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ORIGINAL ARTICLE

Dupilumab-associated ocular surface disease is characterized by a shift from Th2/Th17 toward Th1/Th17 inflammation

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Abstract

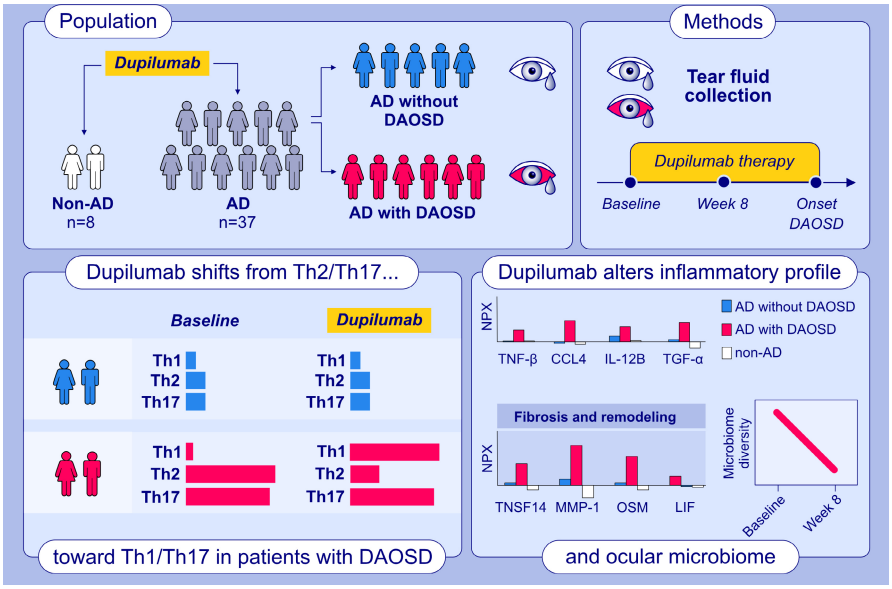
Background: Dupilumab is used for the treatment of atopic dermatitis (AD). Approximately one third of AD patients develop a dupilumab-associated ocular surface disease (DAOSD), of which the pathomechanism is poorly understood. This study aimed at investigating inflammatory markers in tear fluids of patients on dupilumab therapy.

Methods: Tear fluids were collected from AD patients with DAOSD (ADwDAOSD), AD patients without DAOSD (ADw/oDAOSD), and non-AD patients before and during dupilumab therapy, and analyzed using a specialized proteomic approach quantifying inflammatory markers. The ocular surface microbiome was determined by next generation sequencing technology.

Results: Upon dupilumab therapy, an upregulation of 31 inflammatory markers was observed in DAOSD tear fluids compared to baseline in AD patients. While IL-12B was upregulated in both ADwDAOSD and ADw/oDAOSD groups, the pattern of inflammatory markers significantly differed between groups and over time. In the ADwDAOSD group, a shift from a mixed Th2/Th17 pattern at baseline toward a Th1/Th17 profile under dupilumab was observed. Furthermore, an upregulation of remodeling and fibrosis markers was seen in DAOSD. Semantic map and hierarchical cluster analyses of baseline marker expression revealed four clusters distinguishing between AD and non-AD as well as ADwDAOSD and ADw/oDAOSD patient groups. In a pilot study, dupilumab therapy was associated with a decrease in richness of the ocular surface microbiome.

Conclusions: DAOSD is characterized by a Th1/Th17 cytokine profile and an upregulation of markers known to promote remodeling and fibrosis. The expression pattern of inflammatory markers in tear fluids at baseline might serve as a prognostic factor for DAOSD.

KEYWORDS
atopic dermatitis, dry eye disease, dupilumab, ocular surface disease, remodeling, type 1 inflammation



GRAPHICAL ABSTRACT

Dupilumab therapy affects the expression of inflammatory markers in tear fluids and the microbiome of ocular surfaces of AD patients. A high expression of Th2/Th17 inflammatory markers in tear fluids at baseline is characteristic for AD patients later developing DAOSD. In patients developing DAOSD, a shift from a Th2/Th17 toward a Th1/Th17 inflammation pattern at ocular surfaces is observed. AD, atopic dermatitis; CCL, C-C motif chemokine ligand; DAOSD, dupilumab-associated ocular surface disease; IFN, interferon; IL, interleukin; LIF, leukemia inhibitory factor; MMP-1, matrix metalloprotease 1; NPX, normalized protein expression; OSM, oncostatin; TGF, transforming growth factor; Th, T helper; TNF, tumor necrosis factor; TNSF14, TNF super family member 14

1 | INTRODUCTION

Atopic dermatitis (AD) is a common chronic inflammatory skin disease. Both skin barrier dysfunction and T helper 2-predominant inflammation are important pathogenic factors that are determined by an interplay of genetic disposition, epigenetic changes, dysbiosis in the skin and gut microbiome, and external factors.[1,2] AD is frequently associated with ocular surface diseases (OSD) such as conjunctivitis, keratitis, keratoconus, keratoconjunctivitis, dry eye syndrome, and blepharitis with 31.7% in AD versus 13.3% in non-AD individuals.[3] Allergic conjunctivitis is the most common form of OSD in AD patients, reaching a prevalence of 14% in young children with AD.[4] According to a Danish study, 12% and 18% of adults with mild and severe AD, respectively, use anti-inflammatory ocular agents.[5] The lifetime prevalence of OSD in adult patients with AD reached 66.6% for conjunctivitis, 11.0% for blepharitis, 9.7% for keratitis, and 1.1% for keratoconus.[6]

Dupilumab, the first biologic approved for AD, blocks the binding of IL-4 and IL-13 to the IL-4 receptor alpha chain, resulting in an excellent improvement of skin signs and symptoms.[7] The overall tolerability of dupilumab is high, but considerable numbers of patients develop a treatment-associated conjunctivitis (dupilumab-associated OSD, DAOSD). While the frequencies of DAOSD reported from clinical trials were 9% to 22%,[8] higher frequencies up to 38% have been observed in real-life studies.[9,10] However, the pathomechanism of DAOSD is poorly understood.

So far, a scarcity of goblet cells, relative deficiency of mucin production, a mixed inflammatory infiltrate consisting of $CD4^+$ and $CD8^+$ T cells, eosinophils, dendritic cells, monocytes and macrophages, as well as an increased expression of interferon (IFN)-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-10, and IL-17A in the conjunctiva as well as IL-33 in tear fluids of patients with DAOSD have been reported.[11–14] Moreover, a possible role of Demodex mites and IL-17-mediated inflammation has been debated.[15,16]

In order to gain deeper insights into the underlining immune dysregulation responsible for DAOSD, we investigated inflammatory markers in tear fluids of patients on therapy with dupilumab by applying proteomic analyses. Furthermore, we were interested whether dupilumab treatment impacts the microbiome of ocular surfaces in AD patients.

2 | METHODS

2.1 | Patients

In this retrospective study, data of 45 patients (22 females; mean age 44.8 ± 13.8 years) treated with dupilumab (AD, $n = 37$; non-AD, $n = 8$) were obtained. Based on DAOSD manifestation and availability of tear fluids, patients were assigned to the following four groups: group 1, AD with DAOSD (ADwDAOSD, $n = 8$), tear fluids at baseline, at follow-up visit (week 8)/before, and at onset of DAOSD; group 2, AD without DAOSD (ADw/oDAOSD, $n = 15$), tear fluids at baseline and follow-up visit (week 8); group 3, AD with DAOSD, tear fluids taken at onset of DAOSD (ADwDAOSDonset, $n = 14$); group 4, non-AD ($n = 8$), tear fluids at baseline and follow-up visit (week 8) (Table S1). In the non-AD group, patients with chronic hand eczema ($n = 5$), prurigo nodularis ($n = 2$), and eosinophilic esophagitis ($n = 1$) were included. Swabs for microbiome analyses were taken in four AD patients at baseline and week 8, and, an additional swab in one patient at onset of DAOSD. Patients' data including demographics, concomitant diseases, and treatment were extracted from the electronic patient records. All patients provided general consent for further using of data and samples. The study has been approved by the Ethics Committee of the Canton Bern. The procedures followed the tenets of the Declaration of Helsinki and the International Ethical Guidelines for Biomedical Research Involving Human Subjects (Council for International Organizations of Medical Sciences).

2.2 | Sampling tear fluids and ocular surface swabs

Tear fluids were extracted from filter strips used for Schirmer's test that had been applied to each eye for 3 min without prior local anesthesia, and then centrifuged at 13.2×10^3 rpm for 10 min. Tear fluids were immediately frozen at −80°C until further analyses. At all, 84 samples taken from 45 patients were available for Olink analyses.

Swabs along the edges of the lids and swabs of the dorsal conjunctivae using sterile nylon flocked swabs (FLOQSwabs #518CS01, Copan, Brescia, Italy) were collected after anesthesia with tetracaine HCL (tetracaine 1% SDU Faure; Thea Pharma S.A. Schaffhausen, Switzerland). Samples were taken from both eyes of four patients and pooled for each collection site. For negative controls, swabs without and with one drop of tetracaine HCL (each $n = 2$), respectively, were processed as lid and conjunctival swabs. Samples were stored in 2 mL DNA LoBind tubes (Eppendorf, Hamburg, Germany) cooled on ice until DNA extraction at the same day.

2.3 | Olink inflammation panel

Total protein concentration in tear samples were determined using PierceTM BCA Protein Assay Kit (Thermo Fisher) according to the manufacturer's instructions. Tear samples normalized by protein concentration were measured using targeted proteomics via the proximity extension assay by applying the Olink Target 96 inflammation panel (Olink Proteomics, Uppsala, Sweden) according to the manufacturer's instructions.[17]

2.4 | Metagenomic DNA sequencing and annotation

Lid and conjunctival swabs as well as negative controls were processed and metagenomic DNA was isolated at the same day using the QIAamp DNA Microbiome kit (Qiagen # 51704, Hilden, Germany) according to the manufacturer's protocol. Whole-metagenome shotgun sequencing was performed at the Next Generation Sequencing Platform of the University of Bern, Switzerland. The Nextera DNA Flex Library Preparation kit was used for library preparation for sequencing following standard pipelines of the Illumina NovaSeq 6000 Sequencing System on S4 flow cells. To exclude low-quality reads and reads of human origin, the resulting 150 bp paired-end reads were quality filtered using Trimmomatic[18] (version 0.36) and mapped to the human reference genome (Ensembl GRCh38) using Bowtie2[19] (version 2.3.4.1). For taxonomic assignment, the Metagenomic Phylogenetic Analysis tool (MetaPhlAn3)[20] was applied with default settings.

2.5 | Statistical analyses

For descriptive purposes, continuous data were presented as means with standard deviations (SD), while categorical data as absolute numbers with percentages.

For analysis purposes, NPX values of tear fluids in left and right eyes were averaged together. Samples that did not pass the quality control using the platform-specific "Olink NPX manager" software, were excluded, while NPX with >50% of samples below the limit of detection were marked in the tables and figures. All NPX values were expressed on log2 scale.

Differences of variables across patients' groups at baseline were assessed using Pearson's X^2 test, or Fisher's exact test where required, for categorical data and one-way ANOVA for continuous data. Paired-sample *t*-test or repeated measure ANOVA were used to assess differences of variables across time. Linear mixed models were used to assess repeated measures accounting for the effect of patients' groups. Post hoc tests were performed for all variables with *p*-value <.05 and the Šidák correction was adopted to adjust pairwise multiple comparisons. When assessing multiple results of inflammatory markers, the Benjamini–Hochberg controlling procedure, with a false discovery rate (FDR) = 0.1 was also adopted. The log-rank test was used to assess differences in dupilumab suspension rates across groups. Analyses were performed with SPSS software v.26.0 (IBM Corp, Armonk, NY, US).

Associations among inflammation markers and patients' groups were explored by using semantic map analysis.[21,22] This is

a data mining algorithm able to find and display the most important associations in the system. The algorithm uses graph theory and maximum spanning tree (MST) in order to find and show the strongest path of connections between variables.[23] In this analysis, due to the limited number of data available and the presence of continuous data, we used univariate Spearman's rank correlation coefficients as weights in the MST. Since the MST selects only positive associations, for continuous variables, inverse associations were explored by considering the same variables multiplied by −1. Therefore continuous variables are indicated in the map as "+" (high values) and "−" (low values). In order to avoid unstable associations, only connections with a p-value <.10 were considered by the algorithm. In the resulting map variables with many connections (hubs) are the most important for the system, while those with fewer connections or at the periphery (leaves) are the least important. The strength of correlations found by the algorithm can be interpreted as mild, moderate, or strong for values <.6, .6–.79, and ≥.8, respectively.[22]

In addition, clusters of variables in the map were found by using hierarchical clustering algorithm.[24] More specifically, the similarity between each pair of nodes (variables) in the map is first calculated by transforming correlations into distances, and then by computing the shortest path between each node pairs in the map. The resulting distance matrix is used to determine the proximity of nodes using agglomerative hierarchical clustering. The algorithm starts with each node forming its own cluster and repeatedly merges the two closest clusters based on the average distance between all pairs of nodes in the clusters, until a hierarchical tree (dendrogram) is formed. The optimal number of clusters (k) is evaluated using gap criterion, which compares the total intra-cluster variation for different number of clusters with their expected values under null reference distribution of the data (i.e., a distribution with no obvious clustering).[25] The final clustering is produced by finding the smallest height (distance) at which a horizontal cut through the dendrogram will leave k clusters. Analyses were performed with MATLAB v.9.1 software (The MathWorks, Natick, MA, USA).

Quantitative analysis of the microbiome was performed in RStudio using R programming language (version 4.2.2). Relative abundances of microbial taxa above a threshold of 1% were compared between each patient for both, the conjunctiva and the lid margin at the taxonomic levels of kingdom, phylum, genus, and species at baseline and follow-up visits. Associations of microbial abundances with visits (baseline, during dupilumab treatment, at onset of DAOSD) were analyzed using the Microbiome Multivariable Association with Linear Models (MaAsLin2) R package. Significant association was considered below a q value threshold of .20 after adjusting for false discovery rate (Benjamini Hochberg). Changes in richness, defined as number of genera above 1%, were compared using the paired t-test. Statistical significance was considered at a p-value <.05. Data were visualized using the R package ggplot2.

3 | RESULTS

3.1 | Patient characteristics

Patient demographics are provided in Table 1. Among 45 patients included (22 females, mean age 44.8 ± 13.8 years), 37 had AD, predominantly severe AD (mean SCORAD 54.1 ± 18.8) (Table S2). With respect to comorbidities, there were not any differences between groups (Table S3). Among concomitant OSD reported by patients, allergic rhinoconjunctivitis was the most frequent one (Table 2). In our cohort, DAOSD occurred 4.4 ± 4.9 months after the initiation of dupilumab therapy. Schirmer's tests revealed a higher tear production at onset of DAOSD compared to baseline levels, but the increase was not statistically significant (Table S4).

3.2 | Dupilumab treatment alters the expression of inflammatory markers in tear fluids of AD patients

We profiled 92 proteins in tear fluids from 37 patients with AD and eight non-AD patients before and under therapy with dupilumab, respectively. Overall, 23 tear fluids obtained from AD patients before dupilumab therapy and 22 samples taken from AD patients with manifest DAOSD were available. Comparing the expression of inflammatory markers revealed 31 differentially expressed proteins, all of which were upregulated in DAOSD tear fluids compared to baseline samples (Figure 1A, Table S5).

Next, we analyzed the protein expression over time in two AD patient groups, those with and without DAOSD. In tear fluids of ADw/oDAOSD patients taken before and under dupilumab therapy, six differentially expressed proteins could be identified. Five proteins, namely, fibroblast growth factors FGF-21 and FGF-23, proinflammatory markers IL-20 and C-C motif chemokine ligand (CCL) 25, and neurturin (NRTN) were significantly downregulated, while IL-12B, the IL-12p40 subunit of IL-12 and IL-23, was upregulated (Figure 1B, Table S6).

In tear fluids of eight AD patients that developed DAOSD, 25 proteins were upregulated in tear fluids taken under dupilumab therapy compared to baseline samples (Figure 1B, Table S7). Comparison of the protein expression of tear fluids taken at baseline and at week 8, before the onset of DAOSD, revealed a significant upregulation of 12 markers (IL-12B, IL-17C, stem cell factor (SCF), oncostatin (OSM), TNF super family member (TNFSF) 14, TNFSF11 (TNF-related activation-induced cytokine, TRANCE), TNFSF1 (lymphotoxin alpha, LTA), TNFSF receptor (TNFRSF) 9, matrix metalloprotease (MMP) 1, chemokine CCL3, and surface molecules CD6, CD244). After the onset of DAOSD in these patients, IL-12B and five additional proteins (CD8A, CD5, IL-8, leukemia inhibitory factor (LIF), and IL-10RB) were upregulated compared to baseline. IL-12B was significantly upregulated at both time points, before and after onset of DAOSD. Inflammatory markers with significant pairwise differences in tear fluids of AD

TABLE 1 Demographics of patients included in the study, in total and by specific group.

		Total		Group 1 ADwDAOSD		Group 2 ADw/oDAOSD		Group 3 ADwDAOSDonset		Group 4 Non-AD		Group 1 + 3 ADwDAOSDall		
		N = 45		*N* = 8		*N* = 15		*N* = 14		*N* = 8		*N* = 22		*p*[a]
Age (years)	Mean, SD	44.8	13.8	39.0	14.8	42.5	12.1	48.5	9.3	48.6	20.8	45.0	12.2	.61
		N	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%	
Sex	Male	23	51.1	3	37.5	10	66.7	9	64.3	1	12.5	12	54.5	.04
	Females	22	48.9	5	62.5	5	33.3	5	35.	7	87.5	10	45.5	
Disease	Atopic dermatitis	37	82.2	8	100	15	100	14	100	0	0.0	22	100	-
	Chronic hand eczema	5	11.1	0	0.0	0	0.0	0	0.0	5	62.5	0	0.0	
	Prurigo nodularis	2	4.4	0	0.0	0	0.0	0	0.0	2	25.0	0	0.0	
	Eosinophilic esophagitis	1	2.2	0	0.0	0	0.0	0	0.0	1	12.5	0	0.0	
Start of disease (AD; non-AD)	<1 year	8	18.2	1	12.5	2	13.3	5	38.5	0	0.0	6	28.6	.02
	1–12 years	15	34.1	5	62.5	5	33.3	5	38.5	0	0.0	10	47.6	
	13–18 years	6	13.6	0	0.0	3	20.0	1	7.7	2	25.0	1	4.8	
	>18 years	15	34.1	2	25.0	5	33.3	2	15.4	6	75.0	4	19.0	

Abbreviations: AD, atopic dermatitis; DAOSD, dupilumab-associated ocular surface disease; SD, standard deviation.

[a]Overall comparison of groups 1 + 3, 2 and 4. Fisher's exact test was used for categorical data while one-way ANOVA was used for continuous data.

TABLE 2 History of ocular surface diseases in patients included in the study, in total and by specific group.

	Total		Group 1 ADwDAOSD		Group 2 ADw/oDAOSD		Group 3 ADwDAOSDonset		Group 4 Non-AD		Group 1+3 ADwDAOSD all		
Eye diseases[b]	N=45	%	N=8	%	N=15	%	N=14	%	N=8	%	N=22	%	p[a]
Atopic blepharo-conjunctivitis	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	1
Keratoconus	2	4.4	0	0.0	0	0.0	2	14.3	0	0.0	2	9.1	.67
Allergic rhinoconjunctivitis	27	60.0	6	75.0	9	60.0	9	64.3	3	37.5	15	68.2	.31
Ocular Herpes infection	5	11.1	1	12.5	2	13.3	2	14.3	0	0.0	3	13.6	.69
Conjunctivitis sicca	2	4.4	0	0.0	2	13.3	0	0.0	0	0.0	0	0.0	.13
Dry eye	10	22.2	4	50.0	3	20.0	2	14.3	1	12.5	6	27.3	.72

Abbreviations: AD, atopic dermatitis; DAOSD, dupilumab-associated ocular surface disease.

[a]Overall comparison of groups 1+3, 2 and 4. Pearson's chi-squared test, or Fisher's exact test where required.

[b]Multiple conditions per patient were possible.

patients taken before and under dupilumab therapy as well as at onset of DAOSD are shown in Figure 1C. A protein-parallel gene ontology analyses point to an increased expression of proinflammatory and profibrotic mediators (Figure S1).

3.3 | Upregulation of IL-7 upon dupilumab therapy in non-AD patients

Since the incidence of conjunctivitis is not increased in patients treated with dupilumab for asthma, chronic rhinosinusitis with nasal polyps or EoE,[8] we were interested in the expression of inflammatory markers in tear fluids of patients treated with dupilumab for diseases other than AD. Interestingly, in tear fluids of non-AD patients, only a single cytokine, IL-7 was upregulated upon therapy with dupilumab (Table S8).

In order to compare ADwDAOSD, ADw/oDAOSD and non-AD groups, the differences in protein expression before and under dupilumab therapy were analyzed. Significant pairwise differences are shown in Figure 2. A significant upregulation of 16 inflammation markers was observed in the ADwDAOSD group, while a slight upregulation or even downregulation of these particular markers was noticed in ADw/oDAOSD and non-AD groups (Table S9).

3.4 | Distinct clusters of marker expression in AD and non-AD groups

To identify patterns of inflammatory markers in baseline tear fluids that might distinguish between groups, we applied semantic map analysis followed by hierarchical cluster analysis. Indeed, we could identify four clusters that clearly distinguish on the one hand between non-AD and AD groups and on the other hand between ADwDAOSD and ADw/oDAOSD (Figure 3). In baseline tear fluids of ADwDAOSD patients, a relatively high expression of IL-4, IL-5, IL-13, IL-17A, and IL-33, but relatively low expression of TNF-α, IFN-γ, TGF-α, IL-7, IL-8, IL-17C, IL-24, IL-10RA, and PD-L1 was observed (Figure 3, blue dots). In addition, the expression of chemokines such as CCL-13 (MCP-4), CCL19, CCL20, CXCL10, and CXCL11 was lower in ADwDAOSD compared with ADw/oDAOSD.

3.5 | Dupilumab has an effect on the ocular surface microbiome

In a pilot project, we analyzed the ocular surface microbiome of four AD patients. At baseline, *Actinobacteria* and *Firmicutes* were the most abundant phyla, *Cutibacterium* and *Staphylococcus* the most abundant genera, and *Cutibacterium acnes* the most abundant species of the ocular microbiome composition (Figure 4A).

During dupilumab therapy, the genera (lid, $p=.049$; conjunctiva, $p=.011$) and species (conjunctiva, $p=.0047$) richness significantly decreased compared to baseline (Figure 4B, C). One patient who later developed DAOSD, had a distinct profile, the abundance of *Proteobacteria* ($p=.0028$) increased, while that of *Basidiomycota* ($p=.006$) decreased in his lid and conjunctival samples. In his lid samples, the abundance of *Actinobacteria* ($p=.0044$) decreased (Table S10). The genus *Staphylococcus* was absent in conjunctival samples at baseline and before onset of DAOSD. At DAOSD onset, the genera richness was higher compared to baseline and week 8. Unique findings were the presence of the genera *Dolosigranulum*, *Moraxella and Alphapolyomavirus* in samples taken at onset of DAOSD as well as the dominance of the species *Dolosigranulum pigrum* and *Moraxella nonliquefacies* in lid and conjunctival samples, together with *Staphylococcus epidermidis* in the lid microbiome (Figure 4D).

4 | DISCUSSION

Our results clearly demonstrate distinct patterns of inflammation in tear fluids of patients with AD and non-AD patients upon dupilumab

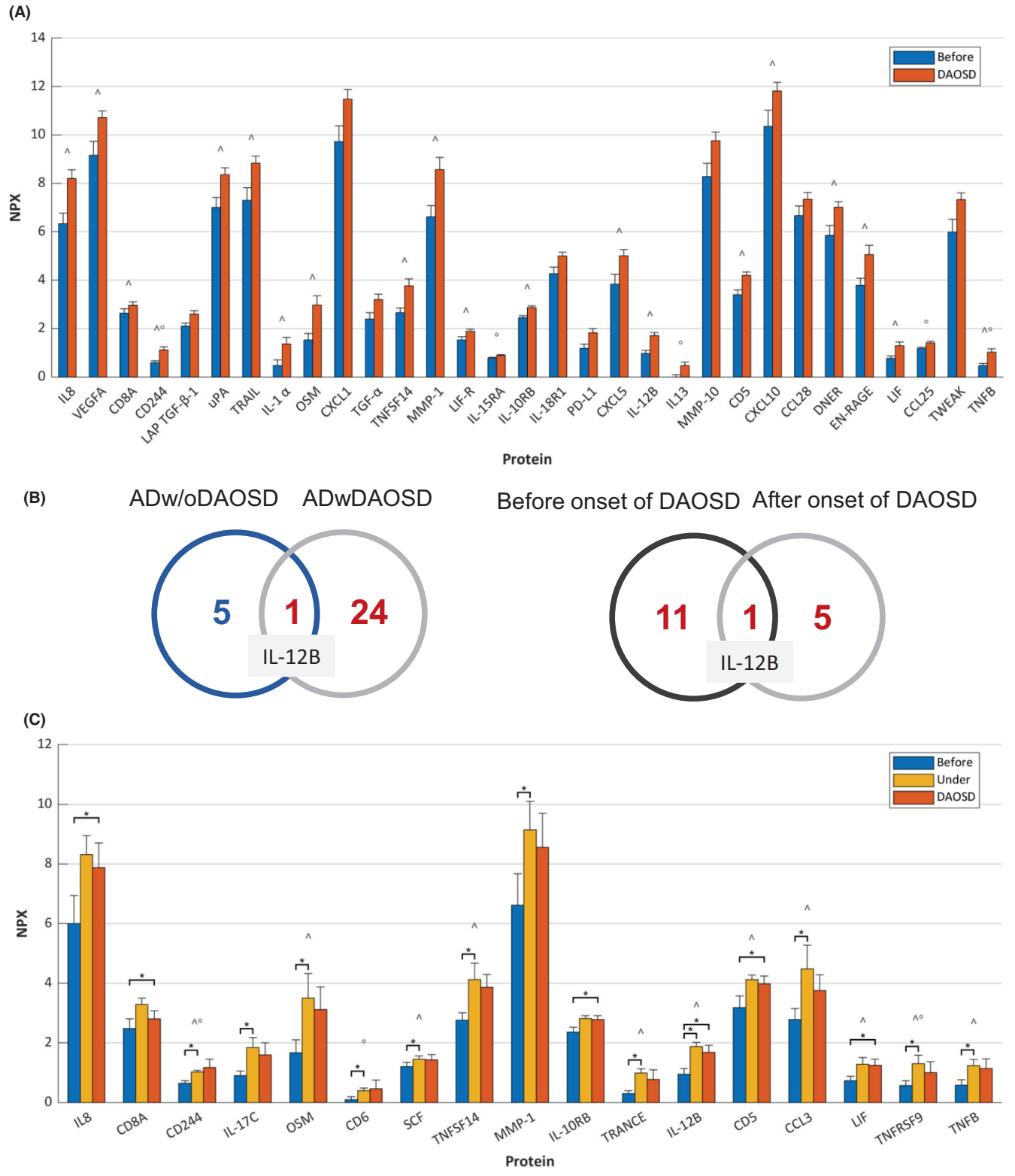


FIGURE 1 Differentially expressed inflammation markers in tear fluids of atopic dermatitis (AD) patients under dupilumab therapy. (A) Selected inflammatory markers with significant differences in tear fluids taken before therapy ($n = 22$) and at onset of dupilumab-associated ocular surface disease (DAOSD, $N = 23$). (B) Graphs show the number of down- (blue) and upregulated (red) markers in AD patients without and with DAOSD (ADw/oDAOSD ($n = 15$); ADwDAOSD ($n = 8$); left panel), as well as in ADwDAOSD patients before (week 8) and at onset of DAOSD (right panel). IL-12B was upregulated in all subgroups. (C) Inflammatory markers with any significant pairwise differences in tear fluids of ADwDAOSD patients ($n = 8$) taken before and under dupilumab therapy as well as at onset of DAOSD. Averages of normalized protein expression (NPX) are presented with error bars representing standard errors. ^ indicates significance after Benjamini–Hochberg FDR controlling procedure; ° specifies >50% of samples with NPX below the limit of detection.

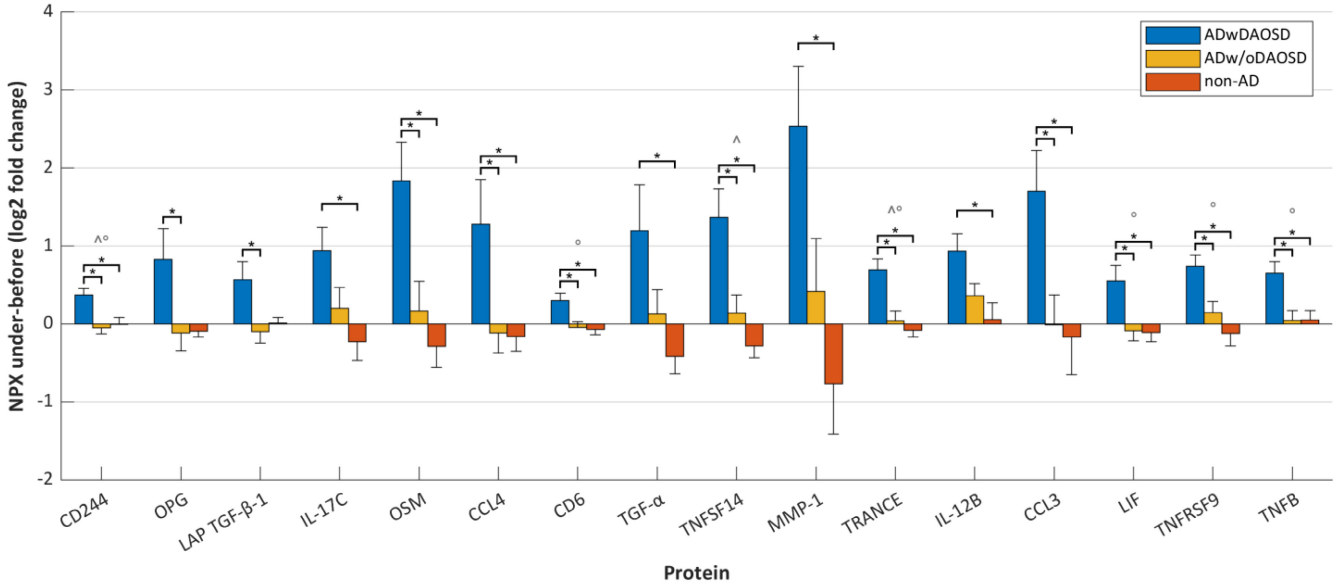


FIGURE 2 Differences in normalized protein expression (NPX) of selected inflammatory markers in tear fluids taken before and under dupilumab therapy with any significant pairwise difference between atopic dermatitis (AD) patients with dupilumab-associated ocular surface disease (ADwDAOSD, $n = 8$) and without DAOSD (ADw/oDAOSD, $n = 15$), as well as non-AD patients ($n = 8$). Average NPX are presented with error bars representing standard errors. * indicates pairwise statistical significance; ^ indicates significance after Benjamini–Hochberg FDR controlling procedure; ° specifies >50% of samples with NPX below the limit of detection.

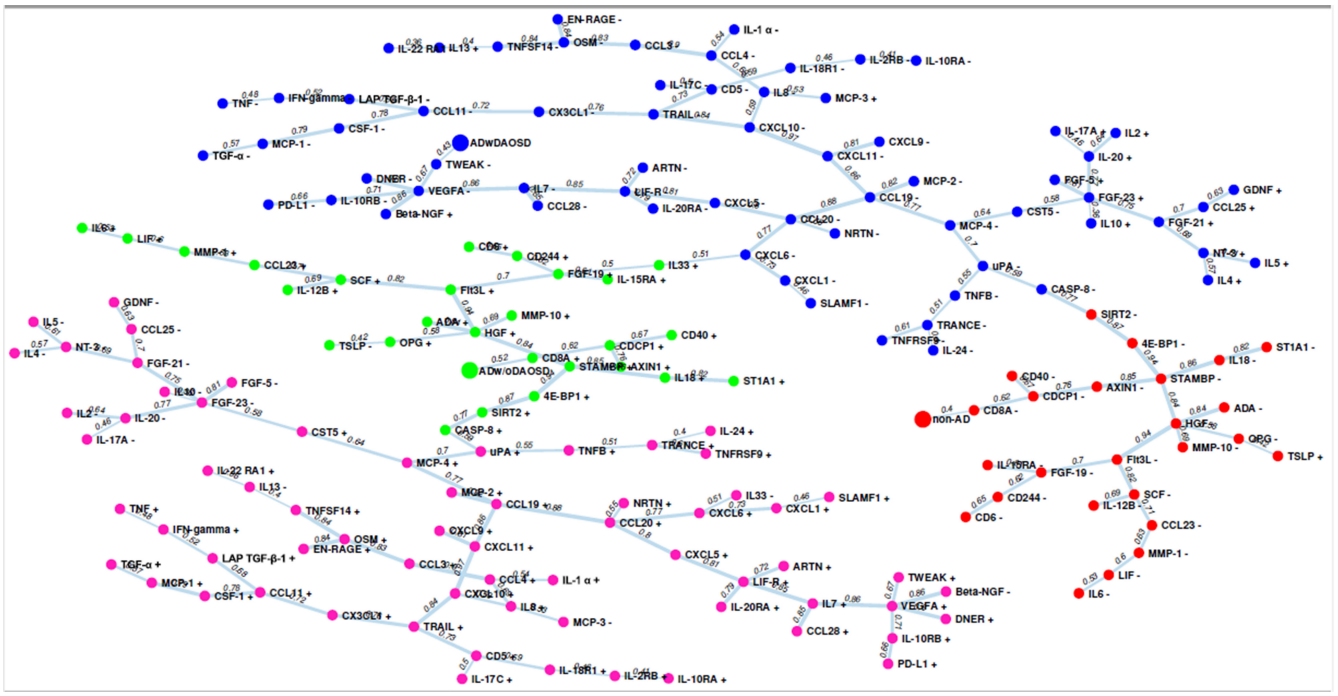


FIGURE 3 Associations among inflammatory markers in baseline tear fluids of atopic dermatitis (AD) and non-AD patients. Semantic map and hierarchical cluster analyses reveal four clusters of markers distinguishing between non-AD (red) and AD (green) as well as between AD patient that later developed dupilumab-associated ocular surface disease (ADwDAOSD; blue) and AD patients without DAOSD (ADw/oDAOSD; pink) under dupilumab therapy. Continuous variables are presented as + (high values) and − (low values). The numbers indicate the strength of correlations (<0.6, mild; 0.6–0.79, moderate; ≥0.8, strong).

therapy as well as of AD patients developing DAOSD compared to those without DAOSD. These differences may help to better understand the pathogenic mechanisms associated with DAOSD and to identify patients at risk for DAOSD.

The hierarchical clustering applied to semantic map analysis of inflammatory markers expressed in tear fluids, revealed four clusters that distinguish between AD and non-AD patients as well as ADwDAOSD and ADw/oDAOSD patients even before therapy. We

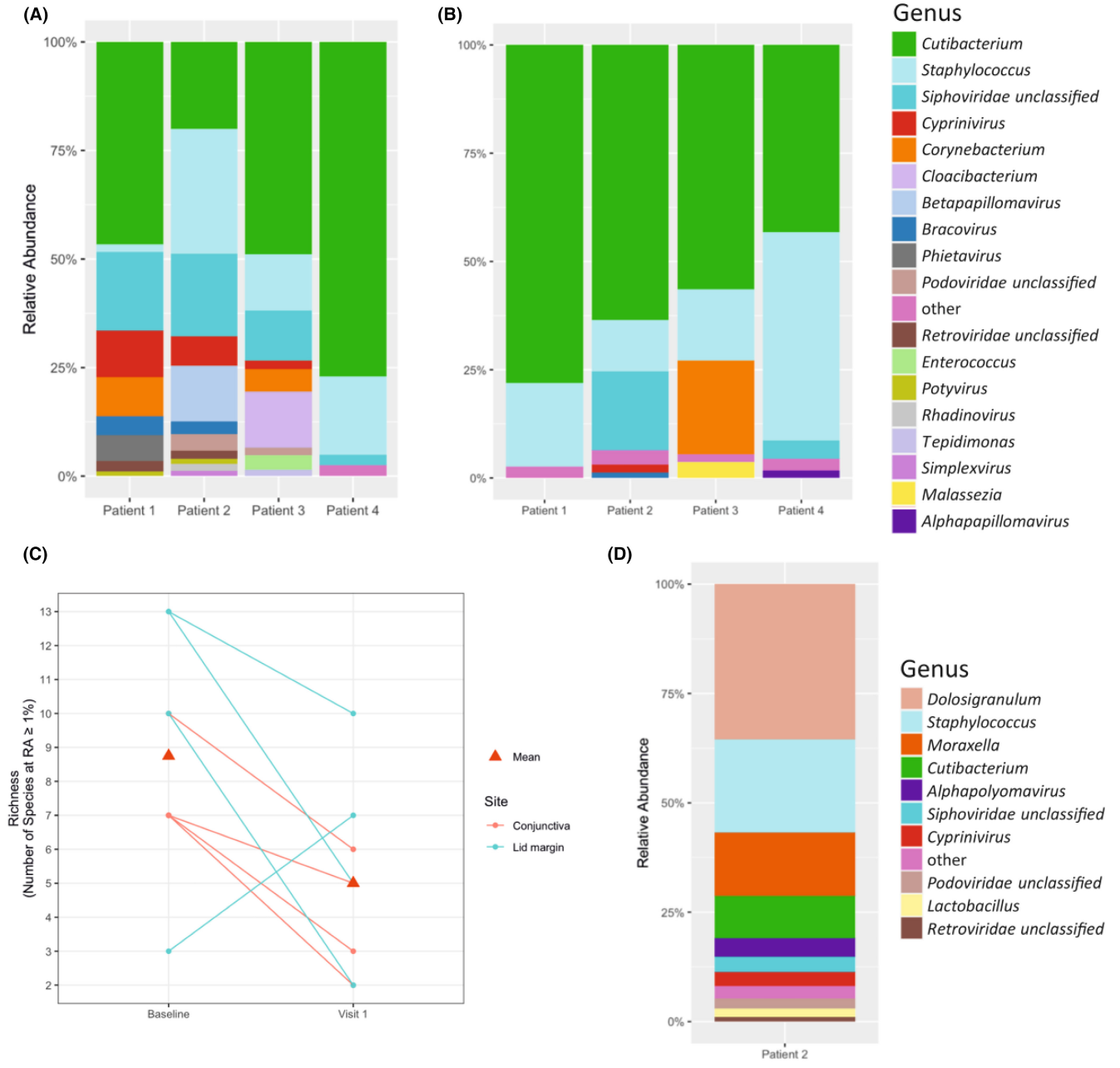


FIGURE 4 Taxonomic composition and richness of the ocular surface microbiome at genus level in atopic dermatitis (AD) patients. Abundances of microbiota with relative abundances >1% in lid samples ($n=4$ each) at baseline (A), under dupilumab therapy (week 8) (B), and at onset of dupilumab-associated ocular surface disease (DAOSD) in patient 2 (D). Changes in genera richness under dupilumab therapy (week 8) compared to baseline in lid and conjunctival samples (C).

observed a cluster with high expression of inflammatory markers in AD patients, including IL-6, IL-12B, IL-18, IL-33, and CASP-8, but low TSLP expression, whereas non-AD patients exhibited low expression of these markers. These findings suggest a preexisting inflammation of the eyes in AD patients, confirming previous observations in AD patients.[6,14,26–28] Moreover, two clusters showed distinct marker expressions in ADwDAOSD and ADw/oDAOSD groups: a relatively high expression of IL-4, IL-5, IL-13, IL-17A, and IL-33, indicating a mixed Th2/Th17 pattern, but relatively low Th1 cytokine expression in baseline tear fluids of patients that later developed DAOSD, and vice versa in those who did not. The expression of chemokines regulating the homeostasis of mucosal surfaces and inflammation was lower in ADwDAOSD compared with ADw/oDAOSD baseline tear fluids, and was associated with relatively low IFN-γ, which in turn is required for stimulating the expression of those chemokines.[29,30] This observation implies the presence of subgroups with different predisposition toward DAOSD among AD patients and might be the starting point for identifying biomarkers predicting DAOSD.

Dupilumab therapy provoked a significant upregulation of inflammatory markers in AD patients that was pronounced in the

ADwDAOSD group and had a distinct pattern compared with non-AD patients. A striking finding was the upregulation of IL-12B in both ADwDAOSD and ADw/oDAOSD groups, which was not observed in non-AD patients.

IL-12B is a subunit of IL-12 and IL-23 that both are strong inducers of Th1 and Th17 immune responses. An inverse correlation between the Th1 cytokine IFN-γ and the number of conjunctival goblet cells has been reported in dry eye conditions.[31] These findings are in agreement with recent reports on a Th1/Th17 profile and goblet cell scarcity in DAOSD.[11,13] Notably, under physiologic conditions, goblet cell proliferation and activation in the conjunctiva are regulated by IL-13.[32,33] Thus, inhibiting IL-13 may result in a depletion of goblet cells followed by decreased mucin secretion, disturbed antigen passage from the ocular surface to dendritic cells in the stroma, reduced production of immunoregulatory factors including TGF-β2, and subsequent Th1 response.[34] Mice that lack conjunctival goblet cells were shown to develop epithelial barrier disruption and an inflammation characterized by increased numbers of $CD11c^+$ and $CD11b^+$ antigen presenting cells, $IL\text{-}12^+$ macrophages and dendritic cells, $IFN\text{-}\gamma^+$ and $IL\text{-}17^+$ T cells, expression of proinflammatory cytokines, such as IL-1α, IL-1β, and TNF-α, but downregulation of Muc5ac.[35,36] Moreover, increased levels of IL-23, IL-17A and IFN-γ, IL-6, TGF-β1 and 2, CCL20 as well as MMP-9 have been observed associated with dry eyes in humans and mice.[32] Thus, the pattern of inflammatory markers in tear fluids of AD patients developing DAOSD has features of dry eye disease. The fact, that we did not observe a decreased tear production in AD patients under dupilumab therapy is probably related to tear sampling without prior local anesthesia. Notably, IL-12B was also upregulated in tear fluids from ADw/oDAOSD patients under dupilumab therapy. However, other inflammatory markers were not upregulated or even downregulated, such as CCL-25 and IL-20, which is why these patients might be protected from clinically relevant inflammation and dry eye disease.[37–39]

A striking finding was the expression of markers associated with fibrosis and remodeling such as TNFSF14 (LIGHT), CXCL-1, CXCL-5, TGF-β, IL-13, IL-6, OSM, MMP-1, and LIF in DAOSD tear fluids.[40–42] TNFSF14 is an important cytokine orchestrating inflammation and remodeling as it regulates the infiltration, survival, and cytokine production of T cells, macrophages and eosinophils, as well as the proliferation of structural cells and their expression of chemokines, growth factors, and metalloproteinases.[40]

IL-7 was the only differentially expressed marker found upregulated in tear fluids taken from non-AD patients under dupilumab therapy. IL-7 has been reported to be produced by epithelial goblet cells in the intestine.[43] By regulating B and T cell function as well as activating innate lymphocytes, IL-7 may promote antiviral and antibacterial responses.[44] As IL-7 was minimally expressed at baseline and not upregulated under dupilumab therapy in ADwDAOSD group, we wanted to know whether DAOSD might be associated with a dysregulated microbiome on the ocular surfaces of AD patients. Even though the number of swabs was limited, we observed a significant decrease in richness at the genera and the species levels. The composition of the ocular surface microbiome, namely most common phyla (*Actinobacteria*, *Firmicutes*) and species (*Cutibacterium acnes*) in ADw/oDAOSD patients was similar to that reported in individuals without OSD.[45,46] By contrast, a different pattern and *Dolosigranulum pigrum* and *Moraxella nonliquefacies*, known to cause conjunctivitis, as dominant species of the ocular surface microbiome were observed in one AD patient who developed DAOSD.[47,48] These observations suggest that dupilumab alters the inflammatory milieu and thereby might have an impact on the microbiome of the ocular surfaces.

Based on our and previously published data, the following pathomechanism of DAOSD is hypothesized:

AD per se is associated with an underlying inflammation of the ocular surfaces. Blocking the functional activity of IL-13 with dupilumab has two major consequences: First, the proliferation and function of goblet cells are inhibited, resulting in decreased mucus production and barrier dysfunction similar to that in dry eye disease. Second, dupilumab causes a shift in the cytokine pattern from a mixed Th2/Th17 toward a Th1/Th17 profile in AD patients developing DAOSD. Both reduced goblet cell function and cytokine shift may have effects on the type and intensity of inflammation, subsequent tissue remodeling as well as the microbiome of ocular surfaces.

AUTHOR CONTRIBUTIONS

KT, DS, MSZ, CAA, DCSB, and HUS were involved in study concept, methodology, and design. KT, ASL, FD, AH, SRH, CB, and ML were involved in acquisition of data. KT, FD, AH, ELH, MK, and CS were involved in analysis and interpretation of data. DCZB and HUS were involved in obtained funding. KT and DS were involved in study supervision. KT, ASL, SC, CS, and DS were involved in drafting of the manuscript. KT, ASL, FD, AH, SC, SRH, CB, ML, CS, ELH, MK, MSZ, CAA, DCZB, HUS, and DS were involved in critical revision of the manuscript for important intellectual content.

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CONFLICT OF INTEREST STATEMENT

HUS is a consultant for GlaxoSmithKline and Sanofi. DS has been an investigator, advisory board member, or consultant for AbbVie, Amgen, AstraZeneca, Galderma, Incyte, LEO, Eli Lilly, Novartis, Pfizer, Sanofi Genzyme. CAA is the Co-Chair for EAACI Guidelines on Environmental Science in Allergic diseases and Asthma and serves on the Advisory Boards of Sanofi/Regeneron, Novartis, GlaxoSmithKline, and SciBase, and is the Editor-in-Chief of Allergy. CS has received honoraria as adviser or speaker for Abbvie, Almirall, BMS, Incyte, LEO Pharma, Lilly, Kiowa Kirin, Novartis, Pfizer, and

Sanofi and has received research funding from PPM Services. DCZB got research funding from the OPOS foundation and Fondation Bertarelli Catalyst Fund. MZ has been a consultant for Bayer, Roche, Alcon Oculis, and got grants from Bayer. SRH has been an advisory board member and lecturer for LEO Pharmaceutics, Sanofi, Blueprint Medicines, Permamed and GSK. The other co-authors have no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon request.

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