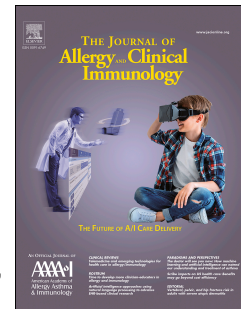


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Confirmation of multiple endotypes in atopic dermatitis based on serum biomarkers

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Conflicts of interest

Marjolein de Bruin-Weller is a principal investigator and advisory board member for AbbVie and Regeneron Pharmaceuticals, Inc; is a principal investigator, advisory board member, and consultant for Sanofi Genzyme; is an advisory board member for Eli Lilly; is an advisory board member for UCB; and is a principal investigator and advisory board member for Pfizer. All other authors declare no relevant conflicts of interest.

ABSTRACT

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

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

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

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

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

Key messages

- In a different population of severe AD patients, we confirmed the biological heterogeneity of AD by once more identifying four patient clusters based on distinct serum biomarker profiles, of which three resembled the previously identified patient clusters. A “skin-homing chemokines/IL-1R1 dominant” cluster, a “Th1/Th2/Th17 dominant” cluster, a “Th2/Th22/PARC dominant” and a “Th2/eosinophil inferior” cluster were identified.
- We additionally developed a prediction model based on a small set of biomarkers which was able to classify patients into the four clusters.
- Stratification of AD patients into distinct biomarker-based endotypes might contribute to more personalized medicine, and may be important to better inform which patient is most likely to benefit from specific targeted therapies.

Capsule summary

By once more identifying four AD patient clusters based on unique serum biomarker profiles, our study provides insight in the immuno-pathological characterization of AD patients, which might contribute to more personalized medicine in future.

Key words: Atopic dermatitis, endotypes, clusters, biomarkers, prediction, personalized medicine, principal components analysis

70 Abbreviations

- 71 AD: Atopic dermatitis
72 EASI: Eczema Area Severity Intensity
73 EGF: Epidermal growth factor
74 IL: Interleukin
75 IQR: Interquartile range
76 OOB: Out of bag
77 PARC: Pulmonary and activation-regulated chemokine
78 PCA: Principal components analysis
79 TARC: Thymus and activation-regulated chemokine
80 Th: T helper
81 TNFSF: Tumor necrosis factor superfamily
82 TSLP: Thymic stromal lymphopoietin

INTRODUCTION

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

In the past decade more and more advances have been made in characterizing the immunologic differences underlying AD. Although AD is considered to be a primarily T helper (Th)2 cell-driven disease, it has now become clear that Th1, Th17, and Th22 cytokine pathways are likely to contribute to AD pathogenesis as well.⁴⁻⁶ Due to the heterogeneity of the disease, it is unlikely that novel molecular therapies targeting specific immunological pathways will be equally effective in all AD patients, which makes the stratification of subtypes of AD patients of increasing importance. The identification of patients by relevant and validated biomarkers is a prerequisite for a more personalized therapeutic approach.⁷ Nevertheless, the distinct molecular mechanisms driving different disease subtypes of AD, previously defined as endotypes⁸, are yet inadequately described.

In a recently published study, we identified four clearly differentiated clusters of patients using a data driven approach on 147 biomarkers measured in 193 moderate-to-severe AD patients⁹. Each cluster was characterized by a specific serum biomarker profile, implying that distinct underlying immuno-pathological pathway drives each cluster. Two of these clusters were characterized by a Th2-dominated biomarker profile⁹, suggesting that the patients in these clusters would be ideal candidates

for Th2 inhibiting therapies, such as the recently developed anti-interleukin (IL)-4R α monoclonal antibody dupilumab or the anti-IL-13 antibodies tralokinumab and lebrikizumab. Stratification of patients into distinct endotypes might contribute to the development of personalized medicine approaches and precision based care in the future. However, the previously defined patient clusters still need to be replicated in an independent patient population. The aim of the present study is to confirm the previously identified AD patient clusters based on distinct serum biomarker profiles, by using the same data driven approach on a new cohort of severe AD patients. Additionally, we aim to build a prediction model enabling the stratification of patients into one of the four previously defined clusters by using a small set of selected markers, which might be incorporated in clinical trials or standard practice as a convenient tool to identify endotypes in the future.

METHODS

Patients and samples

To confirm the endotypes on a clinically well-defined large cohort of severe AD patients, who are most eligible for systemic/biological treatments, we used data of a previously reported cohort including AD patients, who were selected based on AD severity (Eczema Area Severity Intensity score, EASI >21) and only treated with topical corticosteroids, which original aim was to predict the need of systemic therapy.¹⁰ Out of the 152 patients of this cohort, six patients were also included in the cohort from the study of Thijs et al.⁹; in order to lower the risk of bias, these six patients were excluded for the current study, resulting in 146 patients included in the current study. Clinical characteristics were retrospectively extracted from the patients' electronic medical records. For the current study, AD severity was assessed by using EASI score, according to the Harmonizing Outcomes Measures in Eczema (HOME) recommendations.¹¹ Severity scores from the previous cohort⁹ were measured before the availability of these recommendations, and were assessed by using Six Area, Six Sign Atopic Dermatitis (SASSAD)

severity score, which was the standard severity score in our center at that time. Both severity scores incorporate grading of AD signs and assessment of body region involvement. All patients were diagnosed with AD according to the criteria of Hanifin and Rajka.¹² The protocols of this study were approved by the Institutional Review Board of the University Medical Center Utrecht (the Netherlands) adhering to the Declaration of Helsinki Principles.

Serum biomarkers

A panel of 143 serum biomarkers were measured using Luminex technology¹³, using an in-house validated panel of analytes listed in Table E1 (detailed methods in Online Supporting Information). The same biomarker measurements as previously published¹⁰ were used in the current study. Baseline thymus and activation-regulated chemokine (TARC)/C-C motif chemokine (CCL)17 levels, currently the best performing and accepted biomarker for disease severity¹⁴, were measured in routine care using Quantikine® ELISA immunoassays (R&D systems, Inc., Minneapolis, MN, USA).

Statistical methods

Replication of the four distinct patient clusters

Principal components analysis (PCA) followed by unsupervised k-means cluster analysis of the serum biomarker data was used to identify patient clusters. Additionally, PCA followed by k-means cluster analysis was repeated on the pooled serum biomarker data of the current cohort and the original cohort from the study of Thijs et al⁹. Clinical characteristics and averages of serum biomarkers were analyzed between the clusters using one-way ANOVA for normally-distributed variables, Kruskal-Wallis test for non normally-distributed variables, and χ^2 test for percentages.

Prediction model based on previously defined clusters

We built a prediction model based on the biomarker data used in the study of Thijs et al.⁹ to predict in which of the four previously identified clusters the patients in our cohort would belong. The prediction model was built using a supervised random forest approach (package randomForest¹⁵ in R) . The importance of each biomarker in the classification of patients was estimated using the mean decrease in accuracy. The prediction model accuracy, defined as the fraction of correctly predicted cases (1 – model error rate), was studied for several prediction models using all the 140 markers common between the two cohorts, as well as the top 70, top 20 and top 10 biomarkers. A flowchart presenting all the steps of the prediction model can be found as Figure E1 in Online Supporting Information.

Statistical analyses were performed using R Project software version 3.4.1 (R Foundation for Statistical Computing, Vienna, Austria)¹⁶, and the SPSS (Statistical Package for the Social Science) software for Windows version 21.0 (SPSS Inc., Chicago, IL, USA). Serum biomarker data were normalized by Box-Cox transformation before analyses. Before replication of the cluster analysis and building the prediction model, we merged the serum biomarker data of both datasets and corrected them for batch effects concerning two different batches (package sva¹⁷ in R). P-values lower than 0.05 were considered statistically significant.

RESULTS

Patient characteristics and cluster replication

Among the 146 patients, 56% were male, median (IQR) age was 30.5 years (22.0 – 50.0), median EASI score 28.3 (IQR 25.2 – 35.3), median TARC/CCL17 level 4192 pg/mL (IQR 2088 - 8496) and all were treated with topical corticosteroids at the moment of sampling. Patient characteristics from the current and original study⁹ are summarized in Table 1, disease severity in the current cohort differed from the original cohort⁹, in which patients with moderate-to-severe AD (median TARC/CCL17 level 1766 pg/mL,

IQR 874 – 5215, ($p < .001$)) were included. Besides, the current cohort had a significantly higher percentage of patients who were treated with any systemic immunosuppressive drug (not including systemic corticosteroids) within one year before sampling (22.6% vs 11.4%, respectively, $p = 0.010$). Age, sex, atopic comorbidities, and age of onset did not significantly differ between the two cohorts.

A total of 143 serum biomarkers were determined via multiplex immunoassay. After principal components analysis on the Box-Cox transformed serum biomarker dataset, the cumulative percentage of variance showed that 90% of the dataset's variance was described by the first 48 principal components (Figure 1). The first 48 principal components were included in the unsupervised k-means cluster analysis, resulting in the identification of four distinct patient clusters (cluster A, B, C and D, which are displayed in terms of the first three principal components in Figure 2A). The cluster membership per patient was added back to the complete dataset and clinical characteristics were compared between the four clusters (Table 2). Averages of serum biomarker levels were calculated per cluster to characterize the biomarker profiles driving the four clusters (Figure 2B, supplementary Table E2) and were also compared with the previously identified clusters⁹ (reported as cluster 1, 2, 3 and 4; Figure 2B).

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

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

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

Cluster D represented 29.5% of the AD patients, had a median age of 32.0 years (IQR 23.0 – 48.0) and median EASI score of 27.4 (IQR 25.6 – 32.8). It was characterized by a relatively low inflammatory state, particularly distinctive from the other clusters by low serum levels of Th2/severity- (MDC, PARC, TARC) and eosinophil-related markers (RANTES, eotaxin and eotaxin-3) (“Th2/eosinophil inferior” cluster). The biomarker profile of cluster D resembled the profile of the previously identified cluster 2.

Other clinical characteristics including age, sex, atopic comorbidities, age of onset, and hospitalization rate did not significantly differ between the four clusters (Table 2 and Figure 2C).

In addition, we analyzed the merged datasets (previously published and the present one) using a PCA and k-means cluster analysis, and here again we identified four patient clusters. Biomarker profiles of the merged clusters were largely comparable with the original patient clusters⁹. “Merged-cluster” 1 was characterized by the lowest levels of epithelial cytokines and IL-1 related cytokines, “merged-cluster” 2 by the highest levels of Th2 family cytokines, IL-1 related cytokines and TSLP, “merged-cluster”

3 by the highest levels of Th2 family cytokines and pulmonary and activation-regulated chemokine (PARC/CCL18), and “merged-cluster” 4 by the lowest levels of IFN α and apelin. Of the patients who clustered together in the original clusters, 88.3% clustered again together in one of the “merged-clusters” (Table E3). For the replication cohort, 68.5% of the patients clustered together again in one of the merged clusters.

Cluster prediction

As we could (re)confirm three of the four previously defined patient clusters, we next used a supervised random forest approach using the biomarker data of Thijs et al.⁹ to build a prediction model of cluster membership (cluster 1, 2, 3, or 4) for the patients of the current cohort. Only biomarkers determined in both datasets were used for this analysis, resulting in a total of 140 serum biomarkers. Biomarkers were sorted by prediction importance based on the mean decrease in accuracy. The different steps of this analysis are described in Figure E1 in Online Supporting Information.

The top 10 biomarkers were IL-37, IL-1 α , XCL-1, eotaxin/CCL11, IL-1 β , IL-26, LIGHT/tumor necrosis factor superfamily (TNFSF)14, IL-1r1, epidermal growth factor (EGF) and TSLP (Table E4). The accuracy of the prediction model, on the original study⁹, including all 140 biomarkers was 94.1% (Figure 3 and Figure E2) and the out of bag (OOB) estimate of error rate (i.e. number of incorrect classifications) was 5.3% (Table 3). When including only the top 10, top 20 and top 70 biomarkers, the accuracy was 86.7%, 90.4% and 93.6% respectively. The OOB estimate of error rate was 13.8%, 9.6%, and 5.3% respectively (Tables E5 - 7).

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

top 70 biomarkers was 87.0% and 73.3% and 81.5% using the model including the top 10 and the top 20 respectively.

DISCUSSION

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

Since three of the four clusters were confirmed in the current study, we were able to further characterize and name the clusters by their discriminating biomarker profiles. Patients stratified into cluster B were characterized by a relatively high inflammatory state and could be distinguished from the other clusters as being the “Th1/Th2/Th17 dominant” cluster. Cluster C shared relatively high Th2-related cytokine levels with the “Th1/Th2/Th17 dominant” cluster, although separated itself from the other clusters by showing high levels of IL-22 and PARC. Cluster D was characterized by the lowest levels of eotaxins, RANTES, PARC, MDC, TARC and IFN α and was thereby defined as the “Th2/eosinophil inferior” cluster. Patients identified in cluster A showed higher levels of the C-C chemokines CTACK/CCL27, TARC/CCL17, MDC/CCL22 and RANTES/CCL5. CTACK/CCL27, TARC/CCL17, and MDC/CCL22 are known to bind the C-C chemokine receptors CCR10, and CCR4^{19, 20} respectively, thereby

enabling skin-specific homing of CD4+ T-cells.²¹ RANTES/CCL5 is a ligand for CCR3 and CCR5 and is considered to maintain eosinophilic infiltration in chronic inflammation of AD skin.²² Furthermore, patients in cluster A showed higher expression of serum IL-1R1 levels. Previous studies have shown that serum IL-1R1 levels are significantly increased in AD patients compared to healthy controls²³, and that the upregulation of IL-1R1 is associated with frequent exacerbations in asthma patients.²⁴ Based on the underlying biomarker profile, cluster A could be defined as the “skin-homing chemokines/IL-1R1 dominant” cluster.

Although cluster D showed the lowest expression of several severity related markers (TARC, PARC and MDC), this was not reflected by the lowest EASI score. Similar to the previous cohort, the four identified AD patient clusters in the present study were clinically distinguished by disease severity, with the “Th2/Th22/PARC dominant” cluster showing a significantly higher EASI score. However, since all patients had high lesions/signs scores, we consider this difference as not clinically relevant. Furthermore, we were unable to identify an association between the four clusters and other clinical characteristics including age of onset or atopic comorbidities. This result highlights the challenges in identifying patient subgroups based only on clinical features and might indicate that individualized treatment options should not be based on clinical phenotypes of AD, but in fact, on biomarker based endotypes.

One of the four clusters (the “skin-homing chemokines/IL-1R1 dominant” cluster A) identified in the current study could not be matched with one of the previous clusters of the study by Thijs et al.⁹ An explanation for the different fourth cluster could be the difference in the percentage of patients who used immunosuppressive drugs within one year before sampling, as this percentage was higher in the current cohort compared to the original one and, moreover, significantly higher in the non-corresponding “skin-homing chemokines/IL-1R1 dominant” cluster A. The majority of these patients were treated with cyclosporine A (CsA) in the year before sampling. CsA is a calcineurin inhibitor that

selectively acts on T-cells through inhibiting signal transduction mediated by T-cell receptor activation.²⁵ It has been shown previously that CsA treatment in AD patients suppresses the levels of IL-2-, IFN γ - and IL-4/IL-5/IL-13-producing T-cells and T-cell products including TARC/CCL17.²⁶⁻²⁸ However, data on the long-term effects of CsA treatment on serum biomarkers after discontinuation are lacking. In agreement with previous findings in CsA treated AD patients, patients in the “skin-homing chemokines/IL-1R1 dominant” cluster A showed the lowest serum levels of IFN γ , IL-4, IL-5 and IL-13.

The ability to endotype patients based on serum biomarkers has already been demonstrated in asthma, where anti-IL-13, anti-IL-4/IL-13 and anti-IL-5 therapies appeared to be more effective in “Th2-dominant” patients groups. Clinical trials investigating the efficacy of treatment with the anti-IL-4/IL-13 receptor monoclonal antibody dupilumab in AD patients showed that only 35%-40% of the patients achieved clear or almost clear skin²⁹⁻³¹, which corresponds to the percentages of patients in the “Th1/Th2/Th17 dominant” and “Th2/Th22/PARC dominant” clusters. On the other hand, the numbers of non-responders to dupilumab treatment in AD patients are very low^{30, 32}, indicating that Th2 cytokines might not be the most relevant markers to discriminate AD patients in this overall Th2-high population. This might also explain why the top 10 biomarkers based on the mean decrease in accuracy of our prediction model that were found to be distinctive for the four clusters did not include any Th2-related markers. Although two clusters shared a relatively Th2-low cytokine profile compared to the other two clusters, these patients still express higher levels of Th2-related cytokines compared to levels that have previously been reported for healthy controls.^{9, 23} Prediction of treatment response by serum biomarker profiles in AD patients is scarce. A single phase 2b study investigating treatment with tralokinumab (anti-IL-13) suggested that baseline serum DPP-4 levels, reflecting IL-13 activity, might serve as a predictive biomarker for AD patients who may benefit from tralokinumab treatment.³³ Furthermore, a phase 2a study of IL-22 blockade with fezakinumab showed that patients with higher baseline expression of IL-22 had greater disease improvement during fezakinumab treatment³⁴, although IL-22 expression was

measured in skin biopsies, which is hard to implement in daily practice. Theoretically, patients in our “Th2/Th22/PARC dominant” cluster might be optimal candidates for anti-IL-22 treatment.

Both the original and the current study made use of a panel of more than 140 serum biomarkers to confirm the presence of four endotypes within AD patients. Although, for the implementation of serum biomarker based endotypes in clinical trials and daily practice, a smaller set of markers is desired. In the current study we built a prediction model, based on the biomarker data used in the study of Thijs et al.⁹, that could be used to classify our patients into one of the four original clusters with a good accuracy, even when using only the top 10 biomarkers (IL-37, IL-1 α , XCL-1, eotaxin/CCL11, IL-1 β , IL-26, LIGHT/TNFSF14, IL-1r1, EGF and TSLP). Surprisingly, none of those markers are Th2-related cytokines but they consisted of IL-1-, IL-10- and epithelium-related markers. We, hence, hypothesize that markers related to other cytokine pathways might be (more) important to define endotypes in an overall Th2-dominant disease such as AD. In the future, the application of such prediction model, resulting in a small panel of biomarkers, might enhance tailored decision making in the management of moderate-to-severe AD patients and might contribute to more personalized medicine. However, the use of the prediction model should be tested in longitudinal studies and randomized controlled trials with drugs targeting specific pathways first.

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

strength of our study, was the confirmation of three of the four previously identified clusters within a cohort that was not originally designed as a validation cohort.

While we aimed to divide AD patients into distinct endotypes based on blood molecular profiles, previous studies have proposed to integrate models of lesional and non-lesional skin with blood measures, as well as clinical parameters, to reflect disease outcomes in AD. Wen et al.³⁵ demonstrated a Th2/Th22 profile in blood of Asian AD patients, with lower Th1-related cytokine levels compared with European American patients, which was attributed to reciprocal downregulation of this axis by the increased Th17 pathway in the skin³⁶. Zhou et al.⁶ compared AD endotypes among different age groups by evaluating lesional and non-lesional skin, as well as serum biomarkers. This study found decreases in Th2/Th22 activation and increases in Th1/Th17 axes with age in AD patients combined with a normalization of epithelial abnormalities. Although, integrated blood-skin biomarker models might be a more holistic way to build a disease profile, we believe that only serum biomarker endotyping can advance personalized therapeutics and may be more patient friendly. Establishing blood biomarker profiles is particularly crucial in children, in whom skin biopsies are challenging. To confirm our findings in different clinical subgroups of AD patients, it would be very interesting to perform a separate evaluation of endotypes in a cohort of Asian and/or African American and paediatric and/or elderly AD patients. Furthermore, biomarker based cluster analysis in patients with other inflammatory skin diseases, including psoriasis, lichen planus or contact dermatitis might be useful as control for our results in future.

The present study provided the first step in the confirmation of our previously reported serum biomarker based patient clusters⁹, by replicating three of the four clusters in a different retrospective cohort. Additionally, we constructed a prediction model which was able to stratify patients into one of the four clusters by using only 10 serum biomarkers. The use of a small set of biomarkers to predict patients' cluster status may easily be incorporated in clinical trials and standard practice. Given the

365 introduction of new targeted therapies for AD, the use of endotypes may be helpful, since patient with
366 different endotypes might respond differently to the same treatment. Future longitudinal clinical studies
367 are needed to investigate whether the defined endotypes are stable over time and whether patients
368 might switch between clusters over clinical course (after treatment with systemic immunosuppressive or
369 immunomodulating drugs for instance). Subsequently, confirmation of the endotypes and prediction
370 model in clinical trials including AD patients treated with drugs targeting specific pathways will be the
371 final step in the confirmation of endotypes in AD.

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TABLES

Table 1. Baseline characteristics

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

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

Table 2. Clinical characteristics for the four AD patient clusters

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

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

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

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

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

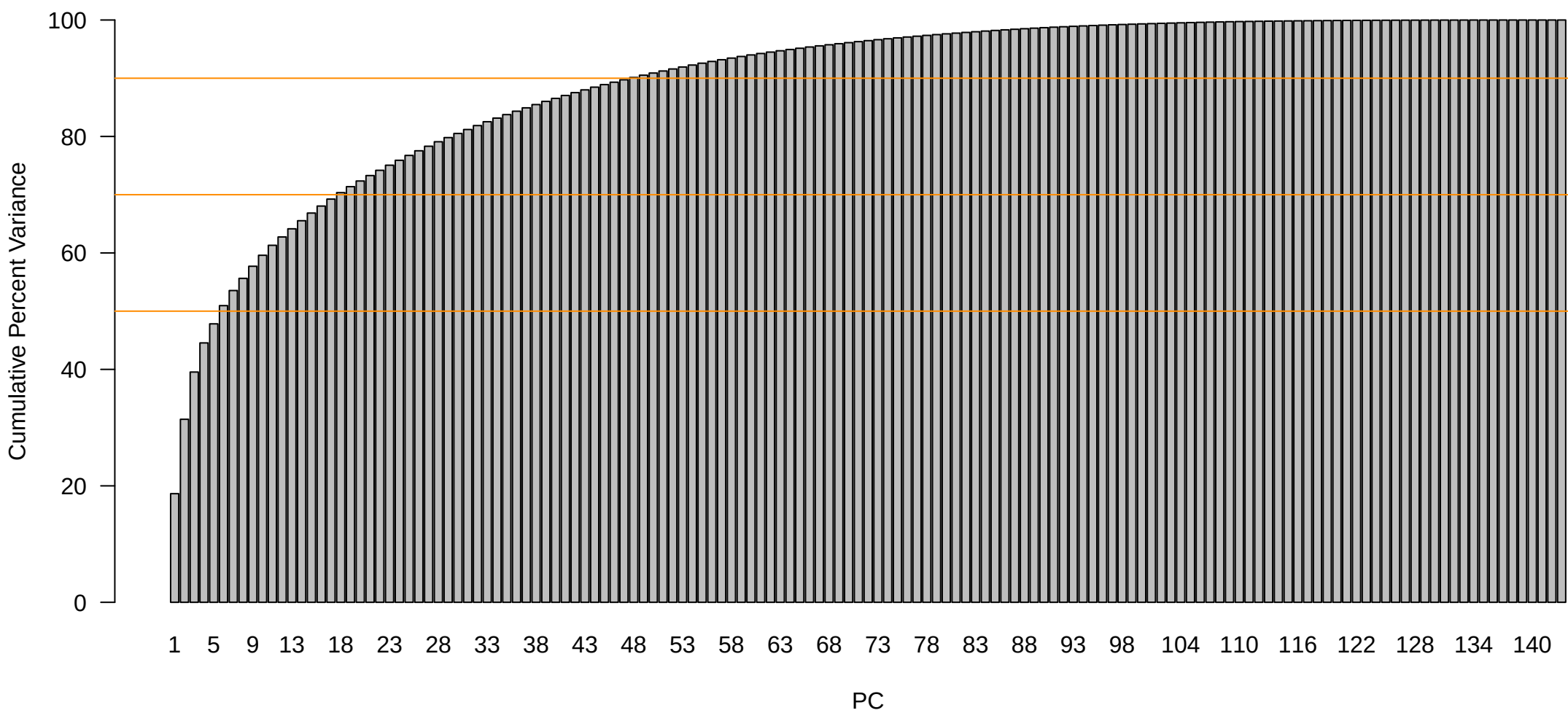
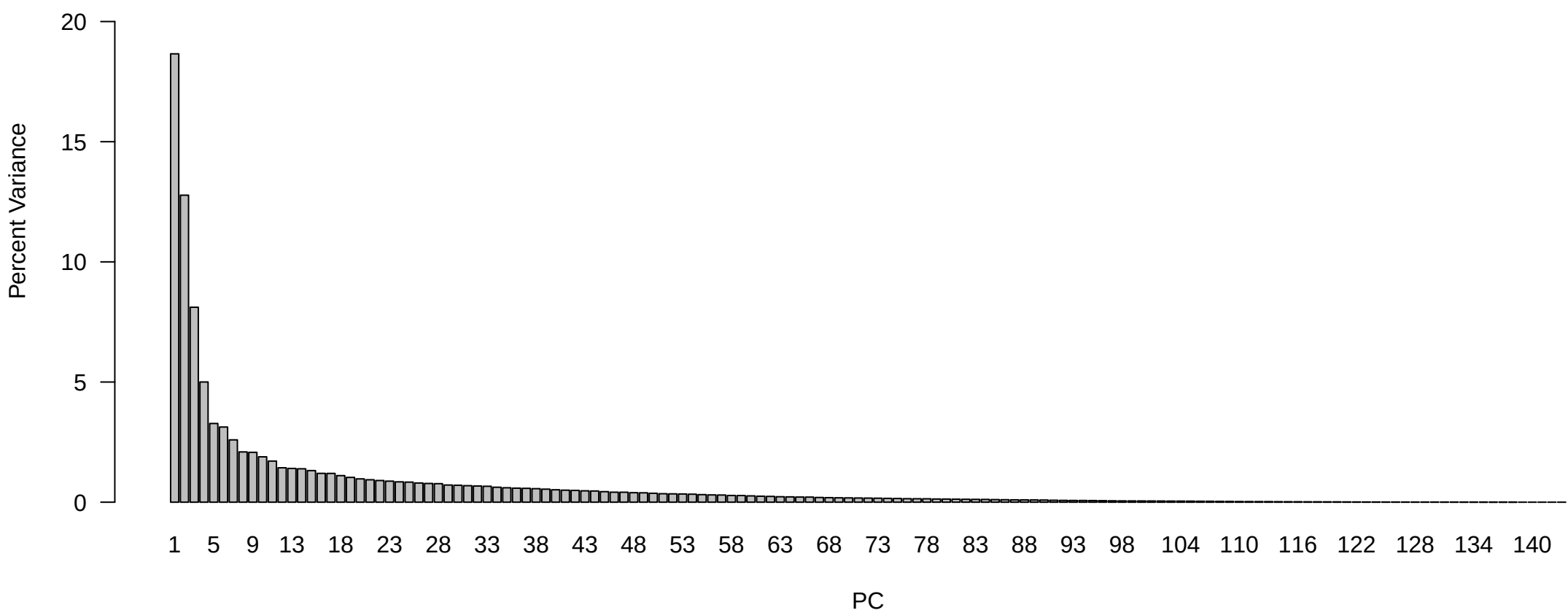
FIGURE LEGENDS

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

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

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

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



Cluster B
high TSLP, epithelial cytokines and
Th2 family cytokines

Cluster C
high PARC and
Th2 family cytokines

Cluster A
high sIL-2r, IL-8 and CXCL6

Cluster D
low RANTES, TIMP-1
and VEGF

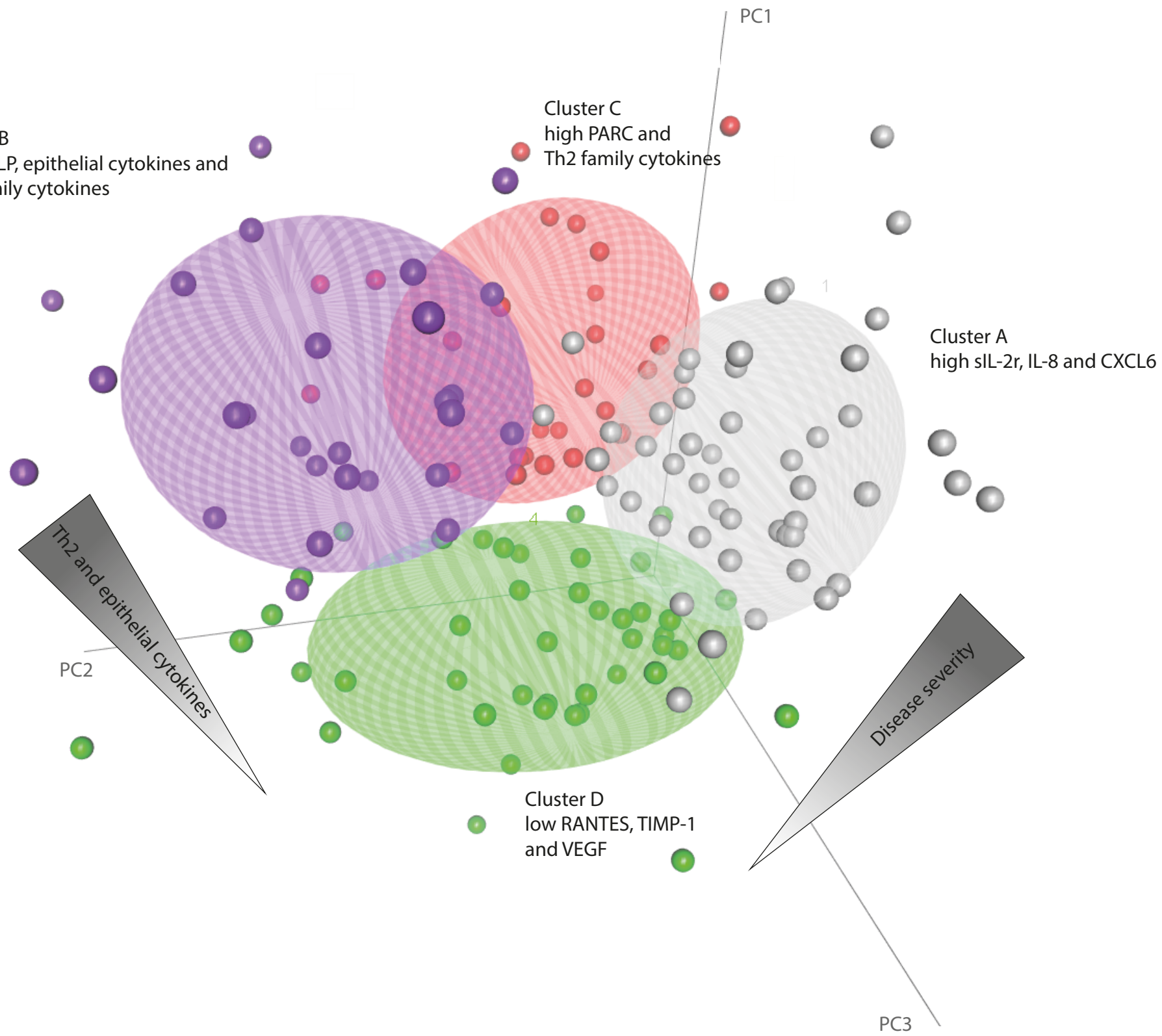
Th2 and epithelial cytokines

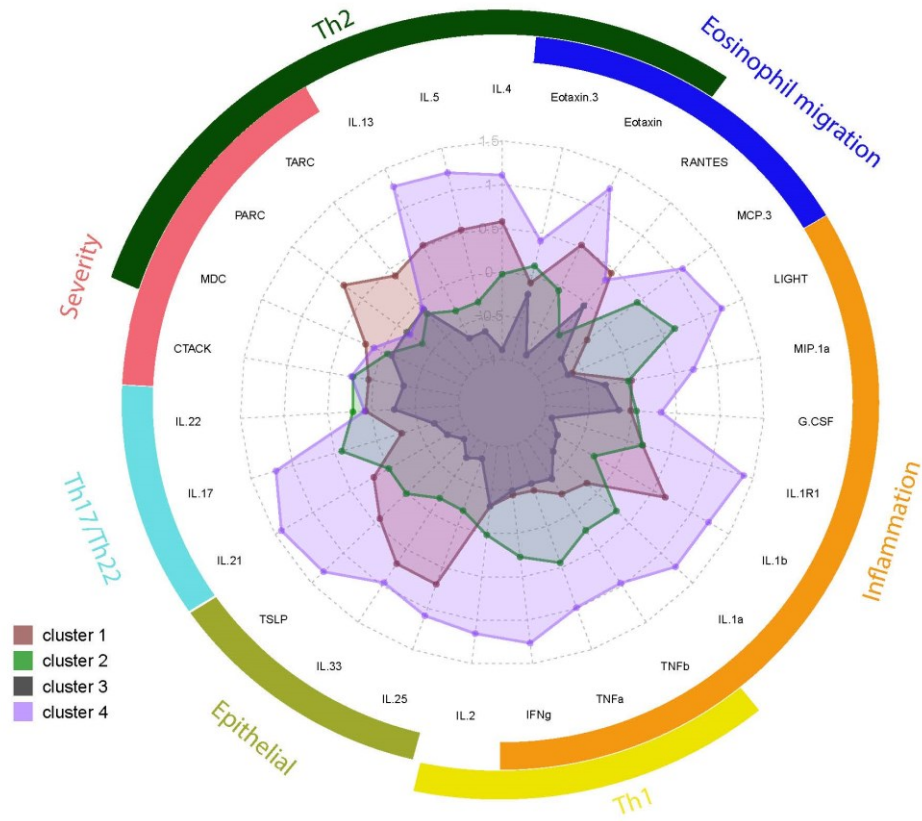
Disease severity

PC2

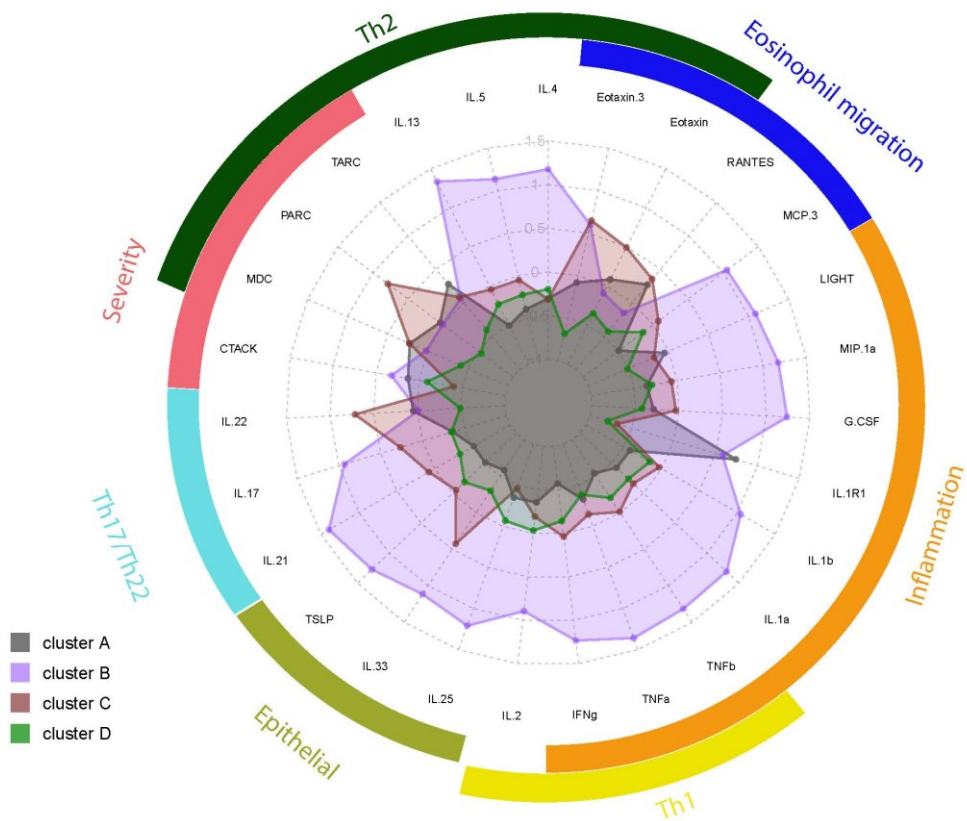
PC1

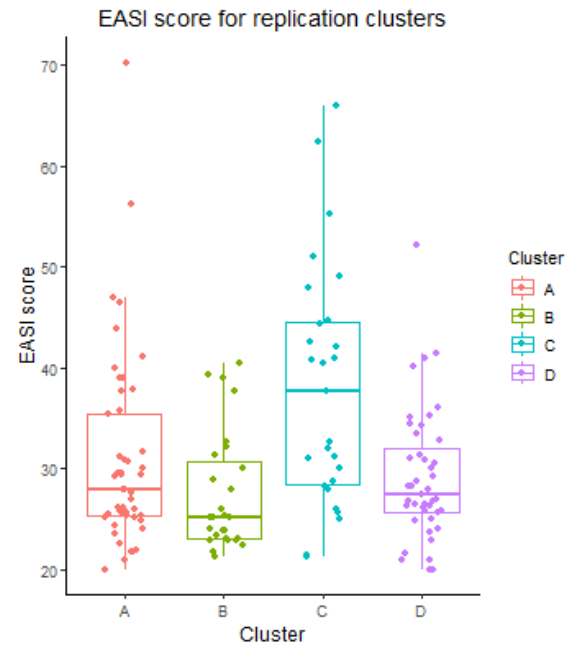
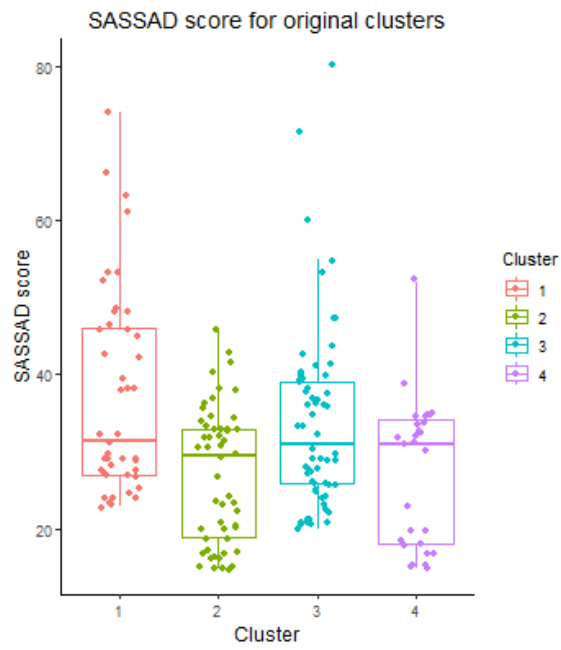
PC3



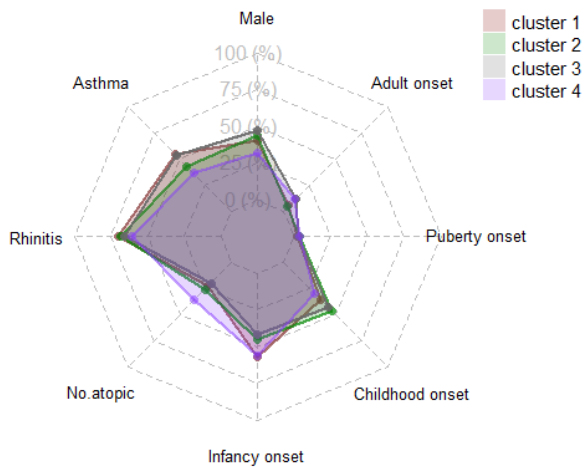
B Original cohort

Replication cohort





Clinical characteristics original cohort



Clinical characteristics replication cohort

