Dissertation

IMMUNE RESPONSE AND CONJUNCTIVAL BACTERIAL FLORA IN PATIENTS WITH MODERATE TO SEVERE ATOPIC DERMATITIS TREATED WITH DUPILUMAB

submitted by

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Department of Ophthalmology
Department of Dermatology & Venereology

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Univ.-Prof. Dr. Peter WOLF

2025

Statutory Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used.

Throughout this thesis and in all related publications I followed the “Standards of Good Scientific Practice and Ombuds Committee at the Medical University of Graz“.

March 12th, 2025

Disclosures

Part of this thesis has been published in Patra V, Woltsche N, Cerpes U, Bokanovic D, Repelnig M, Joshi A, Perchthaler I, Fischl M, Vocanson M, Bordag N, Durdevic M, Woltsche J, Quehenberger F, Legat F, Wedrich A, Horwath-Winter J, Wolf P. Persistent Neutrophil Infiltration and Unique Ocular Surface Microbiome Typify Dupilumab-Associated Conjunctivitis in Patients with Atopic Dermatitis. Ophthalmol Sci. 2023 May 29;4(1):100340. doi: 10.1016/j.xops.2023.100340.

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Foreword

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Abbreviations and Definitions

AD = atopic dermatitis
AKC = atopic keratoconjunctivitis
ANOSIM = analysis of similarities method
APRIL = a proliferation-inducing ligand
ASV = amplicon sequence variant
AUC = area under the curve
BAFF = B-cell activating factor
baseline = baseline visit before Dupilumab initiation
BLC = B lymphocyte chemoattractant
CCL = CC-chemokine ligand
CD = cluster of differentiation
DAOSD = Dupilumab associated ocular surface disease
DNA = deoxyribonucleic acid
EASI = Eczema Area and Severity Index
EASI75 = a 75% reduction from baseline in the EASI
ENA = epithelial neutrophil-activating peptide
ENA = European Nucleotide Archive
FDR = false discovery rate
FGF = fibroblast growth factor
G = group effect
G-CSF = granulocyte colony-stimulating factor
GM-CSF = granulocyte-macrophage colony stimulating factor
GRO-alpha = CXCL1 = C-X-C motif chemokine ligand 1
H&E = haematoxylin and eosin
HGF = hepatocyte growth factor
IFN = interferon
Ig = immunoglobulin
IGA = Investigator Global Assessment
IL = interleukin
IL-4Rα = IL-4 receptor α-subunit
I-TAC = interferon-inducible T cell alpha chemoattractant
l = litre

LDA = linear discriminant analysis
LIF = leukemia inhibitory factor
LTA = lymphotoxin alfa
MCP = monocyte chemoattractant protein
M-CSF = macrophage colony-stimulating factor
MDC = macrophage-derived chemokine
MDC = macrophage-derived chemokine
mg = milligram
MIF = macrophage migration inhibitory factor
MIG = monokine induced by gamma interferon
MIG = monokine induced by gamma-interferon = CXC ligand 9
MIP = macrophage inflammatory protein
ml = millilitre
MMP = matrix metalloproteinase
NA = not applicable
NGF = nerve growth factor
nm = nanometre
NMDS = nonmetric multidimensional scaling
OSM = oncostatin
OX40 ligand = ligand of OX40
PCR = polymerase chain reaction
q2w = once every two weeks
rpm = rounds per minute
SCF = stem cell factor
SCORAD = Scoring Atopic Dermatitis
SDF = stromal cell-derived factor = CXCL12
sIL = soluble interleukin
STAT pathway = signal transducer and activator of transcription pathway
T = time effect
TARC = Thymus and activation regulated chemokine
Th1 = subpopulation of T helper cells
Th2 = subpopulation of CD4 (cluster of differentiation 4)-positive T helper cells
TNF = tumor necrosis factor
TNFSF = TNF super family member
TRAIL = tumor necrosis factor related apoptosis inducing ligand

TRANCE = TNF-related activation-induced cytokine

TSLP = thymic stromal lymphopoietin

TSLP = thymic stromal lymphopoietin

TWEAK = tumor necrosis factor-like weak inducer of apoptosis

visit 1m = first visit 1 month after Dupilumab initiation

visit 4m = final second visit 4 months after Dupilumab initiation

visit conj = visit at the time of DAOSD development

X = time interaction effect

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Figure 26: Serum cytokine profile reproduced from (Patra *et al.*, 2024) with permission of publisher Elsevier, American Academy of Ophthalmology

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Kurzzusammenfassung

Einleitung: Dupilumab-assoziierte Augenoberflächenerkrankungen (DAOSD) sind häufige Nebenwirkungen dieser ansonsten gut verträglichen monoklonalen Antikörper-Therapie bei Patient*innen mit atopischer Dermatitis, wobei die zugrundeliegende Pathogenese bis dato nicht gänzlich bekannt ist.

Material und Methoden: Im Rahmen dieser prospektiven, monozentrischen Pilot-Studie wurden erwachsene Patient*innen mit moderater bis schwerer atopischer Dermatitis, welche für eine 16-wöchige Dupilumab-Therapie vorgesehen waren, inkludiert. Zu mindestens drei verschiedenen Zeitpunkten, vor Dupilumab-Initiierung, 1 Monat nach Therapie-Start und 4 Monate nach Therapie-Start, wurden eine Spaltlampen-Untersuchung, eine dermatologische Ganzkörper-Untersuchung, Bindehaut-Abstriche für Hämatoxylin-Eosin-Färbung und Mikrobiom-Analysen durchgeführt und Blutproben für Serum-Zytokin-Analysen abgenommen.

Ergebnisse: Zwanzig Patient*innen mit moderater bis schwerer atopischer Dermatitis, welche Dupilumab erhielten, und zehn gesunde, erwachsene Kontrollpersonen ohne atopische Dermatitis und Dupilumab-Therapie wurden eingeschlossen. Sechs der zwanzig Patient*innen (30%) mit atopischer Dermatitis entwickelten eine DAOSD und zeigten ein verzögertes Therapieansprechen. Eine ausgeprägte Infiltration mit neutrophilen Granulozyten zeigte sich in den Bindehaut-Abstrichen und erhöhte IL-1β und TNF-α-Levels zeigten sich im Serum von DAOSD-Patient*innen. Das Mikrobiom der Augenoberfläche dieser Patient*innen wies eine diverse und persistente Kolonialisierung auf, besonders durch *Acetobacter aceti*.

Schlussfolgerung: Eine spezifische mikrobielle Besiedelung und eine ausgeprägte Infiltration mit neutrophilen Granulozyten in Verbindung mit erhöhten Werten von systemischen pro-inflammatorischen Zytokinen charakterisieren DAOSD.

Abstract

Introduction: Dupilumab-associated ocular surface disease (DAOSD) is a frequent atopic dermatitis specific side effect of this otherwise well tolerated monoclonal antibody therapy. The underlying pathogenesis has not been clearly understood yet.

Material and Methods: In this prospective single-center study, adult patients with moderate to severe atopic dermatitis scheduled for 16 weeks Dupilumab treatment were enrolled, and a slit-lamp examination, a whole-body dermatological examination, conjunctival smears for haematoxylin and eosin staining, conjunctival swabs for microbiome analyses and blood sampling for serum cytokine profiling were conducted at least at three visits - at baseline before Dupilumab initiation, at one month after treatment start and at the end of Dupilumab treatment after four months.

Results: Twenty patients with moderate-to-severe atopic dermatitis receiving Dupilumab treatment and 10 healthy controls without AD not receiving Dupilumab treatment were enrolled. Six out of the 20 AD patients (30%) developed DAOSD and responded after a delay to treatment. Conjunctival smears revealed massive neutrophilic infiltration and serum analysis showed increased levels of IL-1β and TNF-α in patients with DAOSD. The ocular surface microbiome in DAOSD patients was characterised by a diverse and persistent colonisation, in particular by *Acetobacter aceti*.

Conclusion: A unique microbial landscape and significant neutrophil infiltration on the ocular surface in association with elevated levels of systemic pro-inflammatory cytokines typify DAOSD.

Introduction

Atopic dermatitis (AD) is an inflammatory skin disease manifesting with recurrent, pruritic and localized eczema with increasing prevalence and incidence over the last decades (Asher *et al.*, 2006; Ständer, 2021). In most of the cases, disease onset and resolution are in childhood, however, there are persistent and adult-onset cases, which might present more frequently than assumed (Illi *et al.*, 2004; Weidinger *et al.*, 2016). Today, the estimated prevalence is 15 to 20% among children and up to 10% in adults (Laughter *et al.*, 2021). Up to 20% of the affected children and up to 50% of affected adults show moderate to severe AD significantly reducing their quality of life (Ballardini *et al.*, 2013; Weidinger *et al.*, 2016).

The most relevant risk factor for developing AD is a positive family history (Apfelbacher *et al.*, 2011), and the strongest genetic risk factor are mutations in the skin barrier protein filaggrin (Palmer *et al.*, 2006). The pathophysiology of AD is multifactorial and involves a dysfunctional epidermal barrier, cutaneous microbiome alterations and type-2 immunity dysregulation (Langan *et al.*, 2020). These factors act together in a vicious circle with a dysfunctional epidermal barrier leading to inflammation and T-cell infiltration, *Staphylococcus aureus* colonialization leading to skin barrier damage and inflammation and Th2 immune response leading to a dysfunctional skin barrier and *Staphylococcus aureus* colonialization (Langan *et al.*, 2020). In consequence of epidermal barrier disruption, alarmins which lead to activation of epidermal dendritic cells and type-2 response are released (Langan *et al.*, 2020). After their activation, Th2 cells release interleukin-4 (IL-4) and IL-13, which lead to an immunoglobulin E (IgE) class switch in B cells. B cells then start to produce antigen-specific IgE via the signal transducer and activator of transcription (STAT) pathway (Gandhi *et al.*, 2016). Moreover, Type-2 cytokines such as IL-4, IL-13, thymic stromal lymphopoietin (TSLP) and IL-31 are involved in inducing itch, the characteristic symptom of AD (Langan *et al.*, 2020). The link between type-2 response and neural itch control is the type-2 IL-4 receptor α-subunit (IL-4Rα) of IL-4 and IL-13 on afferent neurons (Oetjen *et al.*, 2017).

AD diagnosis is based on clinical features as there are no specific laboratory or histological findings (Weidinger *et al.*, 2016). To facilitate clinicians' work, diagnostic criteria have been proposed. Table 1 depicts an extraction of a simplified version of AD diagnostic criteria (Eichenfield *et al.*, 2003).

Essential features
Pruritus
Eczema (acute, subacute, chronic)
Chronic or relapsing history
Typical morphology and age-specific patterns Facial, neck and extensor involvement in infants/children Current/previous flexural lesions in any age-group Sparing of groin or axillary regions
Important features
Early age of onset
Atopy Personal and/or family history Immunoglobulin E hyperreactivity
Xerosis

Table 1: AD diagnostic criteria according to (Eichenfield *et al.*, 2003)

Scoring tools were established to quantify disease severity, like the Eczema Area and Severity Index (EASI), Scoring Atopic Dermatitis (SCORAD) and Investigator Global Assessment (IGA), which are widely used in clinical routine and clinical trials (Chopra *et al.*, 2017). EASI quantifies redness, thickness, excoriation (substance defect of the skin, possibly reaching the Stratum papillare of the Dermis), lichenification (secondary skin lesion presenting with skin thickening, hyperpigmentation and exaggerated skin lines) and percentage of affected skin on head, trunk, arms and legs. These scores are summed and range from 0 to 72. A score of 7 or below indicates mild disease, 8 to 21 moderate, 22 to 50 severe and 51 to 72 very severe disease (Chopra *et al.*, 2017). SCORAD quantifies the area of involved skin and the severity of redness, swelling, oozing, crusting, lichenification and dryness separately for head and neck, extremities, anterior trunk, back and genitals (Chopra *et al.*, 2017). In addition, two visual analogue scales ranging from 0 to 100 points for patient-reported sleep loss and pruritus are included in SCORAD. In total, SCORAD ranges from 0 to 103. A score of 25 or below indicates mild disease, 26 to 50 moderate and 51 to 103 severe disease (Chopra *et al.*, 2017). IGA is a 5 point score quantifying morphological skin alterations ranging from 0 to 4 with 0 indicating no disease at all and 4 indicating severe AD (Simpson *et al.*, 2020).

However, AD is not only a cutaneous pathology, but can be regarded as a systemic disease with many other organ manifestations besides the skin (Darlenski *et al.*, 2014). The “atopic

march”, the progression from AD in infancy to asthma bronchiale (in up to 50% of AD patients) and allergic rhinitis in childhood, reinforces this concept (Darlenski *et al.*, 2014). Moreover, there’s a long list of other organ manifestations associated with AD like atopic comorbidities such as asthma bronchiale, food allergy or rhinitis, but also non-atopic comorbidities like ocular, psychiatric, infectious, autoimmune and cardiovascular disorders and certain cancers (depicted in Figure 1) (Thyssen *et al.*, 2023). Knowledge of these is important for clinicians, as they can affect therapeutic decisions, because some comorbidities might improve under certain treatments, whereas others might get worse (Thyssen *et al.*, 2023).

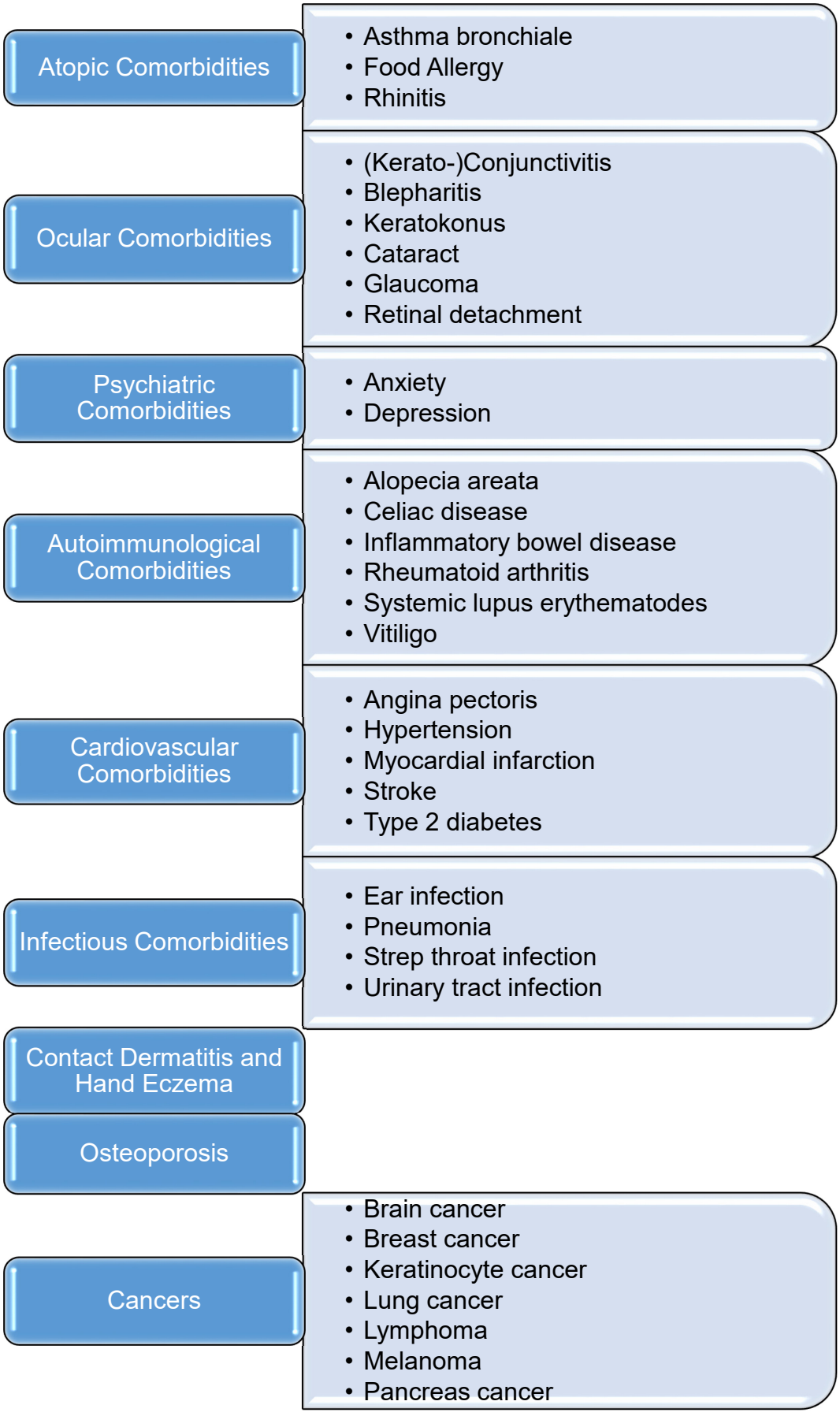


Figure 1: Comorbidities associated with AD according to (Thyssen *et al.*, 2023)

Ocular co-morbidities of AD may be sight-threatening in severe cases and may be associated with rubbing and scratching of the periocular skin (Beck *et al.*, 2019). However, ocular AD manifestations may also be present in patients with well-controlled cutaneous disease or absence of any cutaneous disease (Schneider *et al.*, 2013).

About 20 to 40% of AD patients show periocular skin involvement (Bielory *et al.*, 2010), ranging from eczema, lichenification, periocular hyperpigmentation to de Hertoghe's sign (hair loss in the area of the eyebrow's outer third), Dennie-Morgan folds (creases of the lower or also upper eyelid), but also madarosis (loss of eyelashes), trichiasis (eyelashes misdirected against and rubbing on the ocular surface), ptosis, (often staphylococcal) blepharitis and in cicatrizing cases lagophthalmos (lid closure failure resulting in a tear distribution deficit on the ocular surface), ectropion (lid eversion) or entropion (lid inversion) (Rich *et al.*, 1985; Guglielmetti *et al.*, 2010; Schram *et al.*, 2011; Beck *et al.*, 2019).

Twenty-five to 40% of AD patients (children and adults) show atopic keratoconjunctivitis (AKC), a chronic, non-infectious ocular surface inflammation (Foster *et al.*, 1990). Among the chronic allergic ocular surface diseases, it is the most devastating one, as a severe course of the disease can lead to significant vision loss and even blindness because of the corneal involvement (Foster *et al.*, 1990). AKC is characterized by a Th2 related immune response leading to release of IL-4 and interleukin-5 (IL-5), whereas IL-4 stimulates B cells to release IgE and IL-5 attracts eosinophilic granulocytes (Uchio *et al.*, 2000; Lampinen *et al.*, 2004). In the chronic course of the disease also Th1 cells play an important role (Uchio *et al.*, 2000). AKC is a bilateral, but often asymmetric disease, which presents all year round (Rachdan *et al.*, 2012). Besides blepharitis and eczema on the eyelids, the conjunctiva can present with hyperemia, chemosis (swelling) and a papillary reaction of the subtarsal conjunctiva and moreover, in severe, cicatrizing cases with subconjunctival fibrosis, fornix shortening and symblepharon (Rachdan *et al.*, 2012) (Figure 2). Corneal involvement ranges from superficial keratitis to (shield) ulcers, Trantas dots (accumulations of eosinophilic and neutrophilic granulocytes presenting as yellow-white dots at the corneal limbus), corneal limbal stem cell failure, scars and vascularisation (Rachdan *et al.,* 2012) (Figure 2). There's also a higher risk for microbial and viral, especially herpetic, corneal superinfection (Akova *et al.,* 1994). Furthermore, AD is associated with keratoconus, tear dysfunction, cataract, retinal detachment, glaucoma and uveitis (Beck *et al.*, 2019) (Figure 3). AKC treatment is a step-wise approach starting with topical therapy including preservative-free lubricants, antihistamines, mast cell stabilizers and corticosteroids (for acute exacerbations), followed by topical

calcineurin inhibitors like cyclosporin A and tacrolimus, systemic corticosteroids and systemic immunosuppresants (Sobolewska *et al.*, 2014).

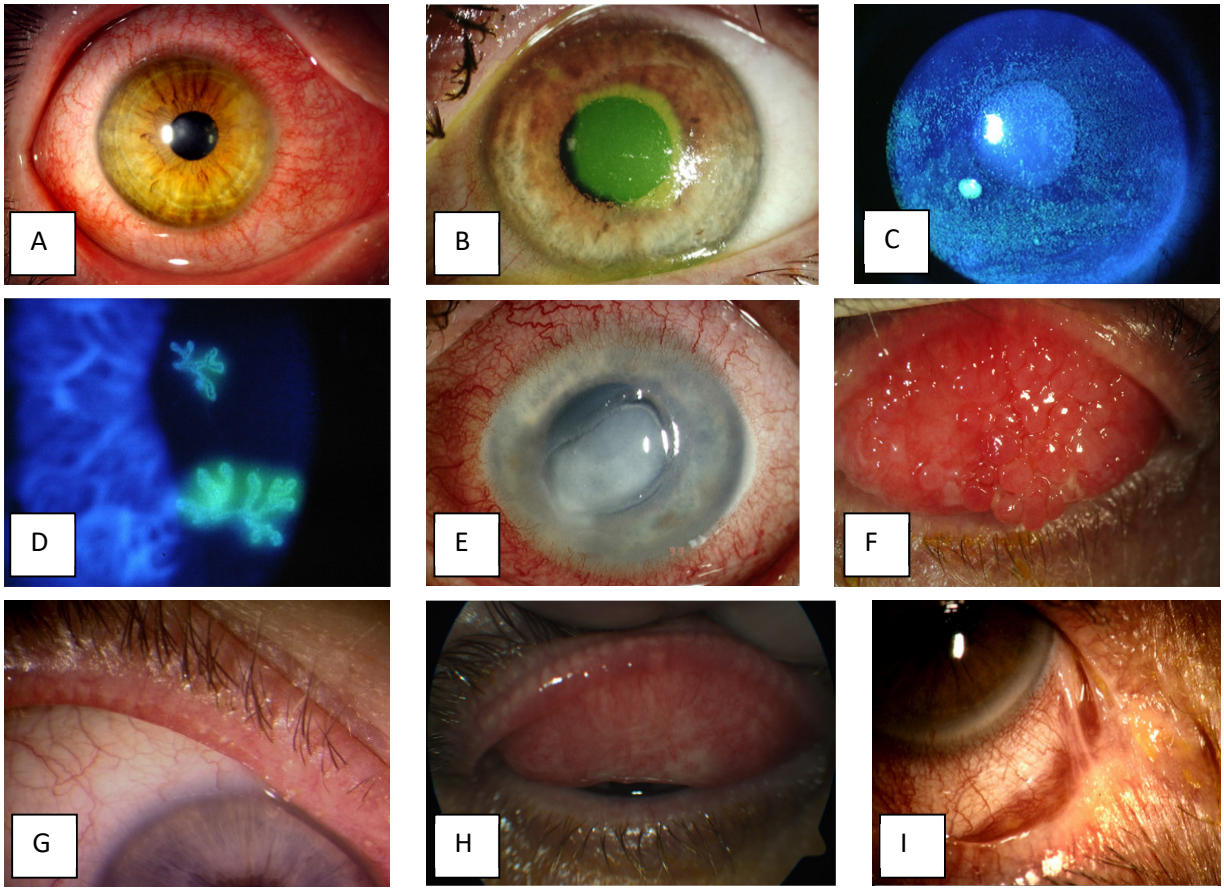


Figure 2: Characteristic features of AKC A – conjunctivitis; B – corneal erosion; C – superficial keratitis; D – epithelial herpetic keratitis; E – corneal ulcer; F – subtarsal papillary reaction; G – meibomian gland disease; H – subtarsal fibrosis; I – symblepharon.

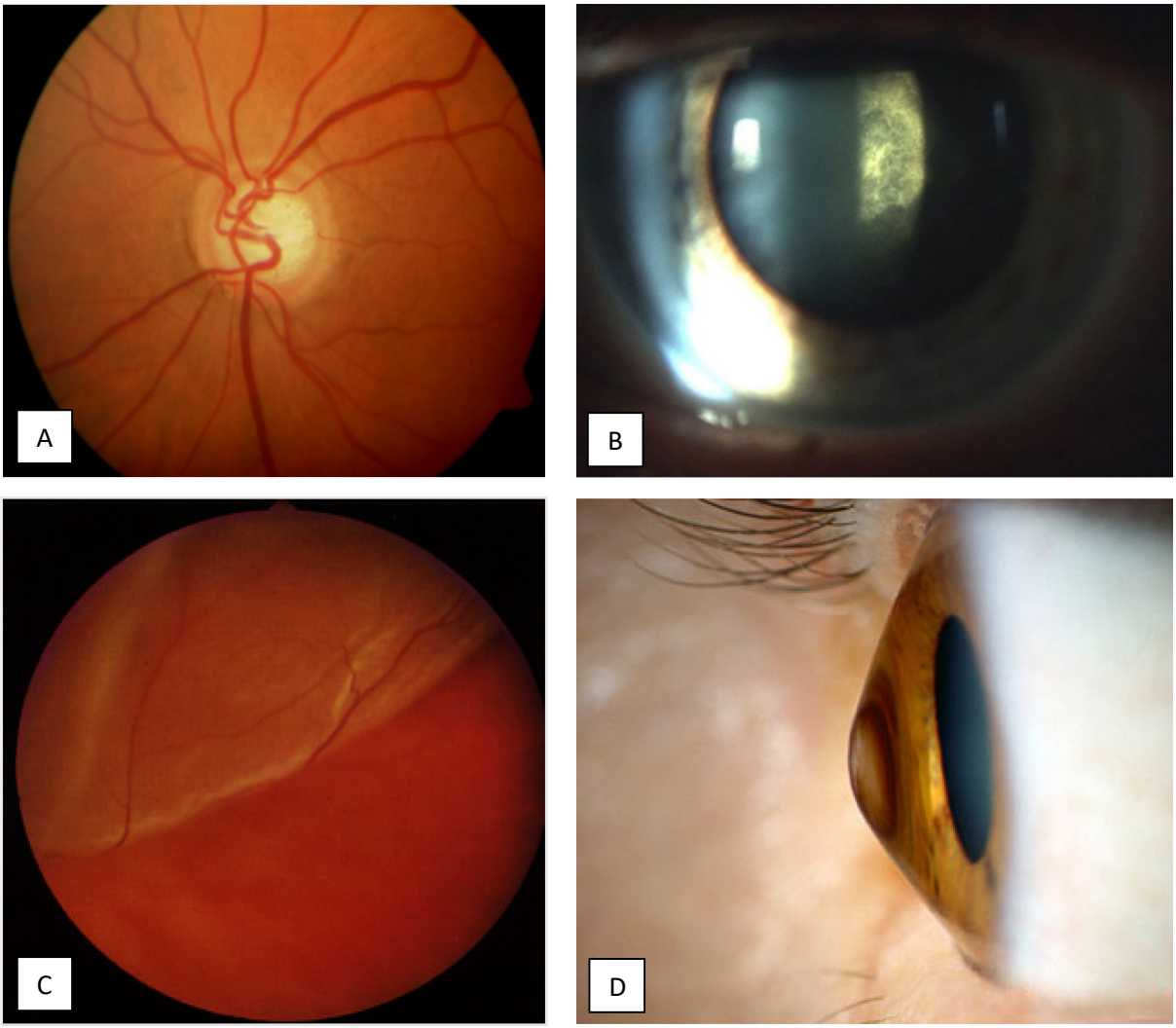


Figure 3: Ocular pathologies associated with AD
A – glaucomatous optic atrophy; B – subcapsular cataract; C – retinal detachment; D – keratoconus

Regarding cutaneous AD treatment, in the past, second-line treatment options for moderate to severe AD patients, who did not respond to lubricants, topical steroids and topical calcineurin inhibitors, were insufficient, as use of systemic immunosuppressants (like ciclosporin), systemic corticosteroids and phototherapy are limited by toxicity (Boguniewicz *et al.*, 2017). Because of that, the first biologic therapy for moderate to severe AD was eagerly awaited (Boguniewicz *et al.*, 2017).

In September 2017, Dupilumab (Dupixent®, Sanofi Genzyme), a human IgG4 monoclonal antibody against IL-4 receptor α-subunit of IL-4 and IL-13, was approved for treatment of moderate to severe AD in adults needing systemic treatment in the European Union. In four randomized, placebo-controlled, double-blind, multinational phase III trials (SOLO I & II,

CHRONOS, CAFÉ) the therapeutic regimen of a loading dose of Dupilumab 600mg and 16 weeks' treatment with Dupilumab 300mg q2w (once every two weeks) alone or in addition to topical corticosteroids led to significant improvement of AD signs and symptoms (Simpson *et al.*, 2016; Blauvelt *et al.*, 2017; de Bruin-Weller *et al.*, 2018). Meanwhile, Dupilumab has also been approved for treatment of moderate to severe AD in adolescents ≥ 11 years (August 2019), severe AD in children ≥ 6 years (October 2020) and severe AD in children ≥ 6 months (March 2023) and as long-term treatment (EMA, 2023). Other medical indications for Dupilumab treatment are specific forms of Asthma bronchiale, chronic rhinosinusitis with nasal polyposis, prurigo nodularis and eosinophilic esophagitis (EMA, 2023). However, besides the therapeutic success in significantly controlling signs and symptoms, Dupilumab has several side effects (EMA, 2023) and one AD-specific side effect: Dupilumab associated ocular surface disease (DAOSD).

While formerly DAOSD was solely defined as conjunctivitis with reported prevalence rates of up to 28% in treatment groups compared to up to 11% in placebo groups in the 4 approval studies (Simpson *et al.*, 2016; Blauvelt *et al.*, 2017; de Bruin-Weller *et al.*, 2018), we nowadays know that it's a broader spectrum also including blepharitis and keratitis with a prevalence of up to 60% in adolescents and adults in real-world analyses (Kamata *et al.*, 2021). In most of the cases, DAOSD presents as mild or moderate blepharoconjunctivitis or superficial keratitis which can be adequately treated with ophthalmological topical therapy without reduction or cessation of Dupilumab (Utine *et al.*, 2021). However, there are also cases presenting with severe ocular surface adverse effects like cicatrizing ocular surface and eyelid alterations, corneal ulcer and even corneal perforation (Nettis *et al.*, 2020; Felfeli *et al.*, 2021; Phylactou *et al.*, 2022; Reddy *et al.*, 2022). Usually, DAOSD develops 2 weeks to 4 months after treatment initiation (Kamata *et al.*, 2021). Known risk factors predisposing to DAOSD development are pre-existing ocular surface or eyelid manifestations as part of AD (Kamata *et al.*, 2021), pre-existing food allergy and higher Thymus and activation regulated chemokine (TARC) and immunoglobulin E (IgE) values before Dupilumab initiation (Uchida *et al.*, 2020; Touhouche *et al.*, 2021).

However, the pathogenesis behind DAOSD is not clearly understood yet. Various hypotheses have been proposed including subclinical ocular atopic disease (Gooderham *et al.*, 2018), infestation with Demodex mites leading to IL-17 mediated inflammation similar to ocular rosacea (de Bruin-Weller *et al.*, 2018), ocular immunodeficiency accompanied by localized infections and concomitant upregulation of proinflammatory molecules (Akinlade *et al.*, 2019;

Kamata *et al.*, 2021), decreased amount of tear fluid (Kamata *et al.*, 2021), mucin deficiency (Barnett *et al.*, 2020), decreased amount and function loss of conjunctival goblet cells (Bakker *et al.*, 2019; R. Achten *et al.*, 2023), eosinophilia (Treister *et al.*, 2018), increased bioavailability of Dupilumab on the ocular surface (R. Achten *et al.*, 2023), a Th1 shift due to the Th2 blockade leading to atopic keratoconjunctivitis-like manifestations (Utine *et al.*, 2021) or an increased activity of OX40 ligand (Neagu *et al.*, 2022). However, none of these hypotheses and findings have fully elucidated the pathogenesis behind DAOSD.

As DAOSD can possibly lead to significantly reduced quality of life and cessation of this otherwise highly effective and well tolerated treatment, further knowledge on pathogenesis and consecutively possible prevention strategies is awaited and a “hot topic” in the field. Because of that, our objective was to dive deeper into the immunological processes especially on the ocular surface and in the blood. We hypothesized that DAOSD might be associated with alterations in the patients’ conjunctival bacterial flora and therefore aimed to investigate the ocular surface microbiome. Besides, our objective was to analyse cellular components on the ocular surface to gain insights into the morphological picture of DAOSD and to analyse the systemic pro-inflammatory response via circulating cytokines in the blood.

Material and Methods

Patient characteristics

Male and female patients older than 18 years with moderate to severe AD scheduled for Dupilumab treatment were enrolled for this prospective single-centre pilot trial. Patients and controls had to be excluded if they were allergic to 0.4% oxybuprocaine hydrochloride eye drops, which are routinely used for local anesthesia of the ocular surface, if they had a history of an ocular pathology like for instance glaucoma requiring regular application of eye drops (except preservative free artificial tear eye drops), if they showed active microbial or allergic inflammation of the ocular surface at baseline, if they regularly wore contact lenses and if they had undergone ophthalmic surgery in the previous 6 months. We obtained informed consent from the whole study and control cohort. The Ethics Committee of the Medical University of Graz approved the study described herein (application number 31-379 ex 18/19) and the conduction conformed to the principles of the Declaration of Helsinki (Patra *et al.*, 2024).

Patient treatment

We asked all study patients for their previous medical history including cutaneous, ophthalmological, and allergic diseases at baseline before Dupilumab initiation. At every study visit, we conducted a thorough slit-lamp examination and photographs of the ocular surface (cornea and conjunctiva) and the eyelids, a thorough whole-body dermatological examination including completion of the 3 scores EASI, SCORAD and IGA, conjunctival smears, swabs and eyelash sampling and blood sampling. In total, 3 study visits were scheduled at specified time points and one possible additional visit was conducted for those patients who developed DAOSD. We conducted a baseline visit before Dupilumab initiation (baseline), a first visit 1 month after Dupilumab initiation (visit 1m) and a final second visit 4 months after Dupilumab initiation (visit 4m). If patients developed DAOSD they were told at the baseline visit to immediately contact the study personnel throughout the whole study period and then an appointment was made for the same or the following day (visit conj). Dupilumab treatment was conducted with the approved regimen of an initial dosage of 600mg followed by 300mg q2w for 16 weeks. The loading dose of 600mg was administered by one of the doctors at the Dermatology department, the following syringes were administered by the patients themselves. In the control group 10 healthy subjects without AD or AD history were enrolled.

At one single time point, we performed a thorough slit-lamp examination, obtained conjunctival smears and swabs and conducted blood sampling (Patra *et al.*, 2024).

Conjunctival smears and swabs collection

One drop of 0.4% oxybuprocaine hydrochloride was applied to the inferior fornix of the right eye for local anesthesia of the ocular surface. Then the inferior fornix of the right eye was gently wiped with a plastic eyelet and the gained material was put on a glass slide and air-dried for the conjunctival smear. For the conjunctival swabs we used the ESwab® device (Copan Diagnostics, California, USA) and also wiped the inferior fornix of the right eye (Patra *et al.*, 2024).

Histological analysis of the conjunctival smears

The air-dried conjunctival smears on the glass slides were stained with haematoxylin and eosin. Two independent investigators (Vijaykumar Patra and Nora Woltsche) quantified the cellular infiltrate at 5 randomly selected different sites in each sample with 40-fold magnification with the light microscope (Patra *et al.*, 2024).

Microbial deoxyribonucleic acid (DNA) extraction out of the conjunctival swabs

Via mechanical and enzymatic lysis total DNA was extracted from the ESwabs®. Mechanical lysis was achieved with bead-beating twice at 6000 rounds per minute (rpm) for 30 seconds with a MagNA Lyser® instrument (Roche, Mannheim, Germany) after thawing the samples and transferring them to MagNA Lyser® Green Bead Tubes (Roche, Mannheim, Germany). Negative controls for sample collection and DNA extraction were unused swabs and unused buffer tubes without swabs. Enzymatic lysis was conducted by incubating the samples with 2.5 µl lysozyme (100 mg/ml: Carl Roth, Karlsruhe, Germany) at 37 ºC for 1 hour. Then the QIAamp DNA microbiome kit (#51704, Qiagen, Germany) was used following the manufacturer's instructions. In 35 µl of the buffer from the kit, total DNA was eluted and stored at -20 ºC until polymerase chain reaction (PCR) amplification. All procedures were conducted under sterile conditions in a laminar flow unit (Patra *et al.*, 2024).

16s microbiome sequencing

16s rRNA library preparation, quantification and sequencing were performed according to Klymiuk et al (Klymiuk *et al.*, 2016). Five µl of total DNA were used for PCR amplification with the target-specific primers 27F 5' - AGAGTTGATCCTGGCTCAG - 3' and R357 5' - CTGCTGCCTYCCGTA - 3' (MWG, Ebersberg, Germany). After an initial denaturation at 95°C for 3 minutes, 32 cycles of denaturation at 95°C for 45 seconds, annealing at 55°C for 45 seconds and extension at 72°C for 1 minute were performed, followed by the final extension at 72°C for 7 minutes. The PCR reactions were conducted in triplicate and pooled. On a 1% agarose gel, 5 µl were visualized to verify the amplification process. According to Klymiuk et al (Klymiuk *et al.*, 2016) normalisation, indexing and pooling of PCR products and purification of the final library were performed. Library quantification was conducted with the QuantiFluor ONE dsDNA kit (Promega, Mannheim, Germany) as instructed by the manufacturer. The library was visualized on an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) with a high-sensitivity DNA assay according to manufacturer's instructions. Sequencing of the 6 pM library was performed with MiSeqII desktop sequencer (Illumina, Eindhoven, The Netherlands) using v3 chemistry for 600 cycles. Data analyses were conducted with 20% PhiX control DNA and FastQ files (Patra *et al.*, 2024).

16s bioinformatic analysis

Illumina paired-end raw sequencing data were controlled regarding quality and present sequencing adapters. Final sequence file analysis was performed with Quantitative Insights into Microbial Ecology (QIIME2 Version 2022.2) (Bolyen *et al.*, 2019). QUIIME2 is a bioinformatic pipeline used to analyse microbiome data in the local galaxy instance (https://galaxyproject.org/) hosted on the MedBioNode HPC cluster of the Medical University Graz in Austria (https://galaxy.medunigraz.at/). With DADA2 denoise pipeline and optimised parameters (Callahan *et al.*, 2016): p-trunc-len-f: 270, p-trunc-len-r: 230, p-trim-left-f: 20, p-trim-left-f: 15 and p-max-ee: 2.0 as implemented in QIIME2 microbiome bioinformatics platform, 1,701,562 raw sequence reads were quality-filtered, de-noised, de-replicated, merged and checked for chimeras. Comparing against the SILVA rRNA database Release 138 at 99% identity, taxonomic assignment of the DADA2 representative sequence set (ASV) was done with the QIIME2 sklearn-based classifier (Quast *et al.*, 2013). R 4.1.0 program for statistical computing (https://www.R-project.org) with the packages vegan, ggplot2, and psych or Microbiome analyst (Team RC, 2021) was used for all further downstream statistical data analyses, including alpha and beta diversity. Data were then uploaded to the European

Nucleotide Archive (ENA), supported by EMBL-EBI, and assigned the primary accession number PRJEB57775 (Patra *et al.*, 2024).

Serum cytokine analysis

Serum concentrations of 65 different cytokines were measured using ProcartaPlex human cytokine 65-plex kit from ThermoFisher Scientific. Twenty-five µl of undiluted serum samples were analysed in 96-well plates according to the manufacturer's instructions. The reference analyte concentration provided from the manufacturer was used to generate standard curves for each of the analytes. For measurements, calibrated Bio-Plex 200 system (Bio-Rad) in combination with Bio-Plex Manager software (version 6.1, Bio-Rad) were used. All samples were measured in duplicates and cytokine concentrations were calculated from the standard curve with 5PL curve fitting. Non-parametric longitudinal analysis was used to analyse raw measurements. False discovery rates (FDR) below 0.05 were assumed as statistically significant. Calculations were performed with R package nparLD (version 2.1). Logarithmic scale (log2 fold change) was used for effect size calculation. Groups effects were transformed to FI scale by exponentiation and delivered as percentages. The delta method was used to calculate standard errors of effects (Patra *et al.*, 2024).

Bacterial culture

Freeze-dried bacterial strains were re-established and grown for 18 hours in media. Optic density of 600 nm (nanometres) was collimated to ~ 1 for *Staphylococcus aureus* (ATCC/BAA-17717) in tryptic soy broth and 0.5 for *Acetobacter aceti* (DSMZ-3508) in yeast peptone mannitol. Afterwards storage at -80°C was conducted until further processing (Patra *et al.*, 2024).

Microplate Growth Curve Assay

Bacterial aliquots were thawed and serially diluted (1:10 2 times, 10^{-2}). One ml (millilitre) of this solution was consecutively diluted in 9 ml of medium containing 2.77 mg/l (milligrams/litre), 5.55 mg/l and 11.11 mg/l of Dupilumab (Dupixent®, Sanofi Genzyme) resulting in a final concentration of 2.5 mg/l, 5 mg/l and 10 mg/l Dupilumab, respectively. Control bacterial suspension without Dupilumab and 200 µl of each concentration were added to sterile Nunc 96 well microtiter plates (Thermo Fisher Scientific) and SPECTROstar omega (BMC labtech)

was used to measure the optical density at 600 nm in regular intervals for 48 hours (Patra *et al.*, 2024).

Statistical Analysis

Statistical analyses were performed with GraphPad Prism (GraphPad Software, 9.3) and R 4.1.0 program (Team RC, 2021). The performed statistical tests are explained in the figure legends. For determination of significance between groups in nonmetric multidimensional scaling, analysis of similarities for the microbiome analysis was performed with R (Patra *et al.*, 2024).

Results

Enrolled Patients and DAOSD Development

Twenty patients with moderate-to-severe AD receiving Dupilumab treatment (13 men, 7 women; mean age: 49 years, range: 20-87 years) and 10 healthy controls without AD not receiving Dupilumab treatment (5 men, 5 women; mean age: 34 years, range: 25-57 years) participated in the study. Six out of the 20 AD patients (30%) developed DAOSD within median time of 54 days (range: 28-103 days) after Dupilumab initiation. All these 6 patients showed blepharoconjunctivitis (Figures 4 and 5) and 2 of them also presented with keratitis. These 6 blepharoconjunctivitis patients showed swollen, erythematous, scaly eyelids, conjunctival injection and increased mucous secretion with symptoms like foreign body sensation, itching and burning. All of them were treated with preservative free lubricants and eyelid hygiene (eyelid margin cleansing, warm compresses, and lid massage) in addition to either topical calcineurin inhibitor therapy (tacrolimus eye ointment 0.03%, twice daily, applied to the periocular skin and into the inferior conjunctival fornices) or topical mast cell stabilizer/antihistamine combination therapy (0,25 mg/ml ketotifenfumarat). One of the two patients with corneal involvement presented with a small corneal infiltrate, which resolved under treatment with preservative free lubricants, antibiotics, and corticosteroids. The other patient showed superior limbitis with Horner-Trantas dots and responded well to preservative free lubricants, corticosteroids, and mast cell stabilizer/antihistamine combination treatment (Figure 6). All the 6 DAOSD patients could be adequately and effectively treated during the study period of 4 months. Every one of them showed complete remission of DAOSD and none of them had to discontinue Dupilumab treatment. Detailed patient and control characteristics are presented in Table 2 (Patra *et al.*, 2024).

	AD patients without DAOSD	AD patients with DAOSD	P value	Controls
Count (%)	14 (70)	6 (30)		10
Age (years, median) *	45	37	0.74	30
Gender: male/female (count) **	9/5	4/2	>0.99	5/5
History of ocular surface disease (%) **	6 (43)	3 (50)	>0.99	0
Ophthalmic therapy at baseline (%) **	4 (29)	2 (33)	>0.99	0
Asthma bronchiale (count) **	4	2	>0.99	0
Visit 1 – Baseline				
No. of patients (count)	14	6		10
EASI (median) *	33,2	27,2	0.43	NA
IGA (median) *	4	4	0.58	NA
Neutrophil Count (median) * ***	1	3	0.47	0
Visit 2 – after 1 month				
No. of patients (count)	11	5		NA
EASI (median) *	13,9	14,6	0.46	NA
IGA (median) *	3	3	0.91	NA
Neutrophil Count (median) * ****	4	7	0.69	NA
Visit 3 – after 4 months				
No. of patients (count)	12	6		NA
EASI (median) *	8,5	10,9	0.35	NA
IGA (median) *	2	3	0.91	NA
Neutrophil Count (median) *	10	65	<0.01	NA
Visit conj				
No. of patients (count)	NA	6		
EASI (median) *	NA	11		NA
IGA (median) *	NA	3		NA
Neutrophil Count (median) *	NA	40		NA

Table 2: Patient and control characteristics reproduced from (Patra *et al.*, 2024) with permission of publisher Elsevier, American Academy of Ophthalmology

(Abbreviations: AD = atopic dermatitis; DAOSD = Dupilumab-associated ocular surface disease.
EASI = Eczema area and severity index; IGA = Investigator Global Assessment; NA = not applicable)

*Wilcoxon rank sum test
**Fisher's Exact Test for Count Data
***not enough sample for one AD patient without DAOSD (13 patients/6 patients)
****not enough sample for one AD patient with DAOSD (13 patients/6 patients)

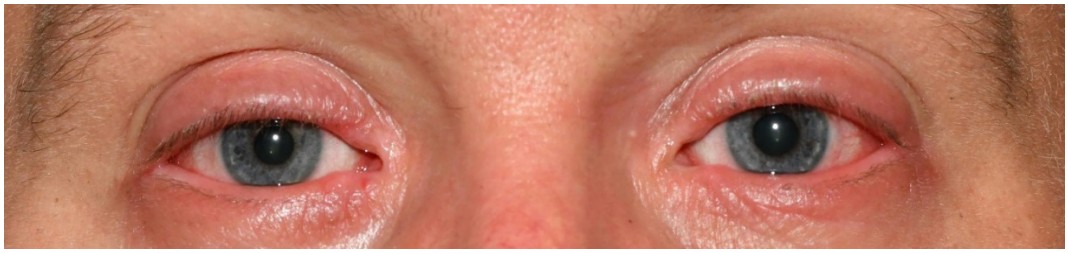


Figure 4: 45-year-old male patient showing blepharconjunctivitis (swollen, erythematous eyelids, conjunctival hyperemia)

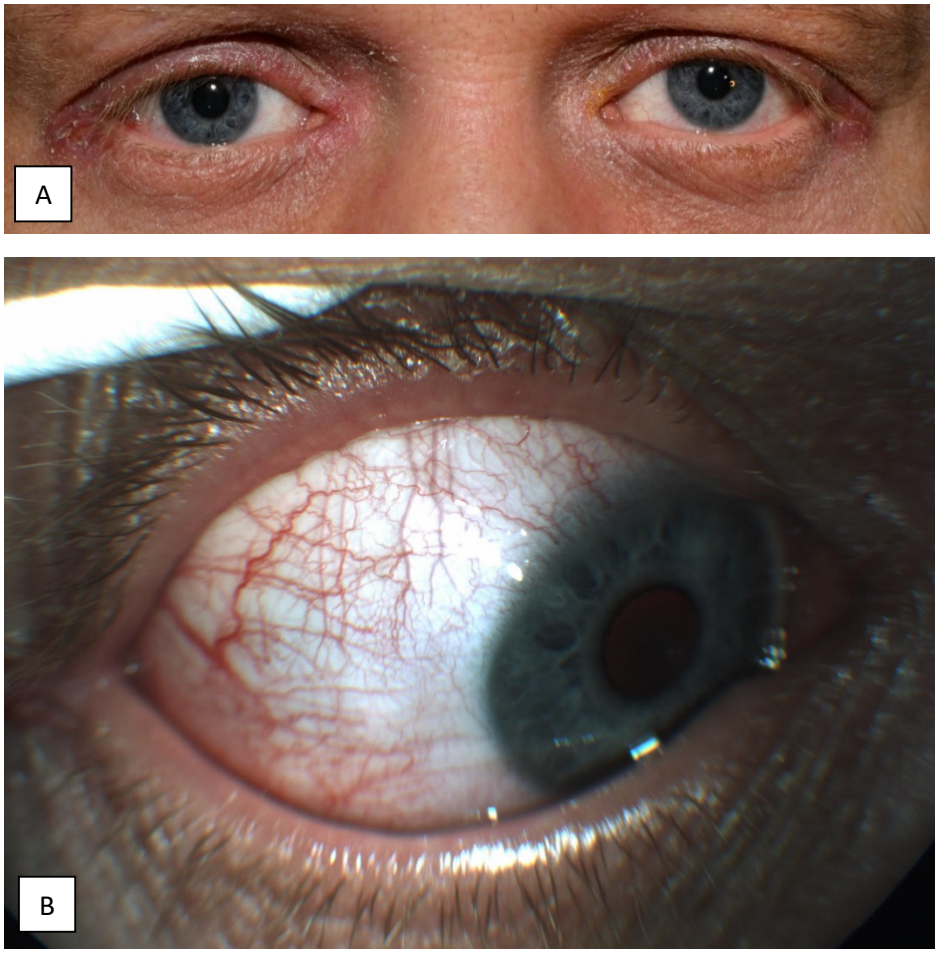


Figure 5: 38-year-old male patient presenting with blepharoconjunctivitis
A – swollen, erythematous, scaly eyelids
B – right eye enlarged showing distinct conjunctival hyperemia

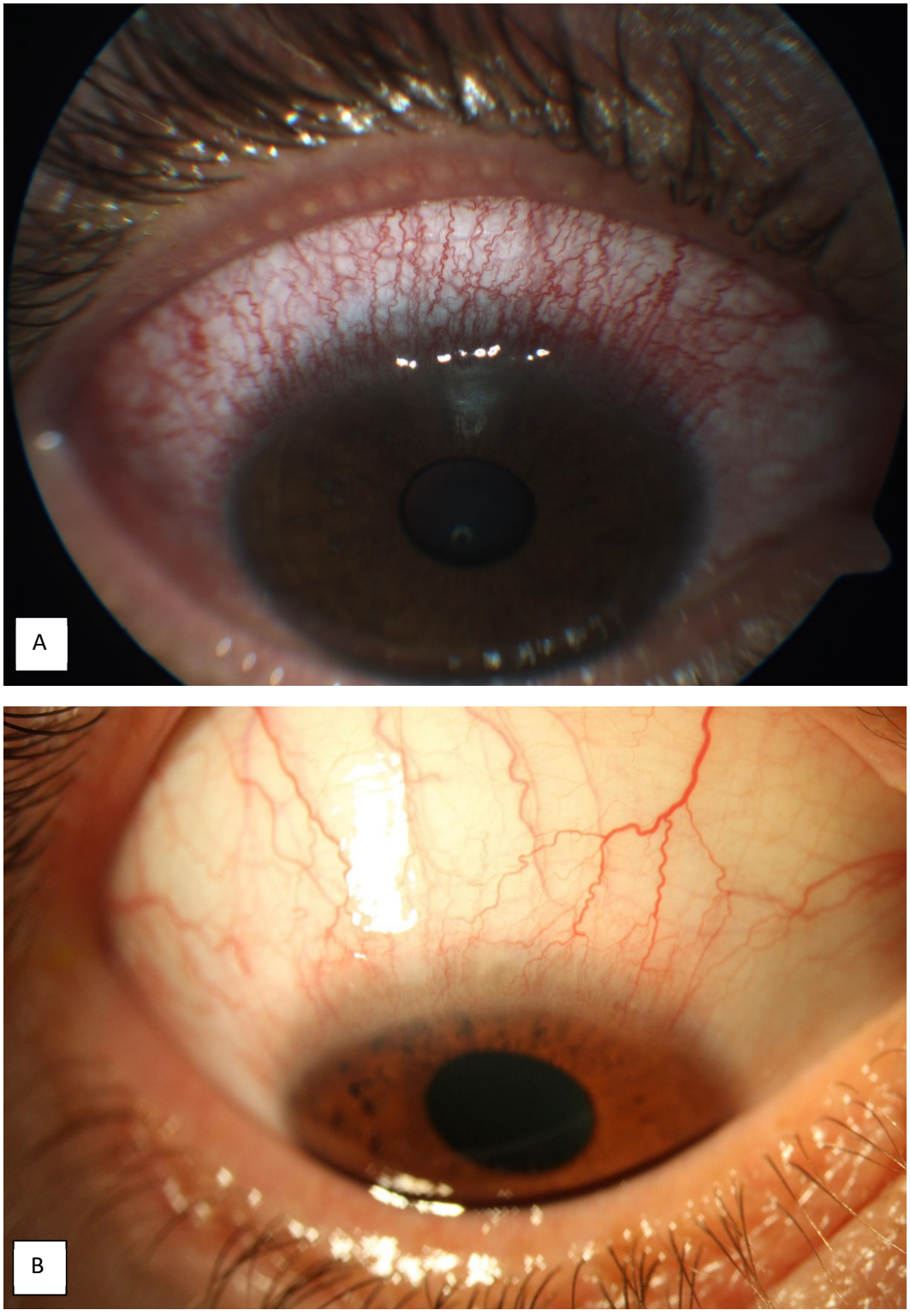


Figure 6: 36-year-old male patient, right eye showing superior limbitis
A - superior limbitis with Horner-Trantas dots (January 29th, 2020);
B - good response to preservative free lubricants, corticosteroids and mast cell stabilizer/antihistamine combination treatment (February 5th, 2020)

AD Treatment Response

The median baseline EASI score of all the 20 enrolled AD patients was 30.2 (range 16 - 52.2). There was no significant difference regarding EASI at baseline between the AD patients developing DAOSD and the AD patients not developing DAOSD (p-value 0.43) (Patra *et al.*, 2024).

The median baseline EASI score of the 14 AD patients not developing DAOSD was 33.2 (range 16 – 52.2), decreasing to 13.9 (range 0.9 – 29.7) at visit 1m and 8.5 (range 0.3 – 35.3) at visit 4m. Compared with baseline EASI this was a significant decrease at visit 1m and visit 4m (p-value < 0.001, respectively) (Figure 7). EASI75 (defined as 75% reduction from baseline EASI score) could be achieved in 3 out of 11 patients (27%) at visit 1m, and 8 out of 12 patients (67%) at visit 4m (Patra *et al.*, 2024).

The median baseline EASI score of the 6 AD patients developing DAOSD was 27.1 (range 17.2 – 43.9), decreasing to 14.6 (range 6.6 – 31.8) at visit 1m and 10.9 (range 3.6 – 25.0) at visit 4m. Compared with baseline EASI there was no significant decrease at visit 1m, however, there was a significant decrease at visit 4m (p-value < 0.001). Median EASI at visit conj was 11 (range 4.1 – 23.1), this was a significant decrease compared with baseline (p-value < 0.001). None of the 6 AD patients developing DAOSD reached EASI75 at visit 1m, however, 1 patient (17%) reached EASI75 at visit conj and 2 patients (33%) at visit 4m (Patra *et al.*, 2024).

A significant IGA reduction was only present in AD patients not developing DAOSD (Patra *et al.*, 2024).

AD patients who started Dupilumab treatment in summer and early fall more frequently developed DAOSD, however, there was no statistical significance regarding seasonal distribution (p-value 0.12) (Figure 8) (Patra *et al.*, 2024).

Overall, in the cohort presented herein, AD patients developing DAOSD showed a delayed Dupilumab treatment response compared with AD patients not developing DAOSD (Patra *et al.*, 2024).

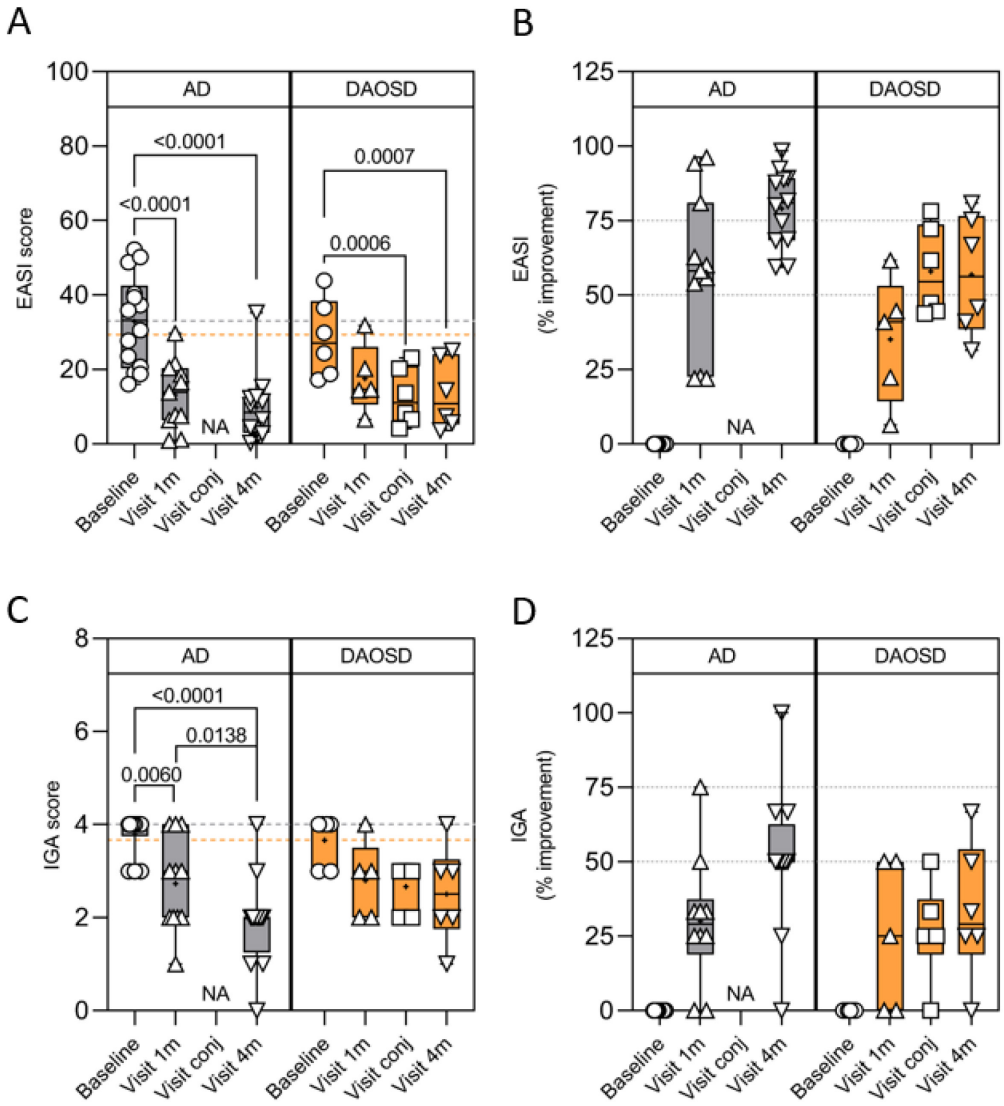


Figure 7: Treatment response monitored with EASI and IGA reproduced from (Patra *et al.*, 2024) with permission of publisher Elsevier, American Academy of Ophthalmology
(A and C showing absolute EASI and IGA values at different visits; B and D showing EASI improvement in % and IGA improvement in % during study period)
Box and whisker plots show minimum, median and maximum values; diminutive + stands for mean values; gray dotted lines show mean values at baseline in AD group and orange dotted lines show mean values at baseline in DAOSD group; n = 6 – 14; one-way analysis of variance was applied; NA stands for “not applicable”

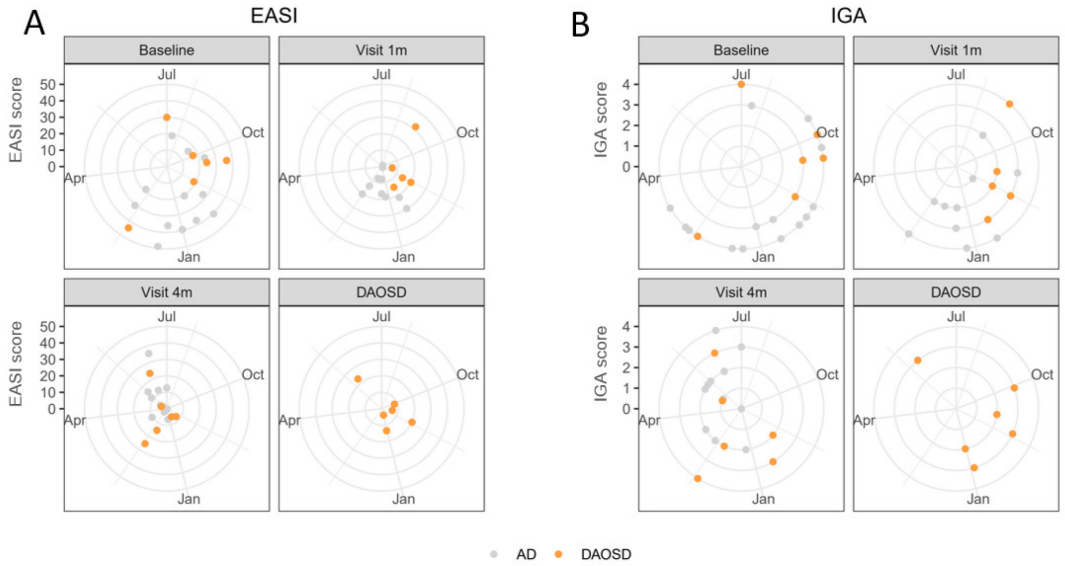


Figure 8: Seasonal effects regarding EASI and IGA scoring and DAOSD development reproduced from (Patra *et al.*, 2024) with permission of publisher Elsevier, American Academy of Ophthalmology
The scale on the left hand side labels the plot's circles starting at the center

Conjunctival Neutrophilic Infiltration in AD Patients with DAOSD

A massive cellular infiltrate mainly consisting of neutrophilic granulocytes could be observed in H&E stained conjunctival smears taken from the inferior fornix of the right eye in DAOSD patients. Neutrophilic infiltration reached statistical significance at visit conj (p-value < 0.05) and at visit 4m (p-value < 0.01) (Figure 9) (Patra *et al.*, 2024).

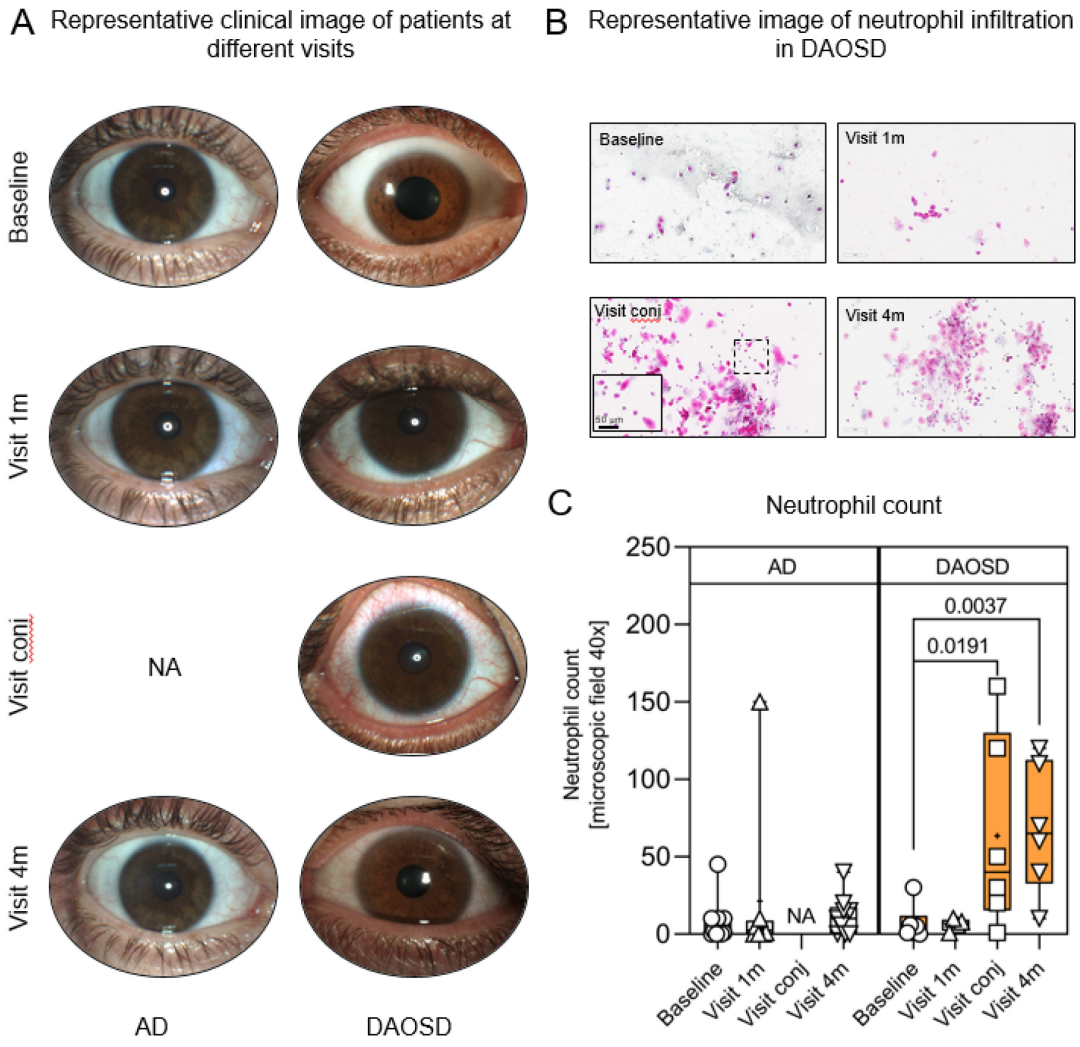


Figure 9: Neutrophil infiltration in conjunctival smears reproduced from (Patra *et al.*, 2024) with permission of publisher Elsevier, American Academy of Ophthalmology
A – macroscopic images of the ocular surface from AD patients not developing DAOSD (AD) and AD patients developing DAOSD (DAOSD) at different visits; B – representative photographs from conjunctival smears showing development of neutrophil infiltration over different visits in a DAOSD patient; C – Box and whisker plots presenting neutrophil count showing minimum, median and maximum values; diminutive + indicating mean values; n = 6 – 14; one-way analysis of variance was applied; NA stands for “not applicable”

Ocular Microbiome in AD Patients with DAOSD

Microbiome sequencing was performed in conjunctival swabs taken from the inferior fornix of the right eye of all patients and controls. After combining all sequence data sets of bacterial 16S amplicon sequencing 2 332 922 reads were yielded. Pre-processing was performed resulting in 1 701 562 reads (73%), depicting average 47 610.65 ± 17 137.13 reads per sample. Sufficient sequencing depth was achieved as all samples reached a plateau at 7000 reads/samples for the rarefaction. Reads were then allocated to 3370 amplicon sequence variants (ASVs). Average lengths of bases was 307.6 ± 17.01. Two samples had to be excluded from further analyses because of their low number of reads (Patra *et al.*, 2024).

Similar median ASV values were observed at baseline in AD patients not developing DAOSD, AD patients developing DAOSD and controls. AD patients not developing DAOSD showed a significant ASV number decrease at visit 1m (p-value = 0.001) and visit 4m (p-value < 0.01) compared to baseline. AD patients developing DAOSD, however, did not show a significant ASV number decrease at any time point compared with baseline (Figure 10A) (Patra *et al.*, 2024).

Shannon index analyses indicating the bacterial diversity showed similar results (Figure 10B). There was a significant decrease in bacterial diversity in the AD patients not developing DAOSD at visit 1m and visit 4m (p-value < 0.05, respectively) compared to baseline. However, AD patients developing DAOSD did not show significant differences between any time points. Interestingly, control subjects presented with slightly lower median levels of bacterial diversity compared with AD patients developing or not developing DAOSD at baseline, and AD patients not developing DAOSD and AD patients developing DAOSD showed similar ASV numbers at baseline, however, AD patients developing DAOSD showed slightly higher median levels of bacterial diversity at baseline compared with AD patients not developing DAOSD. Shannon index decrease correlated with neutrophilic infiltration increase at visit conj (Figure 11) (Patra *et al.*, 2024).

Consecutively, a nonmetric multidimensional scaling analysis based on Bray-Curtis dissimilarity and analysis of similarities statistical testing between different time points was conducted (Figure 10C). An r-value of 0.0045 and a p-value of 0.4448 were identified for AD patients not developing DAOSD compared to an r-value of 0.1246 and a p-value of 0.0496 in AD patients developing DAOSD. So, there were significant differences within the clusters between different time points and also much larger dissimilarities among the bacterial communities in AD patients developing DAOSD (Patra *et al.*, 2024).

Taken together, our data sets showed that AD patients not developing DAOSD presented with a significant ocular microbial diversity reduction after Dupilumab treatment at visit 4m and that

in contrast, AD patients developing DAOSD showed persistently high ocular microbial diversity (Patra *et al.*, 2024).

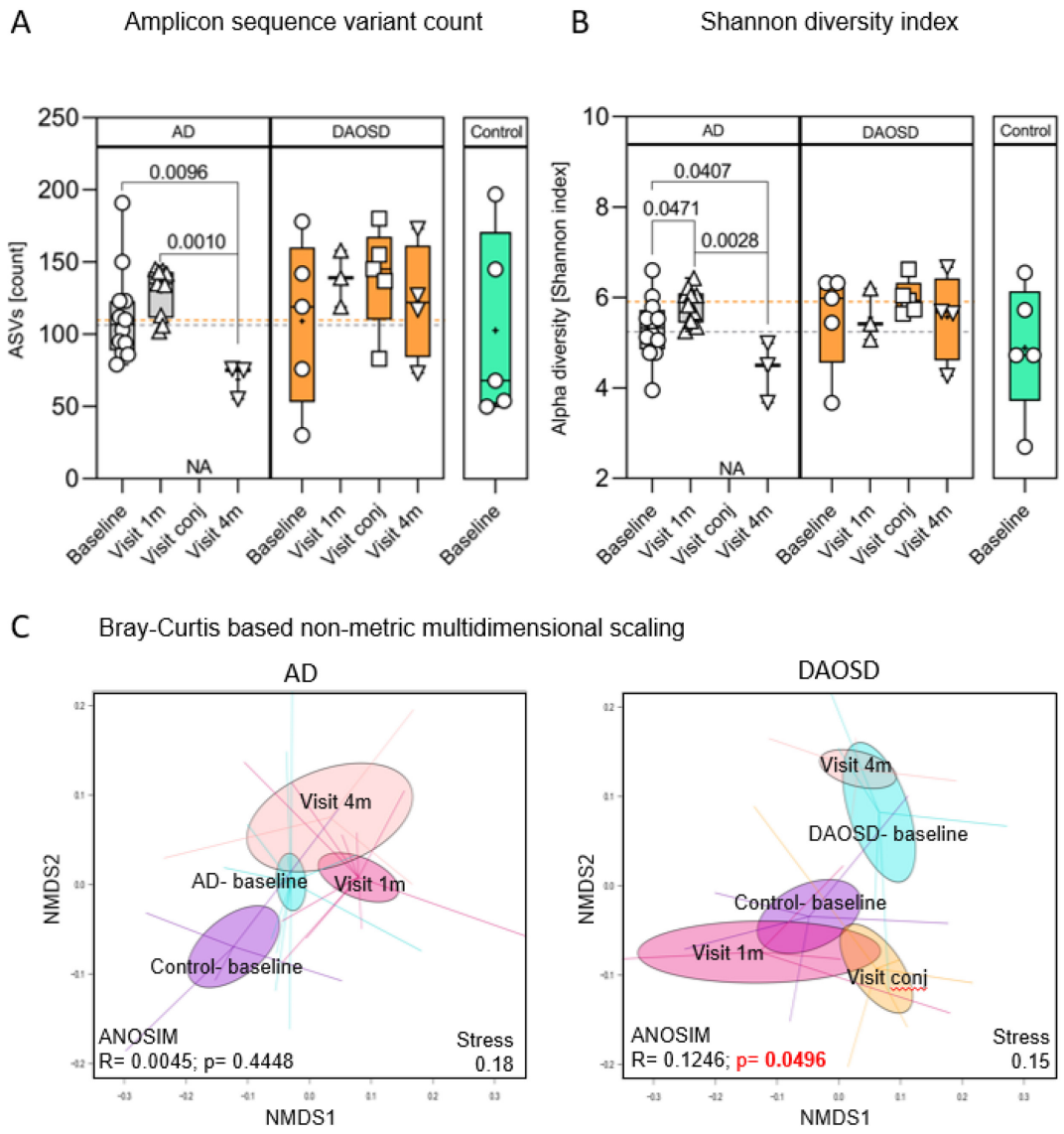
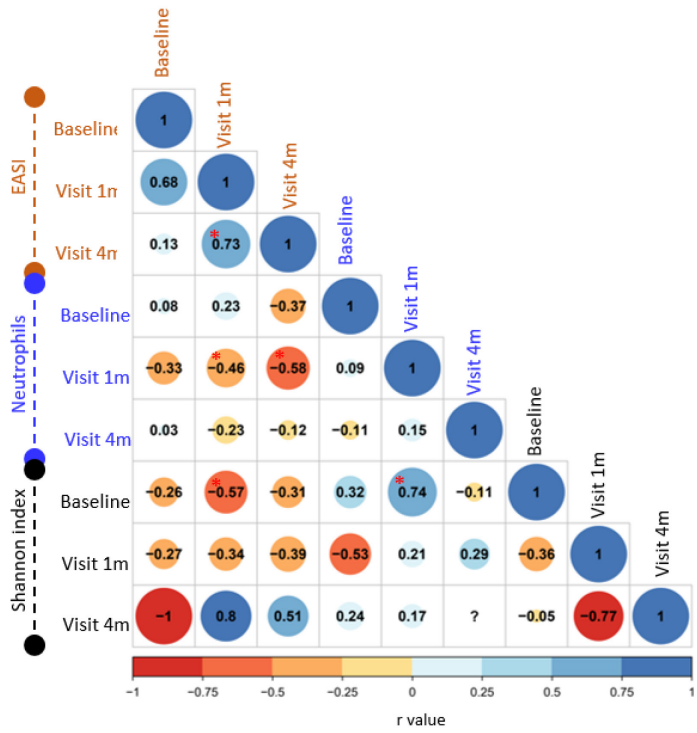
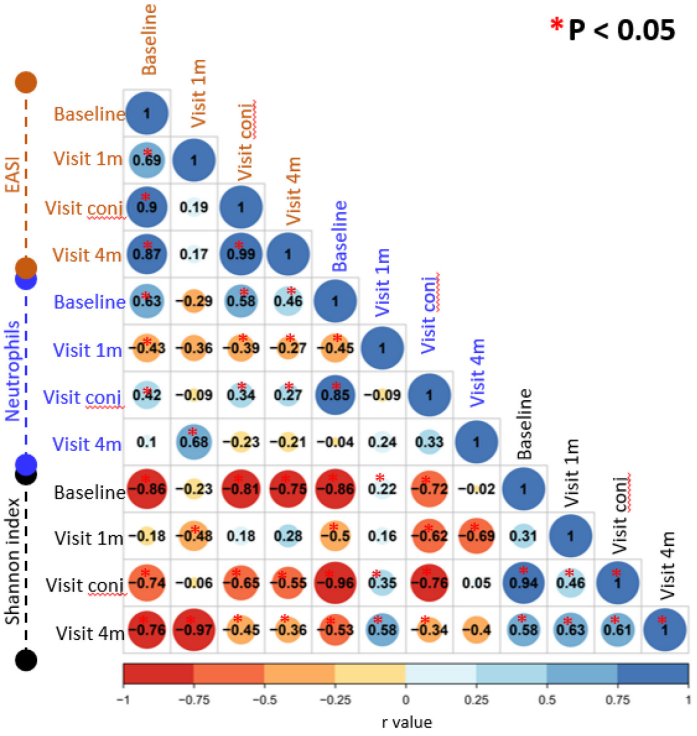


Figure 10: Alterations of the ocular surface microbiome during the study period reproduced from (Patra *et al.*, 2024) with permission of publisher Elsevier, American Academy of Ophthalmology
A – Amplicon sequence variant counts (ASVs) and B – Shannon diversity index, both analysed with sequence analysis from conjunctival swabs
Box and whisker plots showing minimum, median and maximum values; diminutive + indicating mean values; gray dotted lines show mean values at baseline in AD group and orange dotted lines show mean values at baseline in DAOSD group;n = 3 – 14; one-way analysis of variance was applied
C – Bray-Curtis dissimilarity-based nonmetric multidimensional scaling (NMDS) plots for AD patients not developing DAOSD (AD) and AD patients developing DAOSD (DAOSD).
Ellipses are based on standard error of the mean; analysis of similarities method (ANOSIM) was applied; NA stands for “not applicable”

AD


Baseline
Visit 1m
Visit 4m
Baseline
Visit 1m
Visit 4m
Baseline
Visit 1m
Visit 4m
EASI
Neutrophils
Shannon index
r value

DAOSD


*P < 0.05
Baseline
Visit 1m
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Visit 4m
Baseline
Visit 1m
Visit coni
Visit 4m
Baseline
Visit 1m
Visit coni
Visit 4m
EASI
Neutrophils
Shannon index
r value

Figure 11: Correlation analysis of EASI and neutrophil count and Shannon diversity index reproduced from (Patra *et al.*, 2024) with permission of publisher Elsevier, American Academy of Ophthalmology
performed with R studio (RStudio 2021.09.0+351 “Ghost Orchid” Release) and “corrplot” function; variables used were EASI scores, neutrophil counts from conjunctival smears and Shannon diversity indices; r value ranging from -1 to 1, indicating either negative or positive correlation; statistically significant values are marked with *

Species-Level Characterization

Species-level characterization was conducted as previously described (Madhusudhan *et al.*, 2020). Changes regarding the abundance of the genera *Staphylococcus*, *Cutibacterium* and *Corynebacterium* were found in AD patients not developing DAOSD, whereas increased colonization with *Acetobacter*, *Staphylococcus* and *Cutibacterium* according to disease severity were present in AD patients developing DAOSD (Figure 12 – 24) (Patra *et al.*, 2024). To examine differential abundant features of the microbial species, the Linear discriminant analysis Effect Size tool (Segata *et al.*, 2011) was used. AD patients not developing DAOSD showed alterations in abundance of certain species at different time points (baseline, visit 1m, visit 4m) (Figure 13). Interestingly, *Staphylococcus aureus* was significantly less abundant at visit 1m and visit 4m compared to baseline (p-value <0.05, respectively) (Figure 17) (Patra *et al.*, 2024).

In AD patients developing DAOSD, *Staphylococcus aureus* was only observed in 2 out of 6 cases without any alterations regarding abundance between visits. None of the control patients presented with *Staphylococcus aureus* colonization (Figure 17) (Patra *et al.*, 2024).

Furthermore, we observed a decrease in abundance of certain species in AD patients not developing DAOSD over time, whereas abundance of these species increased in AD patients developing DAOSD. Abundance of *Cutibacterium granulosum* was significantly decreased in AD patients not developing DAOSD at visit 4m compared to baseline (p-value < 0.05), whereas it increased in DAOSD patients at visit conj (Figure 20). There was also a decreasing trend of *Liquorilactobacillus mali* in AD patients not developing DAOSD compared to an increasing trend in AD patients developing DAOSD (Figure 21) (Patra *et al.*, 2024).

Five different species were significantly differently abundant over time in AD patients developing DAOSD. Most notably, the abundance of *Acetobacter aceti* was significantly increased at visit conj compared to baseline (p-value < 0.05), whereas *Acetobacter aceti* remained stable over time in AD patients not developing DAOSD (Figure 22). Furthermore, AD patients developing DAOSD showed a significantly increased abundance of *Staphylococcus capitis* at visit 4m compared with baseline (p-value < 0.05), whereas this species did not show significantly increased abundance at any time point in AD patients not developing DAOSD (Figure 22). The abundance of *Staphylococcus caprae* was also significantly increased at visit 1m compared to baseline in AD patients developing DAOSD (p-value < 0.05) and there were no changes at the following visits. In AD patients not developing DAOSD, there were no changes in abundance of *Staphylococcus caprae* during the study period (Figure 23). We could also identify microbes showing decreasing abundance over time as compared with baseline in AD patients developing DAOSD and in AD patients not developing DAOSD, namely

Diaphorobacter ruginosibacter and *Liquorilactobacillus ghanesis* (Figure 23 and 24) (Patra *et al.*, 2024).

As high concentrations of Dupilumab in tear fluid of AD patients developing DAOSD have been reported (R. E. Achten *et al.*, 2023), we generated the hypothesis that certain microbial species, such as *Acetobacter aceti*, might be able to adapt to high concentrations of Dupixent® (Dupilumab and further ingredients), while others like *Staphylococcus aureus* might not. To test this hypothesis, we cultured *Acetobacter aceti* and *Staphylococcus aureus* in different concentrations of Dupixent® (2.5 mg/L, 5 mg/L, 10 mg/L) and monitored their growth over time for 48 hours (Figure 25). Interestingly, *Acetobacter aceti* showed a dose-dependent increasing growth depicted in growth curves and area under the curve for 0 to 48 hours, while the opposite, namely a decrease could be observed for *Staphylococcus aureus* (Patra *et al.*, 2024).

To summarize, we observed different microbial species showing increasing or decreasing abundance over time in AD patients developing DAOSD and in those not developing DAOSD and we could show that certain microbial species, such as increase of *Acetobacter aceti*, could participate in DAOSD development (Patra *et al.*, 2024).

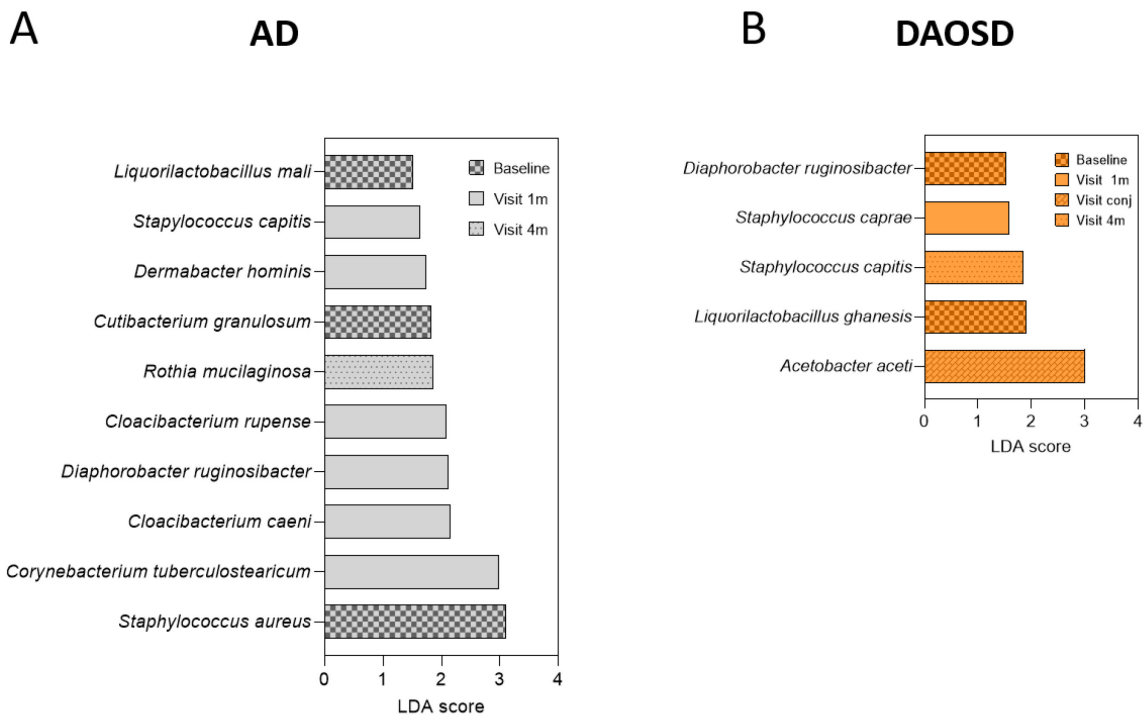

Figure 12: Significantly enriched microbial species at certain time points in A - AD patients not developing DAOSD (AD) and B - AD patients developing DAOSD (DAOSD) reproduced from (Patra *et al.*, 2024) with permission of publisher Elsevier, American Academy of Ophthalmology

Linear discriminant analysis Effect Size tool was used, Linear discriminant analysis (LDA) scores were determined

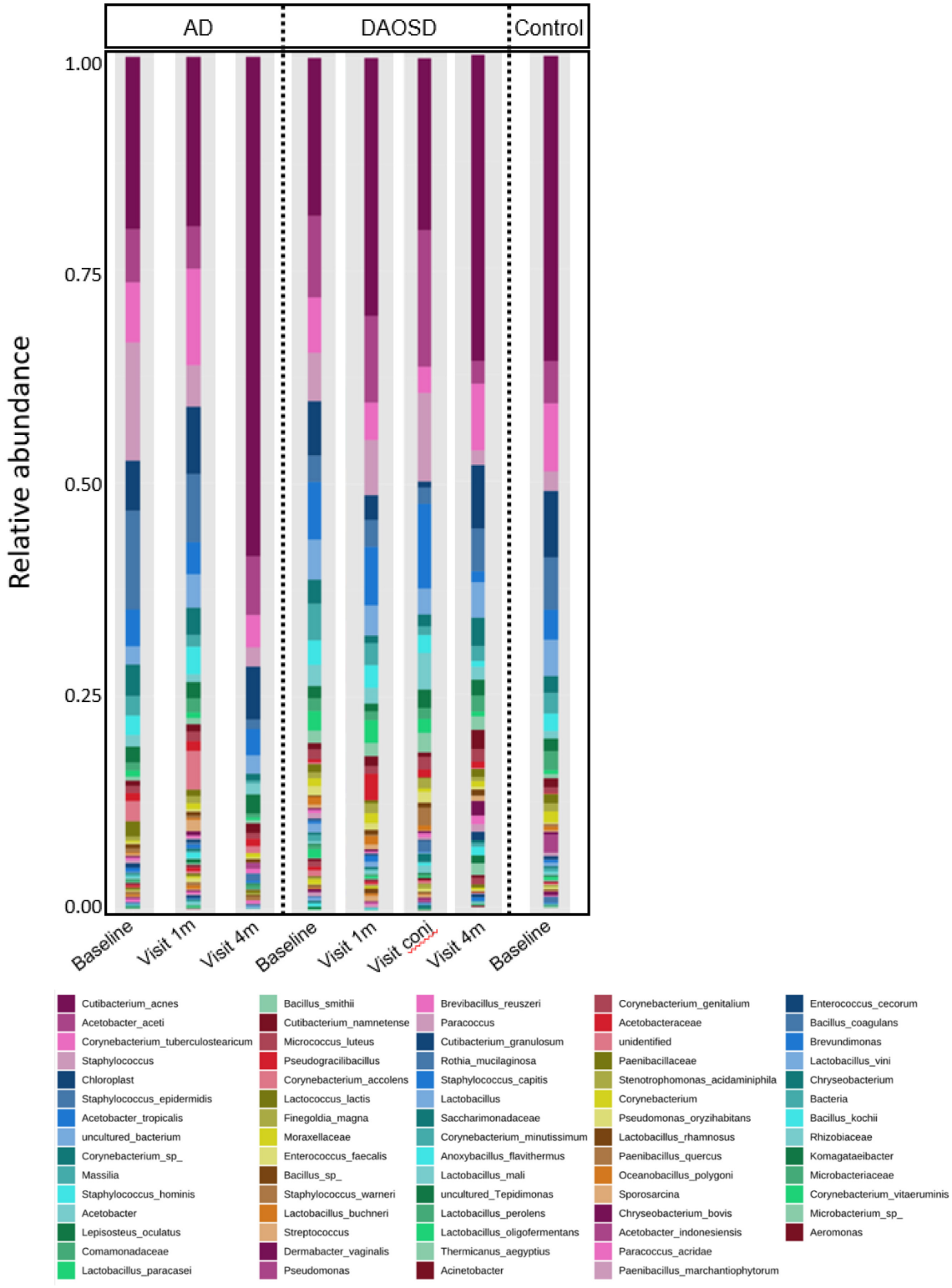

Figure 13: Stacked bar plot showing relative abundance of different microbial species at different time points in AD patients not developing DAOSD (AD), AD patients developing DAOSD (DAOSD) and control subjects reproduced from (Patra *et al.*, 2024) with permission of publisher Elsevier, American Academy of Ophthalmology

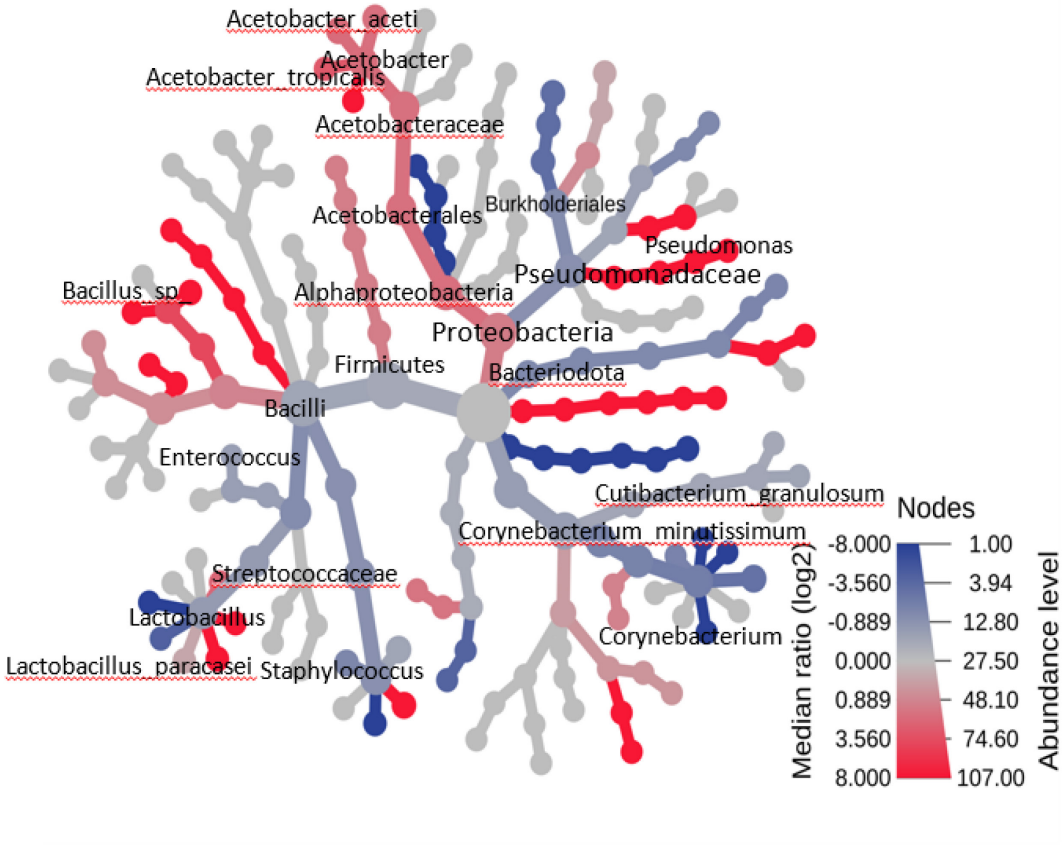

Figure 14: Heat tree showing the relative abundance of various microbial species in AD patients developing DAOSD between “visit conj” and “baseline” reproduced from (Patra *et al.*, 2024) with permission of publisher Elsevier, American Academy of Ophthalmology
Color changes illustrate the difference in $\log_2$ ratio of median proportions of reads in respective comparisons

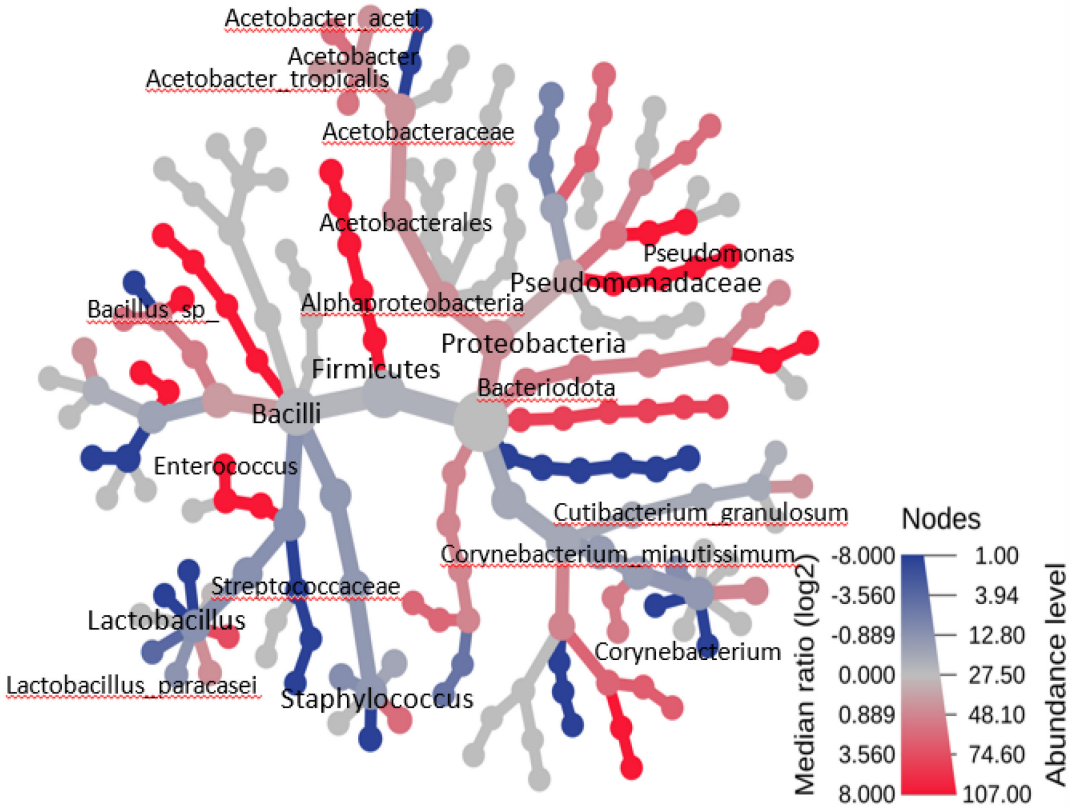

Figure 15: Heat tree showing the relative abundance of various microbial species in AD patients developing DAOSD between “visit conj” and “visit 1m” reproduced from (Patra *et al.*, 2024) with permission of publisher Elsevier, American Academy of Ophthalmology
Color changes illustrate the difference in $\log_2$ ratio of median proportions of reads in respective comparisons

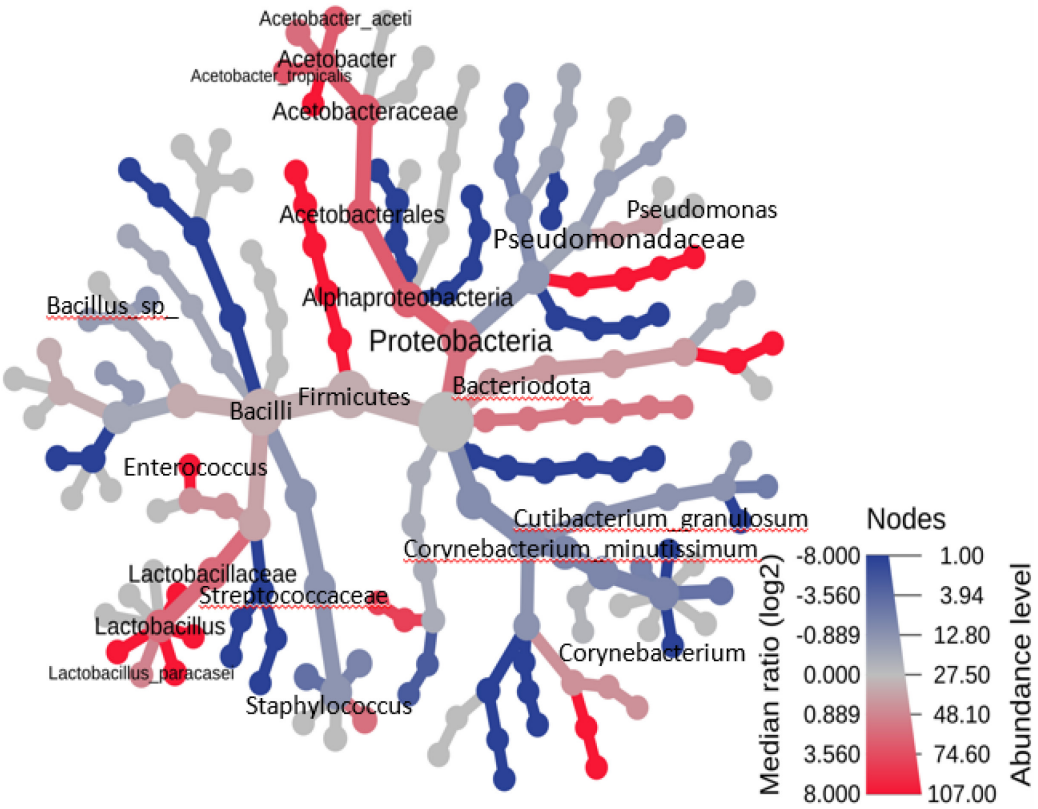

Figure 16: Heat tree showing the relative abundance of various microbial species in AD patients developing DAOSD between “visit conj” and “visit 4m” reproduced from (Patra *et al.*, 2024) with permission of publisher Elsevier, American Academy of Ophthalmology
Color changes illustrate the difference in $\log_2$ ratio of median proportions of reads in respective comparisons

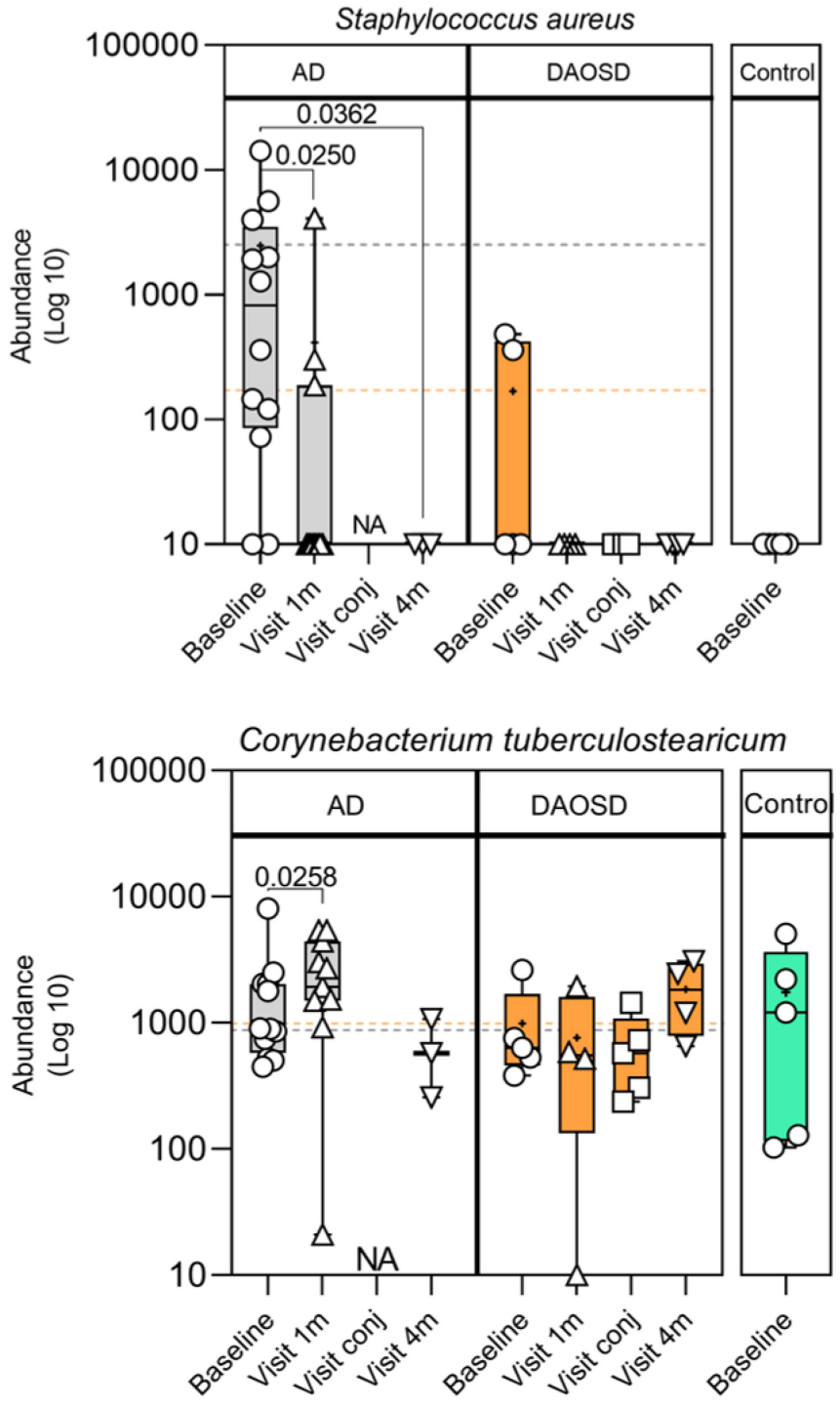

Figure 17: *Staphylococcus aureus* and *Corynebacterium tuberculostearicum*, significantly differentially abundant microbial species at different time points in AD patients not developing DAOSD (AD) reproduced from (Patra *et al.*, 2024) with permission of publisher Elsevier, American Academy of Ophthalmology

Box and whisker plots presenting microbial species in AD patients not developing DAOSD (AD), in AD patients developing DAOSD (DAOSD) and controls showing minimum, median and maximum values; diminutive + indicating mean values; n = 6 – 14; one-way analysis of variance was applied; NA stands for “not applicable”

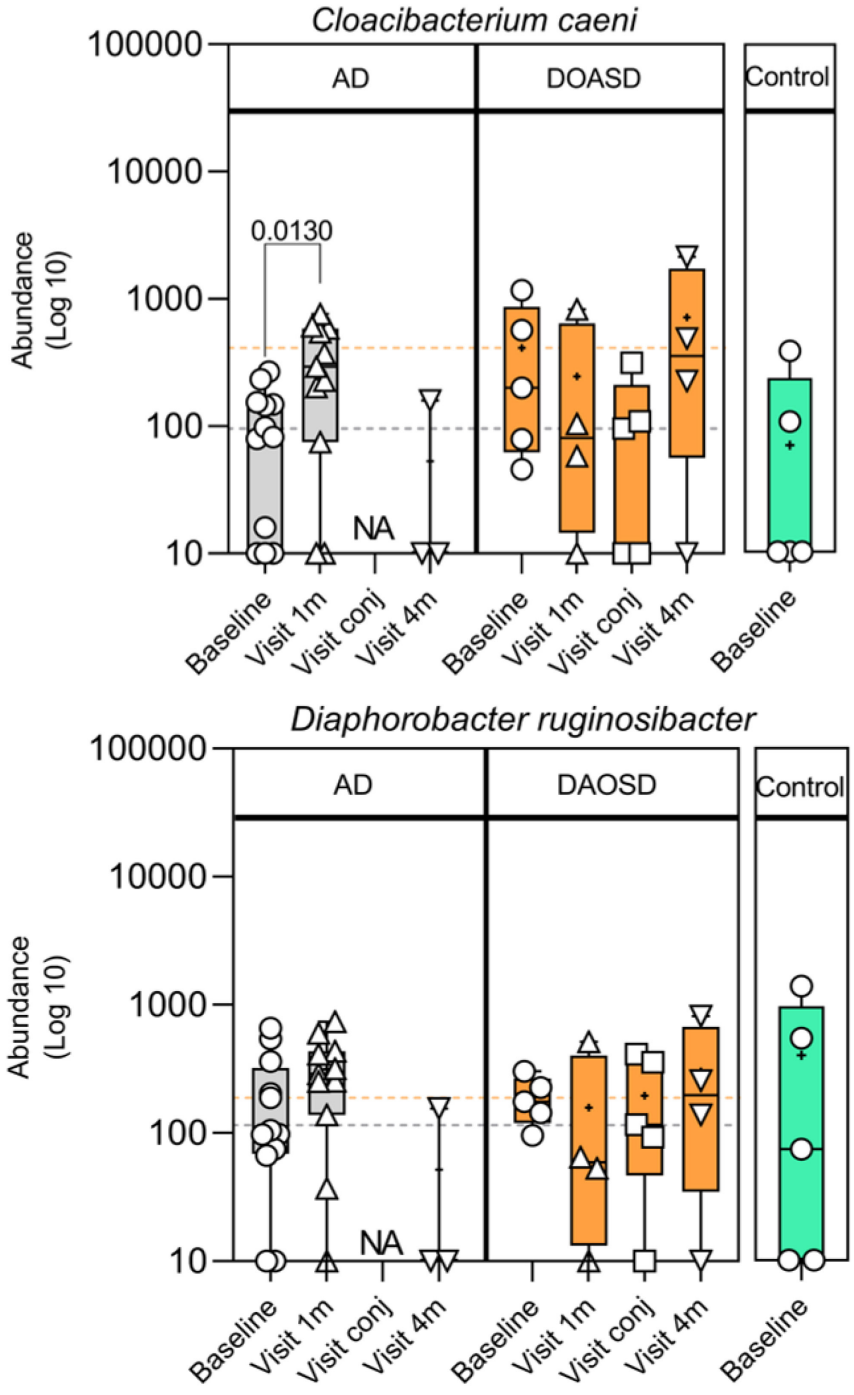

Figure 18: *Cloacibacterium caeni* and *Diaphorobacter ruginosibacter*, significantly differentially abundant microbial species at different time points in AD patients not developing DAOSD (AD) reproduced from (Patra *et al.*, 2024) with permission of publisher Elsevier, American Academy of Ophthalmology

Box and whisker plots presenting microbial species in AD patients not developing DAOSD (AD), in AD patients developing DAOSD (DAOSD) and controls showing minimum, median and maximum values; diminutive + indicating mean values; n = 6 – 14; one-way analysis of variance was applied; NA stands for “not applicable”

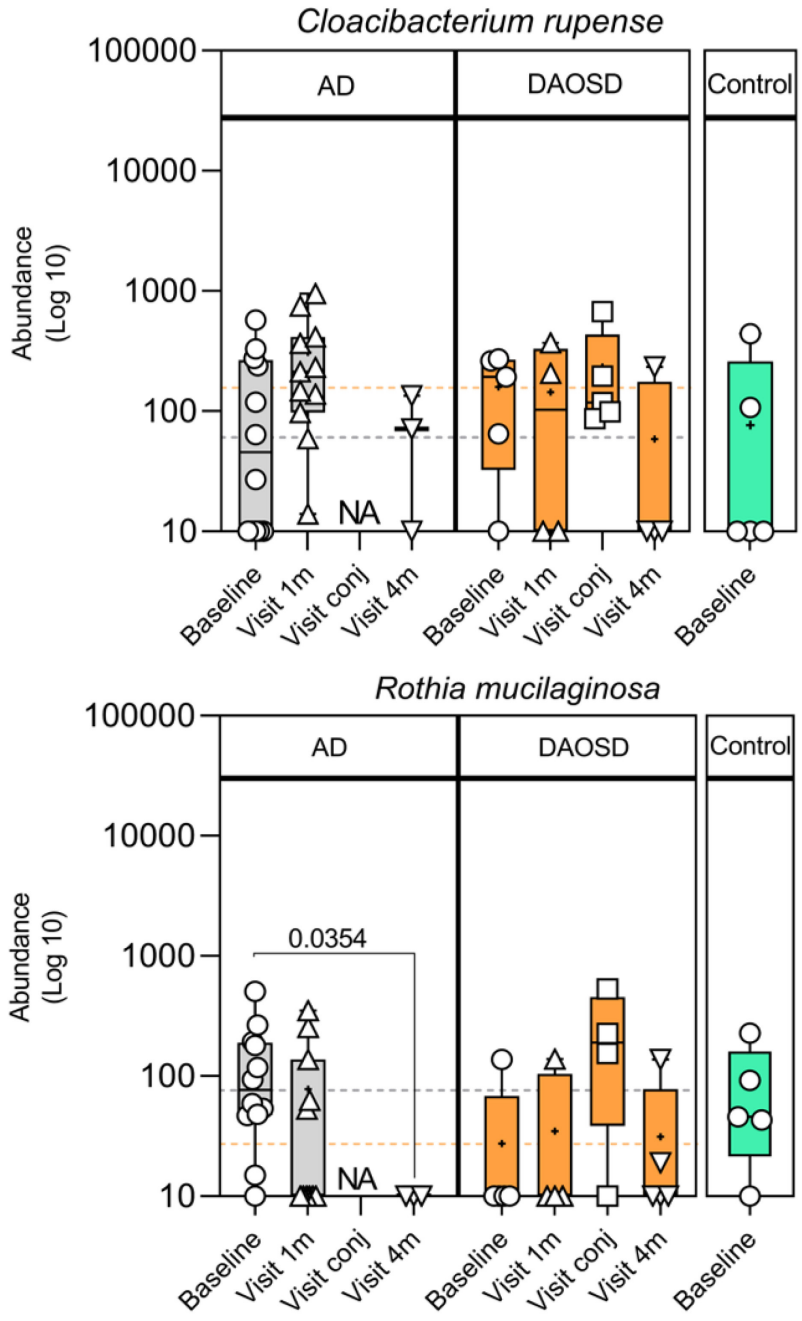

Figure 19: *Cloacibacterium rupense* and *Rothia mucilaginosa*, significantly differentially abundant microbial species at different time points in AD patients not developing DAOSD (AD) reproduced from (Patra *et al.*, 2024) with permission of publisher Elsevier, American Academy of Ophthalmology

Box and whisker plots presenting microbial species in AD patients not developing DAOSD (AD), in AD patients developing DAOSD (DAOSD) and controls showing minimum, median and maximum values; diminutive + indicating mean values; n = 6 – 14; one-way analysis of variance was applied; NA stands for “not applicable”

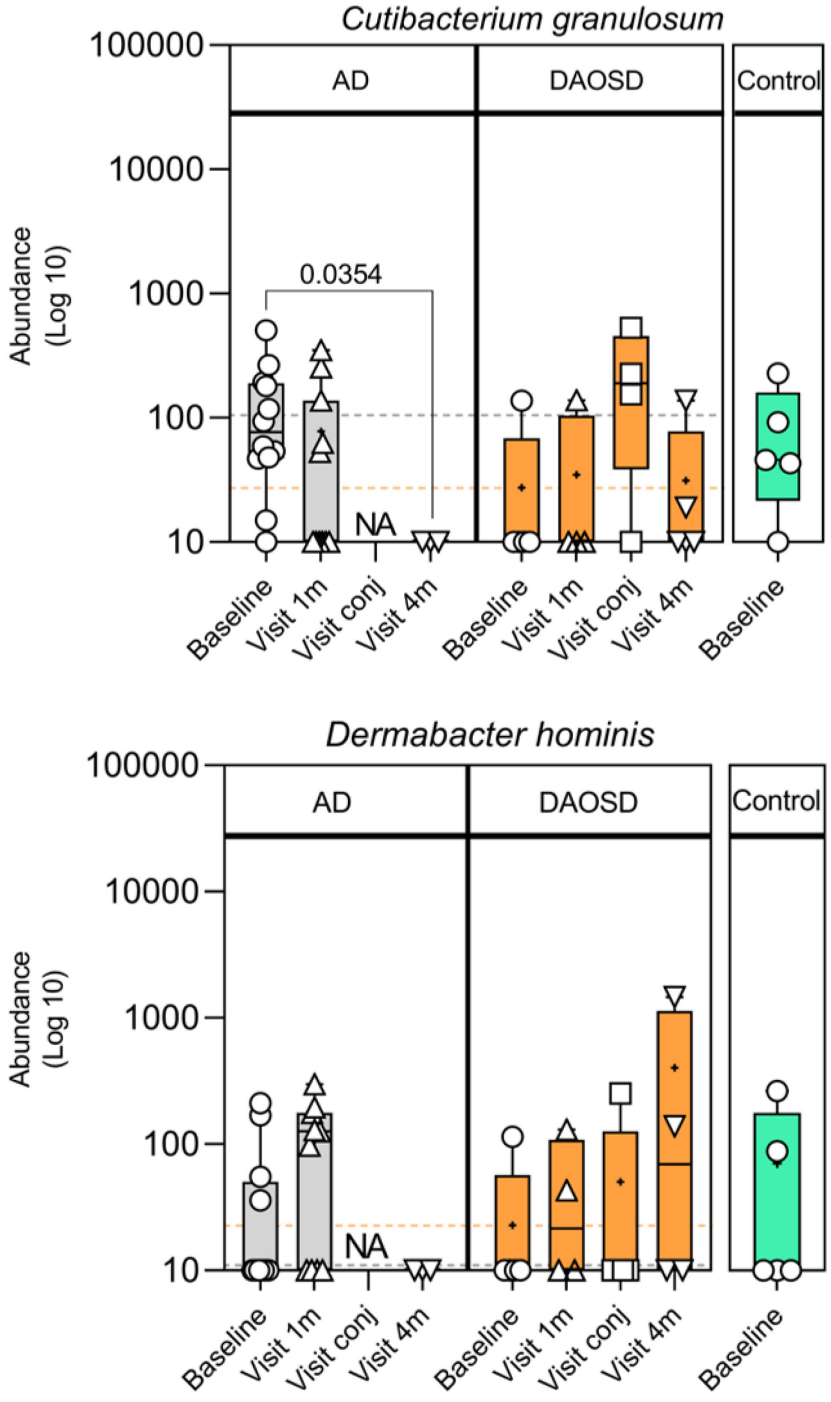

Figure 20: *Cutibacterium granulosum* and *Dermabacter hominis*, significantly differentially abundant microbial species at different time points in AD patients not developing DAOSD (AD) reproduced from (Patra *et al.*, 2024) with permission of publisher Elsevier, American Academy of Ophthalmology

Box and whisker plots presenting microbial species in AD patients not developing DAOSD (AD), in AD patients developing DAOSD (DAOSD) and controls showing minimum, median and maximum values; diminutive + indicating mean values; n = 6 – 14; one-way analysis of variance was applied; NA stands for “not applicable”

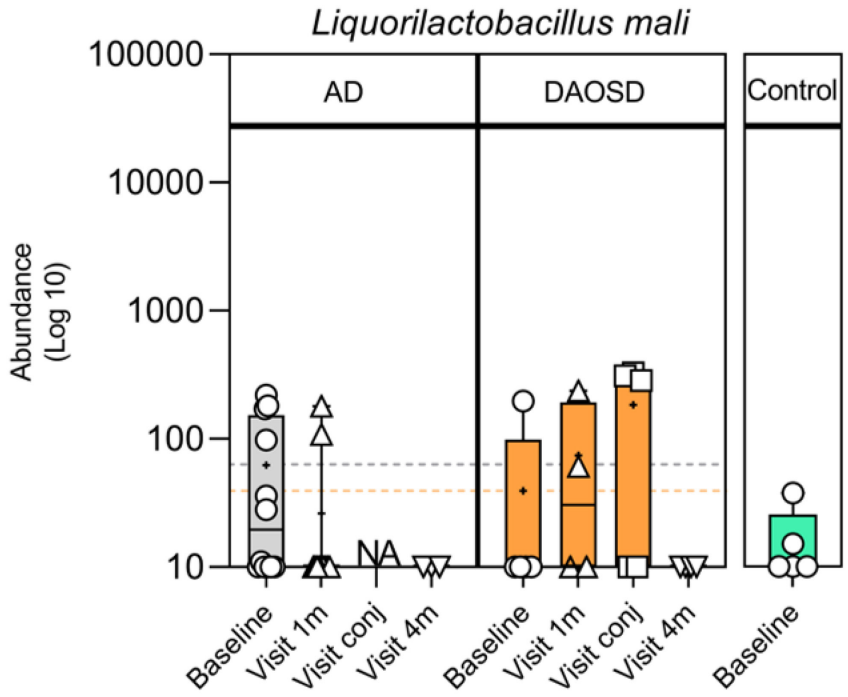

Figure 21: *Liquorilactobacillus mali*, significantly differentially abundant microbial species at different time points in AD patients not developing DAOSD (AD) reproduced from (Patra *et al.*, 2024) with permission of publisher Elsevier, American Academy of Ophthalmology
Box and whisker plots presenting microbial species in AD patients not developing DAOSD (AD), in AD patients developing DAOSD (DAOSD) and controls showing minimum, median and maximum values; diminutive + indicating mean values; n = 6 – 14; one-way analysis of variance was applied; NA stands for “not applicable”

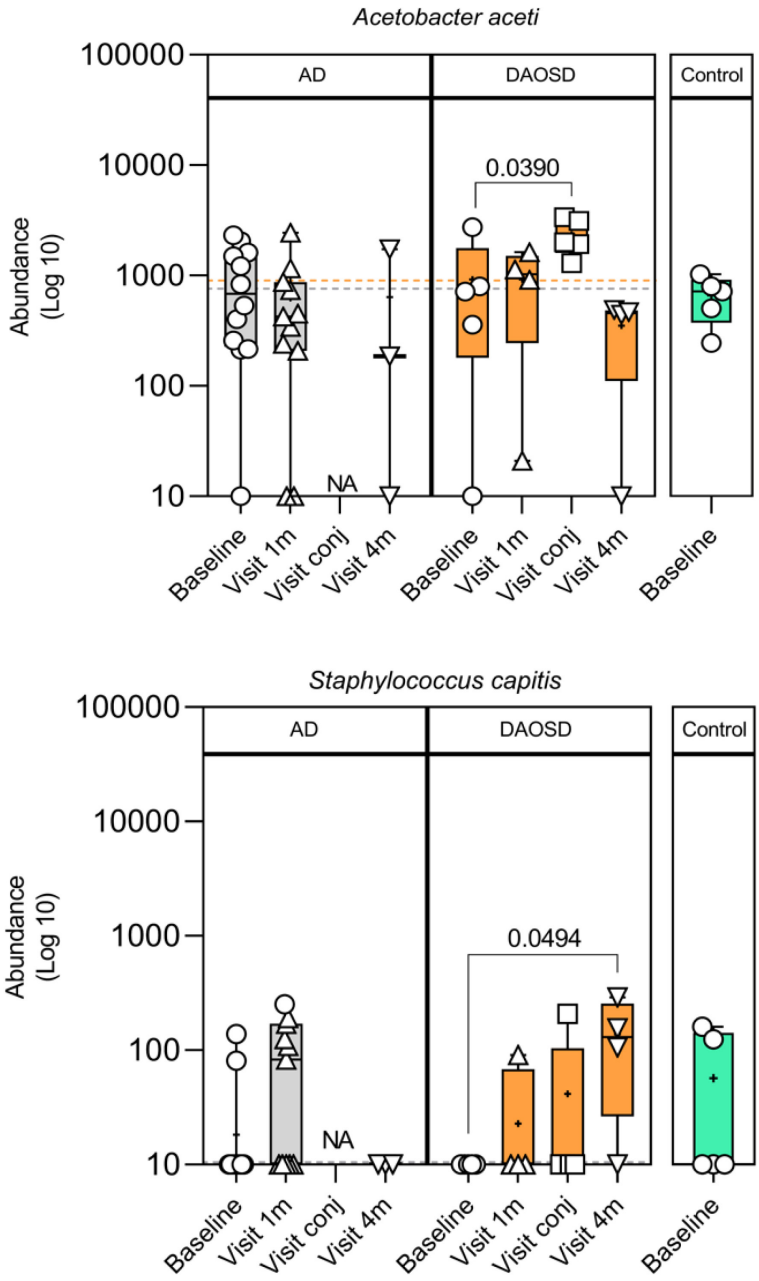

Figure 22: *Acetobacter aceti* and *Staphylococcus capitis*, significantly differentially abundant microbial species over time in AD patients developing DAOSD (DAOSD) reproduced from (Patra *et al.*, 2024) with permission of publisher Elsevier, American Academy of Ophthalmology

Box plots presenting microbial species in AD patients not developing DAOSD (AD), in AD patients developing DAOSD (DAOSD) and controls identified by linear discriminant analysis; Box and whisker plots showing minimum, median and maximum values; diminutive + indicating mean values; gray dotted lines indicating mean value in AD patients not developing DAOSD at baseline; orange dotted lines indicating mean value in AD patients developing DAOSD at baseline; n = 3 – 14; one-way analysis of variance was applied; a pseudo count was added to raw values and Log10 transformation was performed; NA stands for “not applicable”

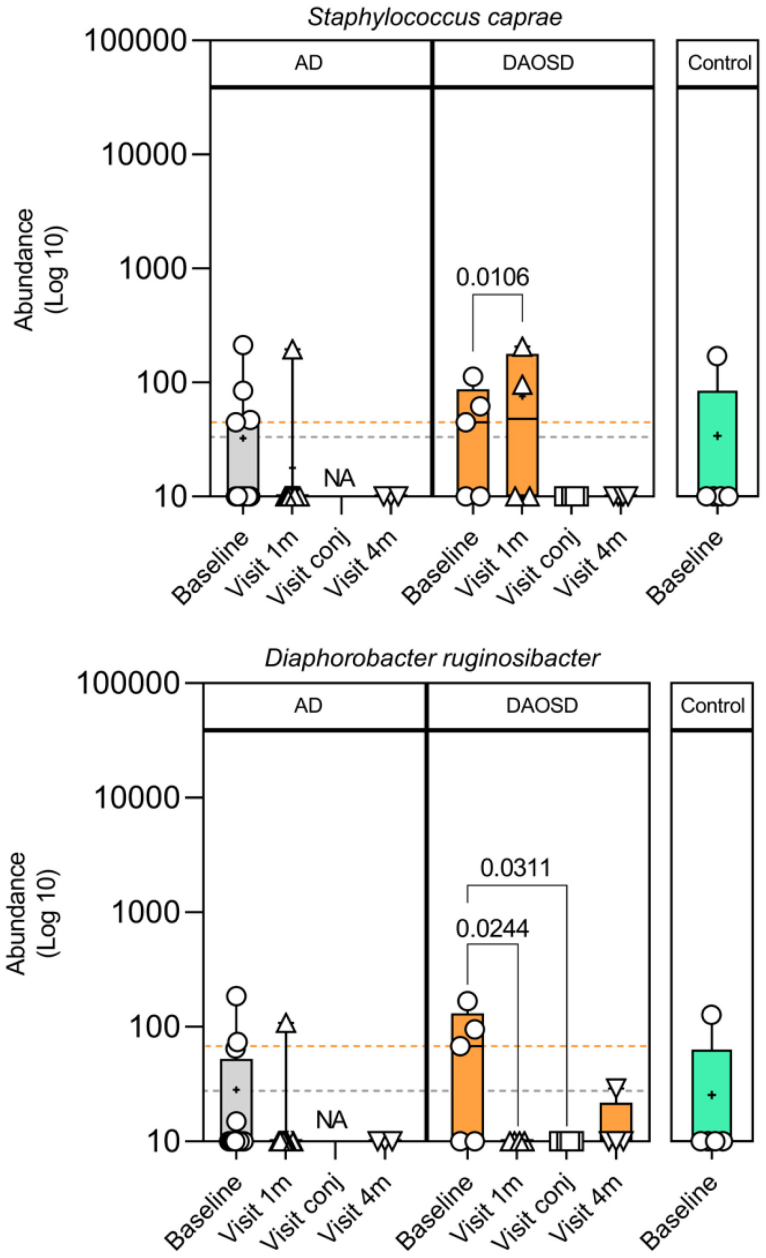

Figure 23: *Staphylococcus caprae* and *Diaphorobacter ruginosibacter*, significantly differentially abundant microbial species over time in AD patients developing DAOSD (DAOSD) reproduced from (Patra *et al.*, 2024) with permission of publisher Elsevier, American Academy of Ophthalmology

Box plots presenting microbial species in AD patients not developing DAOSD (AD), in AD patients developing DAOSD (DAOSD) and controls identified by linear discriminant analysis; Box and whisker plots showing minimum, median and maximum values; diminutive + indicating mean values; gray dotted lines indicating mean value in AD patients not developing DAOSD at baseline; orange dotted lines indicating mean value in AD patients developing DAOSD at baseline; n = 3 – 14; one-way analysis of variance was applied; a pseudo count was added to raw values and Log10 transformation was performed; NA stands for “not applicable

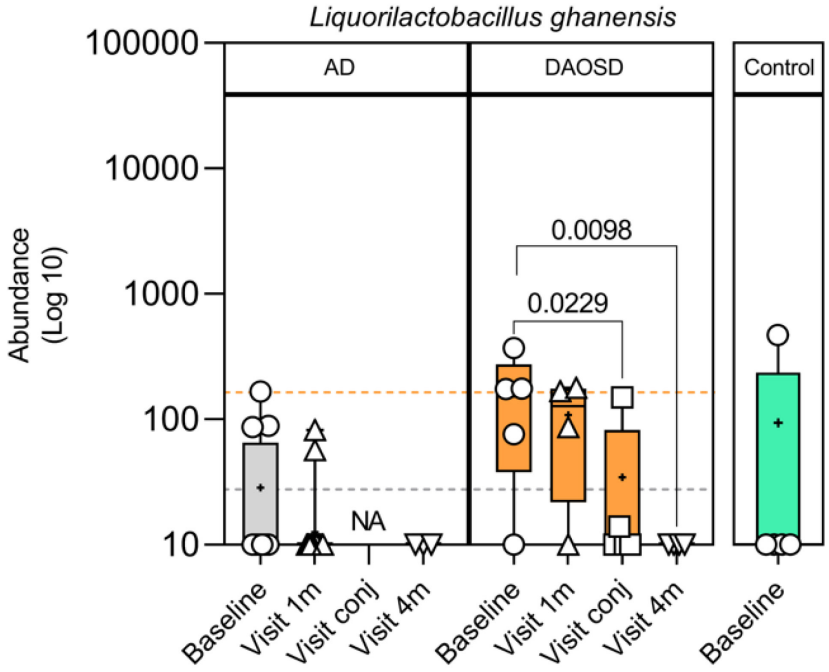

Figure 24: *Liquorilactobacillus ghanesis*, significantly differentially abundant microbial species over time in AD patients developing DAOSD (DAOSD) reproduced from (Patra *et al.*, 2024) with permission of publisher Elsevier, American Academy of Ophthalmology
Box plots presenting microbial species in AD patients not developing DAOSD (AD), in AD patients developing DAOSD (DAOSD) and controls identified by linear discriminant analysis; Box and whisker plots showing minimum, median and maximum values; diminutive + indicating mean values; gray dotted lines indicating mean value in AD patients not developing DAOSD at baseline; orange dotted lines indicating mean value in AD patients developing DAOSD at baseline; n = 3 – 14; one-way analysis of variance was applied; a pseudo count was added to raw values and Log10 transformation was performed; NA stands for “not applicable”

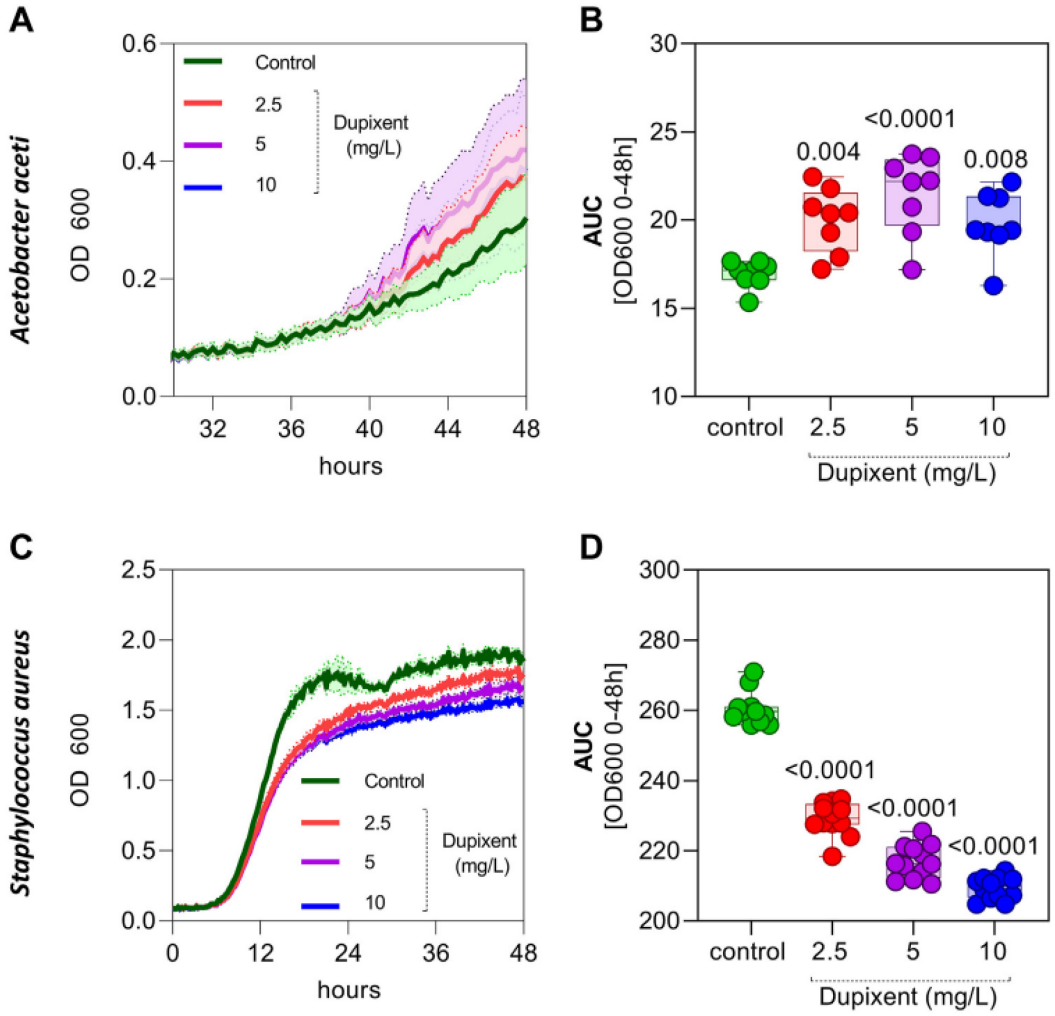

Figure 25: Microbial growth in presence of Dupixent® reproduced from (Patra *et al.*, 2024) with permission of publisher Elsevier, American Academy of Ophthalmology
Growth of *Acetobacter aceti* and *Staphylococcus aureus* cultured in 2.5, 5 and 10 mg/L concentrations of Dupixent® was measured over 48 hours. A and C, Growth curves of *Acetobacter aceti* and *Staphylococcus aureus* cultured with different concentrations of Dupixent®. B and D, Area under curve (AUC) calculated from growth curves from 0 hours to 48 hours, box and whisker plots show maximum, minimum, and median values. N = 8-12. One-way analysis of variance. P values are depicted for respective doses vs. controls. OD means optical density.

Serum Cytokines

The median fluorescent intensity values of 65 different cytokines (group means, 95% confidence interval on logarithmic scale) were analysed in controls at their one examination time point and in AD patients not developing DAOSD and AD patients developing DAOSD at baseline, visit 1m, visit 4m and if present visit conj. Maximum 5% of significant test results could be false-positive expected by the false discovery rate method (Benjamini *et al.*, 1995). The repeated measurements were separated into a group effect, a time effect and a group by time interaction effect via nonparametric longitudinal data analysis with the R package LDpar. There was no significance found for any analysed cytokine regarding the time effect or the group by time interaction effect. A significant higher concentration of B-cell activating factor (BAFF), B lymphocyte chemoattractant (BLC), cluster of differentiation (CD)30, epithelial neutrophil-activating peptide (ENA)-78, eotaxin-1 and -2 and -3, interferon, IL-1α and IL-1β were found at baseline in AD patients overall compared with controls (Figure 26). These findings are in line with previous studies (Yoshizawa *et al.*, 2002; Kiwan *et al.*, 2022). In the DAOSD cohort, there were cytokines showing increasing concentrations over time, like IL-9 and IL-17, while IL-4, IL-13, IL-2R, interferon gamma-induced protein-10, macrophage-derived chemokine (MDC), monokine induced by gamma interferon (MIG) and tumor necrosis factor (TNF)-receptor type II decreased (Figure 26). During the study period (from baseline until visit 4m), AD patients developing DAOSD showed a statistically significant continuous increase of IL-1ß by 22.4% and TNF-α by 17.8% (percentage is average of all visits) compared with AD patients not developing DAOSD (Patra *et al.*, 2024).
Taken together, we observed different cytokine profiles in AD patients and healthy controls. Moreover, IL-1ß and TNF-α might be involved in DAOSD pathogenesis (Patra *et al.*, 2024).

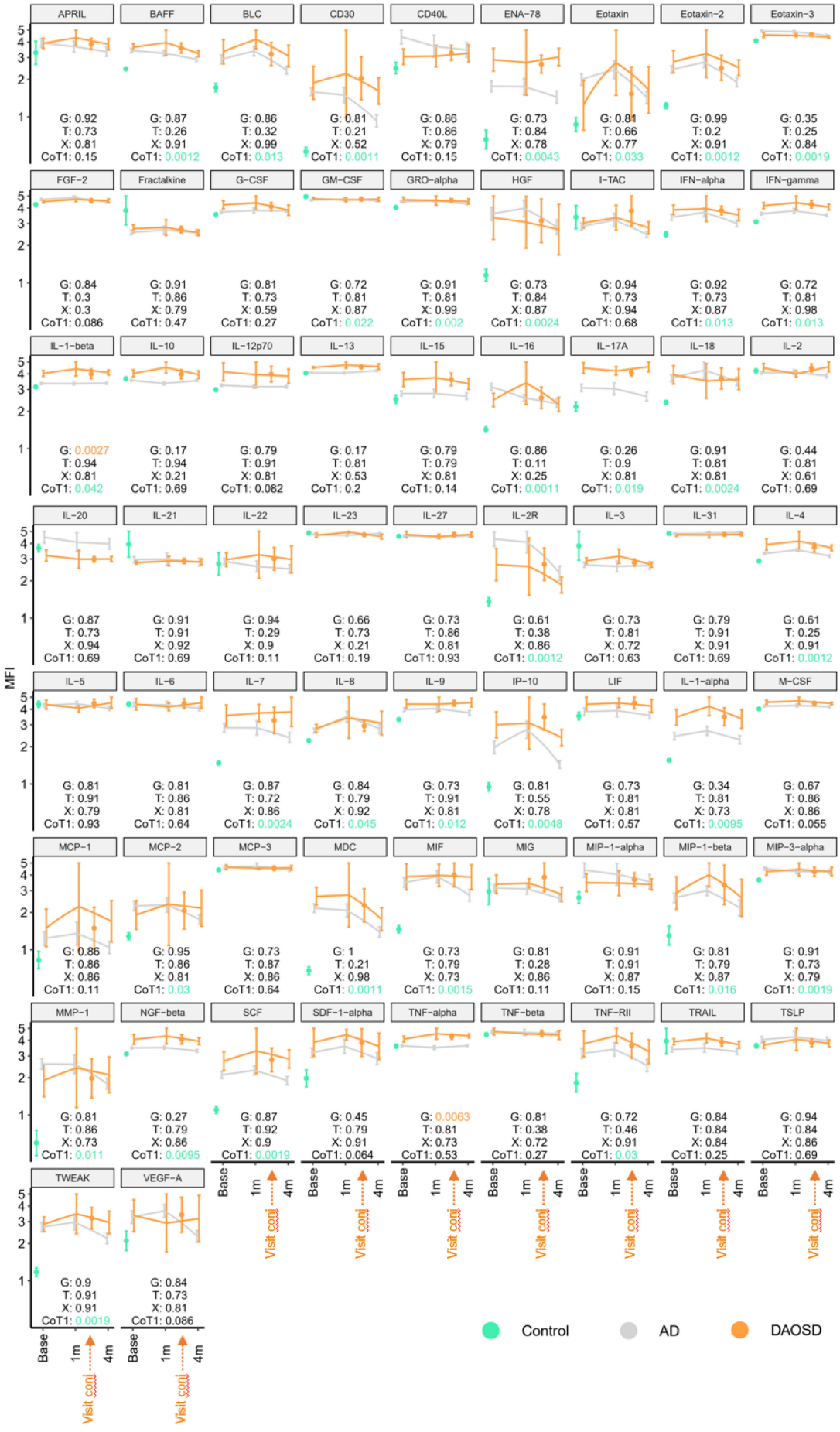
APRIL
G: 0.92
T: 0.73
X: 0.81
CoT1: 0.15
BAFF
G: 0.87
T: 0.26
X: 0.91
CoT1: 0.0012
BLC
G: 0.86
T: 0.32
X: 0.99
CoT1: 0.013
CD30
G: 0.81
T: 0.21
X: 0.52
CoT1: 0.0011
CD40L
G: 0.86
T: 0.86
X: 0.79
CoT1: 0.15
ENA−78
G: 0.73
T: 0.84
X: 0.78
CoT1: 0.0043
Eotaxin
G: 0.81
T: 0.66
X: 0.77
CoT1: 0.033
Eotaxin−2
G: 0.99
T: 0.2
X: 0.91
CoT1: 0.0012
Eotaxin−3
G: 0.35
T: 0.25
X: 0.84
CoT1: 0.0019
FGF−2
G: 0.84
T: 0.3
X: 0.3
CoT1: 0.086
Fractalkine
G: 0.91
T: 0.86
X: 0.79
CoT1: 0.47
G−CSF
G: 0.81
T: 0.73
X: 0.59
CoT1: 0.27
GM−CSF
G: 0.72
T: 0.81
X: 0.87
CoT1: 0.022
GRO−alpha
G: 0.91
T: 0.81
X: 0.99
CoT1: 0.002
HGF
G: 0.73
T: 0.84
X: 0.87
CoT1: 0.0024
I−TAC
G: 0.94
T: 0.73
X: 0.94
CoT1: 0.68
IFN−alpha
G: 0.92
T: 0.73
X: 0.87
CoT1: 0.013
IFN−gamma
G: 0.72
T: 0.81
X: 0.98
CoT1: 0.013
IL−1−beta
G: 0.0027
T: 0.94
X: 0.81
CoT1: 0.042
IL−10
G: 0.17
T: 0.94
X: 0.21
CoT1: 0.69
IL−12p70
G: 0.79
T: 0.91
X: 0.81
CoT1: 0.082
IL−13
G: 0.17
T: 0.81
X: 0.53
CoT1: 0.2
IL−15
G: 0.79
T: 0.79
X: 0.81
CoT1: 0.14
IL−16
G: 0.86
T: 0.11
X: 0.25
CoT1: 0.0011
IL−17A
G: 0.26
T: 0.9
X: 0.81
CoT1: 0.019
IL−18
G: 0.91
T: 0.81
X: 0.81
CoT1: 0.0024
IL−2
G: 0.44
T: 0.81
X: 0.61
CoT1: 0.69
IL−20
G: 0.87
T: 0.73
X: 0.94
CoT1: 0.69
IL−21
G: 0.91
T: 0.91
X: 0.92
CoT1: 0.69
IL−22
G: 0.94
T: 0.29
X: 0.9
CoT1: 0.11
IL−23
G: 0.66
T: 0.73
X: 0.21
CoT1: 0.19
IL−27
G: 0.73
T: 0.86
X: 0.81
CoT1: 0.93
IL−2R
G: 0.61
T: 0.38
X: 0.86
CoT1: 0.0012
IL−3
G: 0.73
T: 0.81
X: 0.72
CoT1: 0.63
IL−31
G: 0.79
T: 0.91
X: 0.91
CoT1: 0.69
IL−4
G: 0.61
T: 0.25
X: 0.91
CoT1: 0.0012
IL−5
G: 0.81
T: 0.91
X: 0.79
CoT1: 0.93
IL−6
G: 0.81
T: 0.86
X: 0.81
CoT1: 0.64
IL−7
G: 0.87
T: 0.72
X: 0.86
CoT1: 0.0024
IL−8
G: 0.84
T: 0.79
X: 0.92
CoT1: 0.045
IL−9
G: 0.73
T: 0.91
X: 0.81
CoT1: 0.012
IP−10
G: 0.81
T: 0.55
X: 0.78
CoT1: 0.0048
LIF
G: 0.73
T: 0.81
X: 0.81
CoT1: 0.57
IL−1−alpha
G: 0.34
T: 0.81
X: 0.73
CoT1: 0.0095
M−CSF
G: 0.67
T: 0.86
X: 0.86
CoT1: 0.055
MCP−1
G: 0.86
T: 0.86
X: 0.86
CoT1: 0.11
MCP−2
G: 0.95
T: 0.86
X: 0.81
CoT1: 0.03
MCP−3
G: 0.73
T: 0.87
X: 0.86
CoT1: 0.64
MDC
G: 1
T: 0.21
X: 0.98
CoT1: 0.0011
MIF
G: 0.73
T: 0.79
X: 0.73
CoT1: 0.0015
MIG
G: 0.81
T: 0.28
X: 0.86
CoT1: 0.11
MIP−1−alpha
G: 0.91
T: 0.91
X: 0.87
CoT1: 0.15
MIP−1−beta
G: 0.81
T: 0.79
X: 0.87
CoT1: 0.016
MIP−3−alpha
G: 0.91
T: 0.73
X: 0.79
CoT1: 0.0019
MMP−1
G: 0.81
T: 0.86
X: 0.73
CoT1: 0.011
NGF−beta
G: 0.27
T: 0.79
X: 0.86
CoT1: 0.0095
SCF
G: 0.87
T: 0.92
X: 0.9
CoT1: 0.0019
SDF−1−alpha
G: 0.45
T: 0.79
X: 0.91
CoT1: 0.064
TNF−alpha
G: 0.0063
T: 0.81
X: 0.73
CoT1: 0.53
TNF−beta
G: 0.81
T: 0.38
X: 0.72
CoT1: 0.27
TNF−RII
G: 0.72
T: 0.46
X: 0.91
CoT1: 0.03
TRAIL
G: 0.84
T: 0.84
X: 0.84
CoT1: 0.25
TSLP
G: 0.94
T: 0.84
X: 0.86
CoT1: 0.69
TWEAK
G: 0.9
T: 0.91
X: 0.91
CoT1: 0.0019
VEGF−A
G: 0.84
T: 0.73
X: 0.81
CoT1: 0.086
MFI
Base
1m
4m
Visit conj
Control
AD
DAOSD

Figure 26: Serum cytokine profile reproduced from (Patra *et al.*, 2024) with permission of publisher Elsevier, American Academy of Ophthalmology
Line chart showing median fluorescent intensity values of 65 cytokines (group means and 95% confidence limits in logarithmic scale) for AD patients developing DAOSD (DAOSD), AD patients not developing DAOSD (AD) and controls at baseline (base), after 1 month (1m) and after 4 months (4m) of Dupilumab treatment. P values false discovery rate corrected for testing 65 cytokines. P values < 0.05 statistically significant. Cytokines with a significant group effect are depicted in the figure. Comparison of controls vs. AD patients receiving Dupilumab at baseline (CoT1) with Wilcoxon test. AD = atopic dermatitis; DAOSD = Dupilumab–associated ocular surface disease; G = group effect; IL = interleukin; T = time effect; X = time interaction effect; APRIL = a proliferation-inducing ligand; BAFF = B cell activating factor; BLC = B lymphocyte chemoattractant; CD30 = cluster of differentiation 30; CD40L = cluster of differentiation 40 ligand; ENA-78 = epithelial neutrophil-activating protein 78; FGF-2 = fibroblast growth factor 2; G-CSF = granulocyte colony-stimulating factor; GM-CSF = granulocyte-macrophage colony stimulating factor; GRO-alpha = CXCL1 = C-X-C motif chemokine ligand 1; HGF = hepatocyte growth factor; I-TAC = interferon-inducible T cell alpha chemoattractant; IFN = interferon; LIF = leukemia inhibitory factor; M-CSF = macrophage colony-stimulating factor; MCP = monocyte chemoattractant protein; MDC = macrophage-derived chemokine; MIF = macrophage migration inhibitory factor; MIG = monokine induced by gamma-interferon = CXC ligand 9; MIP = macrophage inflammatory protein; MMP = matrix metalloproteinase; NGF = nerve growth factor; SCF = stem cell factor; SDF = stromal cell-derived factor = CXCL12; TNF = tumor necrosis factor; TRAIL = tumor necrosis factor related apoptosis inducing ligand; TSLP = thymic stromal lymphopoietin; TWEAK = tumor necrosis factor-like weak inducer of apoptosis

Discussion

Considering the limited knowledge on DAOSD pathogenesis, this thesis work analysed, whether the ocular surface microbiome might play a role in a prospective single-centre study with 20 adult moderate-to-severe AD patients. Six out of these 20 AD patients (30%) showed DAOSD during the study period of 16 weeks (Patra *et al.*, 2024). This percentage is in line with phase III clinical trials showing 5% up to 28% DAOSD occurrence in AD patients, and also with real-life data showing DAOSD in up to 62% of AD patients (Simpson *et al.*, 2016; Blauvelt *et al.*, 2017; de Bruin-Weller *et al.*, 2018; Kamata *et al.*, 2021).

Dupilumab and the Microbiome

In the 6 DAOSD patients in our cohort, conjunctival cytology showed dense neutrophilic infiltration which was linked with elevated systemic levels of IL-1β and TNF-α and increased abundance of certain ocular surface microbes, namely *Acetobacter aceti*, *Staphylococcus capitis* and *Staphylococcus caprae* (Patra *et al.*, 2024). Up to date, it is known that the ocular surface microbiome is paucibacterial with approximately 0.06 bacteria per human cell and that it shows changes depending on environment and age, however, it has not been able to define a certain healthy core ocular surface microbiome (Aragona *et al.*, 2021). According to recent 16S sequencing analyses, *Proteobacteria*, *Actinobacteria* and *Firmicutes* seem to be the major phyla in healthy adults, and *Corynebacteria*, *Staphylococcus*, *Streptococcus* and *Propionibacterium* seem to be the major genera in healthy adults (Cavuoto *et al.*, 2019).

It is known that microbial diversity in lesional AD and non-lesional skin increases - positively correlating with treatment response - during Dupilumab treatment and that *Staphylococcus aureus* decreases (Bieber, 2020; Callewaert *et al.*, 2020; Olesen *et al.*, 2021).

We showed that there are specific alterations in the ocular surface microbial landscape during Dupilumab treatment and in AD patients developing DAOSD. Interestingly, AD patients who did not develop DAOSD showed higher abundance of *Staphylococcus aureus* at baseline and *Staphylococcus aureus* was not highly abundant in AD patients developing DAOSD (Patra *et al.*, 2024). Analogously to the skin microbiome (Bieber, 2020; Callewaert *et al.*, 2020; Olesen *et al.*, 2021, Hartmann *et al.*, 2023), *Staphylococcus aureus* load also decreased on the ocular surface during Dupilumab treatment.

Dupilumab and the Skin Microbiome

Callewaert et al. showed that AD lesional skin had lower microbial diversity and higher abundance of *Staphylococcus aureus* than non-lesional skin. During treatment with Dupilumab, microbial diversity increased and abundance of *Staphylococcus aureus* decreased correlating with improvement of clinical presentation of AD and type 2 immunity biomarkers (Callewaert *et al*., 2020).

Olesen et al. published that relative abundance of the genus *Staphylococcus* and *Staphylococcus aureus* decreased in nose, lesional and non-lesional skin during Dupilumab treatment, while the relative abundance of *Staphylococcus epidermidis* and *Staphylococcus hominis* increased correlating with clinical improvement of AD (Olesen *et al*., 2021).

Also, Hartmann et al. showed that abundance of *Staphylococcus hominis* increased during Dupilumab treatment in lesional and non-lesional skin (Hartmann *et al*., 2023). On the ocular surface we could not find significant changes of *Staphylococcus epidermidis* or *Staphylococcus hominis* during Dupilumab treatment. However, regarding the genus *Staphylococcus*, we found that AD patients developing DAOSD showed a significantly increased abundance of *Staphylococcus capitis* at visit 4m compared with baseline, whereas this species did not show significantly increased abundance at any time point in AD patients not developing DAOSD. The abundance of *Staphylococcus caprae* was also significantly increased at visit 1m compared to baseline in AD patients developing DAOSD and there were no changes at the following visits. In AD patients not developing DAOSD, there were no changes in abundance of *Staphylococcus caprae* during the study period. Furthermore, we showed a significant increase of the abundance of Staphylococcus capitis at visit 4m compared to baseline.

Moreover, Lee et al. also confirmed that cutaneous abundance of *Staphylococcus aureus* decreased during Dupilumab treatment, whereas microbial diversity increased (Lee *et al*., 2021). They also showed the abundance of *Cutibacterium* and *Corynebacterium* increased in correlation with an increase in stratum corneum hydration levels during Dupilumab treatment (Lee *et al*., 2021).

Pazur et al. analyzed *Stapholococcus aureus*, *epidermidis* and *Cutibacterium acnes* in the skin. They observed a decrease of abundance of Staphylococcus aureus, a stable abundance

of *Staphylococcus epidermidis* and also an increase of abundance of *Cutibacterium acnes* during Dupilumab treatment (Pažur *et al*., 2024). On the ocular surface in our patient cohort, abundance of *Cutibacterium granulosum* was significantly decreased in AD patients not developing DAOSD at visit 4m compared to baseline (p-value < 0.05), however, it was increased in DAOSD patients at visit conj. We could also show an increasing abundance of *Corynebacterium tuberculostearicum* at visit 1m compared to baseline in AD patients not developing DAOSD.

Simpson et al. showed how fast *Staphylococcus aureus* abundance decreases after initiation of Dupilumab – after only three days (Simpson *et al*., 2023). Moreover, they could show that patients with the greatest *Staphylococcus aureus* reduction had the best clinical outcomes and that the reduction of the abundance of *Staphylococcus aureus* correlated with reduction of type 2 biomarkers and thymus and activation-regulated chemokine (TARC) (Simpson *et al*., 2023). However, not only the bacterial skin microbiome changes during Dupilumab treatment, also the fungal skin microbiome does, as Umemoto et al. showed (Umemoto *et al*., 2024). According to Staphylococcus aureus and the bacterial microbial diversity, Malassezia yeast species decrease in abundance, whereas non-Malassezia species increase in abundance during Dupilumab treatment leading to greater microbial diversity (Umemoto *et al*., 2024). And interestingly, also the gut microbiome changes during Dupilumab treatment for moderate to severe AD (Yang *et al*., 2024). Gut microbial dysbiosis is shifted to a healthier state along with improved intestinal tryptophan metabolism in Chinese AD patients (Yang *et al*., 2024).

Dupilumab and the Ocular Surface Microbiome

Shortly after our work had been published in 2023, Thormann et al. published bacterial microbiome analyses from conjunctival swabs in a small cohort of four AD patients (Thormann *et al*., 2024). In their cohort, the most abundant phyla at baseline were *Actinobacteria* and *Firmicutes*, the most abundant genera were *Cutibacterium* and *Staphylococcus* and the most abundant species was *Cutibacterium acnes*. During treatment with Dupilumab, the genera and species richness significantly decreased, exept in one patient later developing DAOSD (Thormann *et al*., 2024).
In lid and conjunctival samples of this DAOSD patient the abundance of *Proteobacteria* increased, while the abundance of *Basidiomycota* decreased. Moreover, in the lid samples, also the abundance of *Actinobacteria* decreased. *Staphylococcus* genus was completely absent at baseline and shortly before DAOSD onset. At DAOSD onset, the genera richness

was significantly higher compared with baseline before treatment initiation. Unique findings at DAOSD onset were presence of the genera *Dolosigranulum*, *Moraxella* and *Alphapolyomavirus* and a dominance of the species *Dolosigranulum pigrum* and *Moraxella nonliquefacies* in lid and conjunctiva samples. Moreover, *Staphylococcus epidermidis* was dominant in the lid microbiome (Thormann *et al*., 2024).

In our cohort, there were other microbes, particularly *Acetobacter aceti*, which were much more abundant in AD patients developing DAOSD and especially at visit conj (Patra *et al.*, 2024). *Acetobacter aceti* does not count to the widely investigated microbial species, however it is known that it plays an important role in converting alcohol to acetic acid in an aerobic environment and that it can dissolve insoluble phosphate (Nakano *et al.*, 2008).
Consequently, we hypothesized that *Acetobacter aceti* might be involved in DAOSD development via its production of acetic acid on the ocular surface and that it could be able to metabolize Dupixent® and thus produce even high levels of acetic acid, as one recent publication described the presence of higher levels of Dupilumab in tear fluid in patients with moderate to severe DAOSD compared with no or mild DAOSD (Achten *et al*., 2023). This finding indicated that Dupilumab reaches the ocular surface. Furthermore, Achten et al. found that Dupilumab was present in conjunctival cell suspensions, directly bound to CD45- conjunctival epithelial cells. Consequently, increased drug availability on the ocular surface and AD-induced conjunctival epithelial alterations may play a role for development of DAOSD (Achten *et al*., 2023).

Dupixent® ingredients besides Dupilumab are L-arginine hydrochloride, L-histidine, polysorbate 80, sucrose and sodium acetate (EMA, 2023). Sodium acetate is known to have antimicrobial activity, however, there are some microbes like for instance *Acetobacter aceti* that can easily survive and adapt to high acetate concentrations (Steiner *et al.*, 2001).
We were able to show in vitro that growth of *Acetobacter aceti* increased in culture with Dupixent®, while growth of *Staphylococcus aureus* decreased (Patra *et al.*, 2024). Of course, the question whether sodium acetate administered with Dupixent® injection at a distant body site could reach sufficient levels for these processes at the ocular surface will have to be explored. However, sodium acetate is also a commonly used buffer in various eyedrops. If sufficient levels of sodium acetate were present on the ocular surface, this could explain the persistent colonization with *Acetobacter aceti* in AD patients developing DAOSD and the decreased microbial abundance and diversity in AD patients not developing DAOSD. As consequence for clinicians treating AD patients with DAOSD, antibiotic coverage added to

today's suggested therapeutic regimen with anti-inflammatory and anti-allergic agents might be beneficial. Aminoglycosides would be first choice as *Acetobacter aceti* was reported to be susceptible to gentamicin (Cepec *et al.*, 2022).

Dupilumab and Eosinophilia

It is known that AD patients treated with Dupilumab frequently show transient systemic eosinophilia (Wechsler *et al.*, 2022), however, it was reported that AD patients developing DAOSD showed even higher systemic eosinophil levels 2 months after treatment initiation and higher systemic basophil levels 2 to 3 months later than AD patients not developing DAOSD (Katsuta *et al.*, 2021).

Additionally, another group observed that higher systemic eosinophil levels 4 months after treatment initiation were present in AD patients developing DAOSD or facial redness dermatitis (Ferrucci *et al.*, 2022). And also in a cohort of Japanese AD patients DAOSD occurrence was linked to eosinophil elevation and the authors stated that the eosinophil ratio could serve as a biomarker for DAOSD (Tosuji *et al*., 2022).

We could not repeat these findings in our cohort as we found no significant differences in systemic eosinophil levels between AD patients developing DAOSD and AD patients not developing DAOSD, neither at baseline nor at visit 4m (Patra *et al.*, 2024).

Dupilumab and Conjunctival Cytology and Histology

A research group from the University of Utrecht, Netherlands worked a lot on the pathomechanism of DAOSD. They were the first to observe conjunctival goblet cell loss in DAOSD patients in conjunctival biopsies stained with Alcian blue (Bakker *et al*., 2019). As we only had one conjunctival smear per patient and went for H&E staining, we could not look for conjunctival goblet cells in our cohort. Interestingly, this reported goblet cell scarcity does not correspond to the characteristic histopathology of allergic conjunctivitis and atopic keratoconjunctivitis, where goblet cell quantity is increased, as well as mucus production (Roat *et al*., 1993).

In conjunctival biopsies, Bakker et al. also observed a multicellular immune cell infiltrate consisting of T cells and eosinophil granulocytes in AD patients with DAOSD (Bakker *et al.*,

2019). In contrast, DAOSD patients in our cohort showed only very few eosinophil granulocytes in conjunctival smears, however, lots of neutrophil granulocytes (Patra *et al.*, 2024). Therefore, the type of tissue collection method, which is used (biopsy – smear), might play an important role for the cellular infiltrate you can observe. As it was reported that in allergic conjunctivitis, patients rather show lower counts of neutrophils and eosinophils but higher counts of mast cells at the ocular surface and that infections of the ocular surface are associated with increased neutrophil levels (Anderson *et al.*, 1997; Leonardi, 1999; Mun *et al.*, 2021), we hypothesize that the massive neutrophil infiltration observed in conjunctival smears in AD patients with DAOSD in our cohort might be due to the altered ocular surface microbial landscape as it was reported that neutrophil recruitment in the eye was triggered by bacterial components like for instance lipopolysaccharides (Kolaczkowska *et al.*, 2013; Postnikoff *et al.*, 2017).

Even after anti-inflammatory treatment with calcineurin inhibitors, we observed increased conjunctival neutrophil infiltration at visit 4m in AD patients developing DAOSD. Our hypothesis is that this finding is a consequence of the persistent microbial colonization in DAOSD patients, which was not observed in AD patients not developing DAOSD, who showed a reduction in microbial diversity and abundance (Patra *et al.*, 2024).

Dupilumab and Cytokines

Dupilumab and Cytokine Analyses in Conjunctival Biopsies

Bakker et al. also reported a significant increase of specific cytokines in conjunctival biopsies from patients with DAOSD. They found various infiltrating immune cells including CD4+ and CD8+ T cells, CD11c+ dendritic cells, CD14+ monocytes and CD68+ macrophages and within subepithelial cell infiltrates, they analyzed significantly increased levels of IFN-γ, IL-17A, IL-10 and TNF-α (Bakker *et al.*, 2021). This finding is in line with the significantly higher systemic TNF-α levels we could observe in AD patients developing DAOSD (Patra *et al.*, 2024).

Dupilumab and Cytokine Analyses in Aqueous Humor

Achten et al. analyzed the proteomic profile aqueous humor in Dupilumab-associated non-infectious uveitis, and again TNF-α and IFN-γ were highest (Achten *et al.*, 2024), which is in line with our finding of significantly higher systemic TNF-α and IFN-γ levels in DAOSD patients.

Interestingly, IL-4 and IL-13 are known to reduce ocular inflammation in animal uveitis models (Bridgewood *et al*., 2021). And IL-4 and IL-13 antagonize TNF-α and IFN-γ (Albanesi *et al*., 2007), and thus, IL-4 and IL-13 blockage by Dupilumab may lead to elevated TNF-α and IFN-γ levels and consecutively to non-infectious uveitis.

Dupilumab and Cytokine Analyses in Tear Fluid

To the best of our knowledge, there are only four reports on cytokine profiling in tear fluid of DAOSD patients available. They are inconsistent and none of them showed TNF-α and only one of them showed IFN-γ elevation.
Thormann et al. looked into the cytokine profile of tear fluid in DAOSD patients (Thormann *et al*., 2024). They could not find elevated TNF-α and IFN-γ levels, however, IL-12B, IL-17C, stem cell factor (SCF), oncostatin (OSM), TNF super family member (TNFSF) 14, TNF-related activation-induced cytokine (TRANCE), lymphotoxin alfa (LTA), TNFSF receptor 9, matrix metalloprotease (MMP) 1, chemokine CCL3, CD6 and CD244 were upregulated at week 8 after Dupilumab initiation before development of DAOSD in comparison with baseline. After DAOSD onset, IL-12B, CD8A, CD5, IL-8, leukemia inhibitory factor (LIF) and IL-10RB were elevated compared with baseline. Overall, they found out that tear fluid in DAOSD is typified by a Th1/Th17 cytokine profile and upregulation of markers promoting remodeling and fibrosis (Thormann *et al*., 2024).

Interestingly, Chiricozzi et al. used another cytokine assay and found that DAOSD occurrence was associated with a significant IL-33 increase in the tear fluid (Chiricozzi *et al*., 2023).

Vuillemey et al. also analyzed cytokines in tear fluid, again with another panel, and they could show that tear fluid levels of IL-17A, IFN-γ, IL-10, IL-6 and IL-2 levels were higher in patients developing DAOSD over course of Dupilumab therapy (Vuillemey *et al*., 2022).

Achten et al. again used another technology to measure cytokine levels in tear fluid and they showed that significantly higher IL-22, thymus and activation-regulated chemokine (TARC) and periostin tear fluid levels were present in patients later developing moderate to severe DAOSD compared with patients later developing no or only mild DAOSD (Achten *et al*., 2023).

Dupilumab and Cytokine Analyes in Blood

Again, there are only few publications regarding serum cytokine analyses during Dupilumab therapy and as far as we know, we were the first to look into serum cytokine profiles in patients with DAOSD.

It is known that Dupilumab efficiently suppresses type 2 inflammatory biomarkers in the blood including serum TARC, CCL18, serum periostin, IL-22, plasma eotaxin-1 and -3 (Ariëns *et al*., 2020; Beck *et al*., 2025; Guttman-Yassky *et al*., 2019; Hamilton *et al*., 2021).

Furthermore, Wu et al. found in a cohort of Chinese AD patients that CD25/soluble interleukin (sIL-)2Rα, IL-31 and IL-36β were positive prognostic markers associated with the efficacy of treatment (Wu *et al*., 2023).

Dupilumab and AD Scores

Our reported EASI and IGA counts were in line with previous real-life reports (Halling *et al.*, 2021). We observed a delayed therapeutic response in AD patients developing DAOSD (Patra *et al.*, 2024). Whether that was due to the altered ocular surface microbial landscape, the neutrophil infiltration at the ocular surface or the altered systemic cytokine profile warrants further analyses. A vicious cycle could be initiated by a dysbiotic ocular surface microbiome leading to host immune reaction and immunological stimulation resulting in systemic alterations, which could influence the treatment response (Hur *et al.*, 2021).

Switching from Dupilumab to Tralokinumab

We did not include patients in our cohort who switched from Dupilumab (targeting IL-4 and IL-13) to Tralokinumab (specifically targeting IL-13). However, as we discuss the pathomechanism behind DAOSD in this thesis, we also need to discuss data available on ocular surface disease in IL-13 blockade only. A recent publication showed that IL-4 and IL-13 both contribute to conjunctival goblet cell homeostasis (Hansen *et al*., 2022). And as we discussed before, scarcity of conjunctival goblet cells is a known characteristic of DAOSD.

Results from five clinical trials showed that also Tralokinumab was associated with a higher prevalence of conjunctivitis than placebo, however, the cases were mostly mild and transient

(Wollenberg *et al*., 2022). Overall, in phase III trials with Tralokinumab lower conjunctivitis rates were reported than in phase III trails with Dupilumab (Akinlade *et al*., 2019). As some authors hypothesized that a switch from Dupilumab to Tralokinumab may be favourable in cases of severe DAOSD as Tralokinumab has a more specific working mechanism only affecting IL-13 signalling (Achten *et al*., 2023), Reguiai et al. stated that they would prefer switching to a Janus kinase inhibitor based on their data (Reguiai *et al*., 2024).

Dupilumab and Cutaneous T-Cell Lymphoma

Since Dupilumab's approval there have been increasing numbers of case reports and case series reporting on emergence or exacerbation of cutaneous T-cell lymphoma (mainly mycosis fungoides or Sézary syndrome) associated with Dupilumab use (Beylot-Barry & Staumont-Salle, 2025). Interestingly, as DAOSD, cutaneous T-cell lymphoma increase was only associated with the diagnosis of AD, however, not seen in patients with asthma, chronic rhinosinusitis with polyposis or eosinophilic esophagitis (Lavin *et al*., 2025). In our study cohort, we did not observe a single case of cutaneous T-cell lymphoma – however, our follow-up was finalized after 4 months, and the development of Dupilumab-associated cutaneous T-cell lymphoma is reported to occur around 7.5 months up to one year after Dupilumab initiation (Hasan *et al*., 2024; Park *et al*., 2023). The underlying mechanisms are not clear and have to be elucidated yet, however, there are some hypotheses. On the one hand, increasing availability of IL-13 could promote cutaneous T-cell lymphoma development or progression, on the other hand, initial misdiagnosis of cutaneous T-cell lymphoma as AD is suggested. However, according to a recent analysis, Dupilumab increases the risk of cutaneous T-cell lymphoma in AD patients compared with the risk of AD patients not treated with Dupilumab (Hasan *et al*., 2024). What is known is that it is a very rare event and that risk factors are age > 40 years with no previous history of atopy and atypical clinical features of AD (Beylot-Barry & Staumont-Salle, 2025). In conclusion, in patients with atypical lesions cutaneous T-cell lymphoma has to be ruled out before Dupilumab initiation and in patients with development of atypical lesions during treatment, AD diagnosis has to be reassessed. Although it is reported that Dupilumab alleviates pruritus in certain patients with cutaneous T-cell lymphoma, it is recommended to not prescribe Dupilumab in these cases anymore until the association between Dupilumab and cutaneous T-cell lymphoma in AD patients is further examined (Beylot-Barry & Staumont-Salle, 2025).

Limitations

The biggest limitation of our study is the small sample size and that during the coronavirus disease 19 pandemic some of the scheduled study visits were not able to be conducted (Patra *et al.*, 2024).

As we decided to obtain minimally invasive conjunctival smears rather than conjunctival biopsies, we could only gain cytological but not histological insights and might have missed relevant cellular infiltrates in the lamina propria. However, we wanted to stay minimally invasive to improve patient's adherence to the study protocol and therefore decided to gain only one swab for microbiome analyses and one smear for conjunctival cytology at each visit. As we took only one conjunctival smear, we also performed only one staining and decided to go for haematoxylin & eosin (Patra *et al.*, 2024).

Because of low-microbial DNA yield or PCR or sequencing failures and low-quality reads, we had to exclude some of the conjunctival swabs for microbiome analyses. Because of that, all the samples were rarefied to randomly selected number of reads and diversity metrics which could be calculated independent of sample size in different visits were performed. So, randomized studies with larger study cohorts are needed to reproduce our results with more statistical power (Patra *et al.*, 2024).

We also suggest conducting cytokine profiling in tear fluid and analyses of quantity and quality of metabolites and proteins in the serum to gain more insights into DAOSD pathophysiology.
Our findings should be interpreted with caution because of the mentioned limitations. We could not draw causal connections between the cellular (ocular surface), microbial (ocular surface) and cytokine (serum) alterations we observed and DAOSD (Patra *et al.*, 2024).

In conclusion, we observed a persistent neutrophil infiltration on the ocular surface accompanied by a unique microbial landscape and specific elevated systemic proinflammatory cytokines in AD patients developing DAOSD (Patra *et al.*, 2024).
Particularly *Acetobacter aceti* was much more abundant in AD patients developing DAOSD, especially at visit conj, whereas *Staphylococcus aureus* was not highly abundant in AD patients developing DAOSD (Patra *et al.*, 2024). Furthermore, AD patients developing DAOSD showed elevated systemic levels of IL-1β and TNF-α (Patra *et al.*, 2024).

Based on our findings, antibiotic coverage with gentamicin might be beneficial to be added to today's proposed therapeutic regimen of DAOSD consisting of preservative-free lubricant eye drops, anti-inflammatory eye drops like corticosteroids and calcineurin inhibitors and antihistamine/mast cell stabilizing eye drops.

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