***Stratum corneum* markers of innate and T helper cell-related immunity and their relation to the disease severity in Croatian patients with atopic dermatitis**

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Abstract

Background: Atopic dermatitis (AD) presents with the wide spectrum of clinical phenotypes within and between various populations. Recent study showed low frequency of filaggrin loss-of-function (FLG LOF) mutations in Croatian AD patients. At present, there are no data on biomarkers of immune response in Croatian AD patients that might be useful in the selection and monitoring of novel immune therapies.

Objectives: To investigate levels of cytokines of various signature in the *stratum corneum* (SC) collected from lesional and non-lesional skin of AD patients and healthy controls, and to evaluate their relationship with the severity of disease and skin barrier function.

Methods: SC samples were collected from 100 adult patients with moderate to severe AD and 50 healthy controls. The levels of 21 cytokines were measured by multiplex immunoassay. We conducted machine learning analysis to assess whether a small number of cytokine measurements can discriminate between healthy controls and AD patients and can predict AD severity (SCORAD).

Results: The SC levels of thirteen cytokines representing innate immunity, Th-1, Th-2 and Th-17/22 immune response showed significant differences between healthy and AD skin, the difference being more pronounced in lesional skin. Our analysis demonstrated that as few as three cytokines measured in lesional skin can discriminate healthy controls and AD with an accuracy of 99%, and that the predictive models for SCORAD did not achieve a high accuracy. Cytokine levels were highly correlated with the levels of filaggrin degradation products and skin barrier function.

Conclusions: *Stratum corneum* analysis revealed aberrant levels of cytokines representing innate immunity, Th-1, Th-2 and Th-17/22 mediated immune response in Croatian AD patients. Increased Th-2 cytokines, and their strong association with natural

moisturizing factor (NMF) can explain low NMF levels despite of low frequency of *FLG* LOF mutations in Croatian population. Predictive models for SCORAD identified cytokines associated with SCORAD but warrants further investigation.

INTRODUCTION

Atopic dermatitis (AD) is the most common inflammatory skin disease, affecting up to 20% of children, and up to 10% of adults (1). AD is characterized by generalized barrier dysfunction in both lesional and non-lesional skin. Non-lesional skin shows subclinical inflammation, skin barrier dysfunction, altered levels of cytokines and lymphocytic infiltration (2,3). Mild, but chronic skin inflammation has been demonstrated in AD skin even during the remission (2,4). AD shows large heterogeneity in clinical phenotype, microbiome and involvement of Th-cell subsets (2, 4, 5-15). Differences have been reported between acute and chronic lesions (6-10), skin types (5), age groups (5, 16-21) and genetic background (5, 22), in particular presence of filaggrin gene variants (23-26). Disease heterogeneity of AD poses a challenge in clinical practice urging the need for defining disease endotypes enabling personalized therapy (27).

Need for endotype identification has been potentiated by development of novel therapies which target specific immune pathways and interventions aiming at skin barrier repair in high risk children (5, 16-22). Biomarkers of skin barrier and immune response could facilitate these unmet needs. Recently, we showed that the prevalence of filaggrin loss-of-function (*FLG* LOF) mutations in Croatian patients with moderate to severe AD is approximately 4%, which is much lower than in Northern European countries (28). *FLG* mutations have a strong effect on the skin barrier and immune response (23-28). However, there have been no studies on the profiles of immune mediators in Croatian AD patients and it is yet unclear whether in Croatian patients, AD is driven by the same T-cell subsets as reported in other populations.

Therefore, in the present study we investigated the levels of a broad spectrum of *stratum corneum* (SC) markers including innate immunity, Th1, Th2 and Th17/Th22 cytokines in Croatian AD patients and healthy controls. We further aimed to identify a panel of biomarkers that could discriminate between healthy subjects and AD patients and predict AD severity. The biomarker profiles were determined in lesional and non-lesional skin, as

previously they showed different association with disease severity (29,30). Knowing the profiles of the cytokines mediated by these T-cell subsets and their relation to disease severity might aid in the choice and monitoring of immune therapies.

METHODS AND MATERIALS

Study population

A total of 150 participants (100 AD patients and 50 healthy controls) were enrolled in this study. Our study population included 100 individuals who have been diagnosed with AD and treated at the Allergology outpatient unit Department of Dermatology and Venereology, University Hospital Center Zagreb, School of Medicine University of Zagreb during one year (2016-2017). Diagnosis of AD was confirmed by experienced dermatologists in the Allergology outpatient using Hanifin and Rajka criteria (31).

Exclusion criteria for group of patients were active skin infections, systemic immunosuppressants or phototherapy within last 4 weeks. Topical steroids or immunomodulators had to be excluded within 1 week and moisturizers within 12 hours before evaluation.

The control group consisted of 50 healthy individuals underwent clinical examination in order to exclude milder unrecognized forms of AD. Exclusion criteria for control group were severe inflammation of the skin or severe systemic disorder, personal data of atopic disorders or any kind of eczema in childhood.

All participants provided written informed consent, which complied with the principles of the Declaration of Helsinki. For a patients younger than 18 years of age, both parents were obliged to sign the informed consent. This study was approved by the local Medical Ethical Committee of the University Hospital Centre Zagreb, Croatia.

Clinical assessment

All the patients met the Hanifin and Rajka criteria for the diagnosis of AD (24). The age of onset of AD was recorded. Scoring Atopic Dermatitis (SCORAD) scale was used in order to assess severity. All patients were classified into mild (10-28.9 points), moderate (29-48.9 points), or severe (> 49 points) group of AD defined by SCORAD (32, 33).

Sampling of the *stratum corneum*

The SC was sampled using the previously described method (34). Circular adhesive tapes (22 mm diameter, 3.8 cm², D-Squame Discs; Monaderm, Monaco, France) were placed onto the selected part of the skin and pressed for 10 seconds with a pressure of 225 g cm², using a D-Squame Pressure Instrument D500 (CuDerm Corporation, Dallas, TX, USA). Lesional, non-lesional and healthy skin were collected. Non-lesional and healthy skin was taken from forearm. The non-lesional skin site was chosen approximately 2 cm from the lesion. The tape strips were removed with tweezers, placed individually into prior marker cryo-vials. Sequentially, eight consecutive tape strips were collected and immediately stored at –80 °C.

Determination of filaggrin breakdown products in the *stratum corneum*

Natural moisturizing factor (NMF) was defined as the sum of the concentrations of histidine, 2-pyrrolidone-5-carboxylic acid (PCA) and *trans*- and *cis*- isomers of urocanic acid (UCA). NMF components were determined in the 5th tape strip according to the slightly adopted method described in detail elsewhere (34). Briefly, NMF components were extracted with 600 µl of Millipore water and subsequently analysed by high-performance liquid chromatography (HPLC-UV).

As the amount of the SC on the tape strips varies, the concentration of NMF in the SC on each tape was normalized by the total protein content. Due to incomplete extraction recovery of SC proteins by water, the second extraction of proteins from the tape stripping was performed with 1.0 mol/L KOH solution. Proteins in both extracts were determined using the Pierce Micro BCA Protein Assay Kit (Thermo Fischer Scientific, Rockford, IL, USA), with the bovine serum albumin supplied as standard. The levels of NMF in the SC were expressed as mmol NMF/g protein.

FLG genotyping

All subjects were screened for the three most common filaggrin mutations found in the European population (R501X, 2282del4, R2447X), as previously described (34).

Cytokine analysis in tape strips

Cytokine levels were measured in the 8th tape strip according to the procedure described in details elsewhere (5, 36). Briefly, to each vial 1.2 mL phosphate-buffered saline (Merck, Darmstadt, Germany) containing 0.005 % Tween 20 (Sigma-Aldrich, Zwijndrecht, the Netherlands) was added. Extraction of cytokines and soluble proteins was performed with an ultrasound sonifier equipped with a probe (Salm & Kipp, Breukelen, the Netherlands) for 15 minutes in ice water. Extract aliquots of 200 μ L were distributed in vials and stored at -80°C until further analysis.

In total, concentrations of 21 cytokines were determined using MESO QuickPlex SQ 120 (MSD, Rockville, MA, USA) according to the manufacturer's instructions. Cytokines were measured on preconfigured multiplex panels (U-plex assays, MSD, Rockville, MA, USA). The following cytokines were included CCL11 (Eotaxin), CCL26 (Eotaxin-3), CXCL8 (IL-8(HA)), CXCL10 (IP-10), CCL2 (MCP-1), CCL13 (MCP-4), CCL22 (MDC), CCL3 (MIP-1alpha), CCL4 (MIP-1beta), CCL17 (TARC), IL-17A, IL-1 beta, CCL20 (MIP-3alpha), IL-31, TSLP, IL-21, IL-22, IL-23, IL-27, IL-33. IL-18 was determined using V-plex assay (MSD, Rockville, MA, USA). The extracts were applied undiluted.

As the amount of the SC on the tape varies, the amount of cytokine in the SC on each tape was normalized by the protein content, which was determined using the Pierce Micro BCA Protein Assay Kit (Thermo Fischer Scientific, Rockford, IL, USA), with the bovine serum albumin supplied as standard.

For data analysis, cytokine concentrations that were below the fit curve range were assigned half the value of the lowest sample concentration to maintain the ranking order. The number of samples collected from healthy, non-lesional and lesional skin which were under the fit curve range are presented for each cytokine in Supplementary Table S1. The limit of detection for each cytokine was calculated by Discovery Workbench 4.0 software (MSD, Rockville, MA, USA), as 2.5 standard deviations above the background signal.

Statistical analysis

Statistical analysis was performed using Prism 8 software (GraphPad, San Diego, CA, USA). Cytokine concentrations were log-transformed prior to statistical analysis. The difference in the levels of NMF, transepidermal water loss (TEWL) and cytokines between healthy (Ctrl) and non-lesional (AD_{NL}) skin and between healthy and lesional

skin (AD_L) of AD patients was tested by two-tailed Mann-Whitney test. The difference in biomarker NMF levels between lesional and non-lesional skin was tested by Wilcoxon signed-rank test.

Correlation analysis between SCORAD, TEWL or NMF and cytokine levels in non-lesional and lesional skin of AD patients was performed by two-tailed Spearman's correlation. P -value < 0.05 was considered significant. A total of 179 P -values derived from all statistical analyses were pooled and corrected for multiple testing using Benjamini-Hochberg procedure (37).

Machine learning analysis

We conducted a machine learning analysis for binary classification between AD patients and healthy controls and for regression of SCORAD. We used a panel of cytokines (log-transformed and standardized cytokine concentration with zero mean and unit variance) from 42 healthy controls and 83 AD patients for whom the cytokines were measured in both lesional and non-lesional skin. The missing values for IL-18 in two healthy controls were imputed with the mean value for the other healthy controls. The analysis consisted of two steps (Fig. 1): feature selection to identify a small set of cytokines that are relevant to AD (classification) or SCORAD (regression), followed by prediction of AD status (classification) or SCORAD (regression) by the selected cytokines.

Feature selection (for both classification and regression) was conducted by stability selection (53), i.e. repeated subsampling with elastic net (54) for 200 times with the l_1 ratio (the trade-off between l_1 and l_2 penalty) of 0.5. We selected the top k cytokines ($k=1, 2, 3, \dots$) with a high selection probability and examined their predictive performance, where a selection probability for each cytokine was empirically estimated by the frequency for it being selected out of all iterations of subsampling.

We applied a linear support vector machine (SVM) to predict whether a patient is AD or healthy (classification) or a ridge regression to predict SCORAD of AD patients (regression). Neither linear SVM nor ridge regression selected features since the feature selection was conducted separately. The process was repeated for 50 times, each based

on a different random partition of the training (80%) and test (20%) sets. Optimal parameters were determined by leave-one-out cross-validation on the training data.

The results of the proposed model in the regression analysis were compared with those by non-regularized linear regression (ordinary least squares) and nonlinear models (such as kernel ridge regression with a polynomial kernel and random forest) using all the features. In random forest, we estimated the importance of each feature by its corresponding node on the decision trees, although the actual predictive power of the most important features cannot be estimated.

The baseline accuracy for classification ($69.4 \pm 1.3\%$) was obtained by the average accuracy for all 50 test sets, in which all test samples were predicted to be AD. The baseline root mean squared error (RMSE) for regression (17.5 ± 0.3) was estimated by the average RMSE for all test samples where the predicted SCORAD is the mean SCORAD of AD patients in the training set after removing those with no cytokine measurements.

Machine learning analysis was performed using scikit-learn 0.22 (40) in Python 3.7.

RESULTS

Clinical features

Demographic details of the patients and controls are outlined in Table 1. Most of patients had moderate to severe AD (90%) and developed disease at early age (72% before 3rd year of life, 9% before 3rd and 5th year and 15% between 5th and 18th year), while only 4 patients developed disease in adulthood. The patients had a longstanding disease (minimum duration of disease was 3 years.) There were only four carriers of *FLG* LOF mutation, all of them in the AD patient group. AD patients showed reduced skin barrier associated with significantly higher TEWL readings than healthy controls, even in non-lesional skin (Table 1). 68% (lesional) and 69% (non-lesional) of the AD patients with measured cytokine concentration were female, while the gender was more balanced for healthy controls (52% female). Comparison of the individual cytokine concentration demonstrated no significant differences between female and male participants, apart from for IL-21 in lesional skin (Fig. S1).

Stratum corneum levels of cytokines and NMF: atopic dermatitis vs. healthy controls

The levels of the measured cytokines in healthy and AD skin (non-lesional and lesional) are shown in Fig. 2, separately for the markers of innate activation (CXCL8, IL-18, IL-1 β , TSLP), Th17 markers (IL-17A, IL-21, IL-23), Th2 markers (CCL2, CCL17, CCL22, CCL11, CCL26, IL-33, IL-31), Th1 markers (CXCL10) and other cytokines, CCL13, CCL4, CCL20, CCL3, IL-22 and IL-27.

Majority (16 out of 21) of cytokines showed significantly different levels in AD skin compared to healthy controls even in non-lesional skin (Fig. 2). Most of cytokines showed increased levels in AD skin, and this increase was more prominent in lesional skin. The lower levels in AD skin as compared to healthy skin was found for IL-21, IL-22, IL-23, IL-27, IL-31, IL-33 and TSLP (only in non-lesional skin). The variability in the levels of IL-22, IL-23 and IL-31 was large, especially in non-lesional skin. These cytokines showed also a large number of samples (e.g. for IL-23, respectively 32% and 30%) with the concentrations lower than the fit curve range (Supplementary Table S1).

NMF levels were significantly lower in lesional skin of AD patients compared to healthy skin and AD non-lesional skin (Fig. 3). Difference in median values of NMF between non-lesional and healthy skin amounted to 0.07 mmol/g protein (95% CI -0.03 to 0.18) (P>0.1)

Classification (AD patients vs. healthy controls) by a set of biomarkers

To evaluate whether AD patients and healthy controls can be distinguished by a set of cytokines, and if so, how many cytokines are needed, we calculated the prediction accuracy of the classification (AD vs control) by a linear SVM using cytokines selected by stability selection with elastic net (SS-EN).

The average accuracy of classification was over $99\pm0.2\%$ and $76\pm1.1\%$ with as few as three cytokines in lesional skin and five cytokines in non-lesional skin, respectively (Fig. 4a). The high accuracy suggests the importance of highly selected cytokines, such as IL-18, CCL2, and CXCL8 for lesional skin, in distinguishing AD patients from healthy controls (Fig. 4b, blue). IL-1b, IL-33, CXCL8, IL-18, and CCL22 in non-lesional skin were also highly selected (Fig. 4b, orange), although the predictive power of cytokines in non-lesional skin is much lower than that in lesional skin (Fig. 4a).

***Stratum corneum* biomarkers: correlation with skin barrier function and disease severity**

To investigate the association between biomarker levels and AD severity (SCORAD) as well as biophysical markers of skin barrier function (TEWL and NMF) we performed Spearman correlation analysis. The results of correlation coefficients and corresponding P-values for non-lesional and lesional skin of AD patients are presented as heat-map in Fig. 5.

We found significant association between majority of biomarkers with disease severity (SCORAD) as well as with skin barrier function (TEWL) and NMF.

The association between individual cytokines and SCORAD, TEWL and NMF had different patterns in non-lesional and lesional skin. For example, there was positive correlation between investigated cytokines and SCORAD and TEWL in non-lesional skin, however in lesional skin most of cytokines showed an opposite trend (Fig. 5). Some cytokines such as CXCL8, CXCL10 and CCL2 were positively correlated with SCORAD and TEWL in both lesional and non-lesional skin.

Regarding the strength of the association, the strongest positive correlation between biomarkers and SCORAD in non-lesional skin was observed for Th2-skewed biomarkers CCL2 and CCL17, Th1-skewed biomarker CXCL-10 and furthermore CCL13, CCL4 and

CCL20. For lesional skin, in contrast to non-lesional skin, the strongest negative association was found for CCL13 and CCL11. A significant association for some markers has only been found either in non-lesional or lesional skin. For example, IL-18, IL-1 β , IL-17A, CCL17, and CCL22 were significantly associated with SCORAD in non-lesional but not in lesional skin. An opposite trend was found for TSLP, IL-21, IL-23, IL-27, CCL3 and IL-31.

NMF is significantly correlated with majority of cytokines, and the strength of association was higher in non-lesional skin compared to lesional skin. For a number of biomarkers, significant association was found only in non-lesional (IL-18, IL-1 β , CCL17 and CCL22) or lesional skin (TSLP, IL-27 and IL-21). The opposite direction of significant association between biomarkers and NMF was observed for CCL13, CCL4, CCL20, IL-17A and CCL11, being negative in non-lesional skin and positive in lesional skin.

TEWL showed to be significantly correlated with a lower number of cytokines as compared with SCORAD and furthermore to a lesser degree of strength. The largest strength of association was observed for CXCL8, IL-18 and CCL17 in non-lesional skin and for CCL13, CCL20, CXCL10 and CCL11 in lesional skin (Fig. 5).

The levels of NMF, were inversely correlated to SCORAD and TEWL, in both non-lesional and lesional skin.

Predictability of severity by biomarkers

To examine whether the measurement of a panel of cytokines can predict SCORAD, we evaluated the predictive errors in regression. The SCORAD prediction had RMSE of 15.5 using SS-EN in conjunction with ridge regression, where the RMSE was kept around 15.4 when at least three cytokines from lesional skin were used (Fig. 6a, Fig. S2). A high RMSE was obtained by other models, including non-regularized linear regression (17.0 ± 0.5), kernel ridge regression with a polynomial kernel (15.5 ± 0.3), and random forest (14.1 ± 0.4) (Fig. S3). The RMSE with a fixed number of lesional biomarkers was consistently smaller than that with the same number of non-lesional biomarkers (Fig. 6a). The cytokines with the highest selection probability by SS-EN (CCL11 in lesional skin with $94.6 \pm 0.9\%$ and CCL20 in non-lesional skin with $94.1 \pm 0.7\%$) also had the highest feature importance in random forest (Table S2).

DISCUSSION

This study investigated, for the first time, levels of a wide spectrum of cytokines in the *stratum corneum* in Croatian adult AD patients. The results revealed aberrant profiles of cytokines representing innate immunity activation, Th-1, Th-2, and Th-17/Th-22 immune pathways in lesional as well as in non-lesional skin. The levels of various cytokines were associated with disease severity and skin barrier function, suggesting they might be useful in the selection and monitoring of novel immune therapies which target these immune pathways (7).

In general, differences in cytokine levels between healthy and atopic skin were larger for lesional skin. For example, the levels of IL-18 and CCL17 were respectively 30- and 4-times higher in lesional as compared to non-lesional skin. However, non-lesional skin also showed altered levels in a large number of cytokines supporting the view that immunological aberrations are present even in clinically unaffected skin (41). This holds also for the skin barrier function (TEWL) and NMF levels, which were reduced also in non-lesional skin.

One of the most prominent differences between healthy and atopic skin has been found for markers of innate activation IL-1 β and IL-18 supporting the role of innate immune activation in the pathogenesis of AD (5, 42, 43). This is in a good agreement with findings from a study performed in Irish (5) and Dutch children (22). Interestingly, Kezic *et al.* (25) found higher levels of IL-1 β and IL-18 in non-lesional skin of AD patients with *FLG* mutations as compared to wild-type patients (25,30). One of specificity of Croatian population is low prevalence of *FLG* LOF mutations in healthy population as well as in AD patients (respectively 1% and 4 %) (28,44). However, reduced filaggrin expression can also be acquired e.g. by Th-2 cytokines (24). Indeed, in atopic skin we found reduced levels of filaggrin degradation products (NMF) and increased levels of Th-2 cytokines (CCL17, CCL22, and CCL2). The levels of Th-2 cytokines were strongly correlated with NMF, supporting the role of Th-2 cytokine milieu for acquired deficiency of filaggrin in Croatian AD.

Another representative of innate immune response which showed a large difference between healthy and AD skin was CXCL8, in agreement with previous studies in children (5, 22) and adults with AD (30). This chemoattractant cytokine was suggested, together with CCL17, as a promising candidate biomarker for disease severity assessment and biomonitoring of local therapy in AD (30). In the present study we measured for the first time the SC levels of IL-33, an alarmin cytokine of the IL-1 family known to induce helper T-cells, mast cells, eosinophils and basophils to produce Th-2 cytokines (45). The SC levels of IL-33 were significantly lower in atopic skin in comparison to healthy skin. Lower levels in atopic SC have also been found for IL-21 and IL-27, cytokines preferentially produced by Th-17 cells. IL-33 and IL-17 are targets of recently developed therapies for AD (46, 47). IL-22, IL-23 and IL-31 also showed lower concentrations in AD skin as compared to the healthy skin.

Classification analysis has found several cytokines that are important for distinguishing AD patients and healthy controls. IL-18, CCL2, and CXCL8 had the selection probability of nearly 1 ($99.8 \pm 0.02\%$, $99.3 \pm 0.1\%$ and $95.3 \pm 0.6\%$, respectively) and were able to distinguish AD lesional samples from healthy controls with as high as 99% accuracy. Other cytokines such as IL-1 β , CXCL10, and CCL17 are also likely to be important in classifying AD lesional samples and healthy controls. IL-33, CCL22, IL-31, IL-22, and IL-21, which seemed to be less important to classify AD lesional samples and healthy controls, were commonly selected in AD non-lesional samples. On the contrary, several important cytokines in AD lesional samples, such as CCL2 and CXCL10, were found to be less important in classifying AD non-lesional samples and healthy controls. Cytokines in lesional skin samples are more informative than cytokines in non-lesional skin samples in classifying AD from healthy controls.

Significant correlation with disease severity (assessed by SCORAD) was found for the representatives of innate immune system and all investigated Th-cell subsets, Th-1, Th-2, and Th-17. Interestingly, but in agreement with previous studies, the strength of association with SCORAD was larger for non-lesional skin (48). Furthermore, the direction of associations of the cytokines and SCORAD differed between lesional and non-lesional skin. In non-lesional skin, all cytokines showed significant positive association, while in non-lesional skin an inverse correlation was found for the majority of

cytokines. This might be explained at least partly by the recruitment of specific immune cells and secreted cytokines to the lesions (48,49) and different relative contribution of these cells to the local cytokine milieu in lesional and non-lesional skin.

In the literature, CCL17 (TARC) has been suggested as one of the most promising single biomarker for disease severity in various patient populations (5, 20), biological matrices (*stratum corneum*, interstitial fluid, biopsy)(5,51), and irrespective whether it has been measured at protein (5) or gene expression level (51). CCL17, as well as other representatives of Th-2 immune axis showed strong correlation with SCORAD ($P<0.0001$) also in the present study.

Next to innate immunity and Th2 cytokines, strong association with disease severity was found for CXCL10 (IP-10), a Th1 cytokine. As compared with healthy skin, CXCL10 showed 2- and 10-fold higher levels in respectively non-lesional and lesional skin. In the previous work in adult Dutch patients with chronic AD 10-fold increase in CXCL10 levels in the transdermal fluid collected from lesional AD was found as compared to non-lesional and healthy skin (25, 51), which suggests similar cytokine milieu in two skin compartments, *stratum corneum* and epidermis. The levels of CXCL10 in transdermal fluid were correlated with SCORAD, consistently with our data obtained from the *stratum corneum*. However, in the study performed in Irish (5) and Dutch (22) children, there was no the difference in CXCL10 levels between non-lesional AD skin and healthy skin was not significant. ~~suggesting difference in expression of Th-1 cytokines between children and adults.~~ Consistently, Brunner *et al.*, reported lack of IFN- γ /Th1-associated inflammation in pediatric versus adult patients (49,51).

Interestingly, for some cytokines such as CCL11, CCL13, CCL20, lesional and non-lesional skin show different direction of association with SCORAD. In contrast to other immune markers, these cytokines showed lower levels in AD skin as compared to healthy skin.

Our regression analysis showed that cytokines that are highly correlated with SCORAD are not necessarily predictive. For example, CCL17 has been shown to correlate with SCORAD in our study and many other studies (56, 57). However, the selection probability for CCL17 was the 15th (for lesional skin) and the 14th (for non-lesional skin)

highest out of 21 cytokines in our analysis, and adding CCL17 did not improve prediction of SCORAD in terms of RMSE. SS-EN for regression frequently selected CCL11, CCL13, IL-27, IL-33, and TSLP in lesional skin samples, while CCL20, CXCL10, CCL2, and CCL13 were among the most selected cytokines in non-lesional samples. These cytokines, particularly those received the highest rankings in both SS-EN and random forest (lesional CCL11 and non-lesional CCL20, CXCL10, CCL2, and CCL13) are likely to be relevant to the severity and pathogenesis of lesions of AD. CCL11 and CCL13 (resp. eotaxin and MCP 4) are highly selective chemotactic agents for eosinophils and their involvement in AD has previously been demonstrated (5,52).

Conclusion from the predictive models developed here must be interpreted with caution. The relatively poor predictive performance for SCORAD is not likely to be due to poor modelling choices but rather to the inherent difficulty to build accurate models for this prediction task. It was demonstrated by the high RMSE for SCORAD prediction by various models, including linear models such as simple non-regularized linear regression (RMSE of 17.0 ± 0.5) and more complex stability selection with elastic net in conjunction with ridge regression (15.4 ± 0.3), as well as nonlinear models such as kernel ridge regression (15.5 ± 0.3) and random forest (14.1 ± 0.4). Our regression analysis suggested that no conclusive list of the cytokines can be produced for SCORAD prediction, as small sets of most selected cytokines achieved a high prediction error as well as larger sets of cytokines.

Several recent studies on AD biomarkers in the SC determined gene expression instead of cytokine protein levels. Guttman-Yassky *et al.* detected a large number of genes differentiating health from AD skin in children with moderate to severe AD (41). In contrast to the present study and literature data (5, 22, 30), there was no significant correlation between mRNA expression of CCL17 and SCORAD. However, in agreement with the present study, SCORAD did show a correlation with mRNA expression of other Th-2 cytokines such as CCL22, Th-1 chemokine CXCL10 and Th-17 cytokines. In contrast to the protein levels of some cytokines such as IL-13, IL-22, IL-23, IL-31 and IL-4 which are difficult to detect in the SC, their mRNA levels seem to be easily measured (5, 22, 25). However, disadvantage of gene expression studies is low success rate (i.e. the relative number of samples with detectable mRNA) for transcriptome sequencing (41),

and to achieve sufficient yield of mRNA, deeper SC should be collected which make the collection method more invasive and time consuming. Lastly, mRNA extraction is more labour intensive limiting its widespread use (41).

To summarize, the present study identified involvement of different Th-subsets in pathophysiology of AD in Croatian patients. SC harbours measurable levels of cytokines of different signature which showed ability to discriminate between healthy and AD. Measuring cytokines involved in a specific immune pathway might facilitate monitoring of novel therapies in AD. SC biomarkers were collected in a non-invasive manner, and the analysis could be performed only from a single tape which will facilitate their application in pediatric patients. However, the low predictive performance for SCORAD using the selected cytokines further pose challenges on using cytokines as an alternative to severity measurement.

FIGURE CAPTIONS

Figure 1. Pipeline of our machine learning analysis.

Figure 2. Levels of cytokines in the *stratum corneum* of non-lesional (AD_{NL}, $n = 91$) and lesional (AD_L, $n = 88$) skin of AD patients and healthy controls (Ctrl, $n = 42$). The values are log transformed and shown as boxplots with Tukey-style whiskers depicting first and third interquartile ranges (IQ), median (a line across the box), mean (+) and outliers (the values that lie beyond the whiskers) (•). Differences between healthy controls and non-lesional and lesional skin of AD patients were tested by two-tailed Mann-Whitney test. Benjamini-Hochberg corrected P -values: **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

Figure 3. The levels of natural moisturizing factors (NMFs) in the skin of healthy controls (Ctrl, $n = 49$) and non-lesional (AD_{NL}, $n = 92$) and lesional skin (AD_L, $n = 89$) of AD patients. The median and interquartile ranges are also shown. Differences between healthy controls and non-lesional and lesional skin of AD patients were determined by two-tailed Mann-Whitney test. Differences between non-lesional and lesional skin of AD patients were determined by two-tailed Wilcoxon signed-rank test. Benjamini-Hochberg corrected P -values. The carriers of *FLG* mutations in AD group are presented as black symbols. **** $P < 0.0001$.

Figure 4. Classification of AD patients vs healthy controls by SS-EN. (a) The accuracy of classification with different numbers of cytokines in lesional (blue) and non-lesional (orange) skin. The dashed line indicates the baseline accuracy. The shades represent the 95% confidence interval. (b) Selection probabilities (mean $\pm$ SEM) of cytokines in lesional (blue) and non-lesional (orange) skin for classification.

Figure 5. Spearman correlation coefficients between *stratum corneum* biomarkers and atopic dermatitis severity [Scoring Atopic Dermatitis (SCORAD)], transepidermal water

loss (TEWL) and natural moisturizing factors (NMF). Benjamini–Hochberg corrected P -values: **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$. For the correlation analysis 88 subjects were included with exception of the correlations between SCORAD and NMF in lesional skin (n=89) and between TEWL and cytokines in non-lesional skin (n=87)

Figure 6. Regression of SCORAD by SS-EN. (a) Rooted mean squared error (RMSE) with different numbers of cytokines in lesional (blue) and non-lesional (orange) skin. The dashed line indicates the baseline RMSE. The shades represent the 95% confidence interval. (b) Selection probabilities (mean $\pm$ SEM) of cytokines in lesional (blue) and non-lesional (orange) skin for SCORAD regression.

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Table 1. Demographic details of the study participants.

	AD patients <i>n</i> = 100	Healthy controls <i>n</i> = 50
Sex		
Female	69	27
Male	31	23
Age (years), <i>mean</i> (range)	25 (17-43)	32 (29-45)
SCORAD, <i>mean</i> (range)	40.17 (0-87.3)	-
Clear (0-9.99 points), <i>n</i>	12	-
Mild (< 10-28.9 points), <i>n</i>	18	-
Moderate (29-48.9 points), <i>n</i>	36	-
Severe (49.0-103 points), <i>n</i>	34	-
TEWL		
non-lesional, <i>median</i> (<i>IQ range</i>)	15.3 (10.6-20.7)	11.5 (8.9-16.8)
lesional, <i>median</i> (<i>IQ range</i>)	42.8 (34.4-59.4)	-
FLG LOF mutations	4	0

AD - atopic dermatitis; SCORAD - SCORing Atopic Dermatitis; TEWL - transepidermal water loss

FLG LOF mutations - Filaggrin gene loss-of-function mutations

50 random splits

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Markers of innate activation

Th17 markers

Th2 markers

Th1 marker

Other markers

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		Non-lesional skin			Lesional skin		
		SCORAD	TEWL	NMF	SCORAD	TEWL	NMF
Markers of innate activation	CXCL8-	0.359***	0.345**	-0.594****	0.248*	0.245*	-0.283*
	IL-18-	0.305**	0.304**	-0.514****	0.055	0.089	-0.134
	IL-1 β -	0.236*	0.127	-0.497****	-0.118	-0.057	0.070
	TSLP-	0.160	0.147	-0.166	0.243*	0.147	-0.243*
Other markers	CCL13-	0.448****	0.208	-0.408***	-0.458****	-0.305**	0.357**
	CCL3-	0.009	0.066	-0.068	-0.389***	-0.159	0.142
	CCL4-	0.446****	0.238*	-0.478****	-0.237*	-0.253*	0.259*
	CCL20-	0.438****	0.138	-0.383**	-0.259*	-0.310**	0.344**
	IL-27-	0.008	0.068	-0.030	-0.399***	-0.114	0.276*
	IL-22-	0.119	0.180	-0.169	0.133	0.103	-0.102
Th1 marker	CXCL10-	0.423***	0.258*	-0.514****	0.280*	0.359**	-0.348**
Th17 markers	IL-17A-	0.345***	0.091	-0.279*	-0.208	-0.127	0.242*
	IL-21-	0.047	0.094	0.054	-0.405***	-0.265*	0.321**
	IL-23-	-0.071	0.031	0.053	-0.357**	-0.066	0.088
Th2 markers	CCL2-	0.438****	0.274*	-0.545****	0.259*	0.154*	-0.303**
	CCL17-	0.407***	0.355**	-0.573****	0.208	0.108	-0.150
	CCL22-	0.389***	0.284*	-0.581****	0.048	0.007	0.066
	CCL11-	0.369***	0.144	-0.377**	-0.561****	-0.323**	0.389***
	CCL26-	0.271**	0.030	-0.206	-0.396***	-0.187	0.227
	IL-33-	0.076	-0.072	0.072	-0.295*	-0.128	0.155
	IL-31-	0.243*	0.202	-0.197	-0.358**	-0.153	0.298*
Biophysical marker		NMF-					
		-0.385***	-0.359**		-0.326**	-0.549****

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