Proteomic, miRNA and bacterial biomarker patterns in atopic dermatitis patients and their course upon anti-IL-4Rα therapy

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Abstract

Background: Identification of biomarkers is required for a systems medicine approach and personalized treatment in AD. These biomarkers may not only aid in diagnosing but also might be suitable to predict the effectiveness of targeted treatment. **Objective:** We aimed to identify proteomic, microbial, and miRNA biomarkers in atopic dermatitis patients and investigated their course in relation to the clinical response upon anti-IL-4Rα therapy. **Methods:** Proteomic and miRNA screening was performed in AD patients in comparison to healthy controls. Differentially regulated serum proteins, miRNA, and selected skin microbiota were measured consecutively in 50 AD patients before and upon systemic dupilumab treatment. A random forest classifier was used to predict the outcome of dupilumab therapy based on the initial biomarker patterns. **Results:** We identified 27 proteomic candidates, miRNA, and 3 microbial strains to be dysregulated in AD. Besides the well-known chemokine CCL17 other proteins i.e., CCL13, CCL22, E-selectin and BDNF were differently regulated and significantly associated with treatment response. By contrast neither the microbial changes nor the miRNA pattern were found to be associated with treatment response upon dupilumab treatment. **Conclusion:** AD patients display defined dysregulations regarding their systemic proteomic serum profile, miRNA patterns, and their skin microbiome. The proteomic profile and selekted skin bacteria changed profoundly upon anti-IL-4Rα therapy which was associated with an overall clinical response. This was not seen in miRNA-related biomarkers. Our findings support the hypothesis that biomarker profiles reflect treatment responses and may in the future be used to develop a personalized medicine approach for the treatment of atopic dermatitis patients.

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Results: We identified 27 proteomic candidates, miRNA, and 3 microbial strains to be dysregulated in AD. Besides the well-known chemokine CCL17 other proteins i.e., CCL13, CCL22, E-selectin and BDNF were differently regulated and significantly associated with treatment response. By contrast neither the microbial

changes nor the miRNA pattern were found to be associated with treatment response upon dupilumab treatment.

Conclusion: AD patients display defined dysregulations regarding their systemic proteomic serum profile, miRNA patterns, and their skin microbiome. The proteomic profile and selekted skin bacteria changed profoundly upon anti-IL-4Rα therapy which was associated with an overall clinical response. This was not seen in miRNA-related biomarkers. Our findings support the hypothesis that biomarker profiles reflect treatment responses and may in the future be used to develop a personalized medicine approach for the treatment of atopic dermatitis patients.

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Capsule summary: Serum biomarker profiles and skin microbial colonization patterns correlate well with established clinical severity scores. Baseline CCL17, E-selectin, Notch 1 and CD25s levels may indicate if patients will respond well to dupilumab therapy, however further validation is required.

Keywords: atopic dermatitis; biomarkers; dupilumab; miRNA; microbiome

Abbreviation list

- AD - Atopic dermatitis
- ATCC - American Type Culture Collection
- BSA - Body surface area
- CyA - Cyclosporin A
- DLQI - Dermatology Life Quality Index
- EASI - Eczema Area and Severity Score
- ENA - European Nucleotide Archive
- IGA - Investigator's Global Assessment
- IL-4Rα - Interleukin 4 receptor alpha
- IQR - Inter-quartile range
- miRNA - micro RNA
- NGS - Next-generation sequencing
- NRS - Numeric Rating Scale
- POEM - Patient-Oriented Eczema Measure
- RT-qPCR - Real-time quantitative polymerase chain reaction
- SCORAD - Scoring atopic dermatitis
- Th2 - T helper 2
- VAS - Visual analog scale

Introduction

Atopic dermatitis (AD) is a common inflammatory skin disease[1] that can significantly impact the quality of life in affected patients[2]. Recent advances in biomarker research have allowed for personalized medicine[3]. In oncology, cancer survival rates increased dramatically in patients presenting genetic traits known to respond

to certain therapies[4]. Similarly, the discovery of biomarkers for inflammatory skin diseases could facilitate their personalized care in the future[5]. AD is not a homogeneous entity[6], therefore, the identification of biomarkers for disease endotypes may be of great value for proper diagnosis[7].

Dupilumab is a monoclonal antibody that targets the interleukin-4 receptor alpha (IL-4Rα) and is used to treat moderate-to-severe AD by blocking the signaling pathways that are involved in inflammation and allergic reactions[8]. In clinical trials, dupilumab was effective in reducing the severity of AD symptoms including itch, and improving the quality of life in many patients[9]. However, not all patients respond well to the treatment, and some may experience side effects. The clinical response to dupilumab in AD patients can vary, and there are currently no reliable biomarkers that can predict which patients will respond well to the treatment. Factors that may affect the response to dupilumab include the severity and duration of the disease, certain genetic mutations, and other medical conditions[9].

A thorough review by Renert-Yuval et al. summarized the body of knowledge on the biomarkers in AD[10]. The authors indicated that CCL17 and CCL22 are potential biomarkers that correlate with clinical therapeutic response. One of the more recently suggested biomarkers of AD severity is the skin microbiome[11]. It has been shown that patients suffering from AD display a higher colonization rate with *Staphylococcus aureus* (*S. aureus*) which is further increased during exacerbation of the disease[12]. We previously investigated the microbiome of atopic dermatitis patients[13] but did not evaluate it upon systemic treatment with biologic therapeutics.

In this study, we aimed to identify biomarkers in AD patients and to analyze their profile upon systemic therapy with dupilumab.

Methods

Study design and sample collection

We included adult patients (> 18 years) with the diagnosis of atopic dermatitis based on Hanifin and Rajka criteria made at least 12 months prior to the inclusion, who were indicated for systemic therapy with dupilumab 300 mg s.c. The Ethical Committee of the Charité Universitätsmedizin Berlin approved this non-interventional study (EA1/112/19).

After obtaining informed consent, we gathered data on the clinical presentation of AD, current symptoms before the initiation of the therapy, and six months afterward, along with patient reported outcomes.

In addition, serum, and inter-scapular skin swabs from patients with moderate to severe atopic dermatitis, before and 6 months after initiating systemic therapy, were acquired. The microbiome sampling location was chosen based on the relative stability of microbiota in this anatomic location, precise and straightforward identification of the sampling area, and sufficient surface for biomaterial collection. Serum was prepared as described previously[14].

Serum proteomic screening

Analysis of 440 proteins in sera of patients with moderate to severe atopic dermatitis before systemic therapy and six months after initiating dupilumab was done using Quantibody Human Cytokine Array Q440 chip (RayBiotech, GA, USA). Fluorescent protein arrays were scanned using a PowerScanner (Tecan Group AG). Array microphotographs were quantified using Protein Array Analyzer for ImageJ[15].

Protein biomarker measurement

Human serum samples were analyzed using ELISA kits provided by R&D Systems, Minneapolis, USA (human CCL17, DY364; human CCL13, DY327; human CCL27, DY376; CCL22, DY336; IL22, DY782; IL11, DY218;

CD40L, DY617; E-selectin, DY724; BDNF, DY248; Notch1, DY5317; CD25s, DY223; ADAM8, DY1031; FGF1, DY232; CFD, DY1824 human), following the manufacturer's protocol.

Serum miRNA extraction, profiling and validation

Serum miRNA isolation, library preparation and sequencing were done following manufacturers instructions and are described in detail in the supplement. The differentially expressed miRNAs were further validated using reverse transcription quantitative real-time PCR (RT-qPCR) as described before[14] and in detail in the supplement.

Quantification of selected skin microbiota

We used the data from next generation sequencing (NGS) platform Illumina to identify the main bacterial species informative of the patient's skin status. Based on the results from our previously published paper[13], we decided to further investigate 3 bacterial species in detail, using qPCR analysis.

Cutibacterium acnes (ATCC 6919), *Staphylococcus epidermidis* (ATCC 12228) and *Staphylococcus aureus* (ATCC 29213) cultures were grown as described elsewhere. Skin microbiota were quantified using RT-qPCR as described in the supplement.

Data availability

The sequencing data presented in this study were be deposited in the European Nucleotide Archive (ENA) under the accession number: PRJEB59318.

Classification model

The supervised machine learning classification model was performed using a random forest algorithm with the help of the caret package[16]. All samples with complete observations (without missing values) were included in the training set and divided into good (super and high responders) and low responders groups. Features for the final random forest model were selected based on significant differences in each biomarker. The model fitness was calculated using 10 sets of 10-fold repeated cross-validation.

Statistics

Mann Whitney U test was used for comparing values between unpaired observations, with Holm's p-value correction for multiple comparisons. Paired data were analyzed using paired Wilcoxon's test with Holm's correction where appropriate. Next-generation sequencing-derived data were analyzed using the Wald test with Benjamini-Hochberg FDR to correct for multiple comparisons. P values < 0.05 were considered significant.

Results

Cohort characteristics and clinical response stratification

75 patients were included in this observational study. Of those, 25 patients were not fully analyzed as summarized in Figure 1A.

The final dataset consisted of 50 AD patients (mean age 48.8 years) observed in two time-points (before and 6 months after introducing dupilumab therapy) as well as 39 healthy individuals (mean age 27.2 years) and 15 psoriasis patients serving as controls (mean age 53.9 years). The sex distribution was comparable between the groups (Figure 1B-D).

Three groups of AD patients based on their response to dupilumab were built: 1) low responders if their SCORAD or body surface area (BSA) was reduced by less than 75% from baseline, 2) high responders if the SCORAD or BSA was reduced by more than 75% but less than 90%, 3) super responders with a 90% SCORAD reduction or involvement of only [?] 2% of total BSA. (Figure 1A, F). 15 (30%) patients were considered low responders, 21 (42%) high responders, and 14 (28%) super responders.

The responder status was associated with significant differences in SCORAD. Median SCORAD values after dupilumab therapy for low, high, and super responders were 39.4 (IQR 14.9), 17.9 (IQR 14.5), and 3.85 (IQR 4.875), respectively. The SCORAD decreased proportionally in each group and were 73% +- 34%, 30% +- 14%, and 11% +- 12% of their initial levels in each respective group (Figure 1F). Importantly, there were no differences in SCORAD values before initiating systemic therapy in the respective groups (median SCORAD = 55.9, Kruskal-Wallis rank sum test 3.06, p-value = 0.22, Figure S1).

A similar trend was seen in pruritus, measured on a visual analog scale (VAS, range from 0 to 10). Initially, there was no difference in median pruritus score between groups (low responders 7.6 (IQR 1.5); high responders 6.1 (IQR 3.3); super responders 6.8 (IQR 1.75), Kruskal-Wallis test p = 0.19). The values of pruritus decreased significantly after 6 months of therapy with dupilumab and were 2.3 (IQR 5.2); 1.6 (IQR 1.7); 0.65 (IQR 0.875) in each respective group (Figure 1F).

BSA (0 - 100%) decreased from 34.87 +- 25.61 in low responders; 48.62 +- 25.16 in high responders; 32.86 +- 21.1 in super responders to become 23.93 +- 15.38; 7.88 +- 4.98; 1 +- 0.88 in each respective group (Figure 1F).

Dermatology Life Quality Index (DLQI, ranging 0 - 30 points) followed the same trend (Figure 1F).

Taken together, our results indicate that the groups were comparable regarding their initial severity of AD, and showed significant changes in response to the systemic therapy.

Candidate biomarker identification through screening

We initially performed a proteomic screening with 440 proteins using a microarray and analyzed samples from AD patients. We identified 27 proteomic candidates (|Hedges G| > 0.9, Figure 2A-B). To determine the specificity of the proteomic markers, samples from psoriasis patients and healthy individuals were also studied.

To assess the importance of miRNA as biomarkers, we performed screening from 4 AD patients before treatment, 6 months after therapy, and as controls, from 6 healthy individuals. When unsupervised clustering was performed, AD samples clustered separately from healthy individuals as shown in the dendrogram (Figure 2C).

The analysis of the miRNA patterns before and upon dupilumab therapy largely overlapped, while a clear separation from healthy individuals was determined (Figure 2D). Based on the differential expression analysis (with the DESeq2 package for R[17]), we selected the following miRNAs as candidate biomarkers for further study: hsa-miR-29a-3p, hsa-miR-25-3p, and hsa-miR-378a-3p (Figure 2E). We also included hsa-miR-451a, based on a literature search[18], for further validation by RT-qPCR on the sera of AD and psoriasis patients as well as healthy individuals.

Patients suffering from AD have shown higher colonization rates with*S. aureus* in lesional, but also non-lesional skin[19]. NGS data on 7 AD samples and 7 healthy individuals revealed a high abundance of *C. acnes* , *S. aureus* , and *S. epidermidis* . Although the relative abundance of*C. acnes* and *S. epidermidis* was comparable among the samples, *S. aureus* colonization was significantly increased in AD patients. Therefore, we restricted our further analysis to these three main actors and relativized the amount of *S. aureus* to stable members of the healthy skin microbiota (*C. acnes* and *S. epidermidis*).

Serum proteomic profiles and their course upon Th2 targeted treatment

To validate the screening results, we measured a panel of dysregulated proteins on the entire cohort and performed correlations with the extent of the clinical response upon treatment.

CCL17 (one of the best-described biomarkers of AD) was significantly decreased after 6 months of treatment regardless of the responder status. We also observed a decrease in the chemokines CCL13, CCL22, CCL27, and E-Selectin and an increase in BDNF upon dupilumab treatment (Figure 3A, D, Figure S2A, E, F, I).

To verify if the identified biomarkers reflect the clinical response, we stratified patients according to their treatment outcomes. In low responders, BDNF and ADAM8 increased after therapy, and no alteration was observed in well-responding patients (Figure S2C, E). By contrast, CCL22 and CCL13 did not change significantly in low responders but decreased in high and super responders (Figure S2A and I).

Individual biomarker patterns among the responders were observed before initiating systemic treatment. Super responders had higher levels of Notch1, CD25s, IL11, and lower levels of FGF1 when compared to high responders, (Figure S3).

We observed differences in expression levels of several protein biomarkers in serum between AD and psoriasis patients as well as healthy individuals. BDNF, CCL13, CD25s, CCL17 and E-selectin were exclusively dysregulated in AD patients, when compared to healthy, but also to psoriasis patients (3A, D, I, Figure S2E, I). In addition, CCL22 and CFD were less expressed in healthy individuals compared to AD patients (Figure S2A, D). ADAM8, CD40L, IL22 were lower in psoriasis patients (Figure S2C, G, J).

In summary, CCL17, CCL13, and E-selectin correlated positively with SCORAD, pruritus, and BSA. By contrast, BDNF levels correlated negatively with BSA in AD patients (Figure 3 and Figure S5), indicating their usefulness as a severity-oriented biomarker panel.

Serum miRNA pattern in AD and their alteration upon Th2 targeted therapy

Interestingly, we observed significantly lower expression of all investigated miRNA before therapy when compared to healthy individuals. After therapy with dupilumab, this difference was less prominent in hsa-miR-29a-3p, hsa-miR-25-3p, and hsa-miR-378a-3p. We did not detect any significant differences in the measured miRNA from AD patients before and after therapy nor between AD and psoriasis patients (Figure 4). These results suggest that differences in miRNA profile are rather reflecting the disease as such, than its severity.

Skin microbial composition in AD patients and its alteration upon systemic Th2 therapy

The relative abundance of selected bacteria was determined by RT-qPCR and assessed in relation to clinical symptoms. We observed a relatively stable abundance of *S. epidermidis* throughout the course of therapy (Figure 5D). On the other hand, the ratio of *S. aureus* to*S. epidermidis* decreased significantly after systemic therapy. A similar finding was seen in the ratio of *S. aureus* to *C. acnes* (Figure 5A). Importantly, the ratio of *C. acnes* to*S. epidermidis* remained constant during the observed period (Figure 5A). These changes in bacterial DNA ratio are dependent on the overall decrease in the amount of measured *S. aureus* DNA and a slight increase in *C. acnes* proportion after initiating systemic therapy. The ratio of the measured bacterial DNA of *S. aureus* to*C. acnes* correlated to SCORAD and BSA indicating its association with the clinical status (Figure 5E). Significant differences were also seen in the ratio of these bacteria in the IGA score, with the highest values in *S. aureus* to *C. acnes* ratio observed in grade 4 and lowest in grades 0-1 (Figure 5B). There were no significant differences in the bacterial ratio values before the initiation of dupilumab between the low, high, and super responders (Figure 5C), suggesting that the baseline bacterial skin composition does not seem to associate with treatment outcome in this setting.

Integrative analysis of biomarker composites in good responders

Next, we performed biomarker pattern analysis concerning the treatment effects on severity. Principal component analysis was performed on the whole cohort with the most informative biomarkers. The groups showed a large overlap with the highest differences observed between AD patients before therapy and healthy individuals. AD patients (after therapy) resembled healthy individuals more closely, while psoriasis patients presented in between (Figure 6A). The largest differences among the groups were depicted in Figure 6B.

We investigated the baseline biomarker profiles of AD patients concerning their treatment response. We observed that CCL17, E-selectin, CD25s, and Notch1 consistently changed in all responder groups i.e., they individually showed a consistent pattern in high and super responders (either increasing or decreasing), but the size of the effect was limited. As Notch1, CD25s, IL22, FGF1, CCL27, and CCL17 were strongly correlated they could not be used for further predictive classification modeling (Figure S4A-B).

To increase biomarker sensitivity and to prevent highly correlated variables from distorting random forest accuracy, we subsequently calculated biomarker ratios to one another to form composite biomarkers and evaluated their fitness to predict response to dupilumab (low vs. high and super responders). We observed that the baseline values of Notch1 to CD25s ratio and CCL17 to E-selectin ratio were the best-performing predictors of low response to dupilumab after 6 months of therapy (Figure 6C-D). Subsequently, we analyzed the predictive capability of these two promising composite biomarkers in a random forest classifier. The area under the curve of this prediction model was 0.72 indicating higher than the random probability to correctly predict therapy outcome based on the serum levels of four proteins before initiating systemic therapy (Figure 6E).

As the skin microbial composition before treatment did not show significant differences among the responder groups nor IGA response scores, it did not present predictive capabilities regarding dupilumab therapy outcomes in atopic dermatitis patients after 6 months (data not shown).

Discussion

This study aimed to identify biomarker patterns corresponding to the disease and the extent of clinical improvement upon systemic anti-IL4Rα treatment. We report on known (CCL17), but also somewhat novel (CCL13, E-selectin, CCL22, and CCL27) proteomic biomarkers in severe AD patients. Exploring these biomarkers upon systemic dupilumab treatment CCL17, CCL13, E-selectin CCL22, and CCL27 protein levels decreased in our patients, whereas the BDNF increased. Skin microbial composition showed a decrease in *S. aureus* colonization upon treatment, following the clinical improvement. By contrast, miRNAs could not be classified as potential biomarkers of therapy response.

Our data has led us to propose a panel of composite biomarkers which may assist in predicting clinical responses, defined as a decrease of less than 75% in initial SCORAD values and body surface area affected by atopic dermatitis. Despite the overall positive clinical efficacy of dupilumab, as demonstrated by the high percentage of patients who experienced substantial improvement, a subpopulation of non-responders who may benefit from other treatment options remains.

A report by Hamilton et al. also described changes in biomarkers after initiation of dupilumab systemic therapy in various atopic diseases and found that dupilumab suppressed multiple Th2 mediators such as CCL17, total IgE, periostin, and eotaxin-3, consistent with our findings[20]. Our study adds to the literature by identifying additional biomarker candidates through proteomic screening.

Observed changes of CCL17, CCL13, CCL27, and CCL22 after dupilumab therapy reflect a Th2-inflammation-dependent response. We also observed an increase in the levels of BDNF after 6 months of therapy. BDNF is a protein that is not directly dependent on Th2-pathway and is secreted from eosinophils in patients with atopic dermatitis[21]. Our study found that levels of BDNF were higher in patients with atopic dermatitis compared to non-atopic individuals, which is consistent with previous reports in both children[22]

and adults[21]. The observed increase in BDNF after dupilumab treatment may be a consequence of transient dupilumab-dependent blood eosinophilia but its relevance remains elusive[23].

E-selectin was identified as a potential candidate for a good response to dupilumab therapy in our proteomic screening. E-selectin has been previously reported to be increased in atopic dermatitis and correlated with disease severity[24]. The decrease in both CCL17 and E-selectin may be a result of decreased inflammation in the skin, as these molecules are known to be coexpressed in dermal microvessels[25]. Like CCL17, E-selectin plays an important role in leukocyte migration into inflamed skin. Soluble E-selectin may result from the proteolytic cleavage of its membrane-bound form, and its presence in the serum could reflect the general state of skin inflammation[24].

It is important to note that the observed changes in biomarker levels in our study may be the result of the decrease in clinical severity rather than a direct effect of dupilumab therapy. Other systemic therapeutics have also been shown to decrease cytokines downstream in the inflammatory cascade. For example, previous studies have reported reductions in CCL17, IL13, and IL22 levels after treatment with cyclosporine A (CyA)[26], tralokinumab[27], or fezakinumab[28] in patients with atopic dermatitis. The identification of biomarkers specific to a particular therapy may offer insights into the mechanisms of the disease. However, it is arguably more important to identify biomarkers that specifically represent the severity of the condition for effective clinical management.

Many groups demonstrated a correlation between CCL17 levels and the SCORAD score[29–31]. However, atopic dermatitis is a complex and heterogeneous disease, with multiple contributing factors such as intrinsic and extrinsic pathways, skin barrier defects, and genetic background, involving multiple cell types. This heterogeneity makes it difficult to rely on a single biomarker as an objective indicator of disease severity.

Limitations have been identified with commonly used clinical scores such as SCORAD and EASI[32] for assessing atopic dermatitis, which undermines their use in clinical studies[33,34]. These scores are often semi-quantitative and subjective, leading to a lack of objective indicators for disease severity, making it challenging to monitor the disease in a longitudinal manner[35,36]. Therefore, a set of biomarkers (serological and/or histological) may be needed to objectively address the severity of the disease, as it might be more suitable as a baseline truth than widely used subjective clinical scoring systems[37].

A recently published study by Nakahara et al. underlined the difficulties to identify a biomarker that correlated with the severity of AD as measured by the EASI score during a 16-week therapy period[38]. Notably, the study suggested that two biomarkers, CCL17 and CD25s, have the potential to predict response to dupilumab therapy as measured by POEM and pruritus NRS scales. These findings align with the results of our study, in which CCL17 and CD25s were included in our composite classification model (along with Notch1 and E-selectin) and performed as the best predictors of a good response to therapy.

We did not observe significant changes in miRNA expression after treatment with dupilumab. miRNA levels in the blood change quickly in acute conditions[14] and may reach a state of equilibrium in a relatively short time, which could explain observed similar levels of miRNA before and after therapy. Alternatively, the similarities of miRNome before and after treatment may reflect the fact that dupilumab is managing the downstream effects of Th2 inflammation which does not play a major role in miRNA expression. Nevertheless, we were able to identify differences in miRNA levels between atopic dermatitis, psoriasis, and healthy individuals that may be disease dependent.

In a recent study, miRNA expression levels in the skin and serum of AD and psoriasis patients were compared to healthy individuals[39]. The authors reported differentially expressed levels of (among others): hsa-miR-378a-3p, hsa-miR-146b, and hsa-miR-25-3p in skin and hsa-miR-122-5p in serum. These results are in line with our findings, although the authors used different analytical methods (Affymetrix arrays) and different biomaterials. However, the authors report significantly more differentially expressed miRNA from skin samples than from serum, suggesting that skin biopsies are a more suitable material for identifying mechanistically relevant miRNA biomarkers.

In our study, we identified a treatment-related shift in the composition of microorganisms colonizing the skin of AD patients, which was associated with the severity of the disease (IGA, SCORAD, and BSA). The use of the microbiome as a biomarker for AD severity has been proposed in recent literature[11], but there is currently a lack of consensus on standardized methodology and a need for further large-scale, longitudinal clinical studies to establish its usefulness. A previous study reported that dupilumab treatment decreased skin colonization with *S. aureus* and increased skin microbial diversity in children with AD[40]. Our findings extend these observations to adults. However, we did not find a clear correlation between baseline skin microbial composition and treatment outcomes.

Taken together we identified several proteomic and microbial, but no miRNA, biomarkers in AD patients receiving systemic IL-4Ra targeted treatment. Based on our clinical data, we were able to identify a set of protein-based biomarkers associated with a good clinical response. Nevertheless, these biomarkers need to be validated in a larger cohort during a longer follow-up period. Although the lack of skin based analysis might be regarded as a weakness of our study, the measurement of biomarkers in a patient's serum is more suitable in daily clinical practice and should be further explored to select the optimal treatment in a currently broadening spectrum of targeted therapies.

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Figures

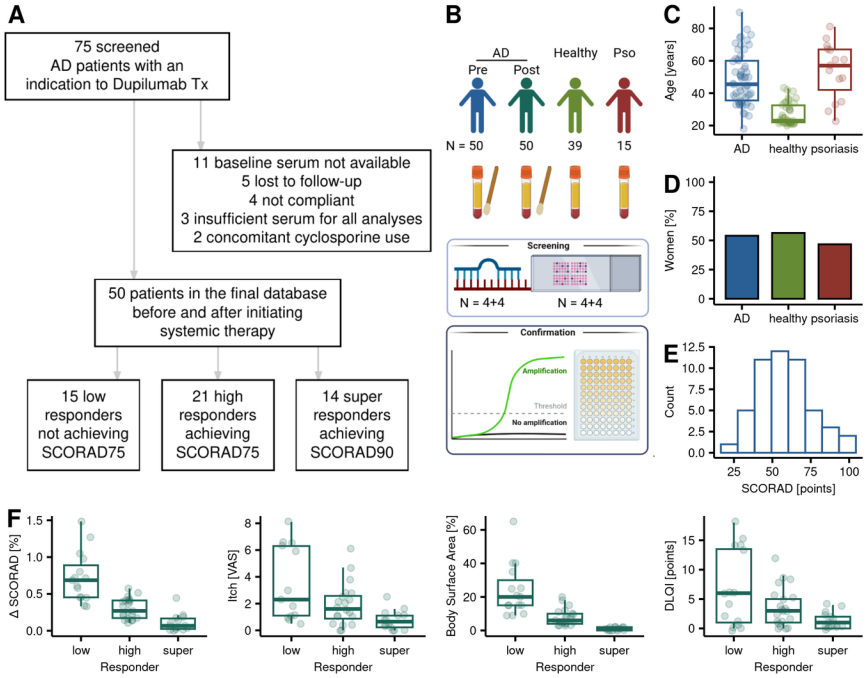

Figure 1: ***Cohort definition and study design.*** ***A)*** *Flow chart summarizing included samples, exclusion criteria, and clinical response.* ***B)*** *Study design, indicating the sample size in each group. Serum samples underwent screening using Next Generation Sequencing (miRNA) and proteomics screening, followed by a confirmation on a larger cohort using RT-qPCR and ELISA. Skin microbiota were quantified by RT-qPCR.* ***C-D)*** *Age and sex distributions in the studied cohort and their corresponding controls.* ***E)****Distribution of SCORAD values in studied AD patients cohort before initiation of therapy.* ***F)*** *Clinical parameters after dupilumab therapy in low, high, and super responders.*

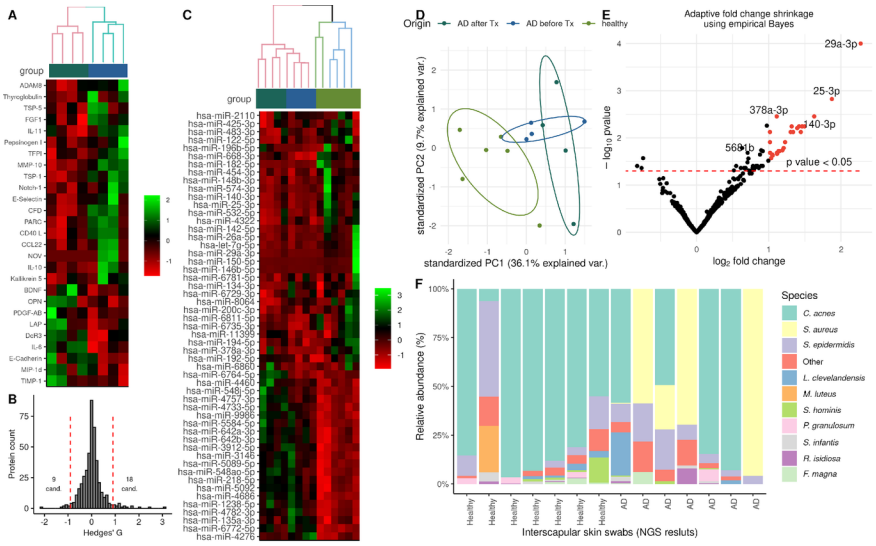

Figure 2: ***Screening for potential protein, miRNA and microbiome biomarkes in patients with atopic dermatitis.*** ***A)*** *Heatmap of 4 atopic dermatitis serum samples before (blue column) and 6 months after (teal column) systemic therapy with dupilumab, unsupervised complete hierarchical clustering with euclidean distances.* ***B)****Histogram of effect size estimates between all 440 screened proteins (pre vs 6-month post initiation of dupilumab therapy) expressed in Hedge's G values. Candidate proteins were identified as showing the Hedge's G value either higher than 0.9 or lower than -0.9.* ***C)****Heatmap of 60 most differentially expressed miRNAs in serum using next generation sequencing in AD patients before (blue) and after (teal) systemic therapy applying unsupervised hierarchical clustering with euclidean distances. Additional 6 healthy control samples (top row green were provided for completeness* ***D)*** *Principal component analysis of the scaled and centered (uncurated) Next-Generation-Sequencing data shows a separation of AD-derived samples from healthy individuals.****E)*** *Volcano plot of differentially expressed miRNAs. Red dots indicate log2 fold change < -1.* ***F)*** *Relative abundance of 10 most abundant skin microbiota in 7 AD patients and 7 healthy individuals measured by sequencing of 3rd to 4th hypervariable regions of 16S ribosomal RNA by NGS.*

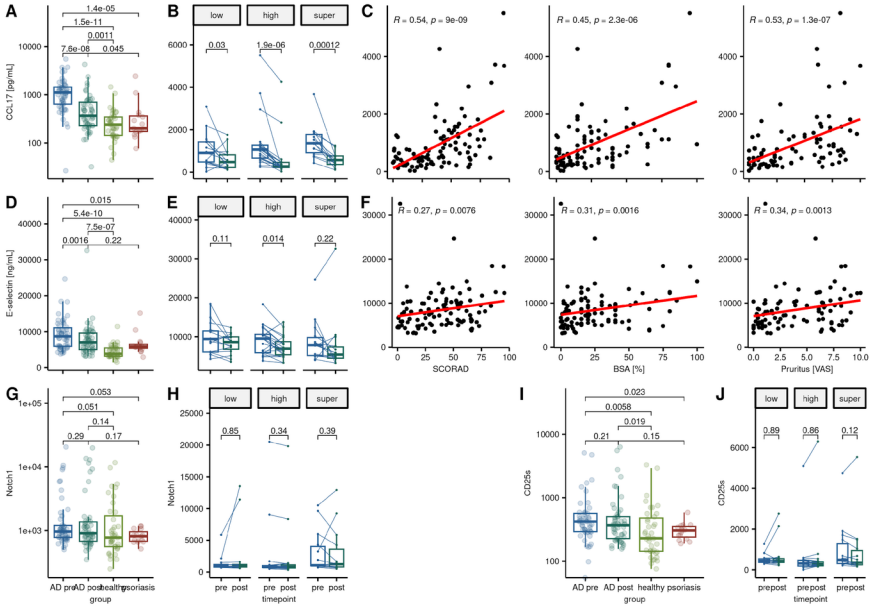

Figure 3: ***Protein predictors in relation to the clinical response to dupilumab.*** *Quantification of selected protein candidates in serum by ELISA.* ***A, D, G, I*** *panels shows protein levels in AD patients (paired samples before and after therapy), psoriasis patients and healthy individuals.* ***B, E, H, J*** *panels show differences in AD patients (paired samples before and after therapy) in three groups of responders (low, high and super responders). Comparisons between unpaired groups were done by Mann-Whitney U test and paired groups by Wilcoxon test.* ***C, F****: Spearman's rank correlation of the selected biomarkers to SCORAD, BSA, and itch on a VAS in AD patients (pre and post therapy).*

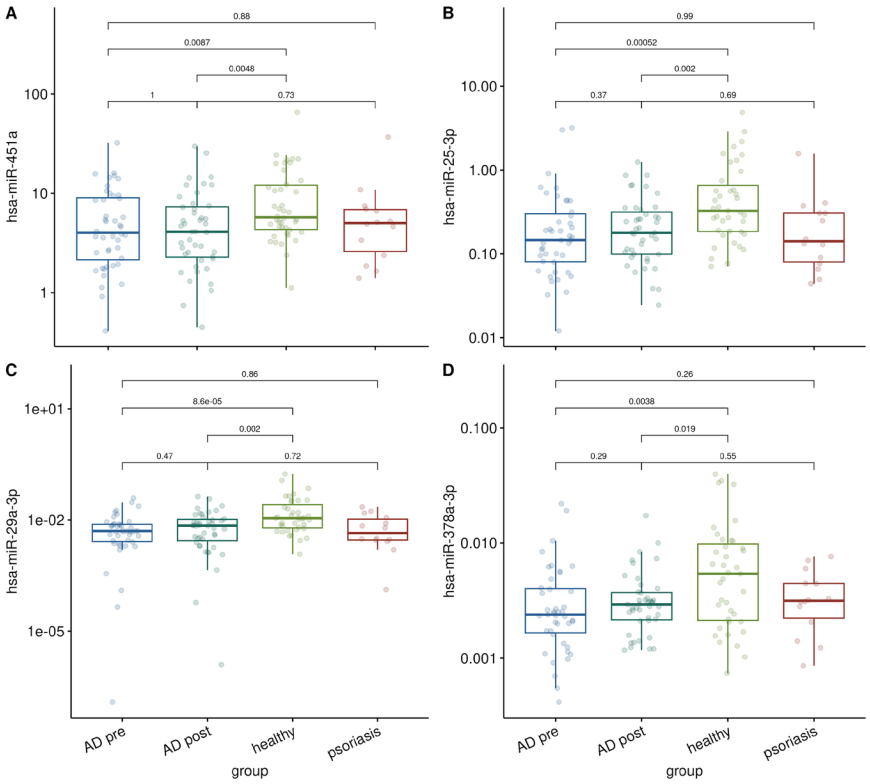

Figure 4: ***Validation of miRNA biomarkers in patients with atopic dermatitis before and after systemic therapy compared to healthy individuals.*** *Validation of selected miRNA candidates using RT-qPCR. The panel shows miRNA levels in AD patients (paired samples before and after therapy), psoriasis patients, and healthy individuals. Comparisons between unpaired groups were done using the Mann-Whitney U test and, in paired groups, using the Wilcoxon test.*

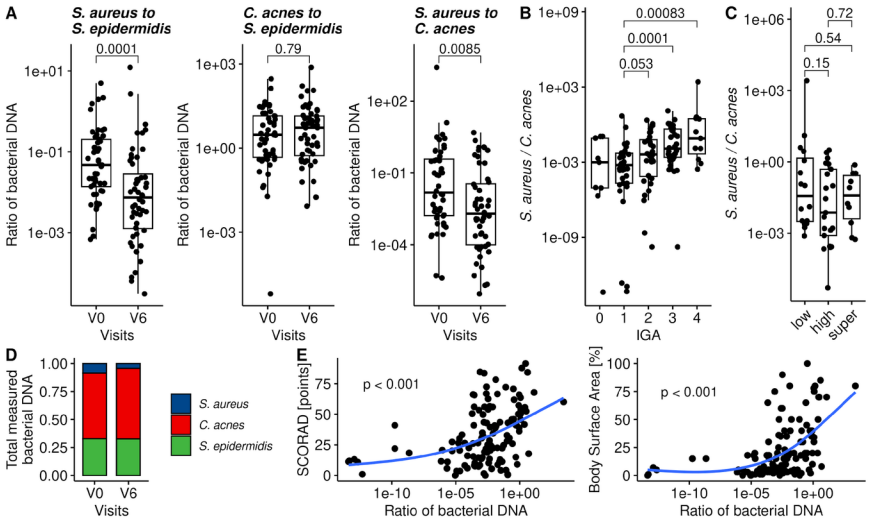

Figure 5: ***Skin bacterial species ratio changes upon dupilumab therapy. A)*** *Ratio of bacterial DNA*

of three bacterial species before and after initiating dupilumab therapy. ***B)*** *S. aureus to C. acnes ratio in 4 grades of IGA score of AD patients.* ***C)****Ratio of S. aureus to C. acnes before therapy in 3 responder groups.****D)*** *Ratio of S. aureus, C. acnes and S. epidermidis in AD patients before and after therapy.* ***E)*** *Correlation of S. aureus to C. acnes bacterial DNA ratio with SCORAD and BSA clinical scores. The blue line represents a generalized additive model's smoothness estimation.*

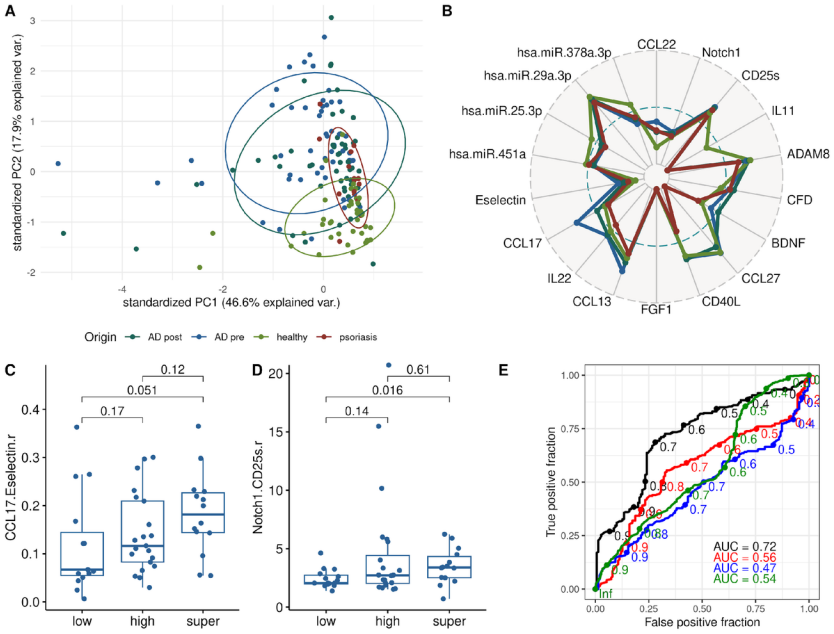

Figure 6: ***Biomarker profiles in the studied cohort and its composite analysis.*** ***A)*** *PCA analysis of the whole cohort with the most informative biomarkers (CCL17, CCL13, CCL22, CCL27, E-Selectin, BDNF, CD25s, Notch1).* ***B)*** *Radar plots depicting changes in median values of each scaled biomarker between AD, psoriasis patients, and healthy individuals.* ***C)-D)*** *Ratios of best performing predictive biomarkers of a good response to dupilumab therapy* ***E)*** *Receiver Operating Characteristic (ROC) in a random forest model predicting good response (high and super responders vs. low responders) to dupilumab based on initial biomarkers with 2 best predictors (CCL17 to E-selectin and Notch1 to CD25s ratios - black curve) compared to models including CCL17 and E-selectin (red curve), Notch1 and CD25s (blue curve) or Notch1, CD25s, CCL17, E-Selectin (green curve).*