti-IL-22 treatment with fezakinumab[19], and high serum concentrations of the IL-13-related markers DPP4 and periostin for tralokinumab (anti-IL-13) treatment[20]. Remarkably, recently published data from three large phase III clinical trials investigating tralokinumab in AD patients do not report DPP4 or periostin analyses.[21, 22] Patients stratified into the "Th1/Th2/Th17 dominant" and "Th2/Th22/PARC dominant" clusters described in **chapter 3**, representing about 40% of the included patients, showed particularly high type 2 (T2)-cytokine levels (including IL-4, IL-5, and IL-13) compared to the other two clusters. These patients could, theoretically, be the most ideal candidates for T2-targeted treatments, including dupilumab[3] (anti-IL-4/IL-13), tralokinumab[23] and lebrikizumab[24] (anti-IL-13). Phase III clinical trials investigating the efficacy of dupilumab treatment showed that 35% - 40% of the AD patients achieved clear or

almost clear skin after 16 to 52 weeks of treatment. In our daily practice studies we recently showed that after 16 weeks of dupilumab treatment, 40% of the patients could be identified as "super-responder" by achieving a clinically relevant improvement in all of the three key domains (EASI-75, Numeric Rating Scale [NRS] itch ≥4, and Dermatology Life Quality Index [DLQI] ≥4), and after 52 weeks 35% of the patients achieved EASI-90.[25, 26] Both these clinical trial as daily practice data correspond to the percentage of patients stratified into the T2-high clusters.[4, 27-29] Recent phase II and phase III clinical trials of tralokinumab and lebrikizumab showed comparable percentages of patients achieving Investigator's Global Assessment (IGA) score of 0 (clear) or 1 (almost clear) at week 16, although placebo response rates were higher compared to dupilumab trials.[21, 24, 30] Still, the percentage of AD patients not responding to dupilumab treatment is very low, suggesting that T2-related biomarkers might not be the most discriminative in an overall T2-high disease as AD.[28, 29] It is important to point out that despite two AD clusters showing a relative T2-low biomarker profile, the levels of T2-related markers in these AD patients were still higher compared to previously reported levels for healthy controls.[12, 31] We therefore expect that, in future, T2-cytokine levels will be useful to identify patients who respond very well to T2-targeted treatment (super-responders) , but not those who will not respond (non-responder).

Both the "skin-homing chemokines/IL-1R1 dominant" cluster and the "Th1/Th2/Th17 dominant" cluster showed significantly higher levels of IL-1R1 and IL-1α.[32] IL-1R1 is the receptor for IL-1α, which has recently emerged as a novel potential therapeutic target in AD patients. Bermekimab, a monoclonal antibody targeting IL-1α is currently under investigation in a phase II clinical trial. Although the exact mechanism of benefit of IL-1α is still unclear, the first results show significant improvement in all disease measures of AD.[33] This confirms that T1 and T17 immunity plays a significant role in adults with (chronic) AD. IL-1 is a proinflammatory cytokine with effects on both innate and adaptive immunity. The IL-1 family includes several ligands and receptors, such as IL-1α, IL-1β, IL-33, IL-36, IL-1Rα, and IL-1R1.[34] IL-1α may drive leukocyte recruitment to the skin, induces breakdown of the skin barrier through production of matrix metalloproteinase, and has shown to stimulate itch by a direct effect on nerves in animal studies.[35, 36] Furthermore, IL-1 was shown to contribute to T17 and T2 cell development.[37] In addition, the IL-1 family member IL-33 is able to induce a T2 skewed response by activation of T2 lymphocytes, increases expression of IL-4, IL-5, and IL-13, and directly disrupts skin barrier in AD by downregulation of fillagrin gene expression.[38-40] IL-36 has been shown to regulate IFNγ, IL-17, and IL-4

production.[41] Thus, the inflammatory effects mediated by IL-1 and its coaction with other cytokine pathways imply its role as a target in AD treatment. Patients stratified into our "skin-homing chemokines/IL-1R1 dominant" cluster and the "Th1/Th2/Th17 dominant" cluster might therefore be an interesting population for anti-IL-1α treatment with bermekimab.

Patients in the "Th2/eosinophil inferior" cluster could not be characterized by upregulation of a specific pathway, but were distinctive from the other clusters by the lowest serum levels of markers related to T2/severity (TARC/CCL17, PARC/CCL18, MDC/CCL22) and eosinophil recruitment (eotaxin-1, eotaxin-3, RANTES/CCL5). Patients in the "Th2/eosinophil inferior" cluster might therefore not benefit from a treatment targeting a particular cytokine pathway, but perhaps need a broader acting drug. Janus kinase (JAK) inhibitors, including upadacitinib[42], abrocitinib[43], and baricitinib[44], are a novel class of small molecules that have been emerged for the treatment of AD. Inhibition of the JAK-STAT pathway modulates a range of immune responses, including multiple downstream cytokine pathways involved in the pathogenesis of AD, such as T2 (IL-4, IL-5, IL-13), T22 (IL-22), T17 (IL-17A, IL-17F, IL-21), and T1 (IFNγ).[45] Both topical and oral JAK inhibitors have shown promising efficacy with acceptable safety profiles, however, long-term data has yet to be elucidated.[42-44, 46, 47] Inhibition of the JAK-STAT pathway may not only mediate inflammation, but also AD-associated pruritus. IL-31, a T2-related cytokine that is thought to play a role in the pathogenesis of AD and, more specifically, the induction of pruritus, signals through three different pathways, including JAK1 and JAK2.[48-53] Besides, neuronal signaling through IL-4Rα and JAK1 was recently shown to be important for chronic itch.[54] The targeting of more than one immune axis suggests the value of JAK inhibition as therapy for multiple AD endotypes.

Although IL-31 was not a significant driver in one of our AD patient clusters, and this cytokine can be hard to measure in serum, IL-31 inhibition by nemolizumab might be a good option for a subgroup of AD with high pruritus scores or the prurigo phenotype, either as monotherapy or in combination with other (targeted) treatments. Future studies are needed to determine the role of IL-31 antagonism in AD treatment, because it is currently not clear if the effects include only symptomatic, antipruritic relief.

The asthma field is running ahead of AD in terms of phenotypic and biomarker-based patient characterization, which becomes more and more clinically useful. The Global Initiative for Asthma (GINA) provides a strategy for personalized management based

on specific clinical and/or molecular characteristics that has formed the basis for many national guidelines. For patients with difficult-to-treat asthma, the decision on which biologic (anti-IgE, anti-IL5/anti-IL-5R, or anti-IL-4R) is appropriate to start first is based on several parameters including the level of blood eosinophils, exhaled nitric oxide (FeNo), the presence of allergen-driven symptoms (sensitization on skin prick testing or specific IgE), total serum IgE, age of onset, the number of exacerbations in the previous year, and comorbidities including nasal polyposis and AD.[55] Learning from these experiences might help to incorporate biomarker-based patient profiling in future treatment guidelines for AD.

While the main outcome of most clinical trials investigating dupilumab and tralokinumab was EASI-75 [21, 27-29], recent data on upadacitinib, an oral JAK-1 inhibitor, showed that 24% - 28% of the patients achieved EASI-100, and 50% - 66% of the patients achieved EASI-90 after 16 weeks of treatment.[42, 56, 57] Abrocitinib, another oral selective JAK-1 inhibitor demonstrated EASI-90 in 39% - 52% of adolescent and adult AD patients after 12 weeks of treatment in recent phase IIb and III trials.[43, 58] According to these results, it is expected that criteria for effectiveness of drugs in the field of AD will become more stringent and treatment goals will shift from reaching EASI-75 to reaching EASI-90, or even EASI-100. Given the different current and emerging (highly) effective treatment options it is essential to determine proper treatment goals for AD. These goals should consider multiple health domains, including clinically established severity scores (EASI) and patient reported outcomes such as NRS itch. A proposed optimal treatment goal for AD could be achievement of EASI-90 and NRS itch ≤2. Instead of the percentage change from baseline that is represented by EASI-90, one might also consider to strive for absolute treatment goals, such as EASI ≤5.

These goals highlight the importance of the identification of patients who will achieve EASI-90/EASI ≤5 and/or low itch scores during treatment with particular (targeted) treatments. Future studies investigating differences in treatment response between AD patient clusters with specific biomarker profiles for the many available and upcoming therapeutic options will help us to predict prognosis and treatment responses, and move towards precision medicine. On the other hand, we should make us all consider whether we should aim for almost complete clearance of AD, as reflected by EASI-90, or even EASI-100. When choosing the optimal drug for the individual patient, the risk of side-effects is maybe even as important to consider as the clinical efficacy of different treatment options. More and more real-life studies

including dupilumab-treated AD patients are being published, and although clinical improvement of AD signs and symptoms is comparable with data from clinical trials[25, 26, 59, 60], various different side-effects seem to be more commonly reported in daily practice. Several adverse events reported during dupilumab treatment in AD, including psoriasis-like skin lesions[61-68], rosacea[69, 70], and alopecia areata [71-75], are known to be driven by activation of the T1 and/or T17 pathway. One might hypothesize that inhibiting the T2 pathway would upregulate other pathways including T1 and T17. Although Hamilton et al.[76] did not observe increases in T1-related gene expressions in skin after 4 weeks of dupilumab treatment, we described signs of skewing towards a more T1/T17 phenotype in peripheral blood mononuclear cells (PBMCs) from some AD patients during one year of dupilumab treatment in **chapter 5**. This difference might be explained by the short duration of the study by Hamilton et al.[76] , as longer dupilumab exposure might be needed to induce change in the T cell functional phenotypes. The fact that Th1/Th17 skewing was especially observed in the skin-homing CD4+ T-cell population in our study, is in line with the mainly skin-related side effects that are observed in dupilumab-treated AD patients. Noteworthy, one of our patients who showed skewing towards the T17 pathway actually developed a severe rosacea after 40 weeks of treatment. These results might indicate that patients with a biomarker profile that is already skewed towards T1/T17, such as the "Th1/Th2/Th17 dominant" cluster, may be at higher risk to develop T1/T17-related side effects during dupilumab treatment. Biomarker-based patient profiling could therefore not only be valuable for deciding which treatment will be the most effective in which patient, but also which one will be the safest.

Besides blood biomarkers, other types of biomarkers can be used to predict possible side effects during dupilumab treatment. As described in **chapter 8**, conjunctivitis, one of the most commonly reported side effects during dupilumab treatment in AD[77], seems to be associated with a scarcity of intraepithelial goblet cells (GCs). Conjunctival GCs are specialized secretory cells that produce mucins, large glycoproteins that lubricate the ocular surface and stabilize the tear film.[78] Proliferation and homeostasis of conjunctival GCs is demonstrated to be mainly regulated by IL-13 expression.[79, 80] By blocking the action of IL-13, dupilumab treatment might cause hypoplasia of conjunctival GCs and decreased mucin production, leading to disruption of the tear film and conjunctival inflammation. We hypothesize that AD patients with preexisting low conjunctival GC density, might be predisposed for developing conjunctivitis during dupilumab treatment. Baseline screening of the amount of conjunctival GCs might therefore be a valuable predictor

for the risk of conjunctivitis, and we are currently investigating this in a prospective clinical study.

Can we predict or even influence the atopic march?

Since the highest prevalence of AD is seen during childhood, applicability of biomarker-based patient profiling in pediatric AD patients is of great interest. Childhood AD is clearly different from adult AD in terms of clinical presentation. Additionally, within the pediatric AD population we see age-dependent heterogeneity of clinical features. The distribution of AD lesions changes from facial, scalp and extensor involvement in infants and young children, to predominant flexural involvement in older children and adults (Figure 1).[81] In terms of morphology, exudative lesions are typically present in infants, while older children exhibit lichenified papules and plaques representing the more chronic disease that is observed in adult AD.[82, 83] Increasing insights into blood and skin profiles of early-onset pediatric AD patients show substantial differences from adult AD.[7, 8, 84, 85] Although both populations show significant T2 activation in skin and blood, early-onset pediatric AD also showed T17/T22 skewing, but lacked the T1 upregulation that is seen in adults (Figure 1).[7, 8, 85] Besides, lesional and non-lesional skin of pediatric AD patients showed higher levels of epidermal proliferation markers (keratin 16 and S100As), and antimicrobial peptides (AMPs).[8] This raises the question whether pediatric AD is, just as adult AD, heterogeneous on the biological level, and whether the biomarker based patient clusters that could be confirmed in adult AD patients in **chapter 3**, can also be identified in pediatric AD patients.

In **chapter 4** we measured a broad panel of 145 serum biomarkers in a cohort of 240 pediatric AD patients aged 0 until 17 years. We confirmed heterogeneity at the level of serum biomarkers in pediatric AD patients and identified four patient clusters based on their unique systemic immune profiles, by using an unsupervised clustering approach. By comparing the driving pathways per cluster, only one out of the four pediatric AD clusters was similar to one of the adult clusters.[12, 32] These results support the previous findings that serum biomarker profiles in pediatric AD differ from adult AD patients. The question is, however, whether the differences in driving biomarker profiles between pediatric and adult AD are disease specific, or whether it is related to age and general maturation of the immune system. Age at moment of sampling and age of onset were not significantly different between our four pediatric AD clusters. While clinical characteristics are clearly age-dependent in pediatric AD[81], our results indicate that the biological heterogeneity does not seem to be age-

related. Future studies including age-matched healthy controls are needed to clarify this, as those were not included in our study.

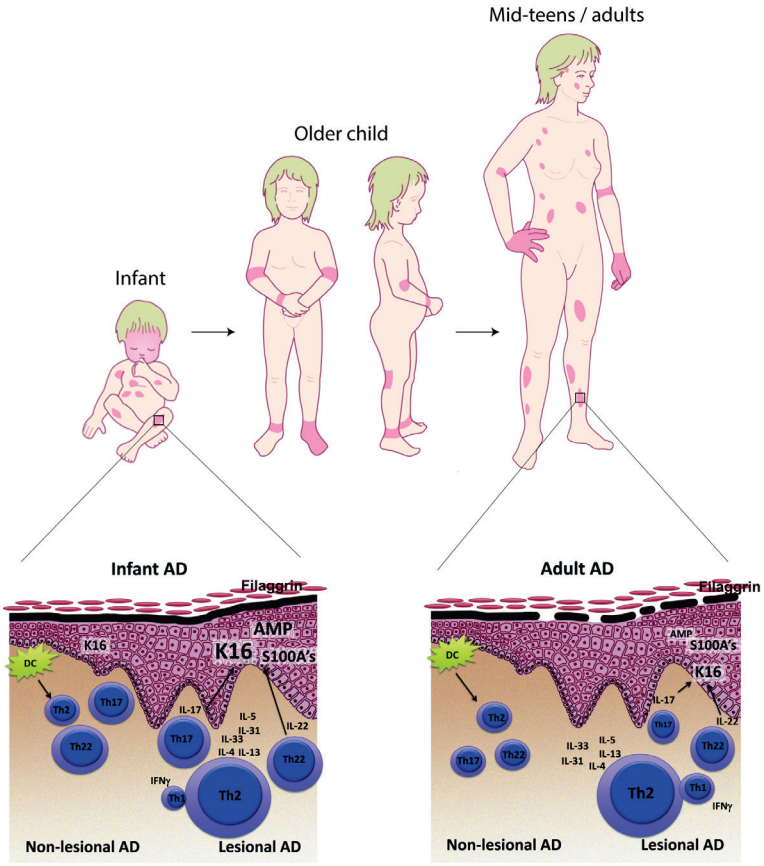


Figure 1. Schematic representation of differences between clinical presentation and selected immune pathways in lesional and non-lesional skin in infant and adult AD. *AMP*, antimicrobial peptide; *DC*, dendritic cell; *K16*, keratin 16. Figure adapted from "The immunology of atopic dermatitis and its reversibility with broad-spectrum and targeted therapies" by Patrick M. Brunner et al. 2017, JACI 139(4): S56 – S76.

One can speculate about the outcomes of these studies based on our results and literature. In the study presented in **chapter 4**, we could demonstrate differences in biomarker expression levels between the three age groups. However, these results were derived from supervised analyses using predefined age groups. The four biomarker-based patient clusters, were identified by using an unsupervised clustering approach, and were found not to be associated with age. Additionally, the

absolute differences in mean biomarker expression levels were less pronounced between the age groups compared to the differences between the biomarker profiles of the four clusters. By contrast, Czarnowicki et al.[86] recently compared systemic immune profiles of different AD age groups ranging from 0 to >18 years, with healthy control subjects. Unsupervised k-means clustering analysis, based on blood flow cytometric marker frequencies, identified three separate age clusters in AD patients. By using the same approach in healthy control subjects, patients were not clearly stratified by age, indicating AD as the driver of the age-related cluster characteristics, rather than age alone. The differences between these results and our findings can be explained by the differences in experimental design of both studies. Czarnowicki et al.[86] used flow cytometry to measure a selected panel of IFNγ, IL-9, IL-13, IL-17, and IL-22 cytokine levels, and T-cell activation within skin-homing/cutaneous lymphocyte antigen (CLA+) versus systemic (CLA-) CD4+/CD8+ T-cells, while we used Luminex multiplex immunoassays to analyze a much broader panel of serum biomarkers. Recently, Wang et al.[87] compared serum cytokine levels between 220 Chinese AD patients divided into four age groups (2-18, 19-40, 41-60, and >60 years) and HCs. Serum levels of the T2 markers IL-4 and TARC/CCL17 were significantly higher in AD patients compared to HCs, but there was no difference between de four age groups. Elderly AD showed higher levels of IL-17A and IL-22 compared with childhood and young adulthood AD, and serum levels of IL-6 was higher in elderly AD compared with childhood AD.[87] Similarly, we found no significant differences in T2-related serum biomarkers between the three pediatric AD age groups, and T17-related markers were higher in the 5-11 and 12-17 years AD children compared to the youngest age group.

Although three out of four of the pediatric patient clusters differ from the previously identified adult AD clusters, our results might indicate that the distinct pathophysiologic mechanisms driving the heterogeneity of pediatric AD cannot be solely assigned to the difference in age or duration of the disease, and argue for more endotype-specific, rather than uniform or age-specific therapeutic strategies. This is strengthened by the fact that correlation coefficients from serum biomarkers with EASI were comparable between the three age groups in our cohort.

In up to 60% of the patients, AD develops within the first year of life, and 85% within the first five years of life.[88, 89] AD predisposes patients to other atopic diseases, including food allergy (FA), asthma, allergic rhinitis, and eosinophilic esophagitis.[89, 90] This phenomenon is known as the atopic march, and AD is often thought to be the

first atopic condition to manifest.[91] However, it is still unclear whether the order of appearance of atopic diseases can be predicted or whether they can be prevented. Different preventing strategies for AD and FA, including emollients, breast feeding, microbial exposures, probiotics, vitamin D and UV light have previously been investigated, nevertheless, the evidence remains limited.[92]

Most children are thought to "outgrow" AD, however, persistence of AD into adulthood is more common than previously recognized.[93] Margolis et al.[93] assessed 7157 children with AD, and found that more than 80% had persistent disease at all ages, although a meta-analysis of 44 studies showed that, overall, less than 5% of childhood AD persisted by 20 years after diagnosis.[94] A recall bias might be a problem in these studies, since patients with only mild AD at very young age might not remember this in adulthood. AD persistence has been associated with later onset, more severe disease, and family history of atopy.[94-99] However, we are still not able to adequately predict the individual course of AD at an early stage in pediatric patients. A possible reason for this is the heterogeneity of the disease. Therefore, the four pediatric AD patient clusters that we described in **chapter 4** could provide a first start for predicting the course of AD and for the development of interventional trials aimed at stopping or minimizing the extent and severity of the atopic march. In addition to our study, Lauffer et al.[99] recently identified three different endotypes within a cohort of AD patients aged 0 – 3 years old, based on multiple clinical parameters and a panel of 33 serum biomarkers, of which 29 were also included in our studies presented in **chapter 3 and 4**. They aimed to predict the clinical course of AD and persistence at the age of 7 years. Cluster 1 of the study by Lauffer et al.[99] was characterized by the highest disease severity at onset, the highest rate of persistence at age of 7 years, and had the highest levels of MIP-1b, IL-9 and IL-17. These markers were also found to be the highest in our "Th1/Th2/Th17/IL-1 dominant" cluster, which was comparable to the "Th1/Th2/Th17 dominant" adult cluster. This suggests that patients stratified into the "Th1/Th2/Th17/IL-1 dominant" cluster might be at higher risk to develop chronic AD. On the other hand, Lauffer et al.[99] again showed that AD persistence was associated with more severe disease, indicating that our severe "skin-homing dominant" cluster may include the patients with persistent disease. Besides, persistence at age of 7 as reported in the study of Lauffer et al.[99] does not necessarily indicate persistence of AD into adulthood.

Early immunomodulatory treatment of (a subgroup of) children with AD might influence the atopic march and persistence of AD into adulthood. For example, IL-

4/IL-13 inhibitory treatment with dupilumab has been associated with reversal of the skin immune and barrier abnormalities, including T2- and T17-related cytokines and markers of epidermal hyperplasia.[76] Epithelial secretion of the T2-promoting cytokines TSLP, IL-25 and IL-31 is involved in early AD initiation and instigation of other atopic disorders.[100, 101] Hence, early inhibition of the T2 axis in children with (severe) AD might not only have the potential to impact the natural history of AD but may interrupt the atopic march as well. On the other hand, we should be very cautious with interfering in young children's immune system. Children have in general a more T2 skewed immune system compared to adults, which is also still developing and changing.[102, 103] Although early treatment with, for example, dupilumab could be beneficial to halt the atopic march, children might be at higher risk of unbalancing the immune system. As previously mentioned, and described in **chapter 5**, dupilumab treatment may induce skewing towards a more T1/T17 profile in a subgroup of patients on the long term. One of our pediatric AD clusters showed already enhanced T17/T1 responses, and children aged 0 – 4 years were characterized by higher levels of T1-related serum biomarkers. Early immune interference in these patients may possibly lead to a higher risk of T1/T17-mediated diseases on the long term. In accordance with this concept, we have recently experienced several cases of children who developed psoriasis-like lesions during dupilumab treatment in our center, comparable to the adult cases that have been described in literature.[61-68] It might therefore be advisable to use short term or pulse therapy with targeted drugs, instead of long term immunomodulatory treatment, in pediatric patients, as this might be enough to interrupt the atopic march and prevent AD persistence into adulthood, but may not skew the immune system. Future longitudinal studies are needed to be able to better predict AD persistence and to clarify the effect of targeted interventions on the immune system of young children.

DUPILUMAB AS AN EFFICIENT IMMUNE MODULATOR IN ATOPIC DERMATITIS

Early immunological effects of dupilumab treatment

Dupilumab treatment has proven its high short- and long term efficacy in large clinical trials and daily practice studies, as evidenced by a significant reduction in EASI scores, as well as improvement in patient-reported outcomes, including itch-Numeric Rating Scale (NRS), sleep-NRS, and Dermatology Life Quality Index (DLQI).[4, 25, 28, 29, 59, 60, 104, 105 106, 107] Studies investigating dupilumab, and emerging other targeted drugs in AD patients will further clarify the role of the different cytokine pathways in the etiology of AD. The high effectiveness of dupilumab treatment confirmed the essential role of the T2 cytokines IL-4 and IL-13 in AD. In parallel with improvements in clinical scores, dupilumab was able to normalize RNA expression of T2-, T-cell, and eosinophil-related genes and to reverse skin barrier abnormalities in lesional biopsies from AD patients after 4 weeks of treatment.[76] Guttman-Yassky et al.[108] showed that dupilumab significantly reduced serum levels of type 2 markers (TARC/CCL17, PARC/CCL18, and periostin) as well as total and allergen-specific IgE concentrations after 4 and 16 weeks of treatment in a phase II clinical trial.[108] In accordance with clinical responses, we recently showed in a daily practice study that disease severity-related serum biomarkers (TARC/CCL17, PARC/CCL18, periostin, and IL-22) significantly reduced after 4 and 16 weeks of dupilumab treatment.[25]

The few mechanistic studies that have been reported all show significant changes in skin and blood already after 4 weeks of dupilumab treatment. In **chapter 5** we demonstrated the very rapid onset of action of dupilumab, by showing that IL-4Rα expression and STAT-6 phosphorylation upon IL-4 stimulation in both B- and T-cells was completely blocked already within two hours after the first dupilumab administration. Receptor saturation remained stable until 52 weeks of treatment. We confirmed the assumption that IL-4Rα was actually occupied by dupilumab antibodies with anti-IgG4 staining on both T- and B-cells, already within two hours after administration, and we could not find signs of receptor internalization. The IL-4Rα is a subunit of two types of receptor complexes for IL-4 and IL-13 (Figure 2): the type I receptor, which is composed of the IL-4Rα chain and a γ chain, and the type II receptor, which is composed of the IL-4Rα chain and the α1 chain of the IL-13 receptor (IL-13Rα1).[109]

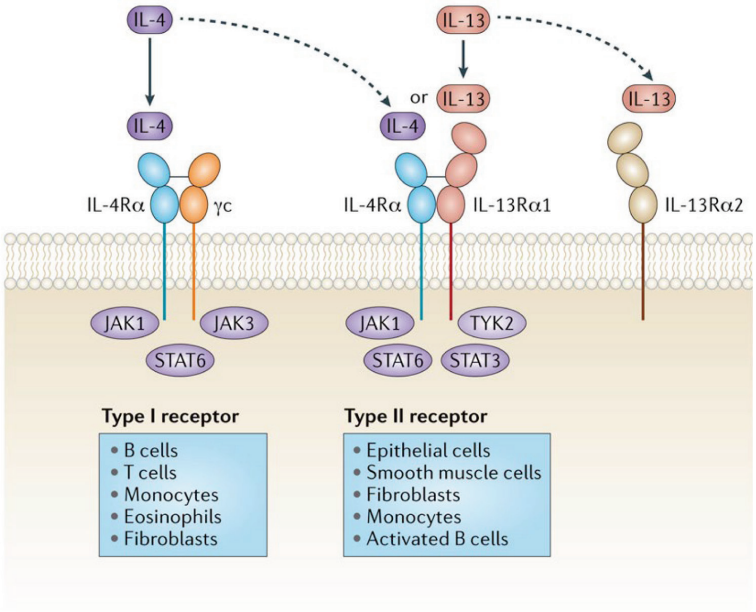


Figure 2. Receptor systems for IL-4 and IL-13. IL-4Rα forms two heterodimeric receptor complexes to mediate the biological functions of IL-4 and IL-13. The type I receptor, comprising IL-4Rα and common cytokine receptor γ-chain (γc), only binds IL-4. The type II receptor complex, comprising IL-4Rα and IL-13Rα1, is the primary receptor for IL-13 but also binds IL-4. In addition, IL-13 can bind to a second receptor, IL-13Rα2. The expression of the type I or type II receptor complexes determines which cytokine or cytokines act on specific cell types. Reproduced in part from Gandhi et al.[110] *Nat. Rev. Drug Discov.* 15, 35–50, 2016.

The type I receptor, activated by only IL-4, is primarily expressed on lymphocytes and controls T2-cell differentiation and isotype class switching of B-cells to produce IgE. The type II receptor, activated by both IL-4 and IL-13, is expressed widely across non-hematopoietic cells, including keratinocytes, hair follicles, epidermal sebaceous, sweat glands, epithelial and smooth muscle cells, and fibroblasts.[109-111] IL-13, but not IL-4, preferentially drives the development of the pathological features of atopic diseases because of the higher amounts of IL-13 produced and higher abundance of IL-13Rα1 at the site of inflammation.[109] This has led to the hypothesis that blocking IL-13 alone may be sufficient to achieve clinical efficacy in AD. Treatment with tralokinumab and lebrikizumab, both anti-IL-13 treatments, have demonstrated positive results in moderate-to-severe AD patients in phase IIb and III trials.[21, 22, 24, 30] The mean difference in EASI scores seems to be higher for dupilumab treatment, but the placebo-adjusted EASI-75 response was similar for lebrikizumab (37%) and

dupilumab (32-36%), while tralokinumab lags somewhat behind (12-22%).[112] However, head to head studies of the different monoclonal antibodies are lacking at his moment.[28, 29] The question remains whether IL-4 blockade has additional positive effects on AD signs and symptoms compared to blockade of only IL-13.

In theory, dupilumab might differentially impact the two IL-4/IL-13 receptor complexes, which may explain the low response of few patients. This differential impact may be influenced by the abundance of the IL-4Rα and IL-13Rα1 subunits on the target cells.[113, 114] If dupilumab preferentially inhibits the type I receptor, then it would primarily suppress T2 cell differentiation, but not necessarily IL-13 driven tissue inflammation, and vice versa. The suppression of tissue inflammation by dupilumab as seen in AD[108] would suggest prominent inhibition of the type II receptor. On the other hand, the fast IL-4Rα blockade presented in **chapter 5** was accompanied by significant decreases in the percentage of peripheral blood proliferating and T helper (Th)2/Th22 cytokine-producing CLA+CD4+ T-cells already within the first 4 weeks of treatment, suggesting an important inhibition of the type I receptor as well. These results were in line with the studies of Hamilton et al.[76] and Guttman-Yassky et al.[108] showing decreases in gene expressions related to the T2 pathway (IL-13, IL-31, TARC/CCL17, PARC/CCL18, MDC/CCL22, eotaxin-3/CCL26), T17/T22 pathway (IL17A, IL-22, and S100As), epidermal hyperplasia (K16, MKi67), and T-cell activation (ICOS) in lesional skin after 4 and 16 weeks of dupilumab treatment. The slight increase of serum IL-4 and IL-13 levels that was shown by Ariëns et al.[25] might be the result of an increase of unbound circulating IL-4 and IL-13 levels, since these cytokines are not able to bind to the IL-4Rα anymore due to the complete blockade. We hypothesize that the increase in serum IL-4 and IL-13 will be temporarily, as we showed that Th2 cytokine producing skin-homing T-cells decreased during dupilumab treatment. The major immunological effects were observed within the first 4 weeks of treatment, after which T-cell cytokine profiles remained relatively stable. The clinical signs and symptoms, however, continued to improve until 52 weeks in our cohort, consistent with the recent study of Ariëns et al.[26] It would hence be interesting to investigate whether the early immunological effects of dupilumab treatment can be used as predictor for the long-term response.

Biomarker-based monitoring of disease severity during dupilumab treatment

Dupilumab is the first targeted therapy registered for adults and adolescents with moderate-to-severe AD patients[115, 116], but several other immunomodulating therapies are currently in development and likely to alter our management of AD. The comparison of results from different clinical studies remains challenging, given the substantial variation in clinician-rated outcome measures that are currently used, and high inter- and intra-observer dissimilarities.[117]. Especially in daily practice, AD severity is often scored by many different physicians, which also results in within- and between-patient variability of severity scores. In **chapter 6** we showed that a previously developed biomarker signature consisting of the serum biomarkers TARC, IL-22 and sIL-2R, named the predicted-EASI (p-EASI)[118], adequately predicts disease severity in AD patients treated with dupilumab. The p-EASI has previously proved to be suitable for prediction and follow-up of disease severity in AD cohorts treated with topical corticosteroids (TCS) and cyclosporin A (CsA) as well.[118, 119] This indicates that the p-EASI can be used to compare effects on disease severity between different treatment options, both topical as well as systemic treatments, and both broad-acting as well as more targeted drugs.

Compared with the results of the previous TCS and CsA cohorts[118, 119], the correlation between EASI and p-EASI was slightly lower in our study including dupilumab-treated AD patients. This difference might be explained by the fact that dupilumab treatment did not influence serum sIL-2Rα levels, while both TCS and CsA treatment did. Our results were based on data measured by Luminex[120] multiplex immunoassays. To rule out an assay-specific explanation for the unchanged sIL-2Rα levels, we performed enzyme-linked immunosorbent assays (ELISA) in two separate cohorts of patients treated with dupilumab in the University Medical Center Utrecht, the Netherlands (n=18), and the Erasmus Medical Center, Rotterdam, The Netherlands (n=11). ELISA measurements confirmed our previous findings by showing stable sIL-2Rα levels from baseline through week 16 of dupilumab treatment (Figure 3). Although both technologies showed the same pattern of sIL-2Rα levels over time, expression levels were not comparable. This might be explained by the difference in antibodies used and the sensitivity of both semi-quantitative technologies.

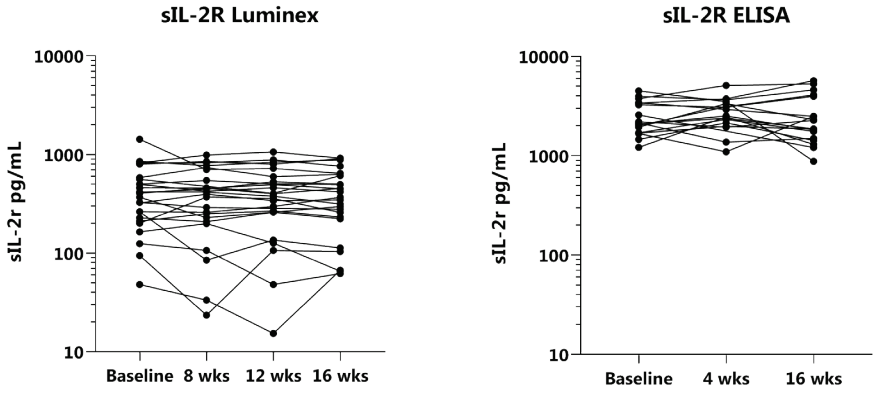


Figure 3. Serum soluble interleukin(IL)-2 receptor levels were not affected by 16 weeks of dupilumab treatment in atopic dermatitis patients, as observed with both Luminex immunoassays and enzyme-linked immunosorbent assays (ELISA).

The IL-2Rα is expressed on the cell membrane of T-cells and released in a soluble form during T-cell activation.[121, 122] Infiltrating cells in lesional skin of AD patients are mainly composed of CD4+ activated T-cells, which have shown to express IL-2Rα.[123] Besides, soluble IL-2Rα is elevated in the serum of AD patients and also highly correlates with AD severity.[124, 125] The release of sIL-2Rα is an indirect effect of T-cell activation by IL-2, which secretion is also dependent on IL-1(α) expression.[126] This suggests a role for T1 inflammation in the etiology of AD, and also explains why dupilumab treatment does not affect sIL-2Rα levels during the first 16 weeks of treatment. Dupilumab specifically downregulates T2 differentiation and inflammation, which was shown by a significant reduction of T2 cytokines and chemokines in lesional skin and serum of AD patients.[25, 76, 108, 113] As few patients in our study did show a clear decrease of sIL-2Rα during dupilumab treatment, one might speculate that this marker plays an important role in a subgroup of AD patients only. For example, the "skin-homing chemokines/IL-1R1 dominant" cluster and the "Th1/Th2/Th17 dominant" cluster described in **chapter 3** showed significantly higher levels of IL-1α[32], a cytokine that was shown to be highly correlated with sIL-2Rα.[126] CsA, and, to a lesser amount TCS, have broad immunosuppressive effects by targeting multiple T-cell phenotypes and related cytokines. Consequently, both treatments significantly reduced sIL-2Rα levels in AD patients in the previous studies of Thijs et al.[118, 119] JAK-inhibitors, the emerging class of small molecules, are likely to be the next generation of FDA approved agents for the treatment of AD. In contrast

to the selective T2-inhibition by dupilumab treatment, JAK-inhibitors target multiple cytokine pathways associated with both acute as well as chronic lesions of AD.[127] As the p-EASI includes serum biomarkers related to T2 (TARC/CCL17), as well as T22 (IL-22), and T-cell activation/T1, we expect that the p-EASI will be usable in AD patients treated with JAK-inhibitors as well. However, future investigations are needed to confirm its usability in AD patients treated with JAK-inhibitors and biologics targeting other cytokine pathways. One could speculate on removing sIL-2Rα from the p-EASI, and optimize the formula for the specific use in dupilumab treated patients. However, we believe that the proven broad applicability is an important strength of the p-EASI. Especially with the many upcoming treatments in the field of AD, an objective, consistent and widely applicable outcome measurement is needed to successfully compare different treatment options and to improve therapeutic decision making.

p-EASI in future studies and daily practice

Our results showed that p-EASI scores decreased more quickly during dupilumab treatment than subjective clinical scores. This indicates that the p-EASI objectively precedes clinical signs and symptoms, and may therefore be a useful tool in daily practice. A higher p-EASI compared to an EASI scored at a specific time point might predict AD flares. Besides, we have the experience from our outpatient clinic that the majority of AD patients with controlled AD under dupilumab treatment, can successfully prolong the interval of dupilumab injections from every two week to every three to eight weeks. The p-EASI score, possibly in combination with NRS itch scores, might be very useful in these patients to decide whether the interval can be further prolonged or not. Besides, the extent of the decrease of p-EASI in the first four weeks of dupilumab treatment, might be used to predict long-term response. Longitudinal studies are needed to investigate this.

As we showed in **chapter 4**, serum biomarkers that correlate the best with disease severity are slightly different in pediatric patients compared to adults. Although TARC/CCL17, currently considered as the most reliable serum biomarker of AD severity[128], showed a positive correlation with EASI, it was not the best performing marker for disease severity in our pediatric AD population. PARC/CCL18, Apelin, and IL-1R2 showed higher positive correlations, and RBP4 and Cathepsin S showed higher negative correlations with EASI.[129] These results are in line with previous studies showing that TARC had a weaker correlation with severity scores in pediatric AD compared to other serum biomarkers, including macrophage-derived chemokine (MDC), CTACK/CCL27, IL1RL1, and IL1RL2.[7, 130, 131] In studies investigating children

younger than 5 years old, Ahrens et al.[132] showed a positive correlation between serum TARC levels and SCORAD only in food- and airborne-sensitized infants, but not in non-sensitized infants with AD, and Brunner et al.[7] found that plasma TARC/CLL17 levels were not correlated with disease activity in early-onset AD patients. Based on these data, one could hypothesize that the performance of the p-EASI might be different in pediatric compared to adult AD. For adequate prediction of severity in pediatric AD, different biomarkers should perhaps be included in the p-EASI, or the existing formula might need to be adjusted, since absolute serum biomarker levels are different in children compared to adults. It is therefore important to further investigate the p-EASI, and perhaps other biomarker combinations for assessment of disease severity in pediatric AD patients.

LONG TERM IMMUNOLOGICAL EFFECT OF DUPILUMAB TREATMENT

Effects of dupilumab on IgE mediated diseases

Our study described in **chapter 5** was the first to investigate immunological changes during long-term dupilumab treatment. We showed that the rapid and functional blockade of the IL-4Rα remained stable until 52 weeks of treatment, on both T- and B-cells. One could hypothesize that the long-term complete blockade of the IL-4Rα might lead to compensatory mechanisms, including upregulation of the receptor itself or other type 2 receptors. Although this could theoretically result in severe flare-ups after stopping dupilumab treatment, we have not experienced this yet in daily practice. Additionally, IL-4Rα saturation might change during long-term treatment. A decreased saturation could reduce clinical efficacy, as it has been hypothesized that maximum efficacy would be observed at doses that achieved dupilumab concentrations in serum sufficient to achieve saturation of the IL-4Rα, as evidenced by linear/dose-proportional pharmacokinetic profiles.[133] It might be very interesting to investigate whether mRNA levels of IL-4 and IL-13 receptors and related cytokines, as well as saturation and unbound dupilumab blood levels change during long term dupilumab treatment.

Although the inhibitory effects on Th2 and Th22 cytokine-producing T-cells were mainly observed within the first weeks of dupilumab treatment, we showed that effects on total serum IgE levels were slower, which steadily decreased until 52 weeks

of treatment (**chapter 5**). Comparable to our results, data from four clinical trials showed that the rate of IgE reduction increased after 4 weeks of therapy, presumably due to the longer half-life of IgE.[3] The magnitude of changes in IgE were not in line with the reduction in EASI score. In addition, patients who were prospectively stratified by baseline IgE levels (reflecting intrinsic versus extrinsic AD) responded all similarly to dupilumab in a 12-week monotherapy study.[3] These data provide validation that AD pathology is independent of IgE. On the other hand, the inhibitory effects of dupilumab treatment related to IgE concentrations are hypothesized to positively influence other atopic comorbidities, including food allergy and allergic rhinitis (AR).[134] Previous studies showed that dupilumab was able to reduce serum levels of allergen-specific IgE concentrations, including food- and respiratory components[108, 135, 136], suggesting either a blockade of the differentiation of short half-life IgE B-cells, or inhibition of the IL-4/IL-13 effect on long-term survival of IgE-producing B-cells. Besides, Abdel-Gadir et al.[137] found that *in vitro* treatment with a neutralizing anti-IL-4Rα mAb restored the suppressive function of peanut reactive Treg cells of peanut-allergic patients. In accordance with these findings, we showed in **chapter 5** that the proportion of circulating CD25+FOXP3+CD4+ regulatory T cells (Treg) significantly increased after 4 and 52 weeks of dupilumab treatment. As Treg are important mediators of tolerance and suppress immune responses through production of immune-regulating cytokines[138], these results highlight the potential disease-modifying effects of anti-IL-4Rα treatment. Three studies investigating the effect of dupilumab treatment in asthma and AD patients with comorbid perennial allergic rhinitis showed significant improvement in AR-associated nasal symptoms and quality of life.[136, 139] However, to date, evidence for the clinical relevance of the inhibitory effects of dupilumab treatment on food allergy is scarce. Only one case has described a patient with severe AD and food allergy, who developed tolerance to two food components that previously induced (anaphylactic) allergic reactions, after three months of dupilumab treatment.[140] Although allergen-specific IgE levels significantly decreased, concentrations remained above the normal threshold in most patients.[108] Alternative mechanisms may maintain IgE synthesis independently of the IL-4Rα chain on long term allergen exposure in established allergy, which are different from the induction phase of allergic responses.[141, 142] Taken together, these results might suggest that dupilumab treatment has low clinically relevant effects on preexisting food allergies, but possibly mainly affect newly developing allergies. Further studies are needed to evaluate the clinical effect on existing food allergies. There are currently two ongoing trials to evaluate the safety and efficacy of dupilumab in food allergy, one involving the use of dupilumab as an adjunct therapy

in peanut oral immunotherapy, the other investigating dupilumab monotherapy in peanut allergy.[143, 144]

Long-term skewing and possibly related side effects of dupilumab treatment

It has been speculated that treatment with a T2 inhibiting agent like dupilumab might induce or aggravate T1- and T17-associated inflammatory diseases as a result of a shift along the T-cell subset spectrum.[145] This hypothesis has been supported by several case reports and case series describing AD patients who developed T1- and/or T17-related side effects during dupilumab treatment, including psoriasis-like lesions[61-68], rosacea[69, 70], alopecia areata[71-75], arthritis[146], and polyenthesitis[147]. In **chapter 5** we demonstrated that, overall, dupilumab does not seem to have long term skewing effects on the peripheral Th subset composition. However, in some individuals IL-17 producing CD4+ T-cells exceeded baseline levels after 52 weeks of treatment. This effect was mainly observed in the specific skin-homing (CCR4+CLA+) T-cell subpopulation, and might therefore explain why most T1/T17-related side effect observed during dupilumab treatment in AD patients are skin-related. A similar mechanism for musculoskeletal-homing T-cells might explain the muscle and joint pain, arthritis, or enthesitis reported in few AD patients during dupilumab.[146-149] Recently, Bridegwood et al.[149] confirmed the expression of IL-4Rα in healthy peri-entheseal bone and enthesis soft tissue, and demonstrated that entheseal-derived T-cells secrete basal levels of IL-4, but not IL-13. Both IL-4 and IL-13 downregulated entheseal IL-23/IL-17 production. Inhibition of the protective function of IL-4/IL-13 on the IL-23/IL-17 axis in entheseal tissue by dupilumab treatment might therefore induce musculoskeletal pathology. Corresponding to current literature, we experienced in daily practice that biopsies of dupilumab-associated psoriasiform dermatitis may not always demonstrate clear psoriasiform changes, even when the lesions clinically appear typically psoriasiform, but may histologically demonstrate characteristics of AD as well, such as spongiosis.[65] The opposite is also possible: in some patients the clinical presentation may not be psoriasiform, while the histology resembles psoriasiform dermatitis.[150] This phenomenon supports the speculation of dupilumab inducing a shift along the spectrum between T1 and T2 immunity. The shift from T2 to T1/T17-associated diseases is not likely to occur in every patient. Some patients may be at increased risk due to (genetic) susceptibilities to other autoimmune diseases. Recently, there was a case that showed facial erythema after treatment with dupilumab for AD accompanied with systemic lupus erythematosus (SLE).[151] This raises the question whether these susceptibilities need to be checked before initiation of treatment. As a pilot study, we recently started measuring HLA-

B27 and autoantibodies (anti-nuclear antibodies) and in all AD patients starting dupilumab treatment and during follow-up.

Abovementioned side effects have not yet been reported in clinical trials evaluating tralokinumab. Although the T1/T17-related side effects associated with dupilumab were only reported in daily practice, it is expected that IL-13 blockade alone will limit the risk of T-cell skewing, since T2 cell differentiation will still be stimulated by IL-4 via the type I receptor. The same applies to treatment with JAK-inhibitors, as these small molecules have a broader inhibitory effect on T-cells, involving multiple downstream cytokine pathways, which might, on the other hand, increase the risk of other side effects, including infections or hematologic abnormalities.[45]

Another more recently described side effect of dupilumab treatment in AD patients in daily practice is a paradoxical head and neck erythema that is clinically and histologically different from their background AD.[150, 152-154] Since the clinical and histological characteristics of dupilumab facial redness (DFR) that are currently described in literature are rather heterogeneous, a clear pathogenesis has not been established yet. Several underlying mechanisms have been proposed, including flaring of allergic contact dermatitis or rosacea, seborrheic dermatitis-like disease, drug-induced photosensitivity reaction, and site specific failure to dupilumab.[150, 154-156] The majority of these hypothesis were associated with a T1/T17/T22-dominated response, indicating again a dupilumab-induced shift in T-cell pathways. In **chapter 11** we described two cases of DFR with hypersensitivity to the *Malassezia* yeast and positive treatment response to itraconazole. We believe that DFR is a heterogeneous and polyform entity, and needs a personalized treatment approach. We have experienced that a subgroup of patients responds very well to treatment with antifungal therapy with itraconazole, while others respond to oral doxycycline. Larger studies involving biopsy testing and different treatment options are needed to further define the underlying pathomechanism of this new entity.

Besides helper T-cell skewing, dupilumab might theoretically increase the risk of helminth infections, as type 2 immunity plays an important role in the protection against parasitic infections by reducing the number of parasites and protecting the host against parasite-mediated damage.[157] Analysis of the pooled safety data from clinical trials revealed no cases of parasitic infections.[158] However, this may have been affected by the very low prevalence of helminth infections in developed countries from which patients were included. Complementary to these findings, our study did

not observe a suppressing effect on the total IL-4 and IL-13 cytokine production by CD4 T-cells (intracellular cytokine staining) and by total PBMC following anti-CD3 stimulation up to 52 weeks of treatment. It cannot be ruled out though that this will eventually occur after longer-term use of dupilumab.

Since IL-4 induces B cell activation and modifies humoral B cell responses to both T cell-dependent and T cell-independent stimuli[159, 160], it may be hypothesized that the complete IL-4Rα blockade during dupilumab treatment that we showed in **chapter 5**, might impact the immune responses to infections and vaccination in patients receiving this drug. IL-4 and IL-13 are involved in B-cell differentiation, proliferation and antibody production, modulating Ig isotype switching toward IgE and IgG4, and away from the more antiviral IgM, IgG1 and IgG3.[161, 162] Atopy and a T2 skewed cytokine milieu has been associated with delayed and impaired responses to early life infections and responses to vaccines.[162] This may imply that dupilumab treatment positively influences the response to vaccines and development of immune responses in pediatric AD patients. Data on the effect of dupilumab treatment on vaccine response a scarce. Only one randomized placebo-controlled trial involving 178 AD patients has been published, and demonstrated that the immune response to the inactivated tetanus and meningococcal vaccines appears to be unaffected by dupilumab treatment.[163] The impact of dupilumab on live vaccines has not been studied yet.

Should AD patient profiling ideally be based on blood, skin or both?

Although the skin is the target organ of AD, early signals of disease activity might be missed when only looking into skin biomarkers, and integrated blood-skin biomarker models might be a more holistic way to build a disease profile. Nevertheless, as AD leads to systemic inflammation[31, 164] the use of serum proteins has been proven effective in identifying different immunological endotypes of AD as well as to objectively score disease severity[12, 25, 99, 118, 119], which we again demonstrated in **chapter 3, 4, and 6** respectively. Due to their systemic representation, blood biomarkers might be useful to predict or monitor comorbidities and side effects, and as we showed in **chapter 6**, serum biomarkers might be ahead of early clinical signs and symptoms. As we have shown in **chapter 5**, peripheral blood T-cells might be good intermediates between skin and serum proteins. Especially the subpopulation of skin-homing (CLA+) T-cells, which recirculate between the skin and peripheral blood, where they might reflect the immunological situation in AD lesions.[165] The easier access to circulating skin-homing T-cells may eliminate the need for skin

biopsies in future studies, which might be particularly beneficial in pediatric AD patients. Another advantage is that since the T-cell compartment is relatively long-lived[166, 167], changes in this compartment may precede clinical effects or might be an early warning sign of imbalance in the immune system. This is especially relevant in the setting of systemic therapies. Blood collection is less invasive than skin biopsies, is in general less time-consuming and laborious to process, and therefore allows for longitudinal follow-up as part of regular care. Recently, tape-stripping has been studied as a less invasive approach to capture the cutaneous immune and barrier abnormalities in AD. Tape-stripping captures the stratum corneum and upper stratum granulosum[168, 169], and was demonstrated to accurately characterize key immune and epidermal barrier biomarkers of the lesional and non-lesional skin of both pediatric and adult AD patients. [169-180] Given these results, tape-stripping can in future be useful across AD endotypes and for monitoring therapeutic responses in longitudinal clinical studies and daily practice. As it is a minimally invasive skin sampling approach, it will particularly be interesting for prediction and follow-up of pediatric AD patients. However, the processing of tape strips is relatively time-consuming and laborious.[181, 182] In addition, the potential variability in depth of tape-stripping makes it necessary to collect large numbers (up to 16) of strips per location. We believe that serum biomarker and skin-homing T-cell inflammatory profiles are the most ideal to use for patient profiling and future personalized therapeutic options in AD, when also taking practical considerations and patient satisfaction into account. Skin biopsies, or tape strips as alternative, will provide added value to mechanistic studies on the complex AD skin immune phenotype and barrier abnormalities. For the implementation of biomarker-driven personalized treatment in daily practice, future studies should develop a smaller serum biomarker panel of maximum 20 markers associated with each patient cluster. The assays used to measure those biomarkers need to be affordable and accessible in the majority of hospital laboratories. Since the Luminex technology that we used in our biomarker studies is not widely accessible, validation of our findings with, for example, ELISA would be an option. However, cost and time saving for ELISA versus multiplex assays such as Luminex will depend on the number of markers to be measured. Luminex has the ability to measure many analytes in single small-volume sample, while ELISA allows for the measurement of only one analyte at a time, which may limit the number of markers per sample. One multiplex panel may cost more than an ELISA test, however, the price per biomarker will be cheaper if an assay contains two or more markers.[183]

CONJUNCTIVITIS DURING DUPILUMAB TREATMENT AND ITS UNDERLYING PATHOMECHANISM

Incidence and management of conjunctivitis during dupilumab treatment

Conjunctivitis is the most frequently reported adverse event in trials evaluating dupilumab treatment for atopic dermatitis.[77] Analysis of pooled data from 11 randomized, double-blinded, placebo-controlled phase II and III clinical trials of dupilumab for AD, asthma, chronic rhinosinusitis with nasal polyposis, and eosinophilic esophagitis, showed a higher incidence of conjunctivitis in the dupilumab treated group (8.6-22.1%) versus the placebo group (2.1-11.1%) in AD but not in the other indications.[77] Moreover, the proportion of AD patients developing conjunctivitis during dupilumab treatment reported in daily practice is even higher (34-38%)[25, 26, 59, 60], which might be the result of increased awareness of ophthalmological symptoms among dermatologists as well as AD patients treated with dupilumab after reports of several studies. This theory is also strengthened by the higher incidence of conjunctivitis in the latest phase III clinical trials (28.0%)[29] compared to an earlier phase IIb trial (7%).[184] Symptoms of conjunctivitis reported in clinical trials were mostly mild to moderate and the majority resolved with topical eye treatment during the studies.

In contrast to previous clinical trial data, we demonstrated in **chapter 8** that after a follow-up of at least 12 months, most AD patients developing conjunctivitis during dupilumab treatment still suffered from mild-to-moderate conjunctivitis despite adequate ocular treatment by an ophthalmologist. In one-third of the patients dose adjustment of dupilumab was necessary to improve or resolve conjunctivitis. Discontinuation of dupilumab due to ocular pathology was needed in three cases. The majority of our patients were treated with several different therapeutics, including anti-inflammatory eye drops. In almost two-third of the patients corticosteroid eye drops were needed. Although the patients in our cohort were a selected group of AD patients in whom ophthalmological symptoms occurring during dupilumab treatment could not be controlled with lubricant drops and/or tacrolimus skin ointment (1mg/g) for the external eyelids, and could therefore be classified as moderate-to-severe conjunctivitis[25], our results highlight the chronic character of conjunctivitis occurring during dupilumab treatment in a subgroup of patients. It is important to be aware of the possible need for long-term ocular anti-inflammatory treatment, since the majority of our patients remained dependent on

ophthalmic medication during the 12 months of follow up. Long-term use of (ophthalmic) corticosteroids is associated with several complications, such as glaucoma, cataracts and central serous chorioretinopathy.[185] Therefore we would recommend to start anti-inflammatory treatment with tacrolimus skin ointment on the eyelids or cyclosporine eyedrops if possible. However, we have the experience that those treatments are insufficient in the majority of moderate-to-severe conjunctivitis cases, eventually leaving no alternative for corticosteroid eye drops.

Eight out of the 33 patients in our cohort developed limbitis during follow up (**chapter 8**). The limbus is the transition zone between the transparent cornea and the opaque conjunctiva, containing epithelial stem cells which are responsible for maintaining the normal homeostasis and wound healing of the corneal epithelium, and the transparency of the cornea.[186] Acute or chronic damage of limbal tissue can cause (irreversible) limbal stem cell deficiency. As a result, the barrier function of the limbus is compromised, resulting in replacement of the corneal epithelium with conjunctival epithelial cells, and neovascularization within the corneal epithelium and stroma. Eventually the cornea becomes opaque, leading to vision loss and even blindness.[187] Remarkably, in six of our eight cases limbitis developed despite adequate ophthalmic anti-inflammatory treatment. This, and the potential risks of (irreversible) complications highlight the significance of adequate monitoring of conjunctivitis in AD patients during dupilumab treatment. We therefore think that it is advisable to refer any dupilumab-treated AD patient with ocular manifestations, not responding to lubricants or tacrolimus ointment on the eyelids, to an ophthalmologist.

Pathomechanism underlying dupilumab-related conjunctivitis

As we confirmed in our study described in **chapter 8**, the management of conjunctivitis occurring during dupilumab treatment in AD patients is challenging. In order to optimize treatment strategies and risk management in clinical practice, a better understanding of the mechanism underlying conjunctivitis occurring during dupilumab treatment is necessary. Patients with AD have a greater risk of ocular comorbidities than the general population.[188-190] Therefore, pre-existing ocular conditions and a specific interaction between dupilumab and AD may be responsible for the higher rate of conjunctivitis among dupilumab-treated AD patients, especially since rates of conjunctivitis were not increased compared to placebo in studies with dupilumab in other type 2 diseases.[191-193] Several pathogenic hypotheses for conjunctivitis occurring during dupilumab treatment have been proposed. For

example, inhibition of IL-4 and IL-13 by dupilumab may increase the OX40 ligand activity, which is known to be involved in the pathogenesis of atopic keratoconjucntivitis.[194] IL-4/IL-13 blockade was also proposed to exacerbate *Demodex*- and IL-17 mediated inflammatory ocular and Meibomian gland dysfunction in AD, leading to rosacea-like conjunctivitis.[195] An eosinophil-mediated response might be another mechanism, as peripheral blood eosinophil counts are observed to increase after dupilumab administration and eosinophilic factors are elevated in the tears of allergic conjunctivitis patients.[188, 196] Adding to this, we showed in **chapter 7** that circulating eosinophils from AD patients exhibit an increased activation state, which is not altered by dupilumab treatment. Another suggestion is that dupilumab induces a dysregulated immune response of conjunctival-associated lymphoid tissue (CALT) in a setting of an altered epithelial barrier.[197] However, it is possible that multiple mechanisms interact and play a role in the development of conjunctivitis during dupilumab treatment.

In the studies presented in **chapter 9 and 10**, we histologically assessed conjunctival biopsies from six AD patients who developed conjunctivitis during dupilumab treatment, and further characterized the infiltrating cells by using imaging mass cytometry. The conjunctival tissue of all patients was characterized by a scarcity of intraepithelial goblet cells and a subepithelial infiltrate consisting of numerous T-cells and eosinophils, ranging in severity from mild to extensive.[198] The combination of our histopathological findings do not correspond with the known histopathological characteristics in other ocular comorbidities, such as atopic keratoconjunctivitis (AKC) and allergic conjunctivitis. These typical T2-mediated eye surface diseases are associated with an increased GC density and mucus production instead of the GC scarcity that was observed in our studies. [199, 200] In addition, allergic conjunctivitis and AKC are associated with increased mast cell and eosinophil infiltration and their activation and degranulation products[200-202], while eosinophils in the conjunctival biopsies of our dupilumab-treated patients showed no signs of degranulation. Low GC density is a hallmark in T1/T17 driven ocular diseases such as dry eyes syndromes and ocular rosacea. [203] Immunohistological analysis of conjunctival epithelium in ocular rosacea is characterized by an inflammatory cell infiltrate, mainly consisting CD4+ cells, phagocytic cells, antigen-presenting cells and mast cells, but not eosinophils.[204] So, the unique combination of low numbers of GCs in the conjunctival epithelium accompanied by an inflammatory cell infiltrate mainly consisting of CD4+ and CD8+ lymphocytes and (non-degranulated) eosinophils, may

imply a new entity of conjunctivitis developing in patients treated with dupilumab for AD.

Conjunctival goblet cells are not only important for the production of mucins, which retain water and keep the ocular surface lubricated, they also produce immunoregulatory factors to maintain immunological tolerance and prevent inflammation on the ocular surface.[205, 206] The loss of GCs is associated with greater ocular surface inflammation and expression of the T1-related cytokine IFNγ, as demonstrated in dry eye diseases.[207] IFNγ produced by CD4+ T-cells and innate cells that are attracted to the conjunctival epithelium during experimental dryness causes apoptosis of GCs and plays an important role in promoting conjunctival squamous metaplasia.[208] In **chapter 10** we showed that subepithelial infiltrates in the conjunctival biopsies of our six patients comprises a diverse panel of infiltrating immune cells, including highly activated and proliferating CD4+ and CD8+ T-cells, but also B-cells, macrophages, monocytes, and dendritic cells. Additionally, increased cytotoxic activity and elevated T1(IFNγ, TNFα) and T17(IL-17) cytokine production was observed within the infiltrates. Since GC differentiation and mucus production is normally stimulated by IL-13[79], we hypothesize that dupilumab treatment may lead to loss of GCs and mucin production, which in turn results in tear film instability and dry eye-like disease. The subsequent loss of the GCs' immunoregulatory function could lead to attraction of T1 cytokine-producing T-cells and innate cells contributing to an irritative conjunctivitis and further loss of conjunctival goblet cells. Inhibition of the IL-4/IL-13 pathway by dupilumab might additionally induce a shift toward a T1/T17 skewed cytokine milieu, further stimulating this process (Figure 4). Unfortunately, we could not compare our findings with samples before initiation of dupilumab from the same patients, but all patients presented with new onset or worsened conjunctivitis during dupilumab treatment. Previous studies have shown that calcineurin inhibitors such as cyclosporin A and voclosporin are able to suppress IFNγ producing CD4+ T-cells, preserve corneal barrier function and increase conjunctival GC density in dry eye disease.[209-212] Calcineurin inhibitors could break the vicious circle by suppressing the conjunctival T1 inflammatory response and restore the development and function of GCs, and should therefore have a prominent position in the treatment algorithm of conjunctivitis occurring during dupilumab treatment. As described in **chapter 8**, the majority of our AD patients with ophthalmologist-confirmed conjunctivitis during dupilumab treatment was treated with tacrolimus skin ointment for the external eyelids or cyclosporine eye drops.

Successful treatment with both skin and ophthalmological application of calcinurin inhibitors has also been described in literature.[77, 213]

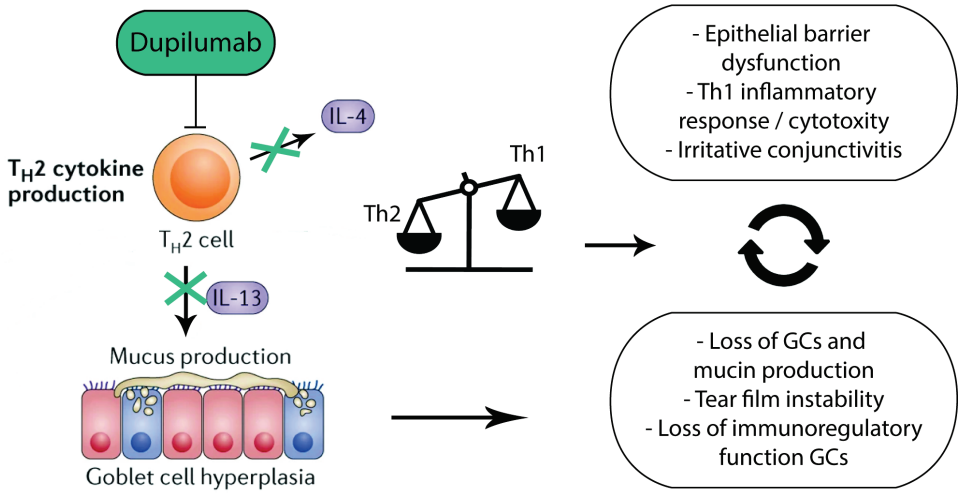


Figure 4. Proposed pathomechanism underlying conjunctivitis occurring in AD patients during dupilumab treatment. Reproduced in part from Gandhi et al.[110] *Nat. Rev. Drug Discov.* **15**, 35–50, 2016.

The fact that conjunctivitis is only reported in AD patients who are treated with dupilumab might be explained by the high incidence of ocular surface disease in AD and its association with GC cell loss.[214] The majority of moderate-to-severe AD patients had previously been treated with oral CsA, which might have simultaneously treated preexisting ocular comorbidities. It may be possible that other oral immunosuppressive drugs, or the more broad-acting JAK-inhibitors, have a positive effect on ocular comorbidities in AD as well, while dupilumab may induce or worsen preexisting ocular diseases by its smaller mechanism of action. More severe AD has been associated with lower GC density, implicating that severe AD patients are at higher risk of developing conjunctivitis during dupilumab treatment. Besides, AD is associated with barrier dysfunction of surface epithelium.[215] Although barrier impairment is also detected in affected mucosal tissues of other type 2 conditions, AD patients more frequently have ocular disease. This may suggest that barrier

abnormalities in AD differentially affect conjunctival tissues compared to other type 2 disease, and could explain in part the higher incidence of conjunctivitis during dupilumab treatment. Pre-treatment and follow-up conjunctival samples from dupilumab-treated AD patients are needed to confirm this.

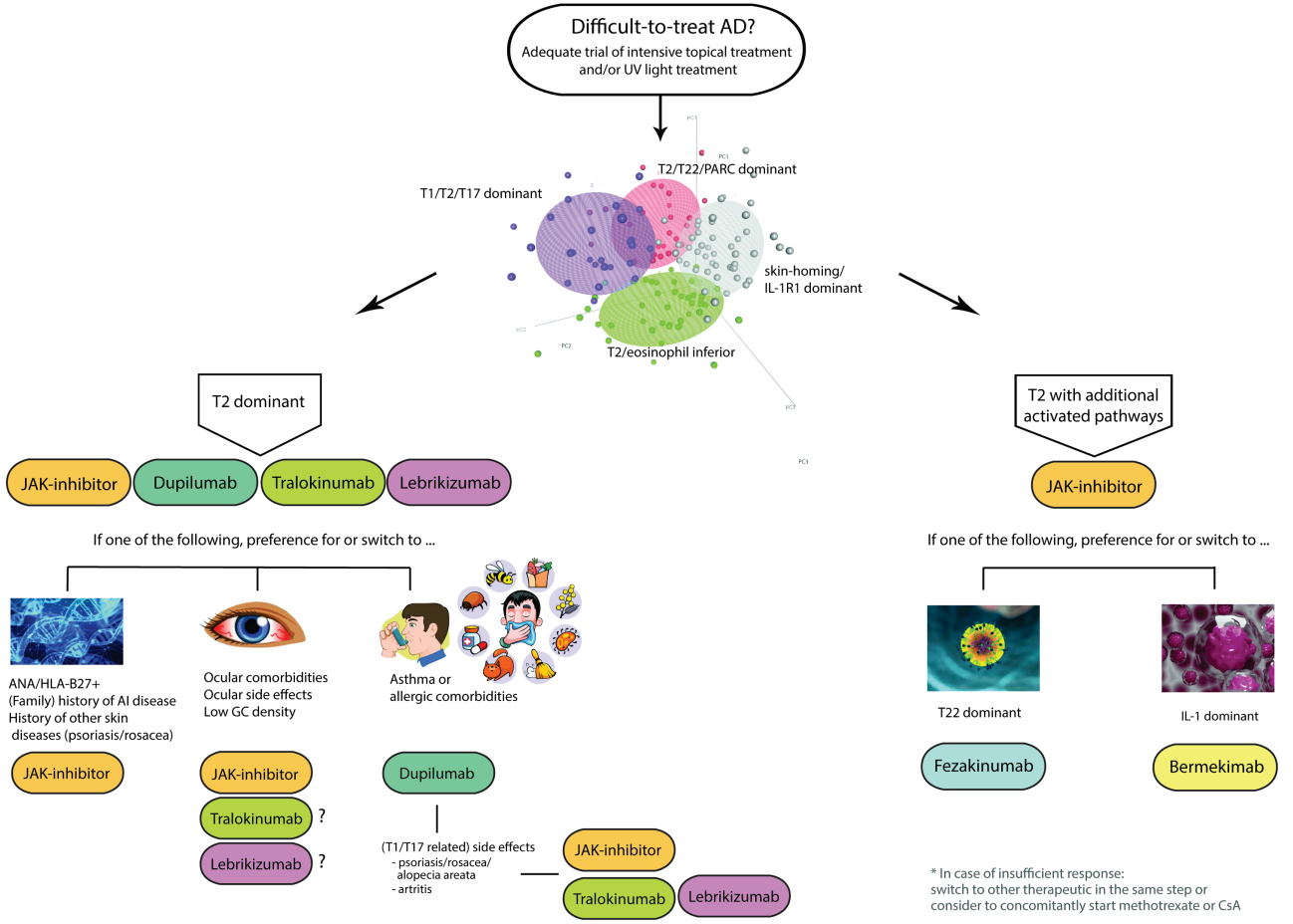
Besides the T1-related cytokines IFNγ and TNFα, increased expression of the immunoregulatory cytokine IL-10 was observed within the subepithelial cell infiltrates compared to non-infiltrated regions and healthy controls. This might be explained as a compensatory mechanism in response to the extensive inflammatory infiltrate, supported by the presence of regulatory T-cells. Increased IL-10 levels were previously reported in tear fluid samples from seasonal allergic conjunctivitis and vernal keratoconjunctivitis samples compared with HCs.[216, 217] High expression of IL-10 has also been suggested to play a role in the development of tissue eosinophilia[218-220], which might explain the presence of the numerous eosinophils in conjunctival tissue as described in **chapter 9 and 10**, as an overflow of eosinophils from the peripheral blood. This, and the fact that eosinophils in our conjunctival biopsies did not show signs of degranulation, would suggest that eosinophilic infiltration in conjunctiva of dupilumab treated patients developing conjunctivitis is more likely to be a bystander effect, instead of playing a pathomechanistic role in this type of conjunctivitis.

The identification of AD patients at risk for developing conjunctivitis during dupilumab treatment by evaluation of the GC density before treatment initiation will be very important in order to prevent conjunctivitis. A recent case series study by Maudinet et al.[221] demonstrated that preventive ophthalmologic examination and, if needed, treatment with artificial tears and antihistamine eye drops before initiation of dupilumab, reduced the incidence of conjunctivitis from 27% to 12%. We are currently running a prospective study in which GC density, ophthalmological characteristics and the effect of (anti-inflammatory) treatment are assessed before and during dupilumab treatment. The first results will be expected in the course of 2021.

PERSONALIZED MEDICINE PERSPECTIVES FOR AD

Due to the increasing knowledge of the inflammatory pathways underlying AD, its treatment is moving toward an era of more targeted and personalized medicine. The high clinical efficacy of dupilumab has affirmed the importance of the T2 pathway in the pathogenesis of AD. However, only approximately one-third of the dupilumab-treated AD patients achieved complete clinical remission[4, 27, 29], confirming the heterogeneous character of the disease. Therefore, other therapeutic targets, including the T17 and T22 axes, as well as broad-acting systemic therapies, including JAK inhibitors are currently on the way.[222] The market authorization of these new therapeutics will highly change the current treatment algorithm for AD patients. Together, based on the adult AD patient clusters, their driving biomarker profiles, (atopic) comorbidities, and risk factors for possible side effects that have been described in this thesis, we propose a possible personalized treatment algorithm for adult difficult-to-treat AD including the current and upcoming systemic treatment options (Figure 5). This algorithm may provide guidance on making the decision which systemic treatment to start in which AD patient. The ultimate goal will be to switch from the current generalized "one-drug-fits-all" to more personalized "patient-endotype-specific" management.

Figure 5. Proposed personalized treatment algorithm for difficult-to-treat adult AD



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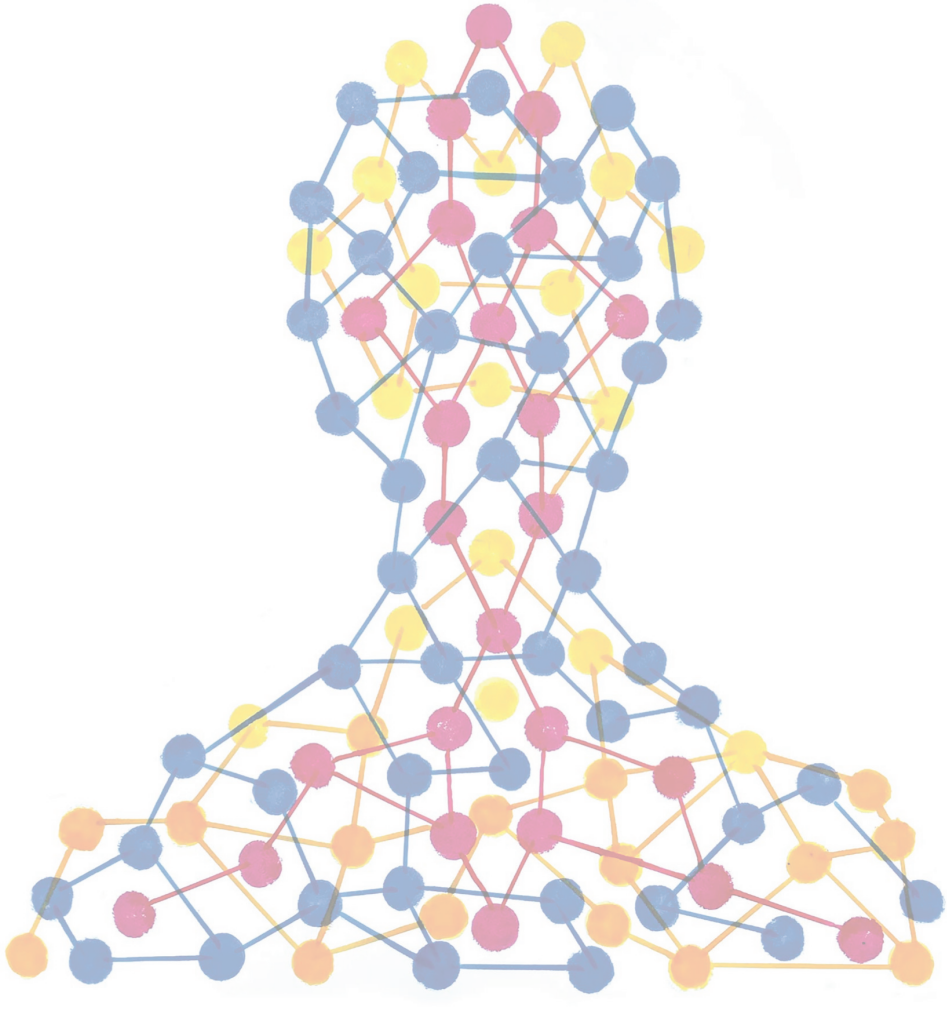


Chapter 13

English summary
Nederlandse samenvatting

English summary

Atopic dermatitis (AD) is one of the most common chronic inflammatory skin diseases worldwide. It usually starts in infancy, but can affect all ages. AD is characterized by exacerbations and remissions of eczematous skin lesions, persistent pruritus and pain, resulting in sleep disturbance and a significantly reduced quality of life. AD is a highly heterogeneous disease on a clinical as well as a biological level. Both genetic and environmental factors contribute to the complex pathogenesis of AD, resulting in immune dysregulation and epithelial barrier disruption.

In the past decade several novel treatments have been developed targeting specific cytokines or cytokine pathways involved in the pathogenesis of AD. These new therapeutics will highly change the treatment of AD. Contrary to the current "one-size-fits-all" approach, there will be a growing need for patient profiling. Given the heterogeneity of AD, it is unlikely that every patient will respond equally to different therapies.

The studies described in this thesis aimed to further identify subtypes of AD patients based on blood biomarker profiles. These profiles can be helpful to find the most optimal treatment for the individual patient. Besides, the studies focused on clarification of early and long term immunological effects of dupilumab, the first biologic treatment for AD, and the pathomechanism underlying different side effects occurring during dupilumab treatment. The main findings were re-explored in the final chapter, to evaluate implications of AD patient profiling and management of moderate-to-severe AD with targeted treatments and their side-effects in clinical practice.

Biomarker-based patient profiling

The majority of AD patients can be controlled with topical anti-inflammatory therapy, but those with insufficient responses to topical steroids, will require treatment with systemic immunosuppressive or immune-modulating drugs. These patients can be defined as 'difficult-to-treat' AD. Early identification of this group might prevent unnecessary treatment delay.

In **chapter 2** we a constructed a predictive signature consisting of eight serum biomarkers, which was able to identify a subgroup of difficult-to-treat AD patients

who are in need of systemic treatment with a sensitivity of 78% and a specificity of 86%. This biomarker signature might in future help clinicians to make the decision whether or not to start systemic therapy in individual AD patients. Validation of our signature in different prospective cohorts is needed to confirm its applicability in daily practice.

After the identification of patients in need of systemic treatment, it is important to define the right medication for the individual patient. The current treatment guidelines for AD do not consider disease subtypes yet, resulting in a high unmet need in individualized treatment options. Since AD is a highly heterogeneous disease, patients have classically been divided into different subgroups based on clinical characteristics, such as age, disease severity, and the presence of other atopic diseases, including asthma, allergic rhinitis and food allergy. However, the stratification of patients into clinical subtypes does not seem to adequately reflect the biologic diversity among AD patients. Given the variety of (upcoming) treatments targeting specific cytokine pathways we believe that it is important to stratify patients based on the most important biological drivers of their AD, rather than subgrouping based on clinical phenotypes.

By measuring a large panel of blood biomarkers in a cohort of 146 severe AD patients, we could identify four AD patient clusters based on their unique serum biomarker profile, as described in **chapter 3**. The biomarker profiles of three out of the four clusters were comparable to previously identified AD patient clusters in a separate cohort of adult AD patients. These results again confirmed that AD is a heterogeneous disease on the biological level. Additionally, we constructed a prediction model which was able to stratify patients into one of the four clusters by using only 10 serum biomarkers. The specific biomarker pathways that were found to characterize the different patient clusters may be helpful for the application of current and upcoming targeted therapies for AD. Since it is expected that patients in different clusters will respond differently to particular treatments, the identification of AD patient clusters may be meaningful for defining the right drug for the individual patient.

It is well-known that the clinical presentation and distribution of the disease is clearly different between pediatric and adult AD patients. Besides, recent studies have proven that profiles of blood and skin biomarkers are substantially different between children and adults with AD. In **chapter 4** we measured the same broad panel of biomarkers as in **chapter 3** in a cohort of 240 pediatric AD patients aged 0 until 17

years. By using the same unsupervised approach, we could again confirm heterogeneity on the biological level and identified four unique patient clusters based on serum biomarker profiles. However, only one out of the four pediatric AD clusters was similar to one of the adult clusters, which supports previous findings that biomarker profiles in pediatric AD differ from adult AD patients.

Pediatric AD cluster membership was not influenced by age or age of onset, but disease severity seemed to be associated with patient clustering. Although most pediatric AD patients will eventually "outgrow" the disease, a significant minority will have persistent AD into adulthood. Besides the potential usefulness in personalized treatment strategies, the identification of biomarker-based pediatric AD patient clusters might also be helpful to define pediatric AD patients at risk of persistent disease.

Effects of dupilumab treatment on molecular parameters and dynamics

Dupilumab is the first targeted therapy that has become available for the treatment of moderate-to-severe AD. It is a fully human monoclonal antibody directed against the interleukin (IL)-4-receptor alpha subunit inhibiting both IL-4 and IL-13 signaling. These cytokines play an important role in the pathogenesis of AD by their contribution to type 2 inflammation and impact on skin barrier dysfunction. In multiple clinical trials and daily practice studies, dupilumab has proven to be effective with limited side effects. Dupilumab significantly improves signs and symptoms of AD and increases the quality of life in difficult-to-treat AD patients.

In **chapter 5** we confirmed the mechanism of action of dupilumab by demonstrating a rapid and stable blockade of the IL-4Rα on B- and T-cells, already within two hours after the first administration. This effect was accompanied by a strong early immunological effect, specifically in skin-homing T-cells of AD patients treated with dupilumab. This immunological effect was characterized by a significant decrease of proliferating and Th2/Th22 cytokine-producing skin-homing T-cells already after four weeks of treatment. Since several recent case-reports have reported Th1/Th17-mediated adverse effects, including psoriasis, alopecia areata, and rosacea developing newly in AD patients during dupilumab treatment, we investigated the long-term effect of dupilumab treatment on (skin-homing) T-cell cytokine production. Overall, dupilumab does not seem to have strong long-term skewing effects on the peripheral T-cell cytokine production. However, in some individuals

the T-cell cytokine profile may shift towards a more Th1/Th17 phenotype, especially within the skin-homing T-cell population, which might predispose patients to Th1/Th17-mediated diseases.

Given the multiple currently available and upcoming treatments in AD, it is important to have an objective and consistent outcome measurement to successfully compare these different therapeutics. Serum TARC is currently the best performing objective biomarker for disease severity in AD. Nevertheless, the correlation of single biomarkers with disease severity is not strong enough to replace clinical outcome measures. In previous studies including AD patients treated with topical corticosteroids and cyclosporine A we showed that a biomarker signature including TARC, sIL-2R and IL-22 was a significantly better predictor of disease severity than a single biomarker. Since this biomarker signature was developed to predict the Eczema Area and Severity Index (EASI) scores, it was named "the predicted-EASI" (p-EASI). In **chapter 6** we demonstrated that the p-EASI adequately predicts disease severity in AD patients treated with dupilumab as well. The use of the p-EASI will help to improve comparability of study outcomes in future clinical trials on new therapies for AD, but may also be helpful as an objective measure for treatment effects in daily practice.

Previous studies have shown that treatment with dupilumab is associated with, mostly transient, increased peripheral blood eosinophil numbers. Based on previous findings it is hypothesized that this eosinophilia observed during dupilumab treatment might be a result of reduced homing of eosinophils to the tissues, resulting in accumulation in the peripheral blood. In **chapter 7** we showed that dupilumab treatment significantly decreased the local presence of eosinophils and expression of the chemokine eotaxin in lesional AD skin. This was accompanied by an increased expression of surface markers related to trafficking (CD193 and CD44) on eosinophils in the peripheral blood after 4 and 16 weeks of treatment. These results support the concept that treatment with dupilumab decreases eosinophil trafficking to the skin.

Side effects of dupilumab treatment and underlying mechanism

Dupilumab has shown a favorable safety profile, with mostly mild side effects being observed. However, higher rates of conjunctivitis have been reported in dupilumab treated patients (5% to 28%) compared to patients treated with placebo (1% to 11%) in clinical trials. Recent daily practice studies have shown even higher rates up to

34%. In our prospective daily practice study presented in **chapter 8**, conjunctivitis was reported in 66 (39.5%) out of 167 moderate-to-severe AD patients treated with dupilumab during 12 months of follow-up. Of these patients, 33 were referred to an ophthalmologist, where conjunctivitis was confirmed. During long-term ophthalmological follow-up, the majority of these patients still suffered from mild-to-moderate conjunctivitis despite ocular treatment. Dose adjustment or discontinuation of dupilumab due to ocular pathology was needed in 10/33 and 3/33 of the patients, respectively. This study showed that dupilumab-associated conjunctivitis can have a chronic character that is sometimes difficult to treat. Dose adjustment of dupilumab treatment might be effective, and ophthalmological follow-up is important.

The exact pathomechanism underlying conjunctivitis during dupilumab treatment in AD patients has not been clarified. Remarkably, increased rates of conjunctivitis were not reported in dupilumab trials in other type-2 inflammatory diseases, like asthma and chronic rhinosinusitis with nasal polyposis, suggesting that AD patients may have a predisposition to develop dupilumab-associated conjunctivitis. In **chapter 9** we found a remarkable scarcity of conjunctival goblet cells together with an inflammatory T-cell and eosinophilic infiltrate in conjunctival biopsies from six AD patients who developed conjunctivitis during dupilumab treatment. In **chapter 10** these infiltrating cells in conjunctival tissue were further characterized by using a new innovative technique (imaging mass cytometry). The study demonstrated that the cellular infiltrate observed in conjunctival tissue of AD patients developing conjunctivitis during dupilumab treatment comprised a diverse panel of infiltrating cells, including highly activated CD4+ and CD8+ T-cells, but also dendritic cells, monocytes and macrophages. Besides, significantly increased signals of T1 and T17-related cytokines and the cytotoxic enzyme granzyme B were observed within subepithelial cell infiltrates in all patient samples compared to healthy controls.

It has been shown that IL-13 normally stimulates goblet cell proliferation and mucus secretion. Based on the results of the two mechanistic studies presented in **chapter 9 and 10**, we hypothesized that dupilumab-associated IL-4/IL-13 signaling inhibition in combination with increased local T1-related cytokine production can underlie the loss of goblet cells and their essential immunomodulatory role in the conjunctiva, hence leading to dry eyes, a highly activated multicellular infiltrate, and tissue damage. Non-invasive measurements of conjunctival GC numbers and tear cytokines

might identify AD patients at risk of developing conjunctivitis who may benefit from early anti-inflammatory ocular treatment.

Another more recently described side effect of dupilumab treatment in AD patients in daily practice is a paradoxical head and neck erythema that is clinically and histologically different from the background AD of patients. The clinical and histological characteristics of this dupilumab facial redness (DFR) that are currently described in literature are rather heterogeneous. Therefore a clear pathogenesis has not yet been established. **Chapter 11** described two cases of AD patients who presented with DFR after 11 weeks and six months of treatment. In both cases DFR was considered to be caused by hypersensitivity to *Malassezia* species, supported by elevated serum levels of *Malassezia*-specific IgE and a positive response to oral itraconazole. Due to the heterogeneous clinical presentation of DFR, other underlying mechanism including rosacea and allergic contact dermatitis should be considered.

Future perspectives

Chapter 12 discussed this thesis' most important findings in the context of other currently available literature on AD and upcoming targeted therapies, which led to recommendations for future research.

As mentioned before, the treatment of AD will move towards an era of more targeted and personalized medicine. The findings in this thesis confirmed that AD is a highly heterogeneous disease. By describing the driving biomarker pathways of the different patient clusters we showed that, besides the T2 pathway, other cytokine pathways may also play an important role in (subgroups) of AD patients. Therefore, other therapeutics targeting for example the T17 and T22 axes, as well as broad-acting systemic therapies, including JAK inhibitors are currently on the way. The driving biomarker pathways of the different patient clusters, together with (atopic) comorbidities, and risk factors for possible side effects that have been described in this thesis will contribute to personalized treatment strategies. We proposed a possible treatment algorithm that may support physicians in making the decision which systemic treatment to start in which AD patient.

Nederlandse samenvatting

Constitutioneel eczeem (CE) is een van de meest voorkomende chronische inflammatoire huidaandoeningen wereldwijd. Het begint meestal op de kinderleeftijd, maar kan op elke leeftijd voorkomen. CE wordt gekenmerkt door periodes van opvlamming en remissies. De ziekte uit zich door middel van een rode schilferende huiduitslag met aanhoudende jeuk en pijn, wat vaak resulteert in slaapstoornissen en een sterke afname van de kwaliteit van leven. CE is een heterogene ziekte op basis van klinische en biologische kenmerken. Zowel genetische als omgevingsfactoren dragen bij aan het complexe ontstaansmechanisme van CE, welke uiteindelijk leiden tot disregulatie van het immuun systeem en verstoring van de huid barrière.

In het afgelopen decennium zijn er verschillende nieuwe therapieën ontwikkeld die aangrijpen op specifieke cytokines of cytokine signaalwegen waarvan bekend is dat ze betrokken zijn bij het ontstaansmechanisme van CE. Deze nieuwe middelen zullen de huidige behandeling van CE drastisch gaan veranderen. In tegenstelling tot de huidige "one-size-fits-all" aanpak, zal er grote behoefte komen aan meer patiëntgerichte behandelstrategieën. Gezien het heterogene karakter van CE, is het namelijk onwaarschijnlijk dat elke patiënt hetzelfde zal reageren op een bepaalde behandeling.

De onderzoeken die in dit proefschrift zijn beschreven hebben als doel gehad om verschillende subtypes van CE patiënten te identificeren op basis van biomarker profielen in het bloed. Deze profielen kunnen ons in de toekomst helpen bij het vinden van de meest optimale behandeling voor een individuele patiënt. Daarnaast hebben wij ons in dit proefschrift gefocust op dupilumab, de eerste geregistreerde biological voor de behandeling van CE. De onderzoeken beschreven in dit proefschrift hebben zich gericht op het korte- en lange termijn immunologische effect van dupilumab en de ontstaansmechanismen van verschillende bijwerkingen optredend tijdens dupilumab behandeling. De belangrijkste bevindingen werden opnieuw besproken in het laatste hoofdstuk, waarbij de klinische implicaties van patiënt profilering binnen CE en de behandeling van CE met verschillende gerichte therapieën in de dagelijkse praktijk en hun mogelijke bijwerkingen werden geëvalueerd.

Patiënt profilering op basis van biomarkers

Voor de meerderheid van de CE patiënten kan de ziekte voldoende onder controle worden gehouden met behulp van lokale anti-inflammatoire therapie (corticosteroïden). Maar de patiënten die onvoldoende reageren op topicale corticosteroïden komen in aanmerking voor behandeling met systemische immunosuppressieve of immuun-modulerende behandelingen. Deze patiënten kunnen worden gedefinieerd als 'moeilijk behandelbaar' CE. Om onnodige vertraging in het starten van de juiste behandeling te voorkomen is het belangrijk om deze groep patiënten tijdig te identificeren.

In **hoofdstuk 2** stelden wij een voorspellende formule samen bestaande uit acht biomarkers gemeten in het bloed, waarmee we in staat waren om de subgroep van moeilijk behandelbare CE patiënten te identificeren. Deze formule had een sensitiviteit van 78% en een specificiteit van 86%, en zou in de toekomst kunnen bijdragen aan de keuze om een CE patiënt wel of niet met systemische therapie te gaan behandelen. Echter, voor de toepasbaarheid in de dagelijkse praktijk is validatie van onze voorspellende formule in verschillende prospectieve patiënten cohorten nodig.

Nadat de CE patiënten die in aanmerking komen voor systemische therapie zijn geïdentificeerd, is het belangrijk om ook de meest geschikte behandeling voor individuele patiënt te vinden. De huidige behandel richtlijnen voor CE houden echter nog geen rekening met verschillende subtypes binnen de ziekte, wat resulteert in een "one-size-fits-all" aanpak.

Omdat CE een uiterst heterogene ziekte is, werden patiënten klassiek verdeeld in subgroepen op basis van hun klinische kenmerken, zoals leeftijd, ziekte ernst en het voorkomen van andere atopische ziekten, zoals astma, allergische rhinitis en voedselallergieën. De indeling van CE patiënten in klinische subgroepen blijkt echter geen adequate weergave van de biologische diversiteit binnen patiënten. Gelet op de verschillende nieuwe gerichte therapieën die aangrijpen op specifieke cytokine signaalwegen, denken wij dat het belangrijk is om patiënten te groeperen op basis van de belangrijkste biologische onderliggende factoren van hun CE, in plaats van te focussen op de klinische fenotypes.

In **hoofstuk 3** hebben wij, door middel van het meten van een grote selectie aan biomarkers in het bloed van 146 patiënten met ernstig CE, vier patiënten clusters kunnen ontdekken op basis van hun unieke biomarker profiel. Het biomarker profiel

van drie van de vier clusters kwam overeen met de clusters die in een eerdere studie zijn gevonden in een onafhankelijk cohort van volwassen CE patiënten. Onze resultaten bevestigen opnieuw dat CE een heterogene ziekte is op biologisch niveau. Daarnaast bouwden we in **hoofdstuk 3** een voorspellend model op basis van 10 serum biomarkers, waarmee we patiënten in één van de vier clusters konden indelen. Het is te verwachten dat patiënten in verschillende clusters anders zullen reageren op de verschillende opkomende gerichte behandelingen voor CE. Het identificeren van de patiënten clusters op basis van hun specifieke biomarker profiel zou daarom in de toekomst kunnen bijdragen aan het vinden van de juiste behandeling voor de individuele patiënt.

Het is bekend dat er tussen kinderen en volwassenen met CE een duidelijk verschil is in klinische presentatie en verdeling van het eczeem. Daarnaast hebben recente studies aangetoond dat ook bloed en huid biomarker profielen substantieel verschillen tussen kinderen en volwassenen. In de studie beschreven in **hoofdstuk 4** hebben we dezelfde uitgebreide selectie van biomarkers als in **hoofdstuk 3** gemeten in het bloed van 240 kinderen met CE met een leeftijd tussen 0 en 17 jaar oud. Door gebruik te maken van dezelfde analyse methode, konden we bevestigen dat CE ook bij kinderen een biologisch heterogene ziekte is door het identificeren van vier unieke patiënten clusters gebaseerd op biomarker profielen. Slechts één van de vier kinder CE clusters kwam overeen met één van de vier clusters die werden gevonden in volwassenen met CE. Dit bevestigde dat biomarker profielen verschillen tussen kinderen en volwassenen met CE.

De clusters die werden gevonden in kinderen met CE werden niet beïnvloed door leeftijd of de leeftijd waarop het CE is ontstaan. Daarentegen bleek ziekte ernst wel geassocieerd te zijn met het cluster waarin patiënten werden ingedeeld. Ondanks dat de meeste kinderen met CE meestal "over de ziekte heen groeien", is er een klein deel van de CE patiënten waarbij het eczeem ook op de volwassen leeftijd zal blijven bestaan. Naast dat het identificeren van biomarker gebaseerde CE patiënten clusters in kinderen nuttig kan zijn in de toepassing van gepersonaliseerde behandel strategieën, kan het daarnaast ook bijdragen aan het opsporen van kinderen die een grote kans hebben op blijvend eczeem.

Effecten van dupilumab behandeling op moleculaire parameters en dynamiek
Dupilumab is de eerste geregistreerde biological voor de behandeling van matig-tot-ernstig CE. Het is een volledig humaan monoclonaal antilichaam gericht tegen de gemeenschappelijke interleukine (IL)-4-receptor-alpha subketen van de IL-4 en IL-13 receptoren en remt daarmee de signaaltransductie van beide cytokines. IL-4 en IL-13 hebben een belangrijke onderhoudende rol in de pathogenese van CE, waarin ze betrokken zijn bij type 2 inflammatie en verstoring van de huid barrière. Meerdere klinische trials en dagelijkse praktijk studies demonstreerden dat dupilumab een effectieve behandeling is voor CE met een gunstig bijwerkingenprofiel. Behandeling met dupilumab leidde in een groot deel van de patiënten tot een snelle afname van het eczeem, vermindering van de jeukklachten en een sterke verbetering van de kwaliteit van leven.

In **hoofdstuk 5** demonstreerden wij een snelle en stabiele blokkade van de IL-4Rα op B- en T-cellen al binnen twee uur na de eerste dupilumab injectie, waarmee we het werkingsmechanisme van dupilumab konden bevestigen. Dit effect ging gepaard met een sterk en vroeg immunologisch effect, met name op de huid homing T-cellen van CE patiënten die werden behandeld met dupilumab. Dit immunologische effect werd gekenmerkt door een sterke afname van het aantal delende en Th2/Th22 cytokine producerende huid-homing T-cellen al in de eerste vier weken van de behandeling. In recente case-reports werden verschillende huid-gerelateerde bijwerkingen tijdens dupilumab beschreven, zoals psoriasis, rosacea en alopecia areata, die in eerdere klinische trials niet duidelijk naar voren zijn gekomen. Omdat bekend is dat dit Th1/Th17-gemedieerde huidaandoeningen zijn, hebben wij in **hoofdstuk 5** ook de lange termijn effecten van dupilumab op de cytokine productie van (huid homing) T-cellen bestudeerd. Over het algemeen leek dupilumab geen verschuivend effect op de cytokine productie van T-cellen te hebben op de lange termijn. In een aantal individuele patiënten werd na 40 tot 52 weken dupilumab behandeling echter wel een toename van de productie van Th1/Th17-gerelateerde cytokines gezien. Dit effect was opnieuw met name te zien in de huid homing T-cel populatie.

Gegeven de verschillende behandelingen die op dit moment beschikbaar zijn voor CE en nog beschikbaar zullen komen, is het wenselijk een objectieve en consistente uitkomst maat te hebben, zodat het effect van de verschillende behandelingen kan worden onderzocht en onderling kan worden vergeleken. De serum TARC concentratie is op dit moment de beste biomarker voor het objectief meten van

ziekte ernst voor CE. De correlatie met ziekte ernst is echter nog niet sterk genoeg om de klinische scoresystemen die worden ingevuld door behandelend artsen of verpleegkundigen te kunnen vervangen. In eerdere studies waarin werd gekeken naar CE patiënten die werden behandeld met lokale corticosteroïden en ciclosporine, hebben we laten zien dat een combinatie van de serum biomarkers TARC, sIL-2R en IL-22 een significant betere voorspeller van ziekte ernst is dan de individuele biomarkers. Omdat deze biomarker combinatie is ontwikkeld om de klinische score 'Eczema Area and Severity Index' (EASI) te voorspellen, noemden we deze uitkomstmaat de 'predicted-EASI' (p-EASI). In **hoofdstuk 6** toonden wij aan dat de p-EASI ook adequaat kan worden toegepast in het voorspellen van ziekte ernst in CE patiënten die worden behandeld met dupilumab. Het gebruik van de p-EASI is van essentieel belang voor de vergelijkbaarheid van toekomstige klinische studies naar nieuwe gerichte therapieën voor CE, en zou mogelijk ook nuttig kunnen zijn als objectieve uitkomstmaat voor ziekte ernst in de dagelijkse praktijk.

Eerdere studies hebben aangetoond dat behandeling met dupilumab is geassocieerd met het optreden van een, meestal voorbijgaande, toename van het aantal eosinofielen en het perifere bloed. Gebaseerd op eerdere bevindingen werd verondersteld dat deze eosinofilie tijdens dupilumab behandeling het resultaat zou kunnen zijn van een verminderd aantal eosinofielen dat zich verplaatst van het bloed naar de weefsels. De afname van verplaatsing naar de weefsels zou vervolgens kunnen leiden tot ophoping van de eosinofielen in het bloed. In **hoofdstuk 7** lieten wij zien dat dupilumab behandeling leidt tot een significante afname van eosinofielen in de lesionale huid. Daarnaast werd er ook een afname in de expressie van de chemokine eotaxine in lesionale CE huid gevonden. Dit ging gepaard met een toename van de expressie van verplaatsing gerelateerde markers (CD193 en CD44) op het celoppervlak van eosinofielen in het perifere bloed van CE patiënten na 4 en 16 weken dupilumab behandeling. Deze resultaten ondersteunen de theorie dat dupilumab behandeling leidt tot verminderde verplaatsing van eosinofielen vanuit het perifere bloed naar de huid.

Bijwerkingen van dupilumab behandeling en hun onderliggende mechanisme

Dupilumab toont een gunstig veiligheidsprofiel, waarbij voornamelijk milde bijwerkingen worden geobserveerd. Het optreden van conjunctivitis werd in klinische studies echter wel vaker gemeld bij dupilumab-behandelde CE patiënten (5% tot 28%) in vergelijking met placebo (1% tot 11%). Recente dagelijkse praktijk studies

rapporteerden zelfs nog hogere percentages van conjunctivitis tot wel 34%. In onze prospectieve dagelijkse praktijk studie beschreven in **hoofdstuk 8** ontwikkelden 66 (39.5%) van de 167 bestudeerde CE patiënten conjunctivitis tijdens dupilumab behandeling gedurende een 12 maanden durende periode. Hiervan werden 33 patiënten doorverwezen naar de oogarts. Gedurende langdurige oogheelkundige follow up bleef de meerderheid van deze patiënten, ondanks anti-inflammatoire behandeling in de ogen, last houden van mild tot matig ernstige conjunctivitis. In 10 van de 33 patiënten was het nodig om het dupilumab interval te verlengen en in 3 van de 33 patiënten werd dupilumab gestaakt vanwege oogklachten. Concluderend kan worden gesteld dat de dupilumab-geassocieerde conjunctivitis een chronisch karakter kan hebben die soms moeilijk behandelbaar is. Intervalverlenging van de dosis kan effectief zijn, en oogheelkundige controle met follow up is belangrijk.

De onderliggende mechanismen van het ontstaan van conjunctivitis tijdens dupilumab behandeling in CE patiënten is tot op heden nog niet opgehelderd. Opvallend is dat conjunctivitis vooralsnog niet gerapporteerd is in klinische studies naar dupilumab voor andere indicaties, zoals allergisch astma en chronische sinusitis met neuspoliepen. Mogelijk spelen CE-specifieke factoren een belangrijke rol bij het ontstaan van conjunctivitis tijdens dupilumab behandeling. Om meer inzicht te krijgen in het onderliggende mechanisme bestudeerden wij in **hoofdstuk 9** conjunctiva biopten die werden genomen van zes CE patiënten die conjunctivitis ontwikkelden tijdens dupilumab behandeling. We vonden een opmerkelijk laag aantal slijm producerende cellen (goblet cellen) en de aanwezigheid van een ontstekingsinfiltraat bestaande uit voornamelijk T-cellen en eosinofielen. In **hoofdstuk 10** werd dit ontstekingsinfiltraat verder gekarakteriseerd doormiddel van een nieuwe innovatieve techniek (imaging mass cytometry). Het ontstekingsinfiltraat in het conjunctiva weefsel van CE patiënten met dupilumab-geassocieerde conjunctivitis bleek te bestaan uit verschillende soorten ontstekingscellen, waaronder sterk geactiveerde CD4+ en CD8+ T-cellen, maar ook dendritische cellen, monocyten en macrofagen. Daarnaast werden er duidelijk sterke signalen van T1- en T17-gerelateerde cytokines en het cytotoxische enzym granzyme B gevonden in vergelijking met gezonde controle samples.

Het is bekend dat IL-13 normaal gesproken de proliferatie van goblet cellen en de aanmaak van slijm door deze cellen stimuleert. Op basis van de resultaten beschreven in **hoofdstuk 9 en 10** stelden wij de hypothese dat remming van IL-4 en IL-13 door dupilumab in combinatie met de daardoor verhoogde T1-gerelateerde

cytokine productie kan leiden tot een afname van het aantal goblet cellen en hun belangrijke immuun-modulerende functie in de conjunctiva. Dit zou vervolgens kunnen leiden tot droge ogen, conjunctivale inflammatie en uiteindelijk weefselschade. Het non-invasief meten van het aantal conjunctivale goblet cellen en cytokines in traanvocht zou mogelijk kunnen bijdragen aan het identificeren van patiënten die risico lopen op het ontwikkelen van conjunctivitis. Deze patiënten hebben mogelijk profijt van vroegtijdige anti-inflammatoire oogheelkundige behandeling.

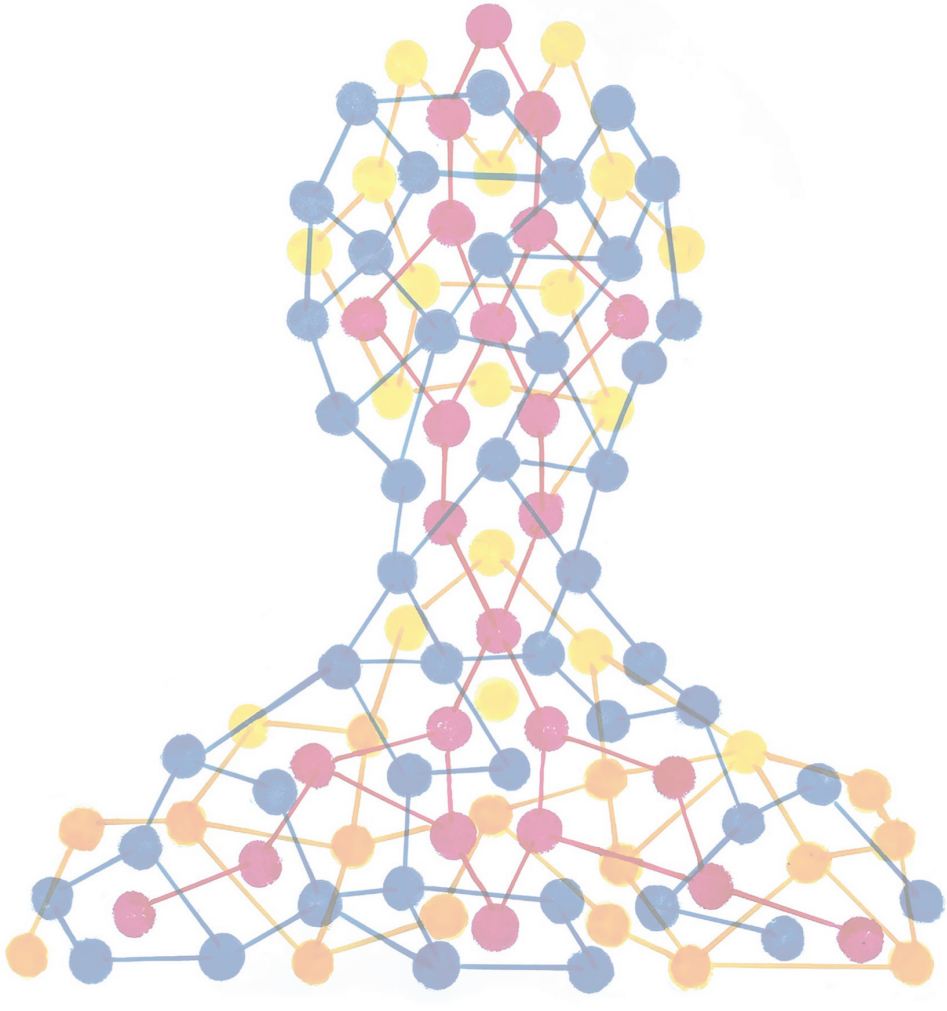
Een andere bijwerking die recent in meerdere dagelijkse praktijk studies is beschreven, is het optreden van erythemateuze huidafwijkingen in het hoofd-hals gebied, welke klinisch en histopathologisch anders lijken te zijn dan het CE waarmee patiënten bekend zijn. De klinische en histopathologische kenmerken van deze 'dupilumab gelaatsroodheid' die worden beschreven in de huidige literatuur lopen erg uiteen. Het is daarom tot op heden nog niet gelukt om het onderliggende ontstaansmechanisme op te helderen. In **hoofdstuk 11** beschreven wij een tweetal casus van CE patiënten die zich presenteerden met nieuwe ontstane roodheid in het gelaat na 11 weken en 6 maanden behandeling met dupilumab. Vanwege de verhoogde waarde van het specifiek IgE voor *Malassezia* in het bloed en de positieve resultaten na behandeling met systemische antimycotica, is in beide casus de gedachte ontstaan dat overgevoeligheid voor *Malassezia* een belangrijke rol speelt in het ontstaan van deze bijwerking. Omdat de klinische kenmerken van dupilumab-gerelateerde roodheid in het gelaat erg verschillend kunnen zijn, is het belangrijk om ook andere onderliggende mechanismen te overwegen, zoals rosacea en allergische contact dermatitis.

Toekomstperspectieven

In hoofdstuk 12 zijn de belangrijkste bevindingen van dit proefschrift bediscussieerd en in de context van de bestaande literatuur geplaatst, waaruit aanbevelingen voor toekomstig onderzoek zijn voortgekomen.

Zoals eerder beschreven zal de behandeling van CE zich in de komende periode gaan verplaatsen richting meer gerichtere en gepersonaliseerde gezondheidszorg. De bevindingen in dit proefschrift hebben bevestigd dat CE een uiterst heterogene ziekte is. Door het beschrijven van de biomarker profielen die de verschillende patiënten clusters onderscheiden, hebben we laten zien dat er voor subgroepen

patiënten naast de T2 cytokines ook andere cytokine routes een belangrijke rol spelen in het ontstaan van CE. Derhalve zijn er verschillende nieuwe therapieën onderweg die aangrijpen op onder andere de T17 en T22 routes, evenals breder werkende systemische middelen, zoals JAK-remmers. De verschillende onderscheidende biomarker profielen zullen in de toekomst, samen met eventuele bekende (atopische) comorbiditeiten en de risico factoren voor mogelijke bijwerkingen die in dit proefschrift werden beschreven, bijdragen aan gepersonaliseerde behandel strategieën. Wij stellen hier een mogelijk behandel algoritme voor welke de behandelaar kan ondersteunen in het maken van een beslissing over welke behandeling het beste gestart kan worden bij welke CE patiënt.



Chapter 14

List of abbreviations

Contributing authors

Acknowledgements

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List of abbreviations

AD	Atopic dermatitis
ACD	Allergic contact dermatitis
AKC	Atopic keratoconjunctivitis
AUC	Area under the curve
CCL	CC chemokine ligand
CCR	C-C chemokine receptors
CLA	Cutaneous lymphocyte antigen
CsA	Cyclosporine A
CTACK	Cutaneous T-cell attracting chemokine
DFR	Dupilumab facial redness
EASI	Eczema Area Severity Index
FDR	False Discovery Rate
GC	Goblet cell
GCP-2	Gamma-tubulin complex protein 2
HC	Healthy control
HE	Haematoxylin and eosin
HND	Head-neck dermatitis
IL	Interleukin
IL-4Rα	Interleukin-4 receptor alpha
IgE	Immunoglobulin E
IHC	Immunohistochemistry
IQR	Interquartile range
JAK	Janus kinase
LAIR-1	Leukocyte associated immunoglobulin like receptor-1
MCP	Monocyte chemoattractant protein
MFI	Mean fluorescence intensity
NPV	Negative predictive value
PARC	Pulmonary and activation-regulated chemokine
PBMC	Peripheral blood mononuclear cells
PCA	Principal components analysis
PPV	Positive predictive value
pSTAT6	Phosphorylated signal transducer and activator of transcription 6
ROI	Region of interest
SASSAD	Six Area, Six Sign Atopic Dermatitis
SD	Standard deviation

sIL-2R	soluble interleukin-2-receptor
SOST	Sclerostin
sPD-1	soluble programmed death-1
SPSS	Statistical Package for the Social Science
TARC	Thymus and activation regulated chemokine
TCS	Topical corticosteroids
Th	T helper
TMA	Tissue Micro Array
TSLP	Thymic stromal lymphopoietin
WSS	Within-cluster sum of square

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List of publications

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Curriculum vitae

Daphne Bakker werd geboren op 1 april 1992 te Alphen aan den Rijn. Na het *cum laude* behalen van haar atheneum diploma aan het Groene Hart Lyceum in Alphen aan den Rijn in 2010, begon zij in hetzelfde jaar aan haar studie geneeskunde aan de Universiteit Utrecht. Haar interesse voor de dermatologie ontwikkelde zij gedurende haar coschappen en resulteerde uiteindelijk in een master onderzoek naar constitutioneel eczeem onder begeleiding van dr. Floor Garritsen en dr. Marjolein de Bruin-Weller op de afdeling Dermatologie en Allergologie. Na het behalen van haar artsenexamen in 2016, begon zij aansluitend als trial-arts op de afdeling Dermatologie en Allergologie van het Universitair Medisch Centrum (UMC) te Utrecht. Na een jaar startte zij met haar promotie traject op dezelfde afdeling. Hierbij mocht zij de lijn van het translationele onderzoek naar constitutioneel eczeem voortzetten onder begeleiding van promotoren dr. Marjolein de Bruin-Weller en prof. dr. Femke van Wijk, en copromotoren dr. Judith Thijs en dr. Julia Drylewicz. Naast haar promotieonderzoek heeft zij meerdere dagdelen per week op het multidisciplinair eczeemspreekuur in het UMC Utrecht gewerkt. Per 1 januari 2021 is zij gestart met de opleiding tot dermatoloog in het UMC Utrecht. Daphne woont samen met Tim Konings in hun huis in Utrecht.