BIOMARKERS IN ATOPIC DERMATITIS

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General introduction

Atopic dermatitis

Atopic dermatitis (AD) is a chronic inflammatory skin disease, characterized by intense itching and a relapsing and remitting course. With a prevalence of 10-20% in developed countries, AD is the most common inflammatory skin disease worldwide.[1,2] The intense itch, resulting in sleep loss and concentration problems, and the social stigmatization causes a profound impact on the quality of life of patients, but also on the lives of their families. AD also has a substantial socioeconomic impact, caused by absence from work, reduced productivity at work, and reduced capabilities of learning at school and university.[3, 4]

The majority of the AD patients can be adequately treated with topical steroids and/or UV-light therapy.[5, 6] However, there is a large group of patients in which oral immunosuppressive drugs are indicated. Cyclosporin A is the only registered oral immunosuppressive drug for AD in the Netherlands and therefore often first choice of treatment in severe AD.[7] Unfortunately, nearly half of the patients have to discontinue treatment with cyclosporin A due to side effects and/or inefficacy.[8] Moreover, cyclosporin A treatment is only approved for a maximum of two years.[9] Various other immunosuppressive drugs are being used 'off-label' in AD, such as mycophenolic acid, methotrexate, and azathioprine.[7] However, studies have shown these drugs are only effective in about half of the AD patients, highlighting the need for more effective treatment options in AD.[10-12]

In contrast to the classically used immunosuppressive drugs, that cause general immune inhibition, the currently tested biologicals or small molecules specifically intervene with Th2 inflammation. Dupilumab, an antibody targeting the IL-4 receptor alpha, is the first biological for AD that has shown promising results in recent phase II trials.[13, 14] Other new antibodies that are currently being tested in clinical trials are targeting key cytokines such as IL-13,[15] IL-22,[16] IL-31,[17] and TSLP.[18] The introduction of these new drugs will probably lead to a new era in AD management, and will hopefully fill the large unmet needs in the treatment of AD.

Precision medicine for atopic dermatitis

AD is recognized as a complex disease, with a wide range of clinical features. Different clinical phenotypes have been described, based on characteristics like age of onset or the presence of other atopic diseases, such as allergic rhinitis and asthma.[26] The complexity of AD is also caused by its multifactorial pathogenesis. The two major pathophysiological factors are disruption of the epidermal barrier and abnormalities in the immune system resulting in an altered T helper 2 (Th2) cell response.[27] Which of these two factors is the key driver of AD, is a matter of debate. However, it is clear that barrier impairment and immune alterations are closely related and affect each other. Disruption of the epidermal barrier increases the permeability for exogenous stimuli, such as allergens, that activate the immune system. Activated keratinocytes release chemokines and cytokines that attract T-cells, which are polarized to Th2 cells. In turn, activation of the immune system leads to an affected epidermal barrier.[28] The Th2 cytokines IL-4 and IL-13,[29] and cytokines driving Th2 polarization, such as thymic stromal lymphopoietin (TSLP),[30] IL-25 and IL-33,[31, 32] lead to an impaired epidermal differentiation and integrity.

Due to the heterogeneous character of disease, it is unlikely that a "one-size-fits-all" treatment approach will be effective in AD. Precision medicine classifies subgroups of patients that differ in their response to a particular treatment. Serum biomarkers can be helpful in the classification of subgroups of AD patients that share the same underlying disease pathway. The identification of these subgroups enables more specific targeting of the underlying disease pathways, which allows treatment being tailored to individual patients. This would not only be beneficial for patients, but would also reduce health-care costs.[33]
Subgrouping patients based on a serum biomarker has already been proven to be useful in asthma, where anti-IL-13 therapy appears to be most effective in the specific subgroup of patients with high serum levels of periostin.[34] We expect that biomarker analyses will become essential with the introduction of new targeted therapies for AD, to enable a better selection of patient populations and optimize therapy.

Outcome measures in atopic dermatitis

There is a need for objective measurement tools in order to compare new treatments to each other and to existing therapies. Currently, there is no gold standard for measurement of disease severity in AD and more than twenty different composite indices have been described.[19] A systematic review of AD severity measurement tools found that 91% of AD clinical trials used an objective severity measure, but less than a third of these scales had been previously published.[20] In addition, Schmitt et al. assessed the validity and reliability of the twenty most commonly used severity measures for AD, and found that only three performed adequately. To address these deficiencies in reporting, experts in the field have established the Harmonizing Outcome Measurements in Eczema (HOME) initiative, an attempt to ensure that investigators employ a core set of outcome measures to enhance comparability between studies.[21] We suggest that in addition to improvements in clinical outcome reporting, an objective serological measure for disease severity would be of great value for clinical research. In contrast to physician assessed outcome measures, serological measures are no subject to inter- and intraobserver variability, but offer an objective outcome measure. Objective serological measures are essential for studying the efficacy of newly tested drugs and for comparison with other new or existing drugs for AD.

Over the past decades, a number of serum and plasma parameters (biomarkers) have been found to correlate with disease severity in AD patients. These biomarkers include a variety of proteins, such as cytokines, chemokines, adhesion molecules and growth factors. Serum thymus and activation-regulated chemokine (TARC/CCL17) was found to show a strong correlation to disease severity in the follow-up of individual patients.[22] However, large differences in serum TARC levels are found between patients with similar disease severity scores in cross-sectional cohorts , consequently showing relatively weak correlations between TARC and disease severity. The low correlations of TARC with disease severity in these cohorts may be the result of the highly heterogeneous character of the disease. Since TARC is a Th2 related chemokine,[23] the correlation of TARC with disease severity may especially be strong in AD patients with a predominant Th2 type inflamma-

tion, while this correlation may be less strong in for instance an Asian patient population in which Th22 type inflammation is thought be more important. The use of a panel of biomarkers might overcome this problem. A panel of biomarkers can assess multiple molecular entities, and might be more suitable for assessing disease severity in AD compared to an individual biomarker.

Most previously described biomarkers were measured in peripheral blood.[24] However, collection of blood is invasive and less suitable for use in the field because of the need for trained personnel. Blood collection by venipuncture is also less favorable in pediatric medicine. Potential alternative sources for biomarker measurements in daily practice and longitudinal studies are dried blood spots (DBS) and saliva. DBS have been used for decades in screening for inherited metabolic diseases in newborns[25] and can be obtained using a simple, minimally invasive, nearly painless procedure that can be done by the patients themselves. Saliva also contains a wide spectrum of biomolecules, which are transported from the blood capillaries through the epithelium of salivary glands.[26] Salivary cortisol levels are for instance routinely used as a biomarker of psychological stress. DBS and saliva may be used as an accurate non-invasive alternative to serum measurements.

Pharmacogenomic biomarkers in atopic dermatitis treatment

In addition to newly developed therapies, implementation of pharmacogenetic biomarkers can optimize the performance of current oral immunosuppressive drugs. Pharmacogenetic research explores the effect of pharmacokinetics, pharmacodynamics, efficacy, and safety of drug treatments in relation to genome variations.[35] The most common genetic variations that have been studied are single-nucleotide polymorphisms (SNPs), genetic copynumber variations (CNVs), and genomic insertions and deletions.[36] All of these genetic variations can influence the response of a patient to a specific drug. The goal of pharmacogenetic research is to predict this response. Pharmacogenetic testing provides a tool that can maximize therapeutic efficacy and safety of drug treatment, with the ultimate goal of creating personalized treatment strategies.[35, 36]

Pharmacogenetic testing was applied in a recent study investigating the effect of extended release tacrolimus in severe AD patients.[37] Based on the genetic markers CYP3A4/CYP3A5 patients could be classified as poor, intermediate or extensive metabolizers of tacrolimus, enabling a personalized dosing scheme for the individual patient.[37] Pharmacogenetic testing of SNPs for CYP3A4/CYP3A5 may also be of use in Cyclosporin A treatment,[38] the most commonly used oral immunosuppressive drug in AD.[7]

Pharmacogenetic testing may also be used to optimize mycophenolic acid treatment. Mycophenolic acid blood levels are known to have a large inter individual variability. This has been observed in kidney transplant recipients, in whom a lower level of mycophenolic acid exposure is closely associated with lower efficacy of drug therapy and acute rejection of the transplanted organ.[39, 40] Low mycophenolic acid exposure and increased enzyme activity of the metabolizing enzyme UGT1A9 correlates to the presence of SNPs in the gene promotor region of UGT1A9.[39, 40] Also for AD patients, low mycophenolic acid exposure due to the presence

of UGT1A9 polymorphisms might contribute to inefficacy.

Although pharmacogenetics are currently scarcely used in dermatological treatment, it enables "personalized medicine" by prescribing drugs based on the genetic makeup of an individual.

Outline of this thesis

In this thesis, we investigated several different applications of biomarkers in AD. Firstly, we focused on biomarkers as an objective measure for disease severity in AD. Current literature on disease severity biomarkers in AD was systematically reviewed, and the performance of known biomarkers was analysed in a meta-analysis described in chapter 2. In a pilot study on 17 AD patients that is described in chapter 3, we investigated if a combination of biomarkers might be more suitable for assessing disease severity in AD compared to an individual biomarker. The performance of a combination of biomarkers was further explored in a longitudinal study including 65 AD patients treated with topical steroids in chapter 4. The applicability of a combination of biomarkers in AD patients treated with CsA is described in chapter 5. In chapter 6, we investigated the performance of immunoglobulin free light chains as a biomarker for disease severity in AD.

Secondly, we focused on biomarkers that enable personalized medicine in AD. Chapter 7 focusses on the presence of systemic inflammation in AD, and the comorbidities that possibly caused by this systemic inflammation. In chapter 8 we tried to dissect the heterogeneity of AD on a biological level using a purely data driven approach. In chapter 9 we investigated if UGT1A9 mutations can be used as a pharmacogenomic biomarker for the prediction of response to mycophenolic acid therapy in AD.

A third aim of this thesis was to improve the practical aspects of the application of biomarkers in AD. The research presented in this thesis is mostly based on biomarker measurement in serum. Chapter 10 provides an overview of alternative sources that can be used for biomarker measurement. Chapter 11 explores the possibility of biomarker measurement in dried blood spots from AD patients.

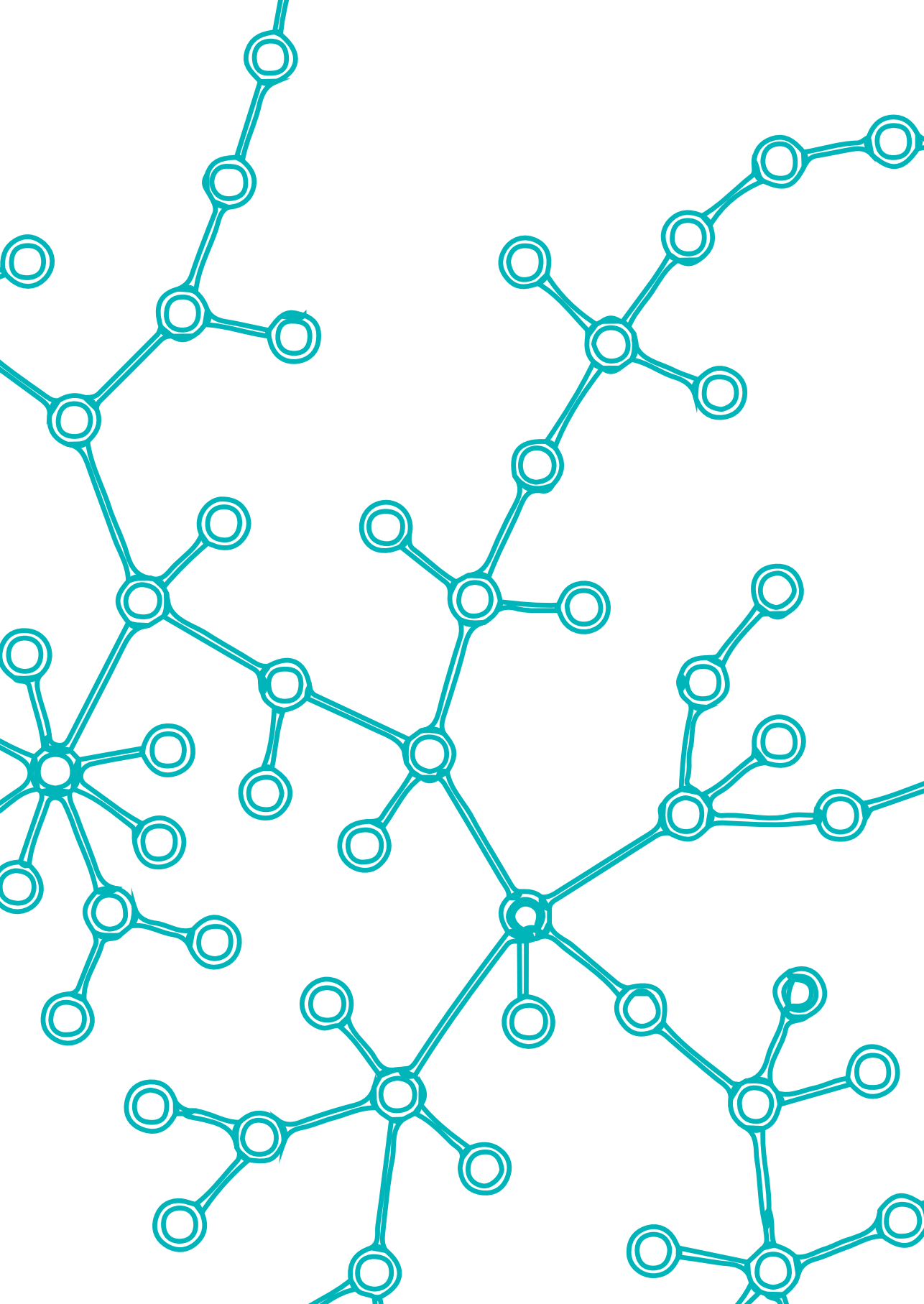
REFERENCES

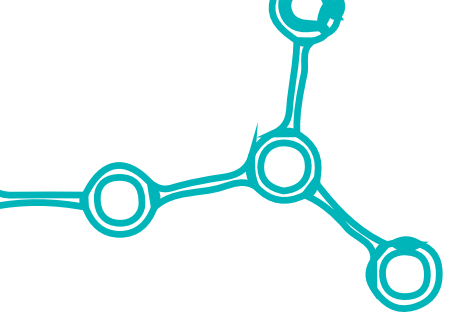
1. Deckers IA, McLean S, Linssen S, Mommers M, van Schayck CP, Sheikh A. Investigating international time trends in the incidence and prevalence of atopic eczema 1990-2010: a systematic review of epidemiological studies. PLoS One 2012; 7:e39803.
2. Williams H, Stewart A, von Mutius E, Cookson W, Anderson HR. Is eczema really on the increase worldwide? J Allergy Clin Immunol 2008; 121:947-54 e15.
3. Simpson EL, Bieber T, Eckert L, Wu R, Ardeleanu M, Graham NM, et al. Patient burden of moderate to severe atopic dermatitis (AD): Insights from a phase 2b clinical trial of dupilumab in adults. J Am Acad Dermatol 2016; 74:491-8.
4. Zuberbier T, Lotvall J, Simoens S, Subramanian SV, Church MK. Economic burden of inadequate management of allergic diseases in the European Union: a GA(2) LEN review. Allergy 2014; 69:1275-9.
5. Gelmetti C, Wollenberg A. Atopic dermatitis - all you can do from the outside. Br J Dermatol 2014; 170 Suppl 1:19-24.
6. Garritsen FM, Brouwer MW, Limpens J, Spuls PI. Photo(chemo)therapy in the management of atopic dermatitis: an updated systematic review with implications for practice and research. Br J Dermatol 2014; 170:501-13.
7. Ring J, Alomar A, Bieber T, Deleuran M, Fink-Wagner A, Gelmetti C, et al. Guidelines for treatment of atopic eczema (atopic dermatitis) Part II. J Eur Acad Dermatol Venereol 2012; 26:1176-93.
8. van der Schaft J, Politiek K, van den Reek JM, Christoffers WA, Kievit W, de Jong EM, et al. Drug survival for ciclosporin A in a long-term daily practice cohort of adult patients with atopic dermatitis. Br J Dermatol 2015; 172:1621-7.
9. Ring J, Alomar A, Bieber T, Deleuran M, Fink-Wagner A, Gelmetti C, et al. Guidelines for treatment of atopic eczema (atopic dermatitis) Part II. J Eur Acad Dermatol Venereol 2012; 26:1176-93.
10. Garritsen FM, Roekevisch E, van der Schaft J, Deinum J, Spuls PI, de Bruin-Weller MS. Ten years experience with oral immunosuppressive treatment in adult patients with atopic dermatitis in two academic centres. J Eur Acad Dermatol Venereol 2015; 29:1905-12.
11. van der Schaft J, Politiek K, van den Reek JM, Kievit W, de Jong EM, Bruijnzeel-Koomen CA, et al. Drug survival for azathioprine and enteric-coated mycophenolate sodium in a long-term daily practice cohort of adult patients with atopic dermatitis. Br J Dermatol 2016.
12. Politiek K, van der Schaft J, Coenraads PJ, de Bruin-Weller MS, Schuttelaar ML. Drug survival for methotrexate in a daily practice cohort of adult patients with severe atopic dermatitis. Br J Dermatol 2016; 174:201-3.
13. Beck LA, Thaci D, Hamilton JD, Graham NM, Bieber T, Rocklin R, et al. Dupilumab treatment in adults with moderate-to-severe atopic dermatitis. N Engl J Med 2014; 371:130-9.
14. Thaci D, Simpson EL, Beck LA, Bieber T, Blauvelt A, Papp K, et al. Efficacy and safety of dupilumab in adults with moderate-to-severe atopic dermatitis inadequately controlled by topical treatments: a randomised, placebo-controlled, dose-ranging phase 2b trial. Lancet 2016; 387:40-52.
15. A Study to Evaluate the Safety of Lebrikizumab Compared to Topical Corticosteroids in Adult Patients With Atopic Dermatitis.] Available from https://clinicaltrials.gov/ct2/show/NCT02465606: Hoffmann-La Roche.
16. A randomized placebo-controlled study to determine the safety, tolerability, pharmacodynamics and clinical efficacy of ILV-094 (an IL-22 antibody) administered intravenously to subjects with atopic dermatitis (AD).] Available from http://clinicaltrials.gov/ct2/show/NCT01941537: Rockefeller University

17. Nemoto O, Furue M, Nakagawa H, Shiramoto M, Hanada R, Matsuki S, et al. The first trial of CIM331, a humanized antihuman interleukin-31 receptor A antibody, in healthy volunteers and patients with atopic dermatitis to evaluate safety, tolerability and pharmacokinetics of a single dose in a randomized, double-blind, placebo-controlled study. Br J Dermatol 2016; 174:296-304.
18. A randomized, double-blind, placebo-controlled, ascending single dose study to evaluate the safety, tolerability, pharmacokinetics and pharmacodynamics of AMG 157 in healthy subjects and subjects with moderate to severe atopic dermatitis.] Available from http://clinicaltrials.gov/ct2/show/study/NCT00757042: Amgen.
19. Schmitt J, Langan S, Williams HC. What are the best outcome measurements for atopic eczema? A systematic review. J Allergy Clin Immunol 2007; 120:1389-98.
20. Charman C, Chambers C, Williams H. Measuring atopic dermatitis severity in randomized controlled clinical trials: what exactly are we measuring? J Invest Dermatol 2003; 120:932-41.
21. Schmitt J, Langan S, Stamm T, Williams HC. Core outcome domains for controlled trials and clinical recordkeeping in eczema: international multiperspective Delphi consensus process. J Invest Dermatol 2011; 131:623-30.
22. Landheer J, de Bruin-Weller M, Boonacker C, Hijnen D, Bruijnzeel-Koomen C, Rockmann H. Utility of serum thymus and activation-regulated chemokine as a biomarker for monitoring of atopic dermatitis severity. J Am Acad Dermatol 2014; 71:1160-6.
23. Imai T, Nagira M, Takagi S, Kakizaki M, Nishimura M, Wang J, et al. Selective recruitment of CCR4-bearing Th2 cells toward antigen-presenting cells by the CC chemokines thymus and activation-regulated chemokine and macrophage-derived chemokine. Int Immunol 1999; 11:81-8.
24. Schulz BL, Cooper-White J, Punyadeera CK. Saliva proteome research: current status and future outlook. Crit Rev Biotechnol 2013; 33:246-59.
25. Li W LM. Dried Blood Spots: Applications and Techniques: Wiley; 2014.
26. Garmhausen D, Hagemann T, Bieber T, Dimitriou I, Fimmers R, Diepgen T, et al. Characterization of different courses of atopic dermatitis in adolescent and adult patients. Allergy 2013; 68:498-506.
27. Eyerich K, Novak N. Immunology of atopic eczema: overcoming the Th1/Th2 paradigm. Allergy 2013; 68:974-82.
28. Elias PM, Steinhoff M. "Outside-to-inside" (and now back to "outside") pathogenic mechanisms in atopic dermatitis. J Invest Dermatol 2008; 128:1067-70.
29. Howell MD, Kim BE, Gao P, Grant AV, Boguniewicz M, DeBenedetto A, et al. Cytokine modulation of atopic dermatitis filaggrin skin expression. J Allergy Clin Immunol 2009; 124:R7-R12.
30. Oyoshi MK, Larson RP, Ziegler SF, Geha RS. Mechanical injury polarizes skin dendritic cells to elicit a T(H)2 response by inducing cutaneous thymic stromal lymphopoietin expression. J Allergy Clin Immunol 2010; 126:976-84, 84 e1-5.
31. Deleuran M, Hvid M, Kemp K, Christensen GB, Deleuran B, Vestergaard C. IL-25 induces both inflammation and skin barrier dysfunction in atopic dermatitis. Chem Immunol Allergy 2012; 96:45-9.
32. Savinko T, Matikainen S, Saarialho-Kere U, Lehto M, Wang G, Lehtimaki S, et al. IL-33 and ST2 in atopic dermatitis: expression profiles and modulation by triggering factors. J Invest Dermatol 2012; 132:1392-400.
33. Jameson JL, Longo DL. Precision medicine--personalized, problematic, and promising. N Engl J Med 2015; 372:2229-34.
34. Corren J, Lemanske RF, Hanania NA, Korenblat PE, Parsey MV, Arron JR, et al. Lebrikizumab treatment in adults with asthma. N Engl J Med 2011; 365:1088-98.
35. Crews KR, Hicks JK, Pui CH, Relling MV, Evans WE. Pharmacogenomics and individualized medicine: translating scienceinto

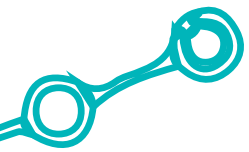
practice. Clin Pharmacol Ther 2012; 92:467-75.

36. Ventola CL. Role of pharmacogenomic biomarkers in predicting and improving drug response: part 1: the clinical significance of pharmacogenetic variants. P T 2013; 38:545-60.
37. van der Schaft J, van Schaik RH, van Zuilen AD, Hijnen DJ, Berg MT, van den Broek MP, et al. First experience with extended release tacrolimus in the treatment of adult patients with severe, difficult to treat atopic dermatitis: Clinical efficacy, safety and dose finding. J Dermatol Sci 2016; 81:66-8.
38. de Jonge H, Kuypers DR. Pharmacogenetics in solid organ transplantation: current status and future directions. Transplant Rev (Orlando) 2008; 22:6-20.
39. Kuypers DR, Naesens M, Vermeire S, Vanrenterghem Y. The impact of uridine diphosphate-glucuronosyltransferase 1A9 (UGT1A9) gene promoter region single-nucleotide polymorphisms T-275A and C-2152T on early mycophenolic acid dose-interval exposure in de novo renal allograft recipients. Clin Pharmacol Ther 2005; 78:351-61.
40. van Schaik RH, van Agteren M, de Fijter JW, Hartmann A, Schmidt J, Budde K, et al. UGT1A9 -275T>A/-2152C>T polymorphisms correlate with low MPA exposure and acute rejection in MMF/tacrolimus-treated kidney transplant patients. Clin Pharmacol Ther 2009; 86:319-27.





PART I



SEVERITY BIOMARKERS

Biomarkers for atopic dermatitis: a systematic review and meta-analysis

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2

ABSTRACT

Purpose of review:

A large number of studies investigating the correlation between severity of atopic dermatitis (AD) and various biomarkers have been published over the past decades. The aim of this review was to identify, evaluate and synthesize the evidence examining the correlation of biomarkers with disease severity in AD patients, something that has not been performed previously.

Findings:

Three electronic databases were systematically searched and relevant studies were selected for inclusion. A total of 222 papers, reporting on 115 different biomarkers in 30.063 patients were critically appraised. Studies were divided into two main groups. The first group consisted of longitudinal randomized controlled trials and cohort studies, which reported measurements at multiple time points. The second contained cross-sectional studies that reported only one measurement per patient. Out of 222 papers, 108 papers reported sufficient data for meta-analysis. Only four biomarkers were eligible for meta-analysis in the longitudinal group, and nine in the cross-sectional group.

Summary:

Serum TARC was found to be the most reliable biomarker studied, showing pooled correlation coefficients of 0.60 (95%CI:0.48-0.70) and 0.64 (95%CI:0.57-0.70) in respectively longitudinal and cross-sectional studies. Additional biomarkers that could prove useful but require additional research include serum CTACK, sE-selectin, MDC, LDH and IL-18.

INTRODUCTION

Atopic dermatitis (AD) is a common, chronic inflammatory skin disease that is frequently associated with a personal or family history of allergic asthma and/or rhinitis. AD can have profound effects on quality of life and imposes a high socio-economic burden.[1] There has been an increasing interest in AD over the past decades, reflected in an exponential increase in publications. However, the use of different criteria for the diagnosis of AD and different outcome measurements hampers study comparability.[2]

Quantifying disease severity using severity measurement tools such as the SCORing of Atopic Dermatitis (SCORAD) and Eczema Area Severity Index (EASI) is time consuming and may be subject to intra- and inter-observer variation.[3] In addition, many severity measurement tools that are used in clinical trials have not been validated. A systematic review of AD severity measurement tools found that 91% of AD clinical trials used an objective severity measure, but less than a third of these scales had been previously published.[4] In addition, Schmitt et al. assessed the validity and reliability of the 20 most commonly used severity measures for AD, and found that only three performed adequately. To address these deficiencies in reporting, leading experts in the field have established the Harmonizing Outcome Measurements in Eczema (HOME) initiative, an attempt to ensure that investigators employ a core set of outcome measures to enhance comparability between studies.[5] We suggest that in addition to improvements in clinical outcome reporting, an objective serological measure for disease severity would be of great value for clinical research.

Over the past decades, a number of serum and plasma parameters (biomarkers) have been found to correlate with disease severity in AD patients. These include a variety of proteins, such as cytokines, chemokines, adhesion molecules and growth factors. In addition, numerous studies investigating the efficacy of various treatments for AD patients have also reported biomarker levels in their patients. To the best of our knowledge, a systematic review of this sprawling literature has not yet been performed. Our aim was to identify, evaluate and synthesize the evidence examining the correlation of known biomarkers with disease severity in AD patients.

2

MATERIALS AND METHODS

Search strategy

This systematic review was performed according to the criteria of the Preferred Reporting Items of Systematic Reviews and Meta-Analyses (PRISMA) statement.[6] Three electronic databases (PubMed, EMBASE and the Cochrane Library) were systematically searched from inception until October 2014. The electronic search was designed to provide optimal coverage for the targeted domain "atopic dermatitis", "biomarkers" as the determinant and "disease severity" as the outcome, and synonyms (Table 1). No language or publication restrictions were used, but studies were limited to research in humans.

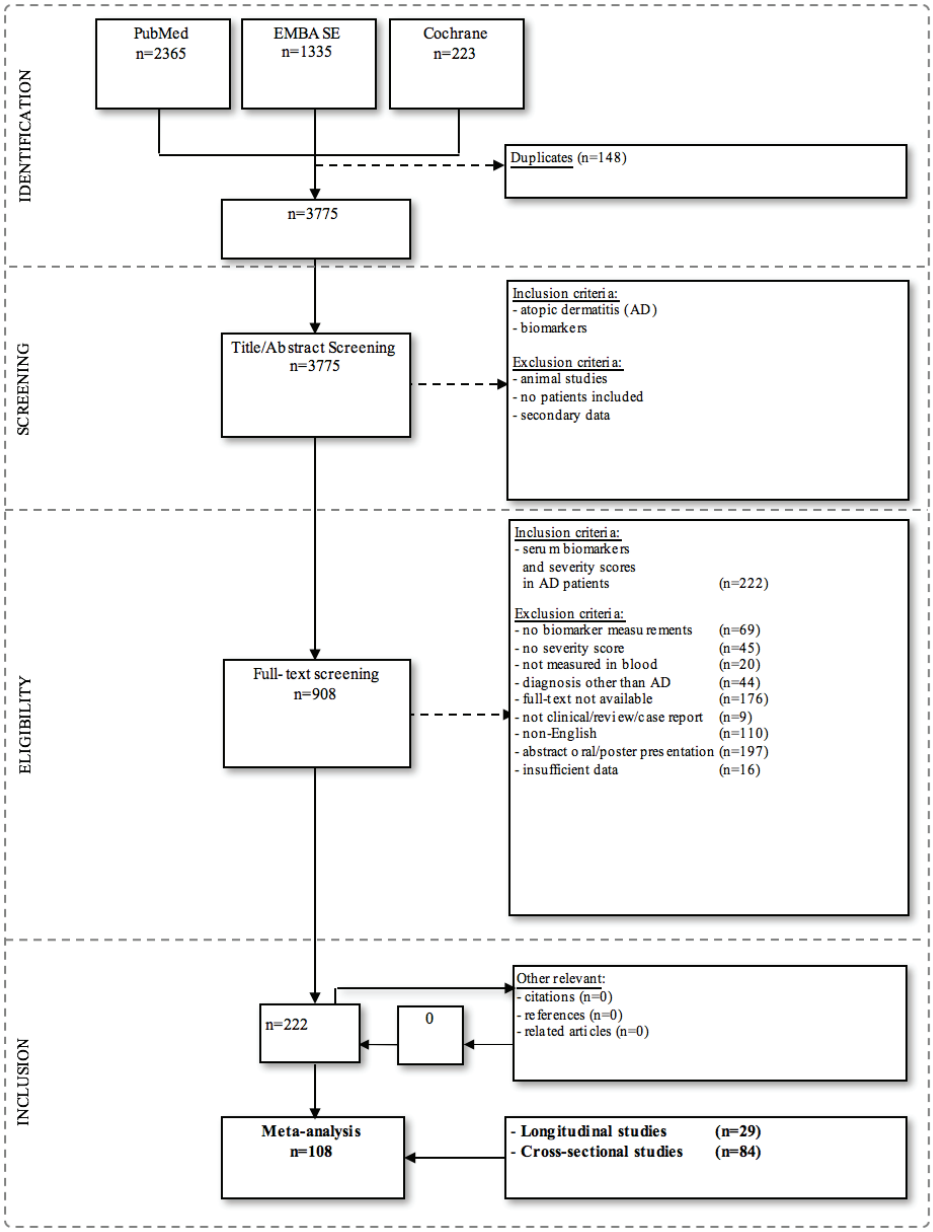
Table 1. Search performed on 25 September 2014 in PubMed, EMBASE and the Cochrane Library databases.

Search
PubMed "atopic eczema"[tiab] OR "atopic dermatitis"[tiab]) AND (soluble*[tiab] OR plasma*[tiab] OR serum*[tiab] OR sera[tiab] OR cytokine[tiab] OR chemokine[tiab] OR serologic*[tiab] OR marker*[tiab]) AND (severity[-tiab] OR scor*[tiab] OR measur*[tiab] OR index[tiab] OR activity[tiab] OR symptom*[tiab] OR correlat*[tiab])
Embase 'atopic eczema':ab,ti OR 'atopic dermatitis':ab,ti AND (soluble*:ab,ti OR plasma*:ab,ti OR serum*:ab,ti OR sera:ab,ti OR cytokine:ab,ti OR chemokine:ab,ti OR serologic*:ab,ti OR marker*:ab,ti) AND (severity:ab,ti OR scor*:ab,ti OR measur*:ab,ti OR index:ab,ti OR activity:ab,ti OR symptom*:ab,ti OR correlat*:ab,ti) NOT ('medline'/exp OR 'medline')
Cochrane library "atopic eczema" OR "atopic dermatitis") AND (soluble* OR plasma* OR serum* OR sera OR cytokine OR chemokine OR serologic* OR marker*) AND (severity OR scor* OR measur* OR index OR activity OR symptom* OR correlat*) in Title, Abstract, Keywords in Trials'

Inclusion and exclusion criteria

All publications on serum and/or plasma biomarkers in AD patients that provided correlations with disease severity were considered. We did not discriminate between randomised-controlled trials, cohorts or case series. Case reports were excluded, as were publications reporting data on less than five patients. All study titles and abstracts obtained from the database searches were screened and reviewed by at least two independent reviewers (JT, TK or DJH). An additional assessment was performed based on the full-text versions of all selected papers and those with insufficient information in the title and abstract.

Figure 1. Flowchart of the study selection process.



Data extraction

A data extraction sheet was developed, pilot-tested on the first included studies and refined accordingly. The adapted version (Table S1) was applied to all subsequent studies and retrospectively to the initially identified subset. The data items extracted from each study are listed in Table S1. In this review, only reported data on biomarker levels with corresponding severity scores in AD patients and the correlation coefficients were extracted for analysis. Because biomarkers were not primary study outcomes in the vast majority of studies, we did not evaluate the risk of bias in individual studies based on whether randomisation, concealment of allocation or the use of control groups were used, as these were less relevant o this investigation. The selected studies were appraised for diagnostic criteria, preferably according to the Hanifin and Rajka criteria[7] or the U.K. Working Party's Diagnostic Criteria for Atopic Dermatitis [8] and for severity scores at the time of blood sampling. In addition, reporting of missing data and loss to follow-up were also assessed.

Data analysis

The principal summary measures of interest were the correlation between the reported biomarker(s) and severity measurement tools, the corresponding p-values and Pearson/Spearman rank correlation coefficients.[9] A meta-analysis was carried out for biomarkers for which correlation coefficient and sample size was reported by at least four studies.

The meta-analysis was performed using the R statistical environment version 3.2.0 (R Core Team. R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing; 2015. Available from: http://www.R- project.org/), using the metaphor package version 1.9-7.[10] For each biomarker, the correlation coefficients were transformed using Fisher's r-to-z transformation, to produce the z value. These were then pooled using a standard random-effects model,[11] regardless of heterogeneity. Heterogeneity was assessed using the I^2 statistic,[12] and by visual inspection of funnel plots of the included studies.[13] The I^2 was considered acceptable if below 60% and the funnel plot was considered unbiased if upon visual inspection the distribution of studies was approximately symmetrical. Forest plots of the included studies and the random-effects pooled estimates were generated and back-transformed to the original correlation coefficients for improved interpretability.

RESULTS

Characteristics of included studies

Figure 1 summarizes the results of the search strategy. A total of 222 publications, examining 115 different biomarkers (Table S2), were included. Studies from all 222 publications were divided into two groups. The first group comprised of randomised controlled trials and cohort studies, which reported multiple measurements in the same patient over time (further referred to as longitudinal studies). Studies that reported only one measurement per patient are referred to as cross-sectional studies. We included 173 longitudinal

studies with data from a total of 5328 patients and an average follow-up between 4 and 3650 days. A total of 56 biomarkers were reported in longitudinal studies. Cross-sectional studies comprised 148 publications with data from a total of 15471 patients, reporting on 65 biomarkers. Some publications describing longitudinal studies also presented cross-sectional study data resulting in overlap between groups. SCORing of Atopic Dermatitis (SCORAD) was used in 132 studies (59.4% of studies) and thereby the most commonly used severity score for AD Other disease severity measures included the Eczema Area and Severity Index (EASI) in eleven studies (5.0%), the Costa score in nine studies (4.1%), and the Six Area Six Sign Atopic Dermatitis (SASSAD) in six studies (2.7%). In total, over 30 different clinical severity scores were used.

One hundred and eight papers, examining 64 biomarkers, reported sufficient data for meta-analysis. Four biomarkers were eligible for meta-analysis in the longitudinal group. In the cross-sectional group, nine biomarkers were eligible for meta-analysis.

Meta-analysis

Thirty biomarkers have been investigated in 29 longitudinal studies providing sufficient data for meta-analysis. Out of 30 biomarkers, only the correlation coefficients of levels of serum Thymus and Activation-Regulated Chemokine (TARC/CCL17), serum total IgE, serum Eosinophil Cationic Protein (ECP), and serum sE-selectin were reported by at least four studies and were therefore eligible for meta-analysis according to our inclusion criteria. The results of the meta-analysis of longitudinal studies are shown in Figure 2a-2d. Eighty-four cross-sectional studies, investigating 49 biomarkers, provided sufficient data for meta-analysis. Biomarkers that were reported by at least four studies and thus included in the meta-analysis, included serum levels of TARC, ECP, total IgE, cutaneous T-cell-attracting chemokine (CTACK/CCL27), CD30, IL-18, Lactate DeHydrogenase (LDH), macrophage-derived chemokine (MDC) and Vitamin D. The results of the meta-analysis of cross-sectional studies are shown in Figure 3-5.

Total serum IgE was found the most frequently studied biomarker. However, pooling of data from the longitudinal studies resulted in a weak correlation coefficient of 0.33 (95% CI: 0.08-0.64). Meta-analysis of the cross-sectional studies showed only a moderate pooled correlation to disease severity of 0.45 (95% CI: 0.32-0.57). Funnel plot asymmetry was present in funnel plots for both longitudinal and cross-sectional studies (Figures S1 and S2). Hence, we conclude that total serum IgE levels are not an appropriate biomarker for the follow-up of disease severity in AD and correlates only moderately to disease severity on a single time point.

TARC/CCL17 is one of the key chemokines involved in homing of CCR4 expressing T cells to the skin.[14] Serum TARC levels were found to significantly correlate to disease severity in AD patients from four longitudinal studies and in patients from 16 cross-sectional studies. Pooled correlation coefficients showed a strong correlation in both longitudinal (r=60, 95% CI: 0.48-0.70) and cross-sectional studies (r=0.64, 95% CI: 0.57-0.70). This suggests that serum TARC/CCL17 could potentially be a valuable biomarker for assessing disease severity in AD as well as evaluating the course of the disease. Although less frequently investigated, reports on plasma TARC levels also show promising results, but require further investigation.

CTACK/CCL27 is another T cell attracting chemokine that is suggested to play an important role in

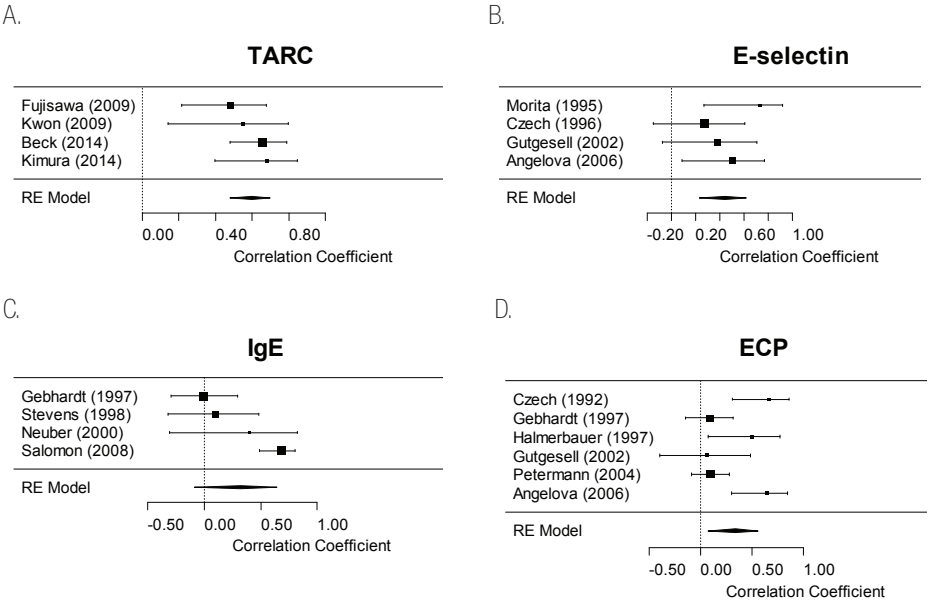
skin inflammation in AD. CTACK is being produced by keratinocytes, and binds to its receptor CCR10 that is expressed on skin homing T cells.[15] Serum CTACK levels have only been reported in two studies, reporting on follow up in 16 patients. However, a large number of cross-sectional studies reported serum CTACK levels, and meta-analysis showed a strong correlation to disease severity (r=0.68, 95% CI: 0.47-0.82). Inspection of the funnel plot revealed asymmetry. Additional data is needed to determine its usability in the follow-up of AD patients.

Serum sE-selectin is a cell adhesion molecule expressed on vascular endothelium, important for the migration of inflammatory cells from blood to tissue.[16] Four longitudinal studies reported correlation coefficients, resulting in a pooled correlation coefficient of 0.44 (95% CI: 0.23-0.62), indicating a moderate correlation. Serum sE-selectin might be a good candidate as a biomarker for disease severity, but requires further investigation.

ECP is a protein that is released during degranulation of eosinophils. Serum ECP was one of the first biomarkers related to disease severity in AD.[17] Serum ECP was frequently measured during follow-up in longitudinal studies, but only showed a pooled correlation coefficient of 0.34 (95% CI: 0.08-0.56). Cross-sectional studies showed correlations varying from -0.06 to 0.76, with a pooled correlation coefficient of 0.43 (95% CI 0.28-0.56). Funnel plots for both longitudinal and cross-sectional studies revealed some asymmetry on inspection. We conclude that the usability of serum ECP as a biomarker for disease severity is questionable.

MDC/CCL22, like TARC, is a chemoattractant for CCR4-expressing skin homing T

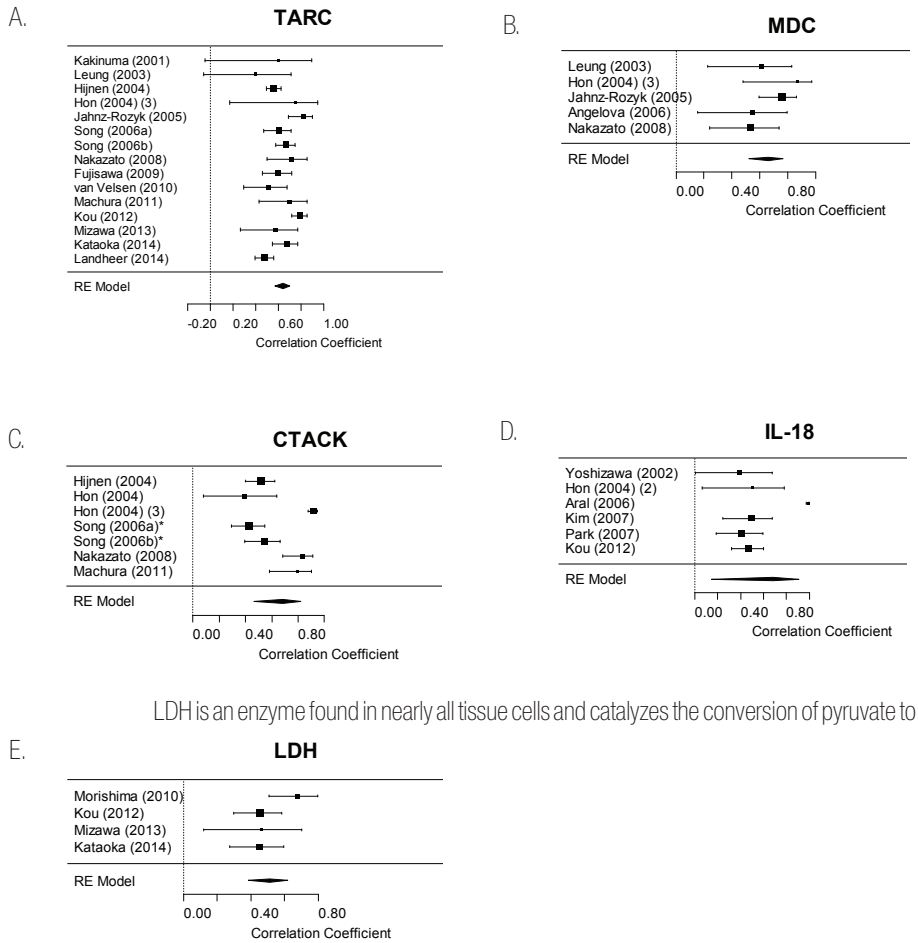
Figure 2A-D. Forest plots of meta-analysis of longitudinal studies.



The heterogeneity (I^2) for TARC was 0%; E-Selectin: 0%; IgE: 78%; and ECP: 74%.

cells.[18] Serum MDC levels showed a strong pooled correlation coefficient in the meta-analysis of cross-sectional studies of 0.66 (95% CI: 0.52-0.77). Correlation coefficients have only been reported in two longitudinal studies, therefore a meta-analysis on longitudinal studies was not possible. We conclude that serum MDC is suitable biomarker for measuring disease severity at a single time point, but additional data is needed to determine its usability in the follow-up of AD patients.

Figure 3A-E. Forest plots of meta-analysis of cross-sectional studies.

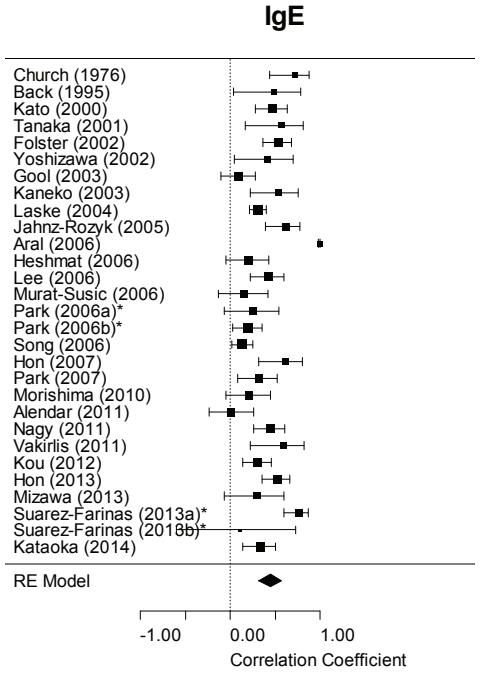


LDH is an enzyme found in nearly all tissue cells and catalyzes the conversion of pyruvate to lac-

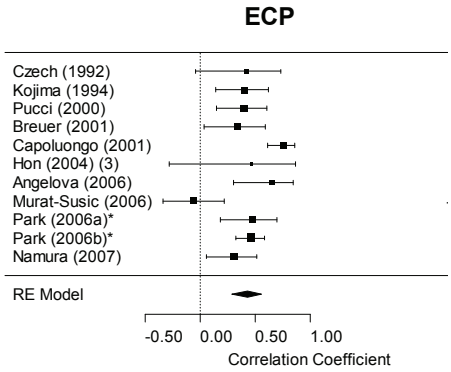
Biomarkers showing a strong correlation with disease severity (heterogeneity (I^2) for TARC: 70%; MDC 22%; CTACK: 93%; IL-18: 97%; LDH: 43%)

Figure 4A-B: Forest plots of meta-analysis of cross-sectional studies.

A.

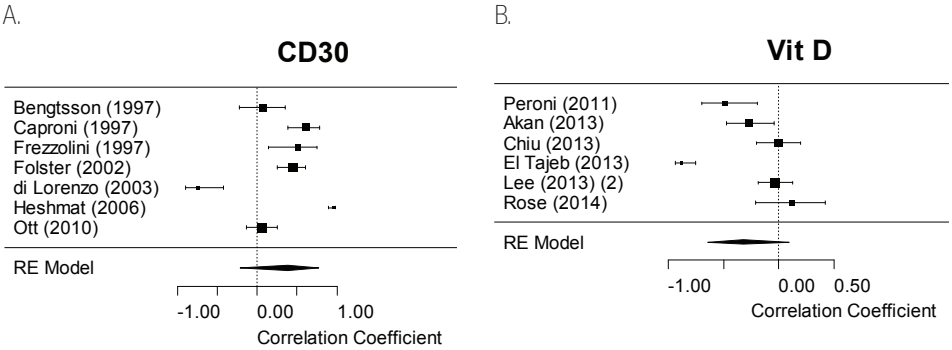


B.



Biomarkers showing a moderate correlation with disease severity (heterogeneity (I^2) IgE: 92%; ECP: 67%).

Figure 5A-B: Forest plots of meta-analysis of cross-sectional studies.



Biomarkers showing a weak correlation with disease severity (heterogeneity (I^2) for CD30: 93%; vitamin D: 94%).

tate. LDH is released during tissue damage, and is therefore commonly used as a marker for malignancies, cardiac diseases, and hemolysis. Four cross-sectional studies reported correlation coefficients of serum LDH levels to disease severity, pooling resulted in a correlation coefficient of 0.51 (95% CI: 0.38-0.62). Inspection of the funnel plot revealed some asymmetry. This makes serum LDH a potential candidate as a biomarker for disease severity.

IL-18 is a member of the IL-1 family and induces production of INF-y by T cells. Furthermore, it stimulates Th1 cells to produce IL-13, and synergistically with IL-12 stimulates the production of IgE and Th2 cytokines.[19-21] Meta-analysis of cross-sectional studies reporting IL-18 as a marker for disease severity, showed a strong pooled correlation coefficient of 0.68 (95% CI: 0.15-0.91). The funnel plot showed asymmetry, but since only four cross-sectional studies reported IL-18 it is hard to distinguish chance from real asymmetry, making the interpretation of a funnel plot rather unreliable. Only one longitudinal study reported a significant correlation between serum IL-18 levels, and disease severity.[22] We conclude that serum IL-18 might be a possible biomarker for disease severity, but requires further investigation.

Serum soluble CD30 levels have been investigated for use in several immune mediated inflammatory diseases. Correlation coefficients of serum CD30 levels with disease severity in AD ranged widely, from an inversed correlation (-0.74) to a very strong correlation (0.96). Pooling the data resulted in a correlation coefficient of 0.39 (95% CI -0.21-0.77), with a fairly asymmetric funnel plot. Serum vitamin D has also been studied as a biomarker in AD. Although some studies showed promising data regarding an inversed correlation with disease severity, there was a wide range of correlation coefficients (0.12 to -0.88). Meta-analysis resulted in a pooled correlation of -0.32 (95% CI -0.64-0.09). Visual inspection of the funnel plot revealed asymmetry. We conclude that CD30, nor vitamin D are suitable as biomarkers for disease severity in AD.

Biomarkers found to show good correlation to disease severity, but studied only in small numbers of studies include serum levels of IL-2R, IL-4R, and IL-31. The remaining biomarkers have been reported in even smaller patient series and show variable or inconclusive results.

DISCUSSION

In search of an objective biomarker for disease severity, this review aimed to synthesize results from all available publications, not limited to studies focusing on the identification of a biomarker. A meta-analysis of both longitudinal and cross-sectional studies revealed that currently serum TARC levels perform best as an objective biomarker for disease severity, showing strong pooled correlation coefficients in both longitudinal and cross-sectional studies. Although serum sE-selectin was reported by only four longitudinal studies, it showed a moderate correlation to disease severity and might also be a good candidate as a biomarker for disease severity. Meta-analysis of cross sectional studies reporting serum levels of CTACK, MDC, LDH and IL-18 showed that these markers are potentially good biomarkers for disease severity. However, these biomarkers were less frequently studied in longitudinal studies, and additional data is needed to determine their usability in the follow-up of AD patients. Although total serum IgE was found the most frequently studied biomarker, we conclude that it is not an appropriate biomarker for longitudinal studies.

Limitations of this study are the occurrence of potential selection- and publication bias. The initial search yielded 222 relevant publications reporting on 93 different biomarkers. Only 16 percent of longitudinal studies, and 57 percent of cross-sectional studies provided sufficient data for inclusion in our meta-analysis. The occurrence of selection bias in the results of the meta-analyses is therefore inevitable. Funnel plot asymmetry was present in a fairly large number of the meta-analysed biomarkers, indicating publication bias. Publication bias may have caused overestimation of the correlation coefficients of the biomarkers reported in this paper. Publication of biomarker data might be influenced by the direction and statistical significance of the correlation between a biomarker and disease severity, as non-significant study outcomes might be less likely published. Although we did not limit our search on language, it was not possible to retrieve data from 110 non-English publications. In addition, 176 out of 908 publications selected for screening were not available at our institutions. Despite these caveats, our synthesis of the disparate and prolific literature has yielded interesting new insights into several promising biomarkers.

Most studies included in this review reported on one, or only a limited number of biomarkers. Because AD is known to be a highly heterogeneous disease and different clinical phenotypes have been described, we hypothesized that it may be useful to simultaneously investigate a panel of biomarkers. From our own experience we know e.g. that a small subset of patients with severe AD have serum TARC levels in the normal range. These patients may represent a distinct immunological subtype for which biomarkers other than TARC may have additional value. Indeed, we recently reported that a combination of serum biomarkers shows a better correlation with disease severity compared to single biomarkers in AD patients.[23]

We conclude that serum TARC is the most reliable biomarker currently available. Serum TARC levels have been determined in relatively large numbers of patients from different studies, showing pooled correlation coefficients of 0.60 (95% CI: 0.48-0.70) and 0.64 (95% CI: 0.57-0.70) in longitudinal and cross-sectional studies, respectively. Additional biomarkers that could prove useful but require additional research include serum CTACK, serum sE-selectin, serum MDC, serum LDH and serum IL-18.

SUPPLEMENTARY DATA

See http://www.dermatologyutrecht.nl/index.php/9-public/115-thijs

REFERENCES

1. Mancini AJ, Kaulback K, Chamlin SL. The socioeconomic impact of atopic dermatitis in the United States: A systematic review. Pediatric dermatology 2008; 25:1-6.
2. Flohr C. Atopic dermatitis diagnostic criteria and outcome measures for clinical trials: still a mess. J Invest Dermatol 2011; 131:557-9.
3. Charman CR, Venn AJ, Williams HC. Measurement of body surface area involvement in atopic eczema: an impossible task? Br J Dermatol 1999; 140:109-11.
4. Charman C, Chambers C, Williams H. Measuring atopic dermatitis severity in randomized controlled clinical trials: What exactly are we measuring? Journal of Investigative Dermatology 2003; 120:932-41.
5. Schmitt J, Langan S, Stamm T, Williams HC. Core Outcome Domains for Controlled Trials and Clinical Recordkeeping in Eczema: International Multiperspective Delphi Consensus Process. Journal of Investigative Dermatology 2011; 131:623-30.
6. Moher D, Liberati A, Tetzlaff J, Altman DG. Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. Journal of Clinical Epidemiology 2009; 62:1006-12.
7. Hanifin JM, Rajka G. Diagnostic features of atopic dermatitis. Acta Derm.Venereol. 1980; 92:44-7.
8. Williams HC, Burney PGJ, Hay RJ, Archer CB, Shipley MJ, Hunter JJA, et al. The Uk Working Party Diagnostic-Criteria for Atopic-Dermatitis .1. Derivation of A Minimum Set of Discriminators for Atopic-Dermatitis. British Journal of Dermatology 1994; 131:383-96.
9. Stewart A. Basic Statistics and Epidemiology: A Practical Guide; 2010.
10. Viechtbauer W. Conducting meta-analyses in R with the metafor package. . Journal of Statistical Software 2010; 36:1-48.
11. Jackson D, Turner R, Rhodes K, Viechtbauer W. Methods for calculating confidence and credible intervals for the residual between-study variance in random effects meta-regression models. BMC Med Res Methodol 2014; 14:103.
12. Higgins JP, Thompson SG. Quantifying heterogeneity in a meta-analysis. Stat Med 2002; 21:1539-58.
13. Sterne JA, Egger M, Smith GD. Systematic reviews in health care: Investigating and dealing with publication and other biases in meta-analysis. BMJ 2001; 323:101-5.
14. Imai T, Nagira M, Takagi S, Kakizaki M, Nishimura M, Wang J, et al. Selective recruitment of CCR4-bearing Th2 cells toward antigen-presenting cells by the CC chemokines thymus and activation-regulated chemokine and macrophage-derived chemokine. Int Immunol 1999; 11:81-8.
15. Homey B, Alenius H, Muller A, Soto H, Bowman EP, Yuan W, et al. CCL27-CCR10 interactions regulate T cell-mediated skin inflammation. Nat Med 2002; 8:157-65.
16. Rossiter H, van Reijsen F, Mudde GC, Kalthoff F, Bruijnzeel-Koomen CA, Picker LJ, et al. Skin disease-related T cells bind

to endothelial selectins: expression of cutaneous lymphocyte antigen (CLA) predicts E-selectin but not P-selectin binding. Eur J Immunol 1994; 24:205-10.

17. Juhlin L, Venge P. Eosinophilic cationic protein (ECP) in skin disorders. Acta Derm Venereol 1991; 71:495-501.
18. Vestergaard C, Yoneyama H, Murai M, Nakamura K, Tamaki K, Terashima Y, et al. Overproduction of Th2-specific chemokines in NC/Nga mice exhibiting atopic dermatitis-like lesions. J Clin Invest 1999; 104:1097-105.
19. Hata H, Yoshimoto T, Hayashi N, Hada T, Nakanishi K. IL-18 together with anti-CD3 antibody induces human Th1 cells to produce Th1- and Th2-cytokines and IL-8. Int Immunol 2004; 16:1733-9.
20. Hoshino T, Wiltrout RH, Young HA. IL-18 is a potent coinducer of IL-13 in NK and T cells: a new potential role for IL-18 in modulating the immune response. J Immunol 1999; 162:5070-7.
21. Hoshino T, Yagita H, Ortaldo JR, Wiltrout RH, Young HA. In vivo administration of IL-18 can induce IgE production through Th2 cytokine induction and up-regulation of CD40 ligand (CD154) expression on CD4+ T cells. Eur J Immunol 2000; 30:1998-2006.
22. Kou K, Aihara M, Matsunaga T, Chen H, Taguri M, Morita S, et al. Association of serum interleukin-18 and other biomarkers with disease severity in adults with atopic dermatitis. Arch Dermatol Res 2012; 304.
23. Thijs JL, Nierkens S, Herath A, Bruijnzeel-Koomen CA, Knol EF, Giovannone B, et al. A panel of biomarkers for disease severity in atopic dermatitis. Clin Exp Allergy 2015; 45:698-701.

A complete list of references to all 222 publications can be found in the supplementary data file.


3

A panel of biomarkers for disease severity in atopic dermatitis

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To the editor,

A large number of biomarkers have been found to correlate with disease severity in atopic dermatitis (AD). The most frequently reported serum biomarkers for disease severity in AD include eosinophilic cationic protein (ECP), total IgE, soluble interleukin-2 receptor (sIL-2R), and thymus and activation-regulated chemokine (TARC/CCL17). In a systemic review on serum biomarkers for disease severity in AD we found that TARC showed the best correlation to disease severity, with weighted mean r-values of 0.51 and 0.63 in longitudinal and cross-sectional studies, respectively.[1]

Serum TARC levels show a strong correlation to disease severity in the follow-up of individual patients. Patients with similar disease severity scores, however, show varying TARC levels in cross-sectional cohorts of patients, consequently showing low correlation between TARC and disease severity. AD is known to be a highly heterogeneous disease and these low correlations may be the result of this heterogeneity. None of the individual (serological) biomarkers that have been studied previously were found to show a better correlation with disease severity than TARC. Therefore, the aim of this study was to explore if a panel of biomarkers shows a better correlation with disease severity compared to individual biomarkers.

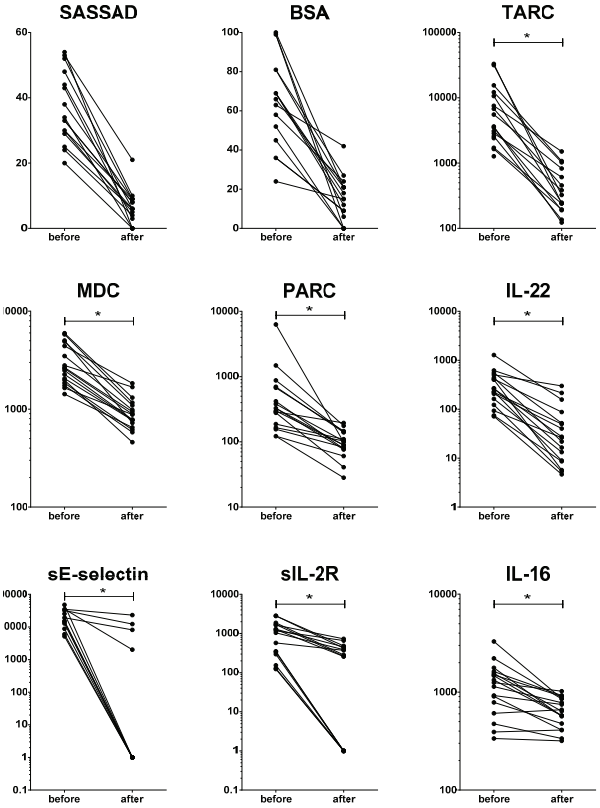
We included seventeen patients (six male and 11 female), mean age 33 years with moderate to severe AD (SASSAD range: 20-54; BSA range: 24-100%) that were admitted to the hospital after exacerbation of eczema. Patients were diagnosed with AD according to the criteria of Hanifin and Rajka.[2] All patients showed elevated total IgE levels (range: 275-76942 IU/mL; mean: 19006 IU/mL). Fifteen patients were also diagnosed with allergic asthma and/or allergic rhinitis. Disease severity was assessed using the Six Area Six Sign Atopic Dermatitis (SASSAD) score, which has been shown to have a good inter-observer agreement.[3] In addition, body surface area (BSA) involvement was determined. All patients were treated with potent topical corticosteroids (class III, European classification system). Patients using oral immunosuppressive medications were not included. Serum samples were obtained at admission to the hospital and after about two weeks of treatment (mean interval: 12.8 days). The protocols of this study were approved by the Institutional Review Board of the University Medical Center Utrecht (Utrecht, The Netherlands), adhering to the Declaration of Helsinki Principles.

Cytokine analyses were performed using a multiplex platform, as described previously.[4] Measurements were performed in undiluted samples, except for RANTES, PARC, TARC, and sE-selectin (1:100). Data was analyzed by 5-parametric curve fitting using Bio-Plex Manager software, version 6.1 (Biorad, Hercules, CA). Multiplex immunoassays were in-house validated and show an intra- and inter assay variability of less than 5% and 20%, respectively, and recovery values of spikes proteins in serum of 99-102%.

All patients showed significant clinical improvement after treatment with potent topical steroids. SASSAD scores decreased from 36.9 at baseline to 8.0 after two weeks of treatment. BSA involvement decreased from 65.4% at baseline to 18.5% after treatment (Fig. 1).

Biomarker levels were analyzed as two paired groups. The difference in biomarker levels between the blood samples before and after treatment was evaluated using the Wilcoxon matched-pairs signed rank test. Of 31 markers studied, seven showed a statistically significantly decrease after treatment (Fig. 1). This included TARC/CCL17, macrophage-derived chemokine (MDC/CCL22), IL-22, pulmonary and activation-regulated chemokine (PARC/CCL18), sIL-2R, soluble E-selectin (sE-selectin) and IL-16. Serum TARC, MDC, sE-selectin, sIL-2R and IL-16 were previously found to show good correlations to disease severity.[1] All determined levels were within lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ) levels of the standard lines except for eight post-treatment levels of sE-selectin and six post-treatment levels of sIL-2R (LLOQ=514 pg/ml and 75 pg/ml, respectively), for which a value of "1" is shown in the graphs. Pre- and post-treatment sIL-2R levels were below detection limit in one patient, and sE-selectin levels were below detection limit in five patients.

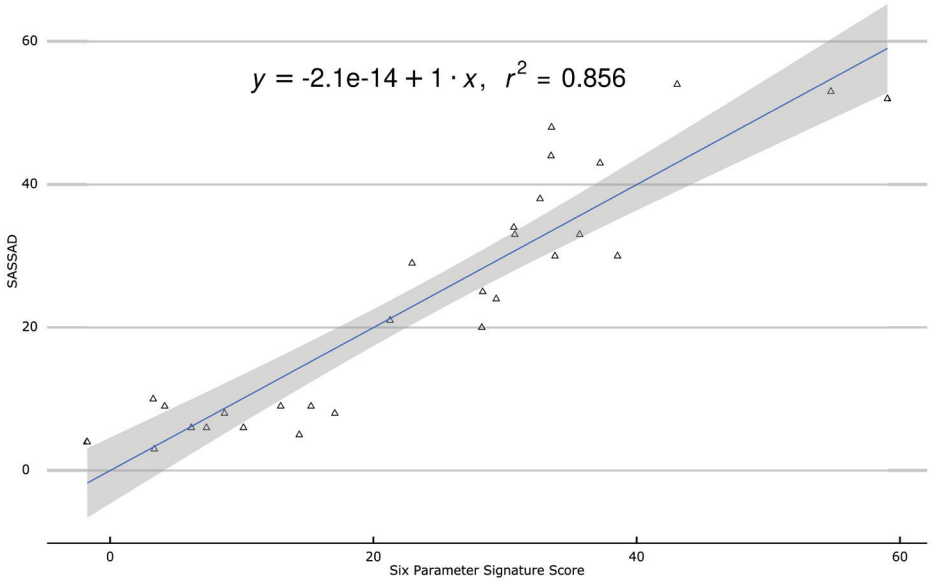
Figure 1. Clinical scores and biomarker levels before and after treatment with topical steroids.



Clinical scores (SASSAD and BSA), and serum biomarkers: TARC (pg/ml), MDC (pg/ml), PARC (ng/ml), IL-22 (pg/ml), sE-selectin (pg/ml), sIL-2R (pg/ml), and IL-16 (pg/ml), significantly decreased after treatment with potent topical steroids. *$p<0.001$.

Stepwise regression analysis with leave one out cross validation was used to assess the best combination of biomarkers to predict disease severity. The analysis was carried out using the R statistical package, Version 3.1. To find the best predictor of disease severity (SASSAD score) a linear combination was explored, using the pre-treatment levels of TARC, MDC, PARC, IL-22, sE-selectin, sIL-2R and IL-16. This established an equation (SASSAD= -39.89 - 6.95 * sex male + 1.78 * log(TARC) + 9.12 * log(PARC) + 4.52 * log(IL-22) - 2.51 * log(sIL-2R)) for predicting disease severity. This five-parameter multivariate signature containing demographic characteristics of gender (sex), and the four molecular mediators of TARC, PARC, IL-22, sIL-2R showed a correlation coefficient of 0.856 (Fig. 2), whereas the correlation coefficient to disease severity for the individual biomarkers ranged from 0.415 to 0.742. This confirms our hypothesis that a panel of biomarkers shows a better correlation to disease severity compared to individual biomarkers.

Figure 2. The relationship between the five-parameter signature score and SASSAD score. AD is a highly heterogeneous disease.



In search of a molecular predictor for disease severity, we have systematically assessed the predictivity of disease severity using a collection of mediators in blood. The five-parameter multivariate signature containing demographic characteristics of gender (sex), and four molecular mediators (TARC, PARC, IL-22, sIL-2R) robustly predicts the SASSAD score. The mathematical relationship between the SASSAD and the five-parameter signature can be denoted by: SASSAD= -39.89 - 6.95 * sex male + 1.78 * log(TARC) + 9.12 * log(PARC) + 4.52 * log(IL-22) - 2.51 * log(sIL-2R).

From our experience we know that some patients with severe AD have serum TARC levels in the normal range, and on the other hand some patients present with mild to moderate disease and high TARC levels. These patients may represent a subset for which TARC as a single biomarker is not suitable. These outliers contribute to the moderate correlations between TARC levels and disease severity in previous publications.[5] These preliminary observations emphasize that not only clinically, but also on serum biomarker level, AD is a heterogeneous disease. The use of a multivariate biomarker signature to predict disease severity seems to overcome this problem as shown here.

The biomarkers included in our panel (TARC, PARC, IL-22 and sIL-2R) are involved in inflammation and have been found to play a role in the pathogenesis of AD. TARC and PARC are members of the CC chemokine family and are involved in the recruitment of T cells into the skin.[6] sIL-2R is synthesized and secreted by T cells, sIL-2R levels reflect the activation state of the T cells in the skin.[6] IL-22 is a member of the IL-10 family. IL-22 induces keratinocyte proliferation resulting in acanthosis, one of the histopathological hallmarks of AD.[7] We recently showed production of IL-22 by both CD4+ and CD8+ T cells isolated from the skin of AD patients.[8] In vivo experiments using cultured human keratinocytes have shown that IL-22 downregulates filaggrin expression and affects expression of profilaggrin processing enzymes, contributing to epidermal barrier dysfunction in AD.[9] Taken together, all four biomarkers play an important role in the pathogenesis of AD. Expression of these biomarkers in skin is reflected by their serum levels and the combination of their serum levels comprises a signature that reflects disease severity in AD patients. In addition, this is the first study that shows correlation between serum IL-22 levels and treatment effect in AD patients.

In conclusion, we found that a combination of serum biomarkers demonstrates a better correlation with disease severity compared to a single biomarker in AD patients. Although confirmation of our results in larger cohorts of patients is needed, it shows that using a panel of biomarkers may be necessary in a multifactorial, complex disease such as AD. With the strong predictive value of biomarkers in treatment of atopic disease with biologicals, the introduction of biologics in the treatment of AD and the lack of consensus on the use of disease severity measures, we believe that these preliminary data are promising and warrant further studies that explore the use of a panel of biomarkers as a disease severity measurement and possibly treatment predictive tool. In addition, we showed that serum IL-22 levels correlate with disease activity in AD patients. These findings further support a role for the IL-22 pathway in AD.

REFERENCES

1. Thijs J, Krastev T, Weidinger S, Buckens CF, de Bruin-Weller M, Bruijnzeel-Koomen C, et al. Biomarkers for atopic dermatitis: a systematic review and meta-analysis. Curr Opin Allergy Clin Immunol 2015; 15:453-60.
2. Hanifin JM, Rajka G. Diagnostic Features of Atopic-Dermatitis. Acta Derm Venereol 1980:44-7.
3. Charman CR, Venn AJ, Williams HC. Reliability testing of the Six Area, Six Sign Atopic Dermatitis severity score. Br J Dermatol 2002; 146:1057-60.
4. de Jager W, te Velthuis H, Prakken BJ, Kuis W, Rijkers GT. Simultaneous detection of 15 human cytokines in a single sample of stimulated peripheral blood mononuclear cells. Clin Diagn Lab Immunol 2003; 10:133-9.
5. Landheer J, de Bruin-Weller M, Boonacker C, Hijnen D, Bruijnzeel-Koomen C, Rockmann H. Utility of serum thymus and activation-regulated chemokine as a biomarker for monitoring of atopic dermatitis severity. J Am Acad Dermatol 2014; 71:1160-6.
6. Walker C, Kagi MK, Ingold P, Braun P, Blaser K, Bruijnzeel-Koomen CA, et al. Atopic dermatitis: correlation of peripheral blood T cell activation, eosinophilia and serum factors with clinical severity. Clin Exp Allergy 1993; 23:145-53.
7. Zheng Y, Danilenko DM, Valdez P, Kasman I, Eastham-Anderson J, Wu J, et al. Interleukin-22, a T(H)17 cytokine, mediates IL-23-induced dermal inflammation and acanthosis. Nature 2007; 445:648-51.
8. Hijnen D, Knol EF, Gent YY, Giovannone B, Beijn SJ, Kupper TS, et al. CD8(+) T cells in the lesional skin of atopic dermatitis and psoriasis patients are an important source of IFN-gamma, IL-13, IL-17, and IL-22. J Invest Dermatol 2013; 133:973-9.
9. Gutowska-Owsiak D, Schaupp AL, Salimi M, Taylor S, Ogg GS. Interleukin-22 downregulates filaggrin expression and affects expression of profilaggrin processing enzymes. Br J Dermatol 2011; 165:492-8.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EASI p-EASI: Utilising a combination of serum biomarkers offers an objective measurement tool for disease severity in atopic dermatitis patients

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ABSTRACT

Background:

Serum biomarkers offer an objective outcome measure for disease severity in atopic dermatitis (AD). Assessing disease severity with a single biomarker may not be sufficient in a complex and heterogeneous disease such as AD. We hypothesized that a combination of biomarkers is more suitable for assessing disease severity than a single biomarker.

Methods:

In a retrospective cohort of 193 AD patients, 147 serum biomarkers were measured to identify biomarkers that correlated with disease severity. Based on the findings in this retrospective cohort we selected ten biomarkers for validation in a prospective cohort of 65 AD patients. During a treatment period with topical steroids of two months, disease severity was assessed by the Eczema Area and Severity Index (EASI) and serum biomarkers were measured. Fourteen psoriasis vulgaris patients and 26 non-atopic subjects were included as controls.

Results:

In the retrospective cohort, IL-18, IL-22, IL-31, TARC, MDC, PARC, sIL-2R, sE-selectin, SDF-1α and I-309 showed correlation coefficients of >0.30 to disease severity. In the prospective cohort, all patients showed significantly decreasing EASI scores during treatment. Serum biomarkers IL-18, IL-22, I-309, MDC, PARC, sE-selectin, sIL-2R and TARC also significantly decreased upon treatment. Linear mixed model analyses in 55 randomly selected patients from the prospective cohort revealed an optimal combination of TARC, IL-22 and sIL-2R as a predictor of EASI scores. This model was validated in the ten remaining patients and showed a correct prediction of EASI scores in 90% of the cases (sensitivity: 100%, specificity: 88.9%).

Conclusion:

Combining serum biomarkers TARC, IL-22 and sIL-2R as a signature offers an objective measurement tool for disease severity in AD patients.

INTRODUCTION

Atopic dermatitis (AD) is recognized as one of the most common chronic inflammatory skin diseases worldwide, associated with a high socio-economic impact.[1-4] In recent years, there has been an increasing number of clinical trials evaluating new treatments for AD, with biologics in particular generating promising results.[5] However, the comparability of study outcomes remains challenging, as different clinician-rated outcome measures are used and these show high intra- and inter-observer differences.[6-8]

In the present study we propose the use of serological biomarkers as objective and reliable outcome measures of disease severity. A recent meta-analysis identified serum thymus and activation-regulated chemokine (TARC/CCL17) as the best biomarker currently available for assessing disease severity in AD.[9] Although TARC strongly correlates with disease activity in individual patients during follow-up, TARC levels vary between patients within cross-sectional cohorts of patients that have similar disease severity scores.[10] The variation in TARC levels between patients may be the result of the large number of biologic pathways involved in the pathogenesis of AD. The use of a panel of several biomarkers from different biologic pathways, representing different phenotypes/endotypes, may overcome this problem. Indeed, we have recently shown that a combination of biomarkers, including serum TARC, pulmonary and activation-regulated chemokine (PARC/CCL18), IL-22 and sIL-2R showed a correlation coefficient of 0.86 with disease severity, whereas the correlation coefficient to disease severity for the individual biomarkers ranged from 0.42 to 0.74.[11] Even though this combination was identified in a small pilot study, it demonstrated that the use of a combination of biomarkers may improve correlation with disease severity in a multifactorial, complex disease such as AD. The aim of the current study therefore was to validate the use of a combination of biomarkers for disease severity in AD patients during treatment with topical steroids.

METHODS

Study design

This study was conducted in two steps: (i) potential new serum biomarkers that correlated with disease severity were identified in a retrospective cohort of 193 moderate to severe AD patients, next (ii) this combination of severity biomarkers was validated in a prospective cohort of 65 AD patients treated with topical steroids. The protocols used in this study were approved by the Institutional Review Board of the University Medical Center Utrecht, adhering to the Declaration of Helsinki Principles.

Patients and samples

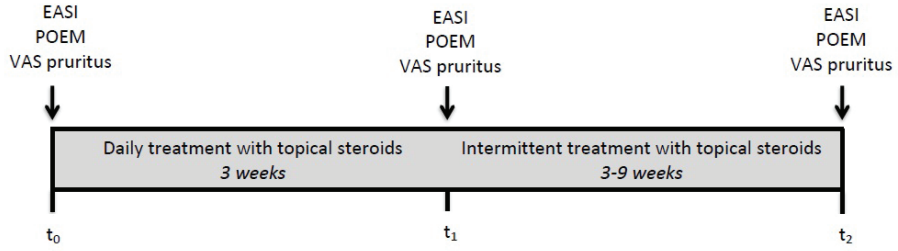
We analyzed the correlation between serum biomarker expression levels and disease severity measured by the Six area, six sign atopic dermatitis (SASSAD) severity score[12] in 193 moderate to severe AD patients, as defined by the criteria of Hanifin and Raijka,[13] from a previously published study.[14] All patients were treated with topical steroids, patients using oral immunosuppressive drugs or UV-therapy within three months before baseline were excluded.

To validate the use of a combination of biomarkers, a prospective cohort of 65 adult AD patients was recruited. Sample size was calculated using correlation coefficients from a previous study,[11] using a Fisher's z-test for Pearson correlation in SAS version 9.2 (SAS Institute, Cary, NC, USA). To compute the sample size we used the following parameters: one-sided, α=0.05, and power=0.8, resulting in a required sample size of 60 patients.

During the first visit (t_0), serum was collected and disease severity was assessed by the Eczema Area and Severity Index (EASI).[15] All clinicians were trained and experienced in using EASI. Additionally, we included Patient-Oriented Eczema Measure (POEM),[16] and visual analogue scale (VAS) for pruritus.[17] At t_0, all patients were instructed by specialized nurses to use potent topical steroids (European classification class III: fluticasone furoate, mometasone furoate or betamethasone dipropionate) according to the fingertip unit.[18]

During a second visit (t_1), after three weeks, blood was collected, EASI, POEM and VAS pruritis were assessed, and patients were instructed to taper the frequency of topical corticosteroid application. Assessment of disease severity and collection of serum was repeated after 6 to 12 weeks (t_2) of treatment. The outline of the study protocol is summarized in Figure 1.

Figure 1. Study design



Patients were included during their first visit to the outpatient clinic (t_0). After t_0 all patients were treated with daily potent topical steroids (European classification: class III). After three weeks (t_1) the use of topical corticosteroids was tapered. Disease severity was assessed by EASI, POEM and VAS pruritis at all three timepoints (t_0, t_1, t_2); serum was collected at the same timepoints. EASI; Eczema Area and Severity Index, POEM; Patient-Oriented Eczema Measure, VAS; visual analogue scale.

Control groups

To determine disease specificity of the serum biomarkers, 14 psoriasis patients treated with topical steroids were included. Disease severity was assesed by the Psoriasis Area Severity Index (PASI),[19] the self-administered psoriasis area and severity index (SA-PASI),[20] and VAS pruritus. We also included 26 non-atopic healthy controls. Healthy controls were approximately age and sex matched to the AD patients (Table 1). Serum was collected at a single time point.

Table 1. Baseline characteristics

	Retrospective AD cohort (n=193)	Prospective AD cohort (n=65)	Psoriasis patients (n=14)	Healthy controls (n=26)
Age, yrs	30.5 (21.0-42.0)	31.6 (22.6-46.7)	39.4 (27.6-67.5)	36.5 (29.5-54.3)
Male, n (%)	81 (42)	31 (47)	8 (67)	13 (50)
SASSAD	31.0 (23.0-37.5)	N/A	N/A	N/A
EASI	N/A	17.4 (10.0-25.7)	N/A	N/A
POEM	N/A	21.0 (17.0-25.0)	N/A	N/A
PASI	N/A	N/A	4.2 (2.3-7.5)	N/A
SA-PASI	N/A	N/A	7.8 (4.8-17.6)	N/A
VAS pruritis	N/A	7.5 (6.0-8.0)	5.0 (0.0-7.0)	N/A

Categorical variables are presented as counts and percentages; continues variables are presented as median (Inter-QuartileRange). ANOVA testing revealed no significant differences in age or gender between the groups. N/A = not available.

Serum biomarkers

In the retrospective cohort consisting of 193 AD patients, 147 serum biomarkers were measured by Luminex analysis (Table S1) as previously described.[14] Total IgE levels were determined using ImmunoCAP (Thermo Fisher, Uppsala, Sweden) according to the manufacturer's instructions. Periostin and DPP4 levels were measured using in-house ELISA-based assays (Abbott Diagnostics, Abbott Park, IL, USA and R&D Systems, Minneapolis, MN ,USA respectively).

In the 65 AD patients that comprised the prospective validation cohort, the biomarkers TARC, PARC, IL-22, sIL-2R, sE-selectin, IL-18, T Lymphocyte-Secreted Protein I-309 (I-309/CCL1), macrophage-derived chemokine (MDC/CCL22), and stromal cell-derived factor-1α (SDF-1α/CXCL12) were measured. For technical reasons S100A12 could not be measured in the prospective AD cohort and was therefore not included. Because serum IL-31 levels have previously been related to pruritis in AD, we also included IL-31. Biomarkers were also measured in psoriasis and healthy control samples.

Statistical analysis

All variables measured in patients from the retrospective cohort were normalised by log-transformation. Pearson correlation coefficients were used to assess the correlation between SASSAD and biomarkers. P-values were adjusted for multiple testing by adjusting the false discovery rate using the Benjamini-Hochberg procedure.

In the prospective validation cohort and control groups, serum levels of TARC, PARC, MDC and sE-selectin were normalised by log-transformation. Differences in biomarker levels between groups were compared with ANOVA. Linear mixed models were used to model the change over time of biomarkers after treatment initiation. This model generalizes the fixed effect regression models by taking into account correlation between measurements in longitudinal data. We investigated the effects of the different biomarkers during treatment using piecewise linear mixed models. For all AD patients, two slopes were considered: one for the first treatment period (between t_0 and t_1) and one for the second treatment period (between t_1 and t_2). Time for the slope's change (t = 22 days) was based on the median duration of the first treatment period. Correlation between individual baseline values and the subsequent slopes was handled through the unstructured covariance matrix of random effects. Models were adjusted for age and gender. The EASI prediction model was built using the Akaike Information Criteria which quantifies the quality of the fit to the data and correct for the number of parameters in the model. A total of 55 randomly selected patients were used to define the best model which was validated using the remaining 10 AD patients by comparing the predicted EASI scores with the measured ones. EASI scores were classified as mild (1.0-7.0), moderate (7.1-21.0) or severe (>21.1).[21] Sensitivity and specificity were calculated to describe the predictive capacity of the

model. We defined false positive as a predicted EASI score classifying a higher disease severity than the observed EASI scores, and false negative as a predicted EASI scores classifying a lower disease severity than the observed EASI scores. To test the robustness of our findings, we repeated the above analysis five times: random selection of 55 AD patients to build the model and use of the 10 remaining AD patients for validation. P-values <0.05 were considered statistically significant. All statistical analyses were conducted using SAS version 9.4 (SAS Institute, Cary, NC, USA) and Prism (version 6;Graphpad).

RESULTS

Identification of new biomarkers for disease severity in AD

A total of 193 AD patients (median 30.5 years InterQuartileRange (IQR) 21.0-42.0, median SASSAD 31.0, IQR 23.0-37.5) were included in the retrospective cohort (Table 1). A significant correlation with SASSAD was found for 32 out of 147 serum biomarkers (Table 2; for a complete list of biomarkers see Table S1). Serum TARC levels showed the strongest correlation with disease severity ($r=0.40$, $p<0.01$). Biomarkers that significantly correlated with disease severity and had correlation coefficients higher than 0.30 (TARC, PARC, IL-22, sIL-2R, IL-18, I-309, MDC, sE-selectin, and SDF-1α) were considered meaningful[22] and included in the prospective study.

A total of 147 biomarkers were measured in the serum of 193 AD patients. 32 biomarkers showed a significant correlation with disease severity measured by SASSAD. Biomarkers marked in bold show correlation coefficients higher than 0.30, and were selected to the panel of biomarkers measured in the prospective AD cohort.

Table 2. Biomarkers showing a significant correlation with disease severity in AD

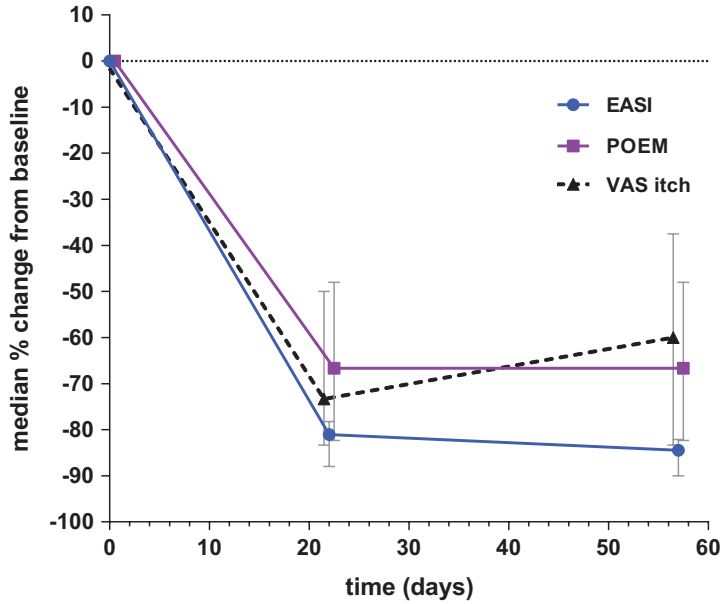
Biomarker	Correlation coefficient	p-value
IL-1ra	-0.19	<0.05
IL-10	-0.25	<0.01
IL-11	-0.24	<0.05
IL-17	-0.22	<0.05
IL-18	0.39	<0.01
IL-22	0.24	<0.05
LIF	-0.23	<0.05
I-309	0.32	<0.01
MCP4	0.21	<0.05
TARC	0.42	<0.01
MDC	0.38	<0.01
XCL1	-0.28	<0.01
HGF	0.23	<0.05
VEGF	0.22	<0.05
TNF-R2	0.25	<0.01
sE-selectin	0.35	<0.01
Trappin2	-0.21	<0.05
Tweak	0.19	<0.05
SOST	-0.21	<0.05
Cathepsin L	0.21	<0.05
S100A12	-0.31	<0.01
TIMP1	0.21	<0.05
MMP1	0.23	<0.01
MMP8	0.25	<0.01
ICAM	0.18	<0.05
SDF1	0.30	<0.01
TPO	0.28	<0.01
Chemerin	-0.19	<0.05
PAI-1	0.25	<0.01
PARC	0.32	<0.01
RANTES	0.20	<0.05
Total IgE	0.29	<0.01

Validation of a combination of biomarkers for disease severity in AD

To validate the use of a combination of biomarkers as surrogate measure for disease severity, 65 AD patients with mild to severe disease (median 31.6 years, IQR 22.6-46.7, median EASI 17.4, IQR 10.0-25.7) were included (Table 1). Patients were followed for median 57 days (IQR 35-107). At baseline (t_0), patients started with daily application of potent topical steroids for a median duration of 22 days (IQR 13-42). At t_1, the use of topical corticosteroids was tapered to two to three times weekly applications, which is considered a safe maintenance scheme.[23] Disease severity significantly decreased ($p<0.0001$) to a median EASI of 3.3 (IQR 1.2- 5.6) at t_1, and remained stable until t_2 (median EASI 2.7, IQR 1.0-4.6). Similarly, POEM and VAS pruritis decreased significantly ($P<0.0001$) during the first treatment period and remained stable until the end of the study (Figure 2).

In addition, 14 psoriasis patients (median 39.4 years, IQR 27.6-67.5, median PASI 39.4, IQR 27.6-67.5), and 26 non-atopic controls (median 36.5 years, IQR 29.5-54.3) were included in the study (Table 1).

Figure 2. Change in EASI, POEM and VAS pruritis in AD patients during topical steroid treatment

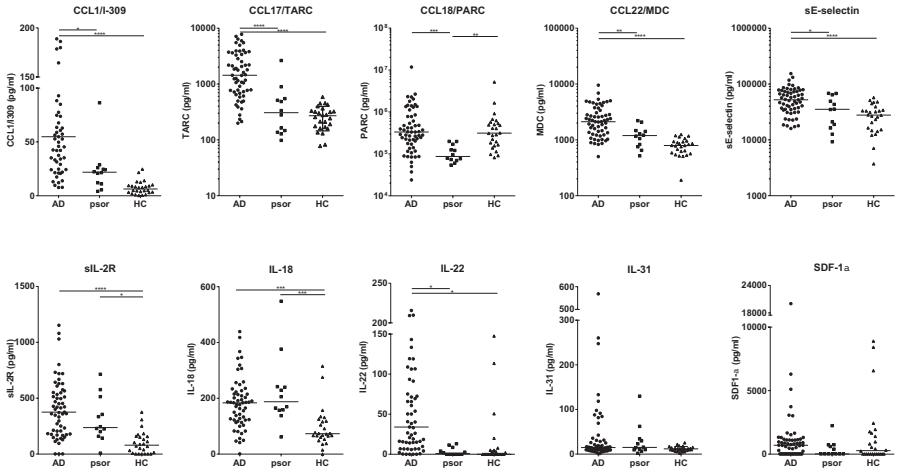


The figure shows the median percentage change from baseline for EASI, POEM and VAS pruritus in AD patients during treatment with local topical steroids.

Serum biomarker changes in prospective patient cohort after initiation of topical steroid treatment

At t_0, serum biomarkers: I-309, TARC, MDC, IL-22, and sE-selectin were significantly higher in AD patients compared to psoriasis patients and healthy controls ($p<0.01$; Figure 3). Serum levels of PARC were significantly higher in AD patients compared to psoriasis patients ($p<0.001$), but not different from healthy controls. Serum levels of IL-18 and sIL-2R were significantly higher in AD patients compared to healthy controls ($p<0.001$), but not significantly different from psoriasis patients. No differences were found in levels of IL-31 and SDF-1α between AD patients, psoriasis patients or healthy controls (Figure 3).

Figure 3. Biomarker levels in AD patients, psoriasis patients and healthy controls.



Differences in biomarker levels between AD patients, psoriasis patients and healthy controls were analysed by ANOVA testing. Horizontal bars represent median biomarkers levels. *$p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$. AD= atopic dermatitis; psor= psoriasis; HC= healthy controls.

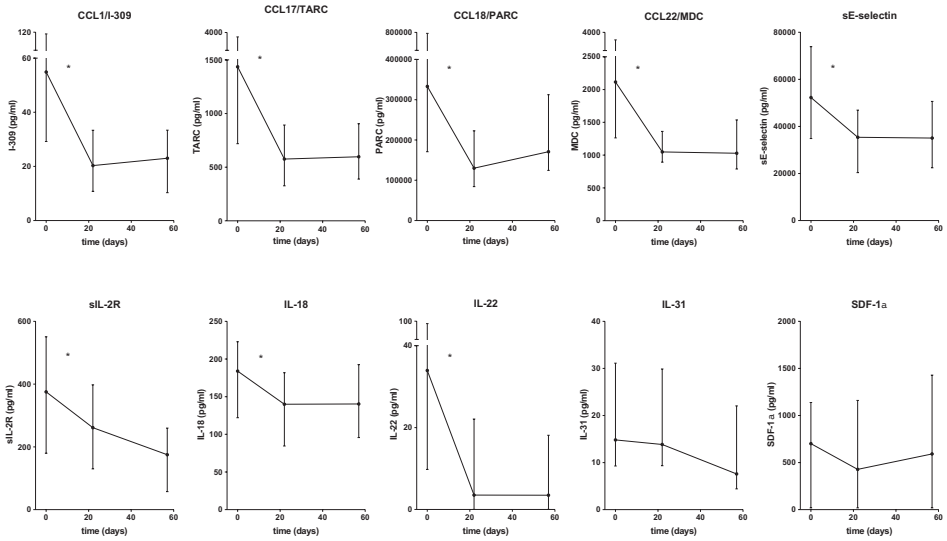
Linear mixed models were used to model the changes over time of biomarkers after treatment initiation with topical steroids. Most biomarkers (I-309, TARC, PARC, MDC, IL-18, IL-22, sE-selectin and sIL-2R) showed a significant decrease during daily treatment with topical steroids (i.e. between t_0 and t_1; $p<0.001$) and remained stable between t_1 and t_2 when patients used topical steroid treatment intermittantly. Only IL-31 and SDF-1α did not change during daily or intermittent treatment with topical steroids (Figure 4, Table 3). After adjusting for gender and age, results remained unchanged.

Table 3. Changes over time of biomarkers after treatment initiation

Biomarker level at baseline	Estimate (95%CI)	P-value
I-309 (pg/ml)	77.57 (58.60, 96.53)	
Log TARC (pg/ml)	3.12 (3.01, 3.22)	
Log PARC (pg/ml)	5.46 (5.34, 5.58)	
IL-18 (pg/ml)	178.13 (155.57, 200.68)	
IL-22 (pg/ml)	59.58 (41.05, 78.11)	
IL-31 (pg/ml)	40.13 (19.15, 61.11)	
Log MDC (pg/ml)	3.29 (3.23, 3.36)	
sIL-2R (pg/ml)	403.75 (342.04, 465.46)	
Log sE-selectin (pg/ml)	4.68 (4.62, 4.74)	
SDF-1α (pg/ml)	7971.95 (1744.21, 14200.00)	
Change/week (between t_0 and t_1)		
I-309 (pg/ml)	-16.12 (-22.16, -10.07)	<.0001
Log TARC (pg/ml)	-0.13 (-0.16, -0.10)	<.0001
Log PARC (pg/ml)	-0.10 (-0.14, -0.06)	<.0001
IL-18 (pg/ml)	-14.26 (-20.53, -7.98)	<.0001
IL-22 (pg/ml)	-13.68 (-18.51, -8.85)	<.0001
IL-31 (pg/ml)	-1.76 (-6.68, 3.15)	0.47
Log MDC (pg/ml)	-0.09 (-0.11, -0.06)	<.0001
sIL-2R (pg/ml)	-57.55 (-76.38, -38.71)	<.0001
Log sE-selectin (pg/ml)	-0.07 (-0.09, -0.05)	<.0001
SDF-1α (pg/ml)	-344.35 (-3730.94, 3042.25)	0.84
Change/week (between t_1 and t_2)		
I-309 (pg/ml)	-0.10 (-1.45, 1.24)	0.87
Log TARC (pg/ml)	0.001 (-0.008, 0.009)	0.80
Log PARC (pg/ml)	0.01 (-0.00, 0.02)	0.05
IL-18 (pg/ml)	-0.23 (-1.85, 1.39)	0.77
IL-22 (pg/ml)	-13.68 (-18.51, -8.85)	<.0001
IL-31 (pg/ml)	-1.76 (-6.68, 3.15)	0.47
Log MDC (pg/ml)	-0.09 (-0.11, -0.06)	<.0001
sIL-2R (pg/ml)	-57.55 (-76.38, -38.71)	<.0001
Log sE-selectin (pg/ml)	-0.07 (-0.09, -0.05)	<.0001
SDF-1α (pg/ml)	904.40 (10.08, 1798.73)	0.06

Linear mixed models were used to model the changes over time of biomarkers after treatment initiation with topical steroids. The biomarkers I-309, TARC, PARC, IL-18, IL-22, MDC, sE-selectin and sIL-2R showed a significant decrease during daily treatment with topical steroids (between t_0 and t_1) and then remained at stable levels during intermittent treatment with topical steroids (between t_1 and t_2). The biomarkers (IL-31 and SDF1α) did not change during daily or intermittent treatment with topical steroids.

Figure 4. Serum biomarkers during treatment in AD patients



Median biomarker levels during treatment of AD patients. Error bars represent 95% confidence intervals. Linear mixed models were used to model the changes over time of biomarkers after treatment initiation with topical steroids. The biomarkers I-309, TARC, PARC, IL-18, IL-22, MDC, sE-selectin and sIL-2R showed a significant decrease (*<.0001) during daily treatment with topical steroids (day 0 until day 22) and then remained at stable levels during intermittent treatment with topical steroids (after day 22). The biomarkers (IL-31 and SDF-1α) did not change during daily or intermittent treatment with topical steroids. P-values are shown in Table 3.

Developing a combination of biomarkers as surrogate for disease severity score

We then aimed to model changes in EASI scores over time by using a combination of biomarkers in a linear mixed model approach. We included all biomarkers that showed a significant decrease during treatment: I-309, TARC, PARC, IL-18, IL-22, MDC, sE-selectin and sIL-2R. Fifty-five randomly selected patients from the prospective AD cohort were used to build the model. Backward selection of the biomarkers, based on Akaike Information Criteria, showed that the combination of TARC, IL-22 and sIL-2R was able to best predict the EASI score at baseline (i.e. before treatment initiation) and after treatment initiation (Table S2). Subsequently, we developed the following signature to predict EASI scores before treatment (Figure 5):

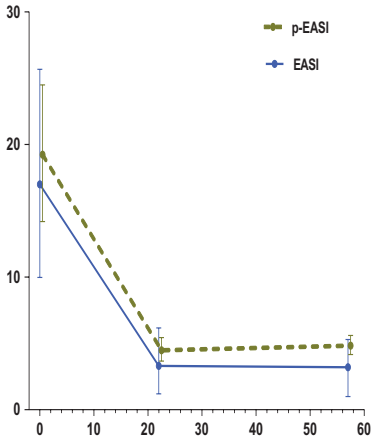
EASI = -36.12+ 18.49 * Log TARC + 0.009 * IL-22 – 0.009 * sIL-2R,

and the following signature to predict EASI scores during topical steroid treatment:

EASI = -5.82 + 4.04 * Log TARC + 0.003 * IL-22 – 0.003 * sIL-2R.

To validate our model, the signature was applied to the ten remaining patients of the cohort. Classification of severity scores according to the predicted EASI using the formulas agreed to the classification of the original EASI scores in 90% of the cases (Table S3), thus validating our model. Moreover, our model showed a sensitivity of 100% and a specificity of 88.9%. To test the robustness of our findings, the above analysis was repeated five times using random selections of 55 AD patients to build the model and using the ten remaining AD patients for validation. The predictive capacity of our model showed a sensitivity ranging from 83.3% to 100.0%, and a specificity ranging from 88.5%-95.2%.

Figure 5. Predicted EASI scores



Median predicted EASI (p-EASI) scores of the 65 patients from the prospective AD cohort. Error bars represent 95% confidence intervals. Serum TARC, IL-22 and sIL-2R levels are used to calculate p-EASI scores before (t0) and after treatment (t1) by the signature:
p-EASI= (-36.12 + 18.49*logTARC + 0.0089*IL-22 - 0.0095*sIL-2R)*(1-treatment) - (5.82 + 4.04*logTARC + 0.0027*IL-22 -0.0028*sIL2R)*treatment (treatment can be no=0, or yes=1).
The strongest predictor in this signature is TARC (Figure 4 and Table S2), but when we compare the effect size and confidence intervals at each stage between the signature and its constituents (e.g.: TARC alone) it demonstrate the high precision that might be achieved in using the p-EASI signature.

DISCUSSION

We have recently shown in a pilot study that a combination of serum biomarkers, including TARC, PARC, IL-22, sIL-2R and gender correlated with disease severity.[11] In the current study we optimized and validated this combination of biomarkers in larger patient populations, and showed that a combination of three serum biomarkers: TARC, IL-22 and sIL-2R predicted disease severity with high precision.

Reliably assessing disease severity in AD patients is a real challenge. Shortcomings of existing clinician rated severity measures have encouraged investigators to develop new, more robust outcome measures that have been used in AD studies.[7] A systematic review on validity and reliability of the 20 most commonly used clinician rated severity measures for AD showed that only the severity measures EASI, SCORAD and POEM performed adequately.[24] To address these deficiencies in reporting, experts in the field have established the Harmonizing Outcome Measurements in Eczema (HOME) initiative, an attempt to ensure that investigators employ a core set of outcome measures to enhance comparability between studies. The HOME initiative recently recommended the use of EASI and POEM as core outcome instruments for measuring disease severity and symptoms in AD.[25, 26] Although assessing EASI is relatively simple, inter-observer variability remains a problem, as was recently highlighted by Zhao et al.[8]

In addition to clinician rated severity measures, a large variety of plasma and serum biomarkers has been assessed for their suitabilty to predict disease severity. Serum biomarkers have the advantage of being objective and less time consuming than clinician rated severity measures. In a recent review on biomarkers for disease severity in AD, we found that over 100 biomarkers have been reported, and that currently serum TARC levels perform best as objective biomarker for disease severity.[9] However, these studies only included a single biomarker for the assessment of disease severity. We hypothesized that a combination of biomarkers may be more suitable than a single biomarker in a highly heterogeneous and complex disease with multiple biological pathways involved in its pathogenesis. Indeed, the current study shows that combining multiple biomarkers in a linear mixed model improves prediction of disease severity compared to a model that only includes serum TARC levels. The combination of serum biomarkers TARC, IL-22, and sIL-2R predicts disease severity in AD patients treated with topical steroids with high precision.

The biomarkers included in our signature: TARC, IL-22 and sIL-2R, are involved in inflammation and have been previously described in the pathogenesis of AD. TARC, a member of the CC chemokine family, is produced by dendritic cells and involved in the recruitment of T cells into the skin.[27] IL-2R is expressed on the T cell surface after stimulation with IL-2.[28, 29] sIL-2R is released into the serum, and sIL-2R levels have been found to reflect the activation state of the T cells in skin.[30] In a recent meta-analysis we reported that sIL-2R levels showed good correlation to disease severity, however it should be noted that only small

patient cohorts have been used in the reviewed studies.[9] In the current study, using significantly larger patient cohorts, we confirmed that sIL-2R is a good biomarker for disease severity. IL-22 is less well known as a biomarker for disease severity in AD, although it has been recognized to play a role in the pathogenesis of AD.[31-34] IL-22 has previously been reported to induce keratinocyte proliferation, and downregulate filaggrin expression, in a similar way to IL-4 and IL-13, thereby contributing to epidermal barrier dysfunction and perhaps increase S. aureus infections associated with AD.[31] Interestingly, Noda and colleagues showed that IL-22 expression was higher in skin of Asian AD patients compared with skin of European American AD patients.[35] This may indicate that the Th-22 axis plays a more important role in Asians with AD. This strengthens our hypothesis that a combination of biomarkers covering multiple biological pathways, is more suitable for follow-up of disease severity than a single biomarker.

In contrast to our pilot study,[11] serum PARC levels and gender are not included in the current signature. Although PARC levels highly correlate with disease severity, PARC showed no added value over TARC in a panel of biomarkers for disease severity and therefore was not a component of our signature. This might be explained by the high correlation between serum PARC and serum TARC levels. The presence of gender in the previously published signature was required in the smaller patient population of the pilot study, but adjustment on gender was not required in the well-balanced, gender matched patient populations of the current studies.[11]

IL-31 has been associated with itch in AD.[36] Indeed, a recent phase II trial studying the effects of a monoclonal antibody targeting the IL-31 receptor A (IL-31RA) showed a strong decrease of itch.[37] IL-31 levels did not correlate to disease severity in our retrospective cohort, however, pruritis was not determined in this cohort. Since VAS pruritis was part of the clinical assesment in our prospective validation cohort we also included IL-31. Pruritis scores significanlty decreased in AD patients during treatment (Figure 2), however, serum IL-31 levels did not show a corresponding decrease (Figure 4). Raap et al. found that serum IL-31 levels correlated to disease severity,[38] however, several other studies failed to confirm these findings.[39-41] Kim et al. found that IL-31 mRNA expression is higher in biopsies from AD patients with high pruritus scores compared to patients with low prutitus scores,[42] suggesting that measurement of IL-31 in skin may be a more relevant measure for itch.

Our group has recently shown that AD patients can be divided into four subgroups with distinct serum biomarker profiles, conforming the biological heterogeneity of AD.[14] Although different combinations of biomarkers may be optimal for these subgroups, the aim of the current research was to develop a surrogate severity biomarker for all AD patients.

To avoid selection bias we included all analytes that were available in our MultiPlex Facility in the retrospective study cohort. Research in our laboratory focusses on chronic inflammatory diseases, which may have resulted in a bias to inflammation related biomarkers available for multiplex analysis. Advances in high-scale proteomics have led to the possibility of measuring over 1000 analytes in small amounts of

serum.[43] Including these techniques in future studies may identify new biomarkers for disease severity in AD.

To make a combination of biomarkers suitable for clinical use, simplicity and ease of interpretation is key. Levels of TARC, IL-22 and sIL-2R should not be interpreted separately, rather it is the combination of these biomarkers as a signature that reflects the disease activity. Combining levels of biomarkers in an algorithm coupled to the electronic patient's file provides a single, easy-to-interpret value. Since the algorithm was developed to predict EASI scores, we named this outcome measure the predicted-EASI (p-EASI). In contrast to EASI, the p-EASI provides an objective outcome measure that is not subjected to intra- and inter-rater variability. Currently, the HOME initiative recommends to use of a core outcome set to measure disease severity in AD clinical trials. This core outcome set comprises of four domains: clinician reported signs, patient reported symptoms, quality of life, and long term control. We believe that adding an objective serum biomarker combination, like the p-EASI, to this core outcome set will highly improve outcome measures in AD. With the increasing number of clinical trials evaluating new biological agents for the treatment of AD, the comparability of study outcomes is of major importance.[5]

A core strength of the current study is its design, including a retrospective cohort for the discovery of new biomarkers and a prospective cohort to validate our findings. Although correlation of serum biomarkers with disease severity has been investigated in many studies, most of these studies only included biomarker measurements on a single timepoint.[9] By including multiple timepoints in the present study, we were able to investigate the changes of biomarkers during treatment. The resulting combination of biomakers has high specificity and sensitivity, demonstrating a very good ability to predict EASI scores. A high specificity and sensitivity was also found during repeated analyses by randomly selecting ten patients for validation of the model, which demonstrates the robustness of our model based on a combination of biomarkers.

In the current study, biomarkers were detected and validated in cohorts of patients treated with topical steroids only, therefore it is of interest to see if the p-EASI biomarker signature is equally strong in predicting disease severity in patients treated with other drugs such as cyclosporin A, methotrexate or azathioprine. Another limitation of the current study is the usage of different clinician rated outcome measures in the retrospective and prospective cohorts, SASSAD and EASI respectively, however, both scores have been shown to be highly correlated.[44] Moreover, the model presented in the current study was based on EASI scores, as recommended by HOME for measuring disease severity in AD.[25]

In conclusion, we have invented a biomarker signature (p-EASI) consisting of TARC, IL-22 and sIL-2R which provides a reliable and objective measure for disease severity in AD patients. We believe that the use of a combination of serum biomarkers will highly improve precision measurement of outcomes and improve comparability of current and future treatments in AD.

SUPPLEMENTARY DATA

See http://www.dermatologyutrecht.nl/index.php/9-public/115-thijs

REFERENCES

1. Deckers IA, McLean S, Linssen S, Mommers M, van Schayck CP, Sheikh A. Investigating international time trends in the incidence and prevalence of atopic eczema 1990-2010: a systematic review of epidemiological studies. PLoS One 2012; 7:e39803.
2. Williams H, Stewart A, von Mutius E, Cookson W, Anderson HR, International Study of A, et al. Is eczema really on the increase worldwide? J Allergy Clin Immunol 2008; 121:947-54 e15.
3. Simpson EL, Bieber T, Eckert L, Wu R, Ardeleanu M, Graham NM, et al. Patient burden of moderate to severe atopic dermatitis (AD): Insights from a phase 2b clinical trial of dupilumab in adults. J Am Acad Dermatol 2016; 74:491-8.
4. Zuberbier T, Lotvall J, Simoens S, Subramanian SV, Church MK. Economic burden of inadequate management of allergic diseases in the European Union: a GA(2) LEN review. Allergy 2014; 69:1275-9.
5. Boyman O, Kaegi C, Akdis M, Bavbek S, Bossios A, Chatzipetrou A, et al. EAACI IG Biologicals task force paper on the use of biologic agents in allergic disorders. Allergy 2015; 70:727-54.
6. Charman CR, Venn AJ, Williams HC. Measurement of body surface area involvement in atopic eczema: an impossible task? Br J Dermatol 1999; 140:109-11.
7. Flohr C. Atopic dermatitis diagnostic criteria and outcome measures for clinical trials: still a mess. J Invest Dermatol 2011; 131:557-9.
8. Zhao CY, Tran AQ, Lazo-Dizon JP, Kim J, Daniel BS, Venugopal SS, et al. A pilot comparison study of four clinician-rated atopic dermatitis severity scales. Br J Dermatol 2015; 173:488-97.
9. Thijs J, Krastev T, Weidinger S, Buckens CF, de Bruin-Weller M, Bruijnzeel-Koomen C, et al. Biomarkers for atopic dermatitis: a systematic review and meta-analysis. Curr Opin Allergy Clin Immunol 2015; 15:453-60.
10. Landheer J, de Bruin-Weller M, Boonacker C, Hijnen D, Bruijnzeel-Koomen C, Rockmann H. Utility of serum thymus and activation-regulated chemokine as a biomarker for monitoring of atopic dermatitis severity. J Am Acad Dermatol 2014; 71:1160-6.
11. Thijs JL, Nierkens S, Herath A, Bruijnzeel-Koomen CA, Knol EF, Giovannone B, et al. A panel of biomarkers for disease severity in atopic dermatitis. Clin Exp Allergy 2015; 45:698-701.
12. Berth-Jones J. Six area, six sign atopic dermatitis (SASSAD) severity score: a simple system for monitoring disease activity in atopic dermatitis. Br J Dermatol 1996; 135 Suppl 48:25-30.
13. Hanifin JM, Rajka G. Diagnostic Features of Atopic-Dermatitis. Acta Derm Venereol 1980:44-7.

14. Thijs JL, Strickland I, Bruijnzeel-Koomen CAF, Nierkens S, Giovannone B, Csomor E, Sellman BR, Mustelin T, Sleeman MA, de Bruin-Weller MS, Herath A, Drylewicz J, May RD, Hijnen DJ. Moving towards endotypes in atopic dermatitis: identification of patient clusters based on serum biomarker analysis. Journal of Allergy and Clinical Immunology in press.
15. Hanifin JM, Thurston M, Omoto M, Cherill R, Tofte SJ, Graeber M. The eczema area and severity index (EASI): assessment of reliability in atopic dermatitis. EASI Evaluator Group. Exp Dermatol 2001; 10:11-8.
16. Charman CR, Venn AJ, Williams HC. The patient-oriented eczema measure: development and initial validation of a new tool for measuring atopic eczema severity from the patients' perspective. Arch Dermatol 2004; 140:1513-9.
17. Phan NQ, Blome C, Fritz F, Gerss J, Reich A, Ebata T, et al. Assessment of pruritus intensity: prospective study on validity and reliability of the visual analogue scale, numerical rating scale and verbal rating scale in 471 patients with chronic pruritus. Acta Derm Venereol 2012; 92:502-7.
18. Long CC, Finlay AY. The finger-tip unit--a new practical measure. Clin Exp Dermatol 1991; 16:444-7.
19. Fredriksson T, Pettersson U. Severe psoriasis--oral therapy with a new retinoid. Dermatologica 1978; 157:238-44.
20. Feldman SR, Fleischer AB, Jr., Reboussin DM, Rapp SR, Exum ML, Clark AR, et al. The self-administered psoriasis area and severity index is valid and reliable. J Invest Dermatol 1996; 106:183-6.
21. Leshem YA, Hajar T, Hanifin JM, Simpson EL. What the Eczema Area and Severity Index score tells us about the severity of atopic dermatitis: an interpretability study. Br J Dermatol 2015; 172:1353-7.
22. Hinkle DE WW, Jurs SG. Applied Statistics for the Behavioral Sciences. 5th ed. Boston: Houghton Mifflin; 2003.
23. Drake LA, Dinehart SM, Farmer ER, Goltz RW, Graham GF, Hordinsky MK, et al. Guidelines of care for the use of topical glucocorticosteroids. American Academy of Dermatology. J Am Acad Dermatol 1996; 35:615-9.
24. Schmitt J, Langan S, Williams HC, European Dermato-Epidemiology N. What are the best outcome measurements for atopic eczema? A systematic review. J Allergy Clin Immunol 2007; 120:1389-98.
25. Schmitt J, Spuls PI, Thomas KS, Simpson E, Furue M, Deckert S, et al. The Harmonising Outcome Measures for Eczema (HOME) statement to assess clinical signs of atopic eczema in trials. J Allergy Clin Immunol 2014; 134:800-7.
26. Chalmers JR, Simpson E, Apfelbacher CJ, Thomas KS, von Kobyletzki L, Schmitt J, et al. Report from the fourth international consensus meeting to harmonize core outcome measures for atopic eczema/dermatitis clinical trials (HOME initiative). Br J Dermatol 2016; 175:69-79.
27. Imai T, Nagira M, Takagi S, Kakizaki M, Nishimura M, Wang J, et al. Selective recruitment of CCR4-bearing Th2 cells toward antigen-presenting cells by the CC chemokines thymus and activation-regulated chemokine and macrophage-derived chemokine. Int Immunol 1999; 11:81-8.
28. Rubin LA, Nelson DL. The soluble interleukin-2 receptor: biology, function, and clinical application. Ann Intern Med 1990; 113:619-27.
29. Gaulton GN, Williamson P. Interleukin-2 and the interleukin-2 receptor complex. Chem Immunol 1994; 59:91-114.
30. Walker C, Kagi MK, Ingold P, Braun P, Blaser K, Bruijnzeel-Koomen CA, et al. Atopic dermatitis: correlation of peripheral blood T cell activation, eosinophilia and serum factors with clinical severity. Clin Exp Allergy 1993; 23:145-53.
31. Gutowska-Owsiak D, Schaupp AL, Salimi M, Taylor S, Ogg GS. Interleukin-22 downregulates filaggrin expression and

affects expression of profilaggrin processing enzymes. Br J Dermatol 2011; 165:492-8.

32. Hayashida S, Uchi H, Takeuchi S, Esaki H, Moroi Y, Furue M. Significant correlation of serum IL-22 levels with CCL17 levels in atopic dermatitis. J Dermatol Sci 2011; 61:78-9.

33. Nograles KE, Zaba LC, Shemer A, Fuentes-Duculan J, Cardinale I, Kikuchi T, et al. IL-22-producing "T22" T cells account for upregulated IL-22 in atopic dermatitis despite reduced IL-17-producing TH17 T cells. J Allergy Clin Immunol 2009; 123:1244-52 e2.

34. Hijnen D, Knol EF, Gent YY, Giovannone B, Beijn SJ, Kupper TS, et al. CD8(+) T cells in the lesional skin of atopic dermatitis and psoriasis patients are an important source of IFN-gamma, IL-13, IL-17, and IL-22. J Invest Dermatol 2013; 133:973-9.

35. Noda S, Suarez-Farinas M, Ungar B, Kim SJ, de Guzman Strong C, Xu H, et al. The Asian atopic dermatitis phenotype combines features of atopic dermatitis and psoriasis with increased TH17 polarization. J Allergy Clin Immunol 2015; 136:1254-64.

36. Mollanazar NK, Smith PK, Yosipovitch G. Mediators of Chronic Pruritus in Atopic Dermatitis: Getting the Itch Out? Clin Rev Allergy Immunol 2016; 51:263-92.

37. Nemoto O, Furue M, Nakagawa H, Shiramoto M, Hanada R, Matsuki S, et al. The first trial of CIM331, a humanized antihuman interleukin-31 receptor A antibody, in healthy volunteers and patients with atopic dermatitis to evaluate safety, tolerability and pharmacokinetics of a single dose in a randomized, double-blind, placebo-controlled study. Br J Dermatol 2016; 174:296-304.

38. Raap U, Wichmann K, Bruder M, Stander S, Wedi B, Kapp A, et al. Correlation of IL-31 serum levels with severity of atopic dermatitis. J Allergy Clin Immunol 2008; 122:421-3.

39. Kyoya M, Kawakami T, Soma Y. Serum thymus and activation-regulated chemokine (TARC) and interleukin-31 levels as biomarkers for monitoring in adult atopic dermatitis. J Dermatol Sci 2014; 75:204-7.

40. Cheon BR, Shin JE, Kim YJ, Shim JW, Kim DS, Jung HL, et al. Relationship between serum 25-hydroxyvitamin D and interleukin-31 levels, and the severity of atopic dermatitis in children. Korean J Pediatr 2015; 58:96-101.

41. Nygaard U, Hvid M, Johansen C, Buchner M, Folster-Holst R, Deleuran M, et al. TSLP, IL-31, IL-33 and sST2 are new biomarkers in endophenotypic profiling of adult and childhood atopic dermatitis. J Eur Acad Dermatol Venereol 2016; 30:1930-8.

42. Kim S, Kim HJ, Yang HS, Kim E, Huh IS, Yang JM. IL-31 Serum Protein and Tissue mRNA Levels in Patients with Atopic Dermatitis. Ann Dermatol 2011; 23:468-73.

43. Gold L, Walker JJ, Wilcox SK, Williams S. Advances in human proteomics at high scale with the SOMAscan proteomics platform. New Biotechnology 2012; 29:543-9.

44. Yang HJ, Jeon YH, Pyun BY. Evaluation of patient's subjective severity using various scoring system in Korean children with atopic dermatitis. Asian Pac J Allergy Immunol 2010; 28:130-5.


5

Levels of serum TARC, IL-22 and sIL-2R predict disease severity in atopic dermatitis patients treated with Cyclosporin A

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CAPSULE SUMMARY

A biomarker signature consisting of serum biomarkers TARC, IL-22 and sIL-2R, adequately predicts disease severity in AD patients treated with CsA. This formula will improve comparability of study outcomes in future clinical trials.

To the editor,

Atopic dermatitis (AD) is the most common chronic inflammatory skin disease and has a profound impact on quality of life. Several new studies with targeted therapies have shown promising results.[1] The introduction of these new drugs will hopefully fill a large unmet need in the treatment of AD. To compare these new drugs to existing therapies and each other, outcome measures that enable objective comparison of treatments are needed.

Serological biomarkers offer objective and reliable outcome measures. A recent meta-analysis identified serum thymus and activation-regulated chemokine (TARC/CCL17) as the best biomarker currently available for assessing disease severity in AD, showing correlation coefficient of around 0.60 with disease severity.[2] Although TARC strongly correlates with disease activity in individual patients during follow-up, TARC levels vary between patients within cross-sectional cohorts of patients that have similar disease severity scores.[3] The variation in TARC levels between patients may be the result of the large number of biologic pathways involved in the pathogenesis of AD. The use of a panel of several biomarkers from different biologic pathways may overcome this problem.

In a pilot study including 17 AD patient we showed that when TARC, PARC, sIL-2R and IL-22 are the correlation coefficient with disease severity raises up to 0.86.[4] In a validation study we confirmed our findings and showed that a prediction model for disease severity that includes the biomarkers TARC, sIL-2R and IL-22 is significantly better than a model with just a single biomarker.[5] Since our model was developed to predict Eczema Area and Severity Index (EASI) scores, we named this outcome measure the predicted-EASI (p-EASI). The predictive capacity of the p-EASI showed a sensitivity ranging from 83.3% to 100%, and a specificity ranging from 88.5%-95.2%.[5] Moreover, the p-EASI provides an objective outcome measure that is not subjected to intra- and inter-rater variability. We believe that the use of an objective outcome measure like the p-EASI will improve utility of results from clinical trials and will lead to more accurate information on the effectiveness of AD treatment. Since the p-EASI was based on data from a population of AD patients treated with only topical steroids, the aim of the current study was to validate the p-EASI in a population of AD patients treated with Cyclosporin A (CsA).

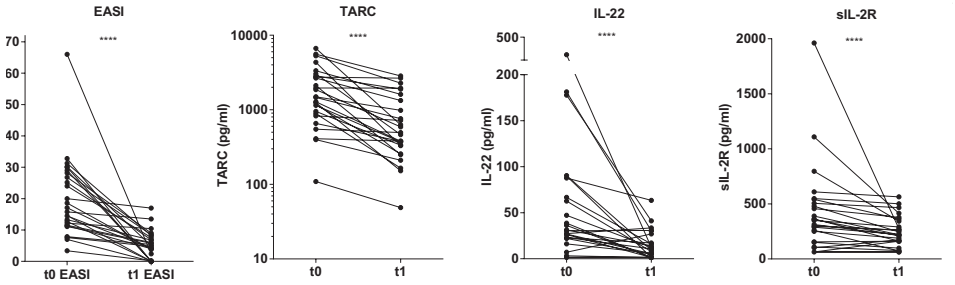
In a retrospective cohort study, 26 patients (median age 39, InterQuartileRange (IQR) 23-52, 20 male) with severe AD, treated with CsA (approximately 4-5mg/kg/day) were included. Disease severity was assessed by EASI, and serum was collected before start of CsA (t_0) and at the first control visit (t_1). Median treatment duration between t_0 and t_1 was 21 (IQR 21-35) days. Serum TARC, IL-22 and IL-2R levels were measured by Luminex using an in-house validated panel of analytes, as previously described.[5] Differences between t_0 and t_1 were tested by Wilcoxon signed-rank test. 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.

During treatment, median EASI scores showed a significant decrease from 16.5 (IQR 11.5-28.4) at t_0 to 4.9 (0.2-7.6) at t_1 ($p<0.0001$, Figure 1). Median serum TARC levels significantly decreased ($p<0.0001$) from 1480.0 pg/ml (IQR 872.3-2883.0) to 479.6 pg/ml (IQR 312.2-1404.0). Although serum IL-22 and sIL-2R levels did not correlate with EASI scores, median serum IL-22 and sIL-2R levels did significantly decrease during CsA treatment from 28.9 pg/ml (IQR 3.9-72.0) and 331.6 pg/ml (IQR 153.8-514.2), to 8.6 pg/ml (IQR 1.4-19.6) and 220.5 pg/ml (IQR 161.0-348.6), respectively ($p<0.0001$, Figure 1).

For the validation of the biomarker signature, EASI scores were binned into mild (≤7), moderate (>7 and ≤21), severe (>21 and ≤50), and very severe (>50).[6] Serum biomarker levels were used to predict EASI scores by the previously published formula[5]: (-36.12+18.49*logTARC+0.009*IL-22-0.009*sIL-2R)*(1-treatment)+(-5.82+4.04*logTARC+0.003*IL-22-0.003*sIL-2R)*treatment, in which treatment can be either No=0, or Yes=1 (Table 1). The p-EASI scores using the formula were in agreement with the clinician reported score in 73% of the cases. The formula showed a sensitivity of 82.6% and a specificity of 88.4% and is therefore considered a good predictor of disease severity.

Figure 1. Disease severity and serum biomarkers significantly decrease during treatment.



EASI scores significantly decreased during treatment with CsA. Serum levels of TARC, IL-22 and sIL-2R showed a similar significant decrease during CsA treatment. Differences in EASI score and serum biomarker levels between t_0 and t_1 were tested by Wilcoxon signed-rank test. ****$p<0.0001$.

This study shows that the biomarker signature p-EASI, consisting of serum TARC, IL-22, and IL-2R levels, accurately predicts EASI scores in AD patients treated with CsA. The predicted quality was similar as in patients treated with topical steroids.[5] This confirms our hypothesis that the biomarkers in our signature truly reflect disease severity in AD, regardless of the treatment that is used.

A recently published study by Ungar et al. also showed that combining multiple biomarkers measured in serum and in skin biopsies better correlates with disease severity than a single biomarker in AD patients treated with CsA.[7] In contrast to our study, the authors did not combine the biomarkers in a formula for the prediction of disease severity. The lack of a formula and the inclusion of biomarkers determined in skin biopsies, which is more invasive then drawing blood, in the latter study makes their approach less suitable for use in clinical practice and studies in children.

Two patients (patient 4 and 26 in table 1) in our study showed notable discrepancies between EASI and p-EASI scores. Patient 4 showed an EASI of 66 at t_0, while the p-EASI was 8.1. This patient had a severe secondary skin infection with S. aureus. However, the patient's eczema was described as relatively mild. We think that the impetiginisation resulted in overestimation of the EASI score at t_0. Patient 26 presented with severe AD that could not be controlled with topical steroids, resulting in start of CsA treatment. Although CsA was given at 5 mg/kg/day, EASI scores only decreased from 20 to 17 during treatment (Table 1). In contrast to the small difference in EASI scores, a significant improvement of AD and a reduction of itch was reported in the patient file. This patient also had prurigo nodules on his upper extremities, which retrospectively, contributed to the high EASI score at both time points. These two patients illustrate pitfalls of physician assessed severity measures such as EASI and the value of an objective outcome measure in AD.

In summary, this study demonstrates that a biomarker signature (p-EASI) consisting of a formula based on serum biomarkers TARC, IL-22 and sIL-2R, adequately predicts disease severity in AD patients treated with CsA. This formula will help to improve comparability of study outcomes in future clinical trials, but may also be helpful in monitoring treatment effects in daily practice.

Table 1. Prediction of EASI scores in AD patient treated with CsA.

Prediction of EASI scores										
patient	timepoint	TARC (pg/ml)	IL-22 (pg/ml)	sIL-2R (pg/ml)	EASI	Severity	predicted EASI	predicted severity	Difference in EASI	Predicted correctly
1	t_0	1282.52	16.13	358.04	11.5	moderate	18.12	moderate	6.62	yes
	t_1	379.16	6.19	210.11	6	mild	4.02	mild	-1.98	yes
2	t_0	886.51	90.33	349.98	28.2	severe	15.89	moderate	-12.31	no
	t_1	165.40	9.37	290.99	9	moderate	2.34	mild	-6.66	no
3	t_0	2745.36	177.85	796.98	15.8	moderate	21.53	severe	5.73	no
	t_1	2659.31	41.15	343.23	13.5	moderate	7.15	moderate	-6.35	yes
4	t_0	1493.94	461.75	1961.16	66	very severe	8.16	moderate	-57.84	no
	t_1	985.24	12.26	274.10	2.4	mild	5.53	mild	3.13	yes
5	t_0	4344.99	89.88	259.85	32.8	severe	29.51	severe	-3.29	yes
	t_1	330.73	1.40	72.72	0	mild	4.16	mild	4.16	yes
6	t_0	951.68	23.06	541.12	7.6	moderate	14.05	moderate	6.45	yes
	t_1	335.29	10.71	364.73	4.8	mild	3.38	mild	-1.42	yes
7	t_0	549.43	30.45	609.19	11.1	moderate	9.06	moderate	-2.04	yes
	t_1	495.15	30.78	564.40	5.8	mild	3.55	mild	-2.25	yes
8	t_0	2670.36	47.22	505.18	18.6	moderate	22.90	severe	4.30	no
	t_1	1335.81	12.39	466.16	3.8	mild	5.52	mild	1.72	yes
9	t_0	3033.73	26.87	101.47	26.8	severe	27.56	severe	0.76	yes
	t_1	464.04	5.50	173.50	8.5	moderate	4.47	mild	-4.03	no
10	t_0	1146.36	31.42	288.19	7.9	moderate	18.02	moderate	10.12	yes
	t_1	370.88	14.10	222.02	5.4	mild	3.97	mild	-1.43	yes
11	t_0	1465.48	1.46	313.20	12.75	moderate	19.48	moderate	6.73	yes
	t_1	759.94	1.40	216.08	1	mild	5.21	mild	5.21	yes
12	t_0	1866.99	27.37	296.57	17.1	moderate	21.82	severe	4.72	no
	t_1	589.73	33.57	262.71	4.2	mild	4.72	mild	0.52	yes
13	t_0	5578.97	22.13	457.26	25.2	severe	29.04	severe	3.84	yes
	t_1	2843.17	4.69	378.03	6.9	mild	7.07	mild	0.17	yes
14	t_0	1964.66	7.15	63.26	14.5	moderate	24.25	severe	9.75	no
	t_1	1954.33	27.03	160.95	4.1	mild	7.09	mild	2.99	yes
15	t_0	2116.12	36.30	363.39	30	severe	22.27	severe	-7.73	yes
	t_1	250.36	1.40	160.95	1	mild	3.42	mild	3.42	yes
16	t_0	1245.58	26.40	302.14	7	mild	18.51	moderate	11.51	no
	t_1	151.99	1.40	259.85	1	mild	2.26	mild	2.26	yes
17	t_0	6661.85	62.75	389.93	29	severe	31.47	severe	2.47	yes
	t_1	630.28	7.90	219.05	1	mild	4.89	mild	4.89	yes
18	t_0	1167.69	66.76	151.40	24	severe	19.77	moderate	-4.23	yes
	t_1	256.56	16.15	97.98	7.6	moderate	3.68	mild	-3.92	no
19	t_0	3339.48	38.72	478.82	13.3	moderate	24.87	severe	11.57	no
	t_1	1610.06	1.40	245.44	6.8	mild	6.44	mild	-0.36	yes
20	t_0	829.84	22.93	251.23	14.6	moderate	15.69	moderate	1.09	yes
	t_1	721.87	17.17	188.93	1	mild	5.24	mild	5.24	yes
21	t_0	2832.57	87.73	548.50	31.25	severe	23.32	severe	-7.93	yes
	t_1	1892.66	63.46	501.43	8.2	moderate	6.17	mild	-2.03	no
23	t_0	409.94	3.18	63.26	3.4	mild	11.63	moderate	8.23	no
	t_1	379.56	1.40	63.26	0.2	mild	4.42	mild	4.22	yes
24	t_0	5292.51	181.28	1109.22	30.15	severe	23.88	severe	-6.27	yes
	t_1	2283.25	30.37	414.79	4.95	mild	6.65	mild	1.70	yes
25	t_0	398.87	1.40	154.59	11.4	moderate	10.53	moderate	-0.87	yes
	t_1	211.05	1.40	160.95	4.05	mild	3.12	mild	-0.93	yes
26	t_0	657.35	1.40	108.38	20	moderate	14.98	moderate	-5.02	yes
	t_1	377.98	1.40	76.42	17	moderate	4.38	mild	-12.62	no

Serum TARC, IL-22 and sIL2R levels in serum of AD patients are used to predict EASI scores before treatment (t_0) and after treatment (t_1) by the formula EASI= (-36.12+18.49*logTARC+0.009*IL-22-0.009*sIL-2R)*(1-treatment)-(5.82+4.04*logTARC+0.003*IL-22-0.003*sIL2R)*treatment, in which treatment can be either No=0, or Yes=1. Both EASI and predicted EASI scores are classified as mild (1.0-7.0), moderate (7.1-21.0) or severe (>21.1). Sensitivity and specificity were determined for the model to describe the predictive capacity of the model. We defined false positive as a predicted EASI score classifying a higher disease severity than the observed EASI scores and false negative as a predicted EASI scores classifying a lower disease severity than the observed EASI scores.

REFERENCES

1. Simpson EL, Bieber T, Guttman-Yassky E, Beck LA, Blauvelt A, Cork MJ, et al. Two Phase 3 Trials of Dupilumab versus Placebo in Atopic Dermatitis. N Engl J Med 2016.
2. Thijs J, Krastev T, Weidinger S, Buckens CF, de Bruin-Weller M, Bruijnzeel-Koomen C, et al. Biomarkers for atopic dermatitis: a systematic review and meta-analysis. Curr Opin Allergy Clin Immunol 2015; 15:453-60.
3. Landheer J, de Bruin-Weller M, Boonacker C, Hijnen D, Bruijnzeel-Koomen C, Rockmann H. Utility of serum thymus and activation-regulated chemokine as a biomarker for monitoring of atopic dermatitis severity. J Am Acad Dermatol 2014; 71:1160-6.
4. Thijs JL, Nierkens S, Herath A, Bruijnzeel-Koomen CA, Knol EF, Giovannone B, et al. A panel of biomarkers for disease severity in atopic dermatitis. Clin Exp Allergy 2015; 45:698-701.
5. Thijs JL DJ, Fiechter R, Strickland I, Sleeman MA, Herath A, May RD, Bruijnzeel-Koomen CAFM, Knol EF, Giovannone B, de Bruin-Weller MS, Nierkens S, Hijnen DJ. EASI p-EASI: Utilising a combination of serum biomarkers offers an objective measurement tool for disease severity in atopic dermatitis patients. Accepted for publication in the Journal of Allergy and Clinical Immunology 2017.
6. Leshem YA, Hajar T, Hanifin JM, Simpson EL. What the Eczema Area and Severity Index score tells us about the severity of atopic dermatitis: an interpretability study. Br J Dermatol 2015; 172:1353-7.
7. Ungar B, Garcet S, Gonzalez J, Dhingra N, da Rosa JC, Shemer A, et al. An integrated model of atopic dermatitis biomarkers highlights the systemic nature of the disease. J Invest Dermatol 2016.


6

Immunoglobulin free light chains in adult atopic dermatitis patients

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ABSTRACT

Background:

Total IgE levels are often postulated as a biomarker for disease severity in atopic dermatitis (AD). But although total IgE levels are increased in the majority of AD patients, total IgE levels do not correlate to disease severity during short-term follow-up. During the synthesis of immunoglobulins, free light chains (Ig-FLCs) are produced in excess over heavy chains. In comparison with IgE molecules, Ig-FLCs have a very short serum half-life. Therefore, Ig-FLCs might be more suitable as a biomarker for disease severity during follow-up. Recent studies showed increased serum levels of kappa Ig-FLCs in infants with AD, correlating with disease severity. The aim of this study was to investigate serum kappa Ig-FLC levels in adults with AD, and their correlation to disease severity.

Methods:

Serum kappa If-FLC and total IgE levels were measured in 82 moderate to severe AD patients and 49 non-atopic controls. Blood was collected from patients before start of treatment with potent topical steroids (European classification: III-IV). 32 patients were treated during a clinical admission, and in this subpopulation a second blood sample was taken after two weeks of treatment. Clinical severity was determined by SASSAD and a panel of serum biomarkers, including TARC.

Results:

Serum kappa Ig-FLCs levels in adult AD patients were not increased compared to non-atopic controls. Moreover, we observed no correlation between kappa Ig-FLC serum levels and disease severity determined by SASSAD and a panel of serum biomarkers, including TARC. Serum kappa Ig-FLC levels did also not decrease during treatment.

Conclusion:

There are no differences in serum kappa Ig-FLC levels between adult patients suffering from moderate to severe AD compared to non-atopic controls. Moreover, serum levels of kappa Ig-FLCs cannot be used as a biomarker for disease severity in adult AD.

INTRODUCTION

Atopic dermatitis (AD) is the most common chronic inflammatory skin disease worldwide.[1] The pathogenesis of AD is multifactorial and involves genetic, immunologic and environmental factors.[2] The role of total IgE in the pathogenesis of AD is controversial. Although the majority of AD patients have highly increased total IgE levels, these levels do not correlate with disease severity.[3] During the synthesis of immunoglobulins, light chains are produced in excess over heavy chains.[4,5] Whereas the serum half-life of IgE molecules is two days, the serum half-life of immunoglobulin free light chains (Ig-FLCs) is only two to three hours.[6] Considering the relapsing and remitting course of AD, this might make Ig-FLCs levels more suitable as a biomarker for disease severity than total IgE. Ig-FLCs have long been considered meaningless spillover from production of immunoglobulins. However, recent data suggest that Ig-FLCs might convey various biological activities.[4,5] Interestingly, increased levels of kappa Ig-FLCs were found in the serum of infants with severe AD compared to infants without AD.[5,6] Moreover, in a cohort of children with severe AD, levels of Ig-FLCs correlated with disease activity.[5]

These reports prompted us to investigate the role of serum kappa Ig-FLCs in adult AD. In this study, serum levels of kappa Ig-FLCs did not differ significantly between adult AD patients and non-atopic controls. In addition, both kappa Ig-FLC and total IgE levels did not correlate to disease severity.

METHODS

Patients and controls

In a retrospective cohort study, 82 patients (50 female;16-65 years) with moderate to severe AD visiting the UMC Utrecht were included. Patients were diagnosed according to the criteria of Hanifin and Rajka.[7] Disease severity was assessed using the Six Area Six Sign Atopic Dermatitis (SASSAD) score (median 21, IQR: 11-32), and Body Surface Area (BSA)(median 33%, IQR: 17-53). After blood was taken, all patients were treated with potent topical steroids (European classification: III-IV), 32/82 were treated during a clinical admission. Patients using oral immunosuppressive medications were excluded. A total of 49 age- and sex-matched non-atopic controls (25 female; age 22-66 years) that did not suffer from any skin disease were included. From the 32 patients that were admitted to the clinic, a second blood sample was taken after a median interval of 11.5 days (IQR: 9.0-13.8). Protocols of this study were approved by the Institutional Review Board of the UMC Utrecht, adhering to the Declaration of Helsinki Principles.

Serum kappa Ig-FLC & total IgE

A fully automated customized kappa Ig-FLC research assay based on ELISA technology was developed (Phadia Thermo Fisher, Uppsala, Sweden) for the Phadia 250® instrument. Kappa Ig-FLC values ≥19.4 µg/ml were considered elevated.[8] A fully automated allergy-testing system (Phadia Thermo Fisher) was used for measurements of total IgE.[9]

Serum biomarkers for disease severity

In addition to clinical severity determined by SASSAD and BSA, disease severity of the 32 admitted patients was assessed by a recently described panel of serum biomarkers.[10] Therefore, serum levels of thymus and activation-regulated chemokine (TARC/CCL17), pulmonary and activation-regulated chemokine (PARC/CCL18), sIL-2R and IL-22 were measured using Multiplex immunoassays at the MultiPlex Core Facility of the Laboratory for Translational Immunology (UMC Utrecht, The Netherlands) as described previously.[11]

Statistical analysis

SASSAD, BSA, and serum biomarker levels were normalized by log-transformation. Statistical comparisons were performed using Pearson correlations, Wilcoxon matched-pairs signed rank tests, and unpaired two tailed t-tests. Prism (version 6; GraphPad) was used for statistical analysis.

RESULTS

Kappa Ig-FLC

Kappa Ig-FLCs levels in AD patients (n=82) did not significantly differ from kappa Ig-FLCs levels in non-atopic controls (n=49) (median: 23.63 µg/ml, IQR: 16.45-30.43, versus 15.66 µg/ml, IQR: 10.95-21.38)(Fig. 1A). Kappa Ig-FLC concentrations slightly decreased to 16.20 µg/ml (median, IQR: 10.00-24.00) after treatment in the 32 admitted patients, although this was not statistically significant (Wilcoxon matched-pair signed rank test)(Fig. 1B). Kappa Ig-FLC levels measured before treatment did not correlate to disease severity measured by SASSAD (r=0.12, p=0.30) and BSA (r=-0.05, p=0.65). Kappa Ig-FLC levels did also not correlate to serum TARC (r=0.19, p=0.30) or any other serum biomarker (data not shown).

Total IgE levels

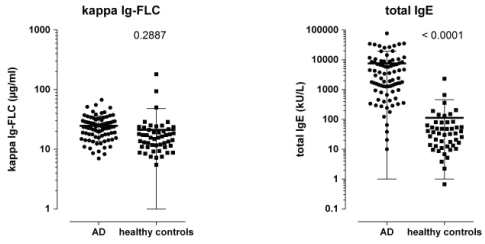
Total IgE levels were significantly higher in AD patients (median: 2702.00 kU/L, IQR: 921.3-8579) than in non-atopic controls (median: 34.05 kU/L, IQR: 12.90-75.05)(Fig. 1A). Total IgE levels did not change after treatment (Fig. 1B). Total IgE levels did not correlate to kappa Ig-FLC levels (r=0.15, p=0.18) (data not shown).

Disease severity

All 32 patients that were treated during a clinical admission, showed significant improvement. SASSAD decreased from 33.0 (median, IQR: 28-44) to 9.0 (median, IQR: 5-16); BSA decreased from 54% (median, IQR: 36-69) to 15.0% (median, IQR: 3.8-23.3) (Fig. 1C). Serum TARC, PARC, sIL-2R and IL-22 levels significantly decreased in all 32 patients (Fig. 1C).

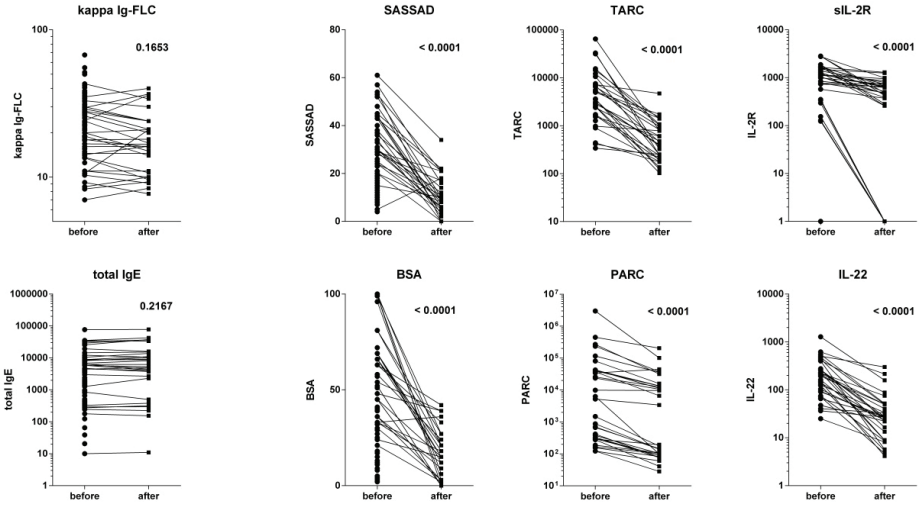
Figure 1. Serum kappa Ig-FLC and total IgE levels in AD patients and non-atopic controls.

A. Kappa Ig-FLC and total IgE levels in 82 AD patients and 49 non-atopic controls



B. Kappa Ig-FLC and total IgE levels in 32 AD patients before and after treatment

C. Disease severity and biomarkers in 32 AD patients before and after treatment



A: A Students' t-test showed no significant differences between the levels of kappa Ig-FLCs in AD patients (n=82; (median: 23.63 µg/ml, IQR: 16.45-30.43) compared to non-atopic controls (n=49; 15.66 µg/ml, IQR: 10.95-21.38). Total IgE levels in AD patients (median: 2702.00 kU/L, IQR: 921.3-8579) were significantly higher compared to non-atopic controls (median: 34.05 kU/L, IQR: 12.90-75.05), according to a students' t-test (p= 0,0001). B-C: Kappa Ig-FLC concentrations showed a small non-significant decrease from 23.63 µg/ml to 16.20 µg/ml (median) after treatment (p=0.17). No significant changes were observed between total IgE levels before and after treatment (p=0.22) (B). Disease severity measured by SASSAD and BSA, significantly decreased during a clinical admission and treatment with topical steroids (n=32). Levels of serum biomarkers TARC, PARC, sIL-2R, and IL-22 also significantly decreased. (C). Data were analyzed using a Wilcoxon matched-pairs signed rank test.

DISCUSSION

This study shows that there are no differences between kappa Ig-FLC levels in adult AD patients and non-atopic controls. In addition, we found no correlation between kappa Ig-FLCs levels and disease severity, BSA or serum biomarker levels.

Previous studies have suggested a role for Ig-FLCs in the pathophysiology of allergic diseases. Serum levels of Ig-FLCs were found to be upregulated in allergic and non-allergic rhinitis [12, 13], and an Ig-FLC antagonist was found to abrogate airway obstruction, hyper responsiveness, and pulmonary inflammation in murine model of asthma.[14] Serum kappa Ig-FLCs levels were shown to be significantly increased in children with AD compared to normal controls.[5, 6] Moreover, a correlation of kappa Ig-FLCs to disease severity was shown in children with severe AD.[5] In contrast to our a priori hypothesis, these findings were not reproducible in adult AD patients. Although kappa Ig-FLCs may play a role in AD in children, in the current research no evidence for Ig-FLC involvement in adult AD was found.

Remarkably, two healthy controls showed high serum kappa Ig-FLC levels (94.0 and 180.9 µg/ml, respectively). Although these high levels may be the result of the presence of another, non-atopic disease, these subjects were apparently healthy and reported no medical conditions. Elevated serum Ig-FLC levels have been shown in multiple myeloma,[15] systemic lupus erythematosus,[16] and rheumatoid arthritis patients,[17] and were also reported shortly after marathon running.[18]

Total IgE levels were analyzed in addition to serum kappa Ig-FLC. Total IgE did not decrease during treatment and is therefore not suitable as a biomarker for monitoring disease severity. Contrary to IgE, serum TARC, PARC, sIL-2R and IL-22 levels significantly decreased during treatment (Fig. 1C). This confirms previous reports, showing that these biomarkers reflect disease severity in AD patients.[10] Considering the heterogeneous character of AD, with multiple immunologic pathways playing a role, we have previously suggested to use a panel of biomarkers, including the above mentioned.[10] This panel may be able to cover multiple immunologic pathways, and may be more suitable for assessing disease severity in AD compared to a single biomarker.

In conclusion, this study shows that there are no differences in serum kappa Ig-FLC levels between adult patients suffering from moderate to severe AD compared to non-atopic controls. Moreover, serum kappa Ig-FLC levels do not correlate with disease severity determined by clinical outcome measures or serum biomarkers. Additionally, serum kappa Ig-FLC levels do not decrease during effective treatment of AD.

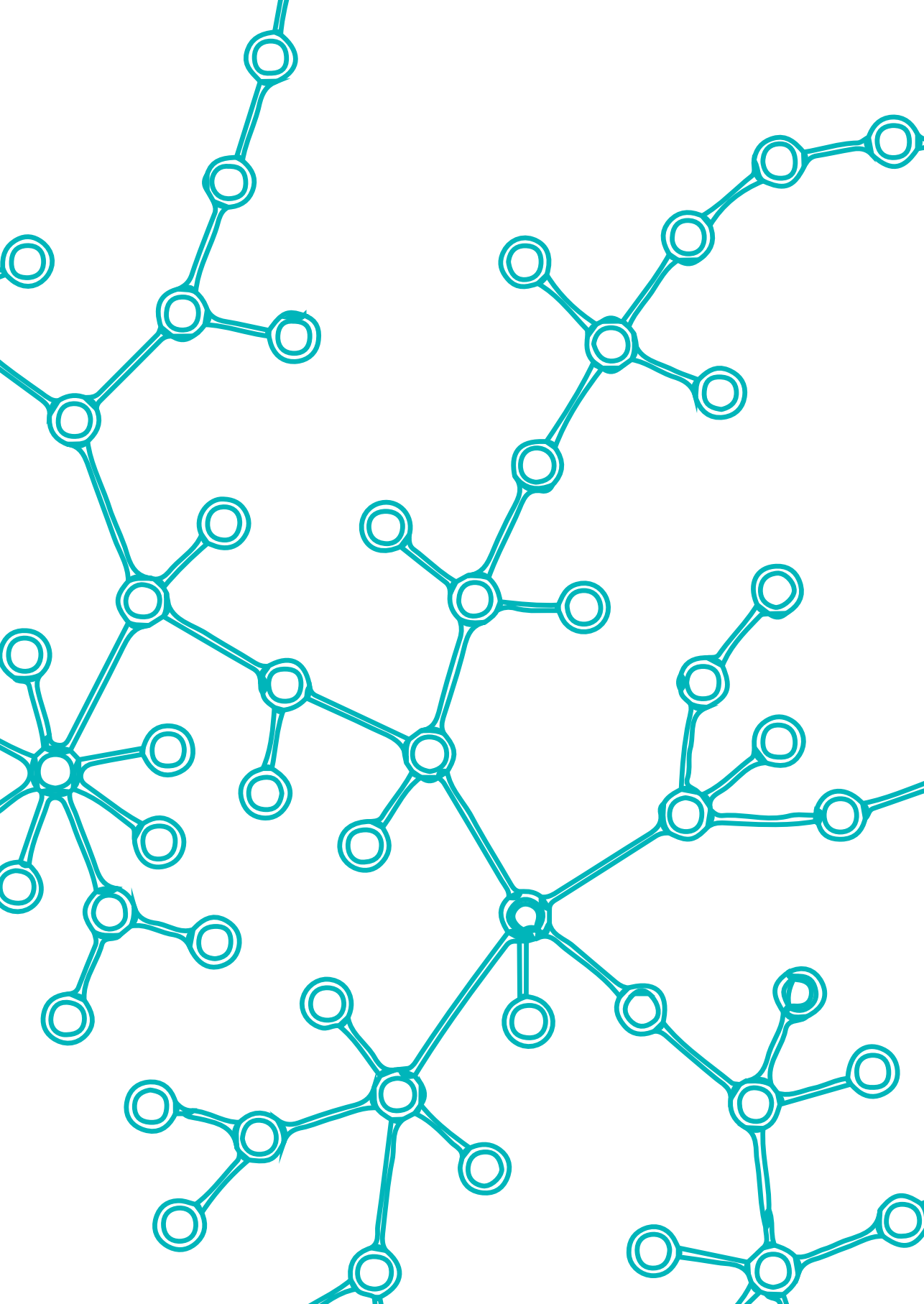
REFERENCES

1. Garmhausen D, Hagemann T, Bieber T, Dimitriou I, Fimmers R, Diepgen T, et al. Characterization of different courses of atopic dermatitis in adolescent and adult patients. Allergy 2013; 68:498-506.
2. Eyerich K, Novak N. Immunology of atopic eczema: overcoming the Th1/Th2 paradigm. Allergy 2013; 68:974-82.
3. Thijs J, Krastev T, Weidinger S, Buckens CF, de Bruin-Weller M, Bruijnzeel-Koomen C, et al. Biomarkers for atopic dermatitis: a systematic review and meta-analysis. Curr Opin Allergy Clin Immunol 2015; 15:453-60.
4. Thio M, Blokhuis BR, Nijkamp FP, Redegeld FA. Free immunoglobulin light chains: a novel target in the therapy of inflammatory diseases. Trends Pharmacol Sci 2008; 29:170-4.
5. Kayserova J, Capkova S, Skalicka A, Vernerova E, Polouckova A, Malinova V, et al. Serum immunoglobulin free light chains in severe forms of atopic dermatitis. Scand J Immunol 2010; 71:312-6.
6. Schouten B, van Esch BC, van Thuijl AO, Blokhuis BR, Groot Kormelink T, Hofman GA, et al. Contribution of IgE and immunoglobulin free light chain in the allergic reaction to cow's milk proteins. J Allergy Clin Immunol 2010; 125:1308-14.
7. Hanifin JM, Rajka G. Diagnostic Features of Atopic-Dermatitis. Acta Derm Venereol 1980:44-7.
8. Katzmann JA, Clark RJ, Abraham RS, Bryant S, Lymp JF, Bradwell AR, et al. Serum reference intervals and diagnostic ranges for free kappa and free lambda immunoglobulin light chains: relative sensitivity for detection of monoclonal light chains. Clin Chem 2002; 48:1437-44.
9. Bousquet J, Chanez P, Chanal I, Michel FB. Comparison between Rast and Pharmacia Cap System - a New Automated Specific Ige Assay. Journal of Allergy and Clinical Immunology 1990; 85:1039-43.
10. Thijs JL, Nierkens S, Herath A, Bruijnzeel-Koomen CA, Knol EF, Giovannone B, et al. A panel of biomarkers for disease severity in atopic dermatitis. Clin Exp Allergy 2015; 45:698-701.
11. de Jager W, te Velthuis H, Prakken BJ, Kuis W, Rijkers GT. Simultaneous detection of 15 human cytokines in a single sample of stimulated peripheral blood mononuclear cells. Clin Diagn Lab Immunol 2003; 10:133-9.
12. Powe DG, Groot Kormelink T, Sisson M, Blokhuis BJ, Kramer MF, Jones NS, et al. Evidence for the involvement of free light chain immunoglobulins in allergic and nonallergic rhinitis. J Allergy Clin Immunol 2010; 125:139-45 e1-3.
13. Meng C, Sha J, Li L, An L, Zhu X, Meng X, et al. The expression and significance of immunoglobulin free light chain in the patients with allergic rhinitis and nonallergic rhinitis. Am J Rhinol Allergy 2014; 28:302-7.
14. Kraneveld AD, Kool M, van Houwelingen AH, Roholl P, Solomon A, Postma DS, et al. Elicitation of allergic asthma by immunoglobulin free light chains. Proc Natl Acad Sci U S A 2005; 102:1578-83.
15. Drayson M, Tang LX, Drew R, Mead GP, Carr-Smith H, Bradwell AR. Serum free light-chain measurements for identifying and monitoring patients with nonsecretory multiple myeloma. Blood 2001; 97:2900-2.
16. Draborg AH, Lydolph MC, Westergaard M, Olesen Larsen S, Nielsen CT, Duus K, et al. Elevated Concentrations of Serum Immunoglobulin Free Light Chains in Systemic Lupus Erythematosus Patients in Relation to Disease Activity, Inflammatory

Status, B Cell Activity and Epstein-Barr Virus Antibodies. PLoS One 2015; 10:e0138753.

17. Deng X, Crowson CS, Rajkumar SV, Dispenzieri A, Larson DR, Therneau TM, et al. Elevation of serum immunoglobulin free light chains during the preclinical period of rheumatoid arthritis. J Rheumatol 2015; 42:181-7.

18. Campbell JP, Eijsvogels TM, Wang Y, Hopman MT, Jacobs JF. Assessment of serum free light chain levels in healthy adults immediately after marathon running. Clin Chem Lab Med 2015.



PART II

BIOMARKERS ENABLING PRECISION MEDICINE IN ATOPIC DERMATITIS

Serum biomarker profiles suggest that atopic dermatitis is a systemic disease

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Submitted

CAPSULE SUMMARY

By using a purely data-driven analysis we have shown that biomarker expression profiles of AD patients are clearly different from healthy controls, confirming the presence of systemic inflammation in AD patients and supporting the hypothesis that AD is a systemic disorder.

To the editor,

Atopic dermatitis (AD) is one of the most common chronic inflammatory skin diseases and is associated with other atopic diseases such as asthma, allergic rhinitis and food allergy. Recent studies have also shown associations between AD and alopecia areata, neuropsychiatric and cardiovascular diseases (CVD).[1] This suggests that systemic inflammation in AD may contribute to the development of these comorbidities over time.[1] The aim of the current study was to characterize systemic inflammation in AD patients compared to healthy controls by studying expression levels of inflammatory biomarkers in serum.

In a population of 193 moderate to severe AD patients (median SASSAD 31.0, IQR 23.0-37.5; median age 30.5 years IQR 21.0-42.0) and 30 healthy controls (mean age 39.1 years, IQR 34.3-44.6) we measured serum concentrations of 144 analytes (see Tables S1-S4 in the Online Repository) via multiplex immunoassay, and serum total IgE, periostin, and DPP4, via ELISA-based assays as described in this article's methods section in the Online Repository. Hierarchical cluster analysis followed by principal component analysis (PCA) was performed to visualize the biomarker expression profiles from AD patients and healthy controls. As shown in the heat map (Figure 1A), healthy controls cluster in the dendogram on the y-axis and can clearly be distinguished from AD patients based on their serum biomarker expression profile. In addition, when we applied PCA, the AD patients and healthy controls were separated into distinct groups based on combinations of the first three principal components (Figure 1B and Movie S5).

The component loadings showed that principal component 1 is primarily driven by IL-5, IL-1β, IL-7, IL-1R1, and IL-15, principal component 2 by IFN-β, IL-20, IL-1Rα, TNF-β, and MCP. Serum biomarker levels in AD patients were compared with levels in healthy controls using an unpaired t-test (Tables S1-S4). Serum levels of the biomarkers driving the principal components were significantly different between AD patients and controls (Figure 1C).

We found highly increased levels of inflammatory biomarkers in the serum of AD patients compared to healthy controls, suggesting systemic inflammation. This contributes to the hypothesis that in a chronic inflammatory skin disease, long term exposure of distant organs to systemic inflammation could have detrimental effects.

Associations between AD, cardiovascular risk factors and CVD have been reported in several population-based studies from North America and Asia.[1] However, recent publications from Europe found no association, or only an association between severe AD and CVD.[2, 34] This suggests that the association between AD and CVD might only be present in the more severe patients. In contrast to most of the epidemiological studies that included mild to severe patients, we only included patients with moderate to severe disease in the current study. One could also hypothesize that only prolonged exposure to systemic inflammation of distant organs leads to pathology. In the current study disease severity was defined by EASI on a single time

point, and long term disease severity was not taken into account. To study effects of (long term) systemic inflammation on distant organs we would prefer to use multiple measurements of disease severity over a longer period of time rather than a single measurement.

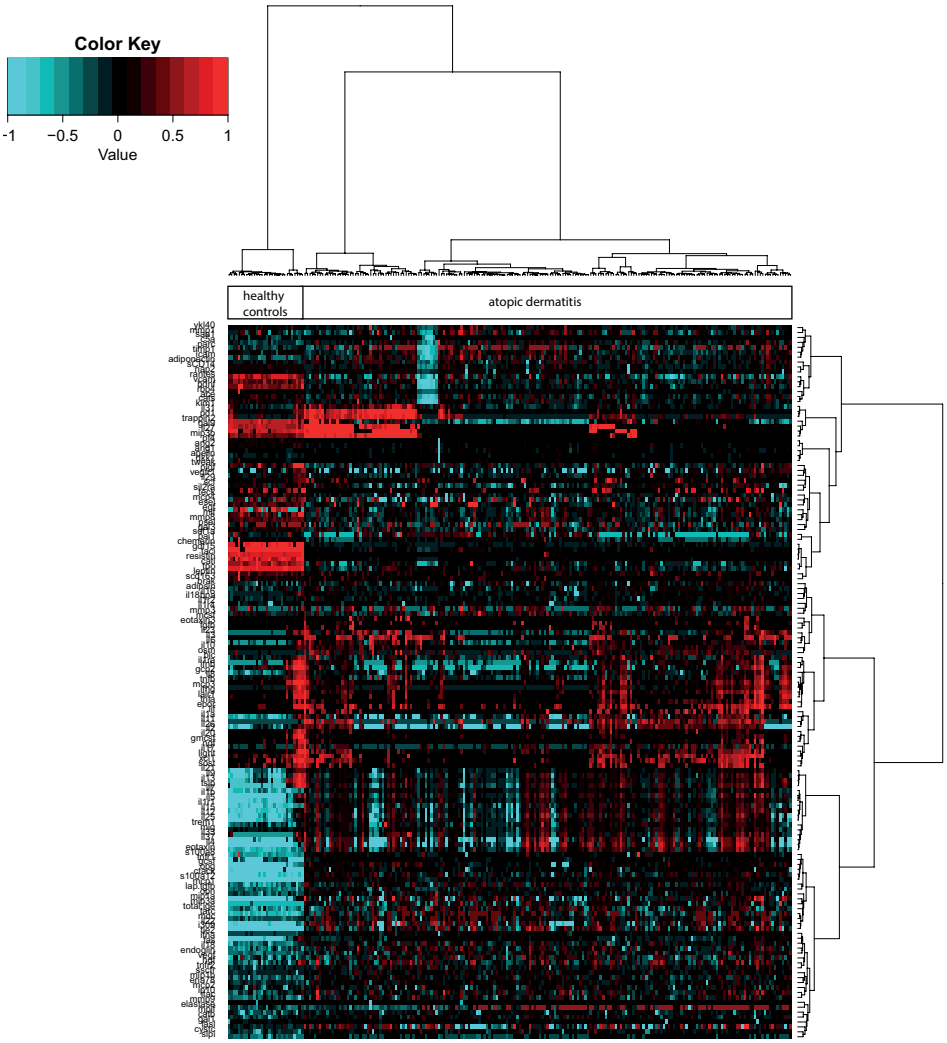
The association between adult AD and CVD, would be consistent with a recent study that showed the occurrence of severe CVD and metabolic abnormalities in a mouse model of severe dermatitis resulting from persistent release of IL-1 family cytokines from the skin.[5] Interestingly, both skin and systemic pathologies were ameliorated by treatment with anti–IL-1α- and anti–IL-1β neutralizing antibodies. The present study is the first study to show that serum IL-1Rα, IL-1β and IL-1R1 levels in AD patients are significantly increased in AD patients compared to healthy controls (Figure 1C and see Tables S1-S4). Taken together, these data support the concept that circulating IL-1s derived from inflammatory skin lesions, could affect distant organs, including the heart and cause cardiovascular comorbidities.[5, 6]

Interestingly, IL-5, one of the key drivers of type 2 eosinophilic inflammation was found to be one of the key drivers in differentiating AD patients from healthy controls. Previously, treatment with a humanized monoclonal anti-IL-5 antibody (mepolizumab) failed to demonstrate clinical efficacy in early phase AD trials.[7] It was hypothesized that in these studies, treatment with anti-IL-5 may have been too short to reduce eosinophils in the skin and have clinical efficacy. In asthma, mepolizumab has been shown effective in a subset of patients with severe, steroid refractory, eosinophilic asthma. Further studies will be required to understand the interrelationship between skin and lung inflammation.

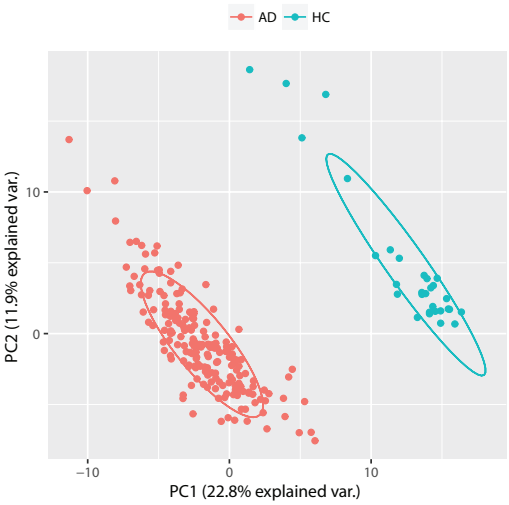
This study has, for the first time, demonstrated elevated serum IL-7 levels in AD patients, as compared to controls. IL-7 is known to be produced by a number of cell types including keratinocytes and has been shown to be essential for T cell development, survival and proliferation of memory and naive T cells and Th17 cells.[8, 9] Transgenic mice constitutively expressing IL-7 develop severe dermatitis, and massive infiltration of T cells in the dermis.[10] In addition, a polymorphism in the IL-7R gene leading to enhanced IL-7 bioactivity, was genetically associated to AD susceptibility.[11] Critically, the same IL-7R polymorphism is associated with higher risk of type I diabetes, rheumatoid arthritis, sarcoidosis, multiple sclerosis, and asthma. These findings make the IL-7 axis interesting as a therapeutic target for AD, but also highlight that skin derived IL-7 could be involved in distant organ pathologies.

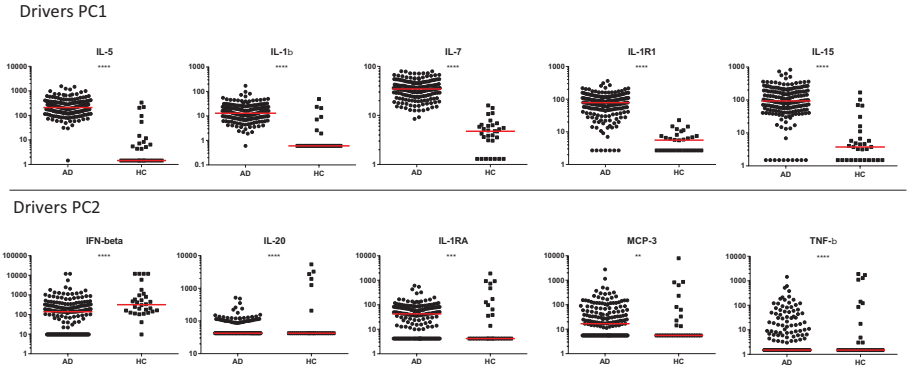
In conclusion, we have shown that biomarker expression profiles in moderate to severe AD patients are clearly different from healthy controls, which confirms the presence of systemic inflammation in AD patients and supports the hypothesis that AD is a systemic disorder. More mechanistic studies are needed to prove that systemic inflammation indeed contributes to comorbidities like asthma and cardiovascular diseases in AD patients.

Figure 1. Unsupervised clustering of serum biomarker expression and principal component analysis of moderate and severe AD patients versus healthy controls.



A. Hierarchical cluster analysis. Mean serum biomarker levels are displayed across the y-axis. Healthy controls (n=30) and AD patients (n=193) are displayed across the x-axis. The heat-map reveals clear differences between biomarker expression profiles from healthy controls and AD patients.





B. Principal component analysis of moderate and severe AD patients versus healthy controls. Plotting of the first two principal components reveals clear separation between healthy controls and AD patients. AD patients are represented by red dots, healthy controls are shown as turqoise dots. The first two principal components explain 34.7% of the variation in the dataset.

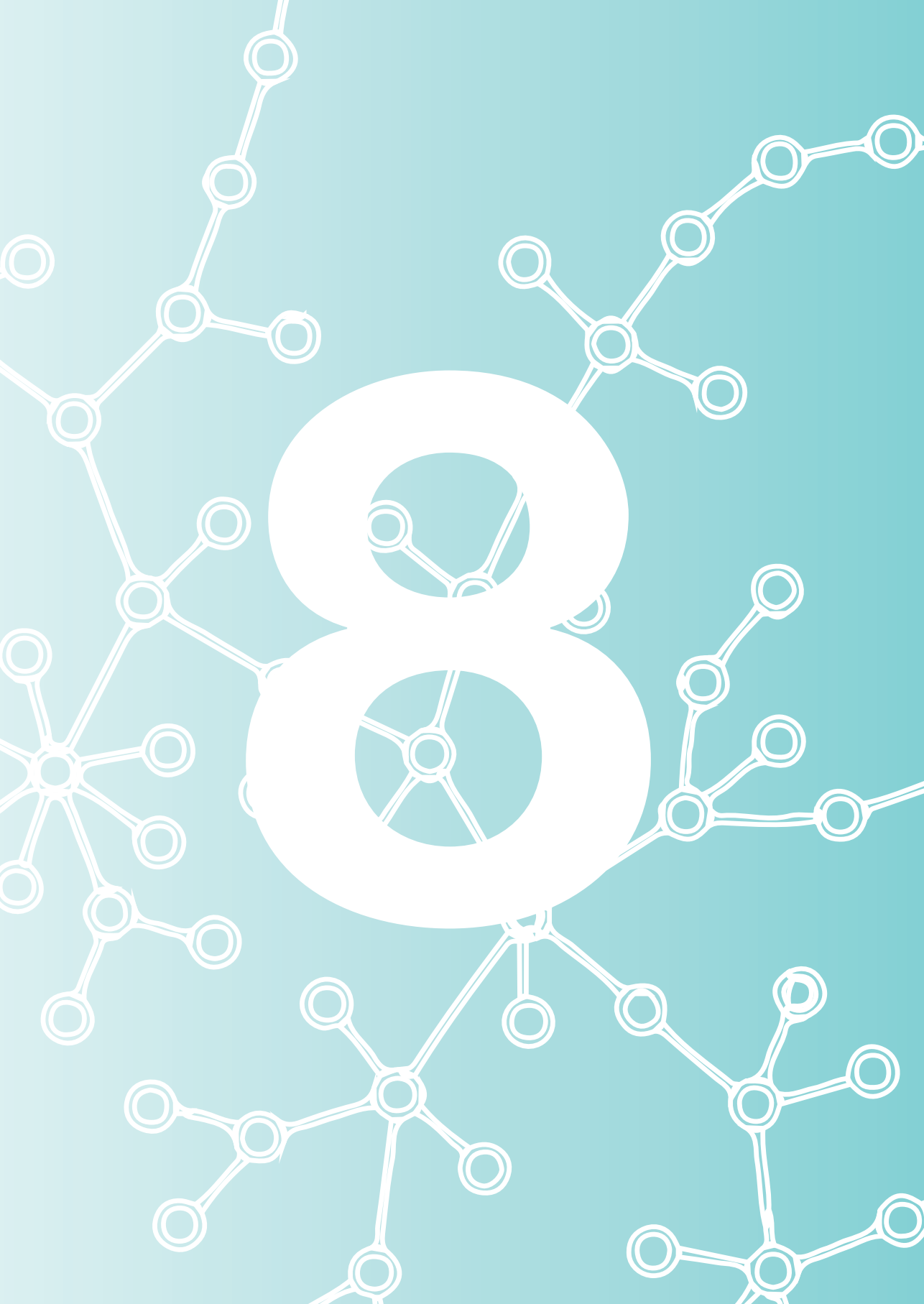
C. AD patients can be distinguished from healthy controls based on the serum biomarkers that drive each principal component. Differences in serum biomarker levels were tested using an unpaired t-test (*P<0.05;**P<0.01; ***P<0.001; ****P<0.0001). All driving biomarkers are significantly different between AD patients and healthy controls. Serum biomarker levels are shown in pg/ml on the Y-axes, red lines represent the median serum levels. Serum samples with analyte levels that were out of range were replaced by upper or lower limits of quantification.

SUPPLEMENTARY DATA

See http://www.dermatologyutrecht.nl/index.php/9-public/115-thijs

REFERENCES

1. Brunner PM, Silverberg JI, Guttman-Yassky E, Paller AS, Kabashima K, Amagai M, et al. Increasing Comorbidities Suggest that Atopic Dermatitis Is a Systemic Disorder. Journal of Investigative Dermatology 2017; 137:18-25.
2. Andersen YM, Egeberg A, Gislason GH, Hansen PR, Skov L, Thyssen JP. Risk of myocardial infarction, ischemic stroke, and cardiovascular death in patients with atopic dermatitis. J Allergy Clin Immunol 2016; 138:310-2 e3.
3. Standl M, Tesch F, Baurecht H, Rodriguez E, Muller-Nurasyid M, Gieger C, et al. Association of Atopic Dermatitis with Cardiovascular Risk Factors and Diseases. J Invest Dermatol 2017; 137:1074-81.
4. Thyssen JP, Skov L, Hamann CR, Gislason GH, Egeberg A. Assessment of major comorbidities in adults with atopic dermatitis using the Charlson comorbidity index. J Am Acad Dermatol 2017.
5. Yamanaka K, Nakanishi T, Saito H, Maruyama J, Isoda K, Yokochi A, et al. Persistent release of IL-1s from skin is associated with systemic cardio-vascular disease, emaciation and systemic amyloidosis: the potential of anti-IL-1 therapy for systemic inflammatory diseases. PLoS One 2014; 9:e104479.
6. Boehncke WH, Boehncke S, Tobin AM, Kirby B. The 'psoriatic march': a concept of how severe psoriasis may drive cardiovascular comorbidity. Exp Dermatol 2011; 20:303-7.
7. Oldhoff JM, Darsow U, Werfel T, Katzer K, Wulf A, Laifaoui J, et al. Anti-IL-5 recombinant humanized monoclonal antibody (mepolizumab) for the treatment of atopic dermatitis. Allergy 2005; 60:693-6.
8. Heufler C, Topar G, Grasseger A, Stanzl U, Koch F, Romani N, et al. Interleukin-7 Is Produced by Murine and Human Keratinocytes. Journal of Experimental Medicine 1993; 178:1109-14.
9. Mazzucchelli RI, Riva A, Durum SK. The human IL-7 receptor gene: deletions, polymorphisms and mutations. Semin Immunol 2012; 24:225-30.
10. Uehira M, Matsuda H, Hikita I, Sakata T, Fujiwara H, Nishimoto H. The Development of Dermatitis Infiltrated by Gam-



Moving towards endotypes in atopic dermatitis: identification of patient clusters based on serum biomarker analysis

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ABSTRACT

Background:

Atopic dermatitis (AD) is a complex, chronic, inflammatory skin disease with a diverse clinical presentation. It is however unclear whether this diversity exists at a biological level.

Objective:

To test the hypothesis that AD is heterogeneous at the biological level of individual inflammatory mediators.

Methods:

Serum from 193 moderate to severe adult AD patients (geomean (95%CI) SASSAD of 22.3 (21.3, 23.3) and 39.1 (37.5, 40.9) respectively) and 30 non-AD healthy controls was analysed for 147 serum mediators, total IgE and 130 allergen specific IgEs. Population heterogeneity was assessed by principal component analysis (PCA) followed by unsupervised k-means cluster analysis of the principal components.

Results:

AD patients showed pronounced evidence of inflammation compared to healthy controls. PCA of AD serum data revealed the presence of four potential AD patient clusters. Fifty-seven principal components (PCs) described approximately 90% of the variance. Unsupervised k-means cluster analysis of the 57 largest PCs delivered 4 distinct clusters of AD patients. Cluster 1 had high SASSAD and BSA with the highest levels of PARC, TIMP-1 and sCD14. Cluster 2 had low SASSAD with the lowest levels of IFN-α, TIMP-1 and VEGF. Cluster 3 had high SASSAD with the lowest levels of IFN-β, IL-1 and epithelial cytokines. Cluster 4 had low SASSAD but highest levels of inflammatory markers: IL-1, IL-4, IL-13 and TSLP.

Conclusion:

AD is a heterogeneous disease both clinically and biologically. Four distinct AD patient clusters have been identified that could represent endotypes with unique biological mechanisms. Elucidation of these endotypes warrants further investigation and will require future intervention trials with specific agents such as biologics.

INTRODUCTION

Atopic dermatitis (AD) is the most common inflammatory skin disease worldwide. The underlying pathological mechanisms are incompletely described and AD presents clinically with a broad range of features. Different clinical AD phenotypes have been described that are based on characteristics such as age of onset, or the presence of other atopic diseases such as allergic rhinitis and asthma.[1, 2] Despite efforts to better understand AD pathogenesis through defining phenotypical subsets, for example patients with intrinsic as compared to those with extrinsic AD,[3, 4] AD pathogenesis remains poorly understood. Indeed it is hypothesised that AD cannot be explained by one mechanism alone; this hypothesis also suggests that it is unlikely that newly developed biological drugs that target highly specific biological axes of the immune or skin barrier development systems will be effective in all AD patients. Consistent with this thinking recent clinical trials with the anti-IL4Rα monoclonal antibody dupilumab have been very encouraging, but it is clear that this treatment is not equally effective in all patients, and that an alternative therapeutic approach will be required in some patients. Indeed, in the recently published phase 3 dupilumab trials the primary outcome of reduction in IGA to 0 or 1 occurred in 38% of patients.[5] In the exciting future of personalised healthcare it is critical to be able to provide the right drug to the right patient. Hence, it is essential to gain better insight into the heterogeneous nature of the disease at the biological level. We hypothesised that the heterogeneity in AD could be described via differential serum biomarker profiles and set out to test this using a broad Luminex panel assessed in a large 200-patient cohort of moderate and severe AD patients. When these data were subjected to principal component analysis (PCA) followed by unsupervised cluster analysis, we were able to identify four patient clusters with distinct serum biomarker profiles and overlapping clinical phenotypes that may represent distinct disease endotypes.

METHODS

Study design

We hypothesised that AD is heterogeneous at the biological level of individual serum inflammatory mediators. To test this hypothesis we measured 147 analytes in the serum of 200 moderate to severe AD patients and 30 non-atopic dermatitis healthy controls via Luminex-based multiplex immunoassays, total IgE and allergen-specific IgEs (ISAC) were also assessed. Clinical characteristics were extracted from the patients' electronic files. Principal component analysis (PCA) of the serum biomarker data (including total IgE) followed by unsupervised k-means cluster analysis was used to identify heterogeneous patient clusters. ISAC data was not included in PCA analysis since it was comprised of semi-quantitative data only (detailed methods related to measurement of serum mediators and statistical analysis are available in the Methods section in this article's Online Repository).

Patients and controls

The rule of thumb adopted for sizing populations for multivariate principal components is five times the number of independent variables measured.[6] The number of independent variables mentioned above is the number of unique variables that are not correlating to any other variable. Our experience from asthma research suggests[7] that analytes are often highly correlated; extrapolating this suggested that within 150 analytes there are likely to be about 20 unique independent variables. Following the guidance above, the required sample size was 100 (5*20) moderate, and 100 severe patients.

From a biobank of over 1000 AD patients, 200 patients who were all treated with only topical corticosteroids, were selected. To select patients they were first grouped based on the Six Area, Six Sign Atopic Dermatitis (SASSAD) severity score[8] into six groups; moderate patients with SASSAD scores ranging from (i) 15-20 (ii) 21-25 (iii) 26-30 and severe patients with SASSAD scores ranging from (i) 31-35 (ii) 36-40 (iii) >40. Then, as previous analysis revealed that gender and age were factors of interest in AD[9], the six groups were normalised on sex and age prior to final selection. Final selection included equal numbers from each of the six SASSAD groups. Thirty healthy controls without a previous history of AD, allergic asthma or allergic rhinoconjunctivitis, and approximately age and sex matched to the AD patients, were recruited from UMC Utrecht and included in the study.

All AD patients were diagnosed with AD according to the criteria of Hanifin and Rajka.[10] However, AD patients present with a wide spectrum of clinical features that can vary considerably between individuals. From our observations in the clinic we have the impression that there are two mutually exclusive subsets of patients; patients with a predominantly chronic lichenified eczema, and patients with a predominantly

erythematous eczema. In order to correlate clusters with clinical features, we labelled patients as chronic lichenified eczema or erythematous eczema.

The protocols of this study were approved by the Institutional Review Board of the University Medical Center Utrecht (Utrecht, The Netherlands), adhering to the Declaration of Helsinki Principles.

RESULTS

Patient demographics, clinical characteristics and serum biomarker profiles

From the 200 serum samples, seven were different time points from the same individuals and hence were not included in the final patient selection. Therefore a total of 193 moderate to severe AD patients were studied, made up of 95 moderate and 98 severe patients. Thirty adult controls with no history of AD, allergic asthma or allergic rhinitis were also recruited for the study, although they were older than the AD patients (geomean (95%CI) age of 39.1 (34.3, 44.6) years versus 30.6 (28.3, 33.2) years for moderate and 31.1 (28.5,34.0) years for severe patients). Moderate AD patients had a lower SASSAD and lower median body surface area (BSA) involvement than severe AD patients (geomean (95%CI) SASSAD 22.3 (21.3, 23.3) versus 39.1 (37.5, 40.9), respectively and median (Q1, Q3) BSA of 33% (21, 48) versus 54% (39, 72), respectively). All AD patients had elevated levels of serum TARC compared to healthy controls (median (Q1, Q3) of 3950 (2104-8944) pg/mL in severe AD, 1505 (654, 3200) pg/mL in moderate AD and 97 (68, 137) pg/mL in controls). Patient characteristics are summarised in Table 1.

For all patients and healthy controls, serum concentrations of 147 analytes were determined via multiplex immunoassay. Serum total and specific IgEs, periostin, DPP4, anti-Staphylococcus aureus alpha toxin IgG levels were also measured. A bioassay was used to measure the neutralising ability of Staphylococcus aureus alpha toxin antibodies in AD and healthy control serum. For technical reasons, IL-19, GRO-α and SR-PSOX could not be measured in healthy controls. In AD patients, all analytes were detectable (Tables S2-S7). In serum of healthy controls, IL-3, leukaemia inhibitory factor (LIF), eotaxin, I309, and macrophage colony-stimulating factor (M-CSF) were undetectable (Tables S2-S7). In general AD patients had a clearly different serum biomarker profile from healthy controls with large upregulation of key inflammatory biomarker families in AD versus control including Th2, epithelial and IL-1 family cytokines as well as total IgE and specific IgEs (Tables S4-S8).

Principal component analysis and unsupervised cluster analysis reveals four clusters of AD patients To test the hypothesis that AD was biologically heterogeneous we performed PCA on the Box-Cox transformed serum biomarker dataset which revealed the likely presence of four clusters (Fig 1A). Summation of the

variances showed that the first 57 principal components described 90% of the dataset variance (Fig 1B). Therefore the first 57 principal components were included in an unsupervised k-means cluster analysis which delivered four AD clusters (Fig 1C and movie S10).

Table 1. Patients' baseline characteristics.

Clinical Characteristics	Healthy controls (n=30)	Moderate AD patients (n=95)	Severe AD patients (n=98)
Age (y)^{GM}	39.1 (34.3-44.6)$^{>MOD,>SEV}$	30.6 (28.3-33.2)$^{<HC}$	31.1 (28.5-34.0)$^{<HC}$
Female, n (%)	15 (50%)	57 (60%)	55 (66%)
SASSAD^{GM}	NA	22.3 (21.3-23.3)$^{<sev}$	39.1 (37.5-40.9)$^{>mod}$
Body Surface Area (%)^{Med}	NA	33.0 (21.0-48.0)$^{<sev}$	54.0 (39.0-72.0)$^{>mod}$
TARC (pg/ml)^{Med}	96.5 (65.5 - 141.1)$^{<mod,<sev}$	1499.0 (619.7-3245.0)$^{>HC,<sev}$	3950.0 (2094.0-9460.0)$^{>HC,>mod}$
Total IgE (kU/L)^{Med}	37.2 (13.5 - 93.9)$^{<mod,<sev}$	1041 (251.0-3210)$^{>HC,<sev}$	4166.0 (1015.0-9385.0)$^{>HC,>mod}$
Atopic diseases, n (%)			
- allergic rhinitis	NA	30 (32%)	22 (22%)
- allergic asthma	NA	8 (8%)	8 (8%)
- allergic asthma and rhinitis	NA	36 (38%)	36 (37%)
- no atopic disease reported	NA	21 (22%)	29 (30%)
- missing data	NA	0 (0%)	3 (3%)
Age of onset AD			
0-1 yrs	NA	42 (44%)	46 (47%)
1-12 yrs	NA	42 (44%)	32 (33%)
12-18 yrs	NA	3 (3%)	5 (5%)
>18 yrs	NA	5 (5%)	10 (10%)
missing data	NA	3 (3%)	5 (5%)
Clinical label			
- predominantly lichenification	NA	43 (45%)	54 (55%)
- predominantly erythema	NA	6 (6%)	11 (11%)
- no clinical label	NA	46 (48%)	43 (44%)

Categorical variables are presented as counts and percentages; log-normally distributed variables are presented as geomean (95% CI); non-normally distributed data are presented as median (25th-75th percentile) of logged data. Variable types are shown in superscript next to variable name; Med (median), GM (geometric mean). Superscript letters after measure of central tendency are groups that are significantly different at a level of $p<0.05$; <mod, sev, HC = statistically less than moderate (mod) AD, severe (sev) AD, healthy controls (HC); >mod, sev, HC = statistically greater than moderate AD, severe AD, healthy controls. No superscript letters denotes no statistical difference between groups.

Characterisation of AD clusters

AD cluster membership, or control status, was added back to the complete dataset and all variables were compared by cluster versus the healthy controls. Clinical characteristics (not included in the clusters analysis) were analysed between the four clusters and are shown in Table 2. Averages of serum analytes per cluster were calculated and tabulated to characterise the clusters (Table S2 and S3). As expected more frequent sensitization to allergen components measured by ISAC was seen in AD patients compared to healthy controls (Table S8). However, no clear differences in sensitization patterns were observed between the patient clusters (Table S9). Measurement of anti-S. aureus alpha toxin IgG was used as a systemic marker of AD patient S. aureus exposure, however this showed no difference between clusters. Total IgE levels did also not show any significant differences between the four clusters (Table S3). Key characteristics, both clinical and serum profiles, for each AD cluster are described below.

Cluster 1 (high SASSAD/BSA, PARC/TIMP-1/sCD14 high) represented 23% of the AD population and was 59% female. Cluster 1 patients had a geomean (95%CI) age of 31.2 (27.7, 35.1) years and, alongside cluster 3, the highest severity score. Cluster 1 geomean (95%CI) SASSAD was 35.3 (31.9, 39.0), which was greater than clusters 2 and 4, and cluster 1 median (Q1,Q3) BSA score was 54 (40.4, 69), which was greater than clusters 2 and 3. Cluster 1 was uniquely defined by having the highest levels of PARC, TARC and TIMP-1 (both greater than clusters 2, 3, 4 and healthy controls) and was distinguished from clusters 2, 3 and 4 by having the highest level of sCD14 (Table S2). Clinically cluster 1, alongside cluster 3, had the highest incidence of asthma (54% vs 53%, respectively) which was greater than that in clusters 2 and 4 (43% vs 36%, respectively) although cluster 1 was unique in having the highest incidence of asthma and rhinitis (47% vs 38%, 38%, 32%, respectively). Cluster 1 patients had a similar frequency of erythematous phenotype to cluster 4 (15% vs 19%, respectively) which was above that observed in clusters 2 and 3 (8 and 4%, respectively). Cluster 1 patients also shared with cluster 4 patients the highest frequency of infant onset AD (57% vs 56%, respectively) which surpassed those in clusters 2 and 3 (45% and 42%, respectively) (Table 2).

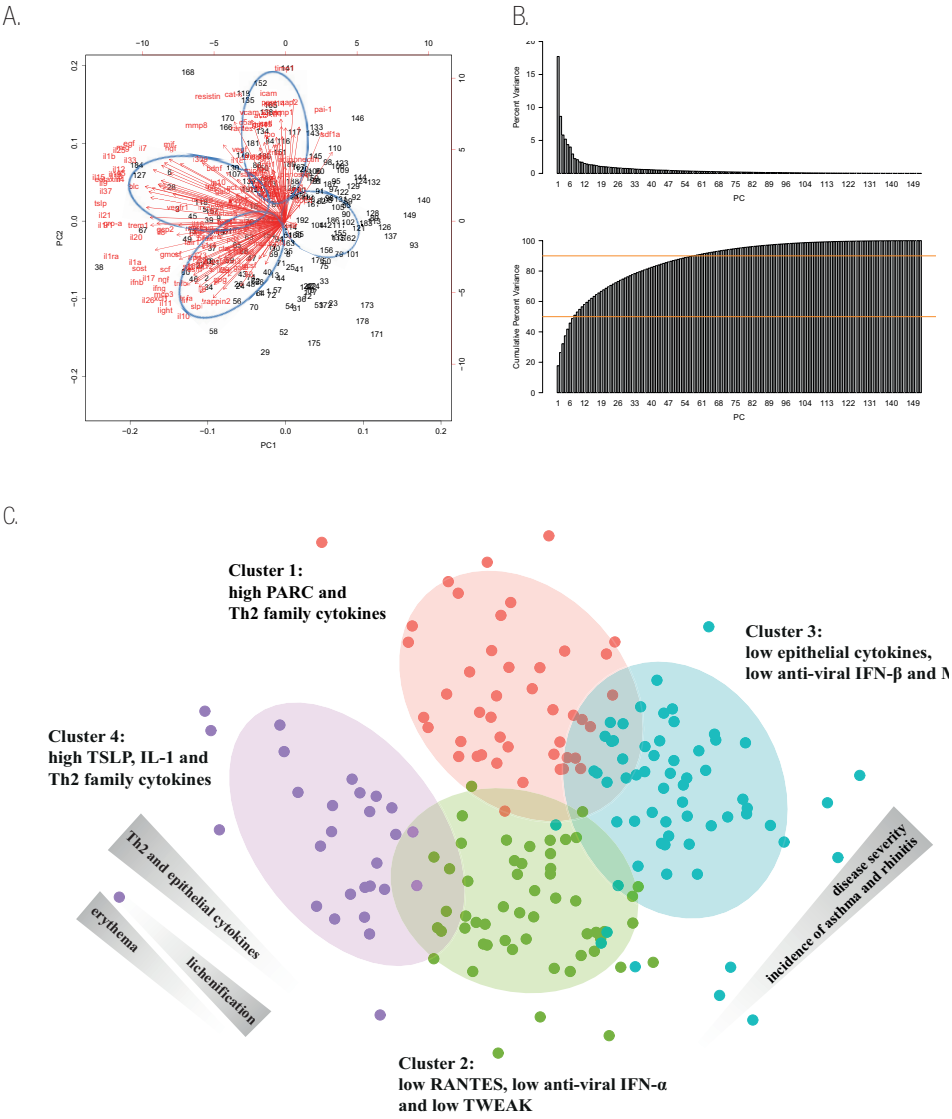
Cluster 2 (low SASSAD, IFN-α/TIMP-1/VEGF low) represented 29% of the AD population and was 56% female. Cluster 2 patients were younger than cluster 3 patients with a geomean age (95%CI) of 28.1 (25.3, 31.1) years and, alongside cluster 4, the lowest severity score. Cluster 2 geomean (95%CI) SASSAD was 25.5 (23.2, 28) which was lower than both clusters 1 and 3, and cluster 2 median BSA (Q1, Q3) was 36 (24, 54) which was less than cluster 1 (Table 2). Cluster 2 was uniquely defined by having the lowest levels of TIMP-1 (lower than clusters 1,3,4 and healthy controls), VEGF and IFN-α (lower than clusters 1, 3, 4, but higher than healthy controls). Clinically cluster 2 patients had a similar frequency of lichenified phenotype as cluster 3 (74% vs 76%, respectively) which was above that observed in clusters 1 and 4 (60%

and 43%, respectively). Cluster 2 patients also had the highest frequency of childhood onset AD (47%) which surpassed those in clusters 1, 3 and 4 (36%, 43% and 30%, respectively).

Cluster 3 (high SASSAD, IFN-β/IL-1/epithelial cytokine low) was the largest cluster, representing 33% of the AD population, and was 53% female. Cluster 3 patients were older than both cluster 2 and 4 patients with a geomean (95% CI) age of 35.0 (31.4, 39.0) years. Cluster 3, alongside cluster 1, had the highest severity score. Cluster 3 geomean (95% CI) SASSAD was 32.1 (29.7, 34.8), which was higher than both clusters 2 and 4. Cluster 3 median BSA (Q1, Q3) was 40.5 (27, 54) which was less than cluster 1. Cluster 3 was uniquely defined by having the lowest levels of IFN-β, IL-1Ra, IL-26, MIG (all less than control and clusters 1, 2, 4), eotaxin, IL-1β, IL-1R1, IL-4, IL-9, IL-12, IL-15, IL-21, IL-25, IL-33, TREM1, TSLP (all more than controls but less than clusters 1,2,4), CTACK, IL-1α, Gro-α, IL-19, IL-37 (all less than clusters 1, 2, 4). Clinically, cluster 3 patients had the lowest frequency of erythematous phenotype (4% vs 15%, 8%, 19%, respectively) as well as, together with cluster 4, the highest frequency of adult onset AD (12% vs 5%, 4%, 11%, respectively) (Table 2). Cluster 3 patients also had the highest frequency of comorbid asthma alone (15% vs 7%, 6%, 4% respectively) as well as, together with cluster 1, the highest frequency of comorbid asthma alone or with rhinitis (53% vs 54%, 43%, 36%, respectively) (Table 2).

Cluster 4 (female predominant, low SASSAD, IL-1/4/13 and TSLP high) represented 15% of the AD population and was the most female predominant group (68%). Cluster 4 patients were younger than cluster 3 patients with a geomean (95% CI) age of 27.4 (23.3, 32.1) years and, alongside cluster 2, the lowest severity score. Cluster 4 geomean (95% CI) SASSAD was 25.8 (22.4, 29.7) which was lower than both clusters 1 and 3 and cluster 4 median BSA (Q1, Q3) was 43.5 (33, 55). Cluster 4 was uniquely defined as profoundly inflamed by having the highest levels of BLC, elastase, eotaxin, EPOR, FGF-b, GCP-2, GM-CSF, IFN-γ, IL-1α, IL-1β, IL-1Ra, IL-1R1, IL-4, IL-8, IL-9, IL-11, IL-13, IL-15, IL-17, IL-19, IL-20, IL-21, IL-37, LAIR1, LIGHT, MCP-3, MIP-1α, NGF, SOST, sVEGF-R1, TNF-α, TNF-β, TREM-1, TSLP, XCL-1 (all more than clusters 1,2,3 and healthy controls), GRO-α, IFN-β, IL-2, PLGF (all more than clusters 1,2,3) (Table S2). Clinically, cluster 4 patients had the highest frequency of erythematous phenotype (19% vs 15%, 8%, 4%, respectively). Cluster 4 patients also had the lowest frequency of comorbid asthma (36% vs 54%, 43% and 53%, respectively) and the highest frequency of no comorbid asthma or rhinitis (36% vs 23%, 26% and 20%, respectively) (Table 2).

Figure 1. PCA and cluster analysis of AD patients.



A: PCA of Box-Cox normalized serum data reveals presence of four likely clusters. Blue ovals are illustrative annotation representing four likely clusters.

B. Eight PCs describe 50% of the variance and 57 PCs describe 90% of the variance in the Box-Cox normalized serum dataset

C. Generation of four clusters of AD patients by unsupervised k-means clustering of the 57 most important PCs into four clusters using a predefined seed value. Clinical characteristics (not included in the clusters analysis) were analysed between the four clusters (Table 2), and averages of serum analytes per cluster were calculated and tabulated to characterise the clusters (see Table E2 and E3 in the Online Repository). This figure shows the characterisation of the four AD clusters.

Table 2. Key demographic data for the four AD clusters and healthy controls.

N	44	54	62	28	30
(% AD patients)	-23	-29	-33	-15	(NA)
Age^{GM} (y)	31.2	28.1$^{<HC,3}$	35.0$^{>2,4}$	27.4$^{<HC,3}$	39.1$^{>2,4}$
	(27.7, 35.1)	(25.3, 31.1)	(31.4, 39.0)	(23.3, 32.1)	(34.3, 44.6)
Sex (%female)	59.1	55.6	53.2	67.9	50
SASSAD score^{GM}	35.3$^{>2,4}$	25.5$^{<1,3}$	32.1$^{>2,4}$	25.8$^{<1,3}$	NA
	(31.9, 39.0)	(23.2, 28.0)	(29.7, 34.8)	(22.4, 29.7)	
BSA scoreMed	54.0$^{>2,3}$	36.0$^{<1}$	40.5$^{<1}$	43.5	NA
	(40.4, 69.0)	(24.0, 54.0)	(27.0, 53.5)	(33, 54.7)	
Comorbidities (%)					
- none	23.3	26.4	19.7	35.7	NA
- asthma (A)	7	5.7	14.8	3.6	NA
- rhinitis (R)	23.3	30.2	27.9	28.6	NA
- A & R	46.5	37.7	37.7	32.1	NA
- all asthma	53.5	43.4	52.5	35.7	NA
Age of onset (%)					
- infancy	57.1	45.1	41.7	55.6	NA
- childhood	35.7	47.1	43.3	29.6	NA
- puberty	2.4	3.9	3.3	3.7	NA
- adult	4.8	3.9	11.7	11.1	NA
Clinical labels (%)					
- predominantly lichenification	60	73.7	76.1	42.9	NA
- predominantly erythema	15	7.9	4.3	19	NA
- no clinical label	25	18.4	19.6	38.1	NA
- predominantly erythema	15	7.9	4.3	19	NA
- no clinical label	25	18.4	19.6	38.1	NA

Variable types are shown in superscript next to variable name; M (mean), Med (median), GM (geometric mean). Healthy controls (HC). Categorical variables are presented as counts and percentages. Numbers shown in brackets after measure of central tendency are 95% confidence intervals (for mean and geomean) or interquartile range (median). Superscript numbers after measure of central tendency are groups that are significantly different at a level of $p<0.05$.

DISCUSSION

Atopic dermatitis is recognized as a complex and highly heterogeneous disease, characterised by a diverse clinical manifestation, but little work has been performed to characterise the biological differences underlying this clinical heterogeneity. Classically, clinical characteristics such as disease severity, age of onset, and the presence of atopic comorbidities are used to divide AD into different disease phenotypes.[2] However, these phenotypical characteristics do not seem to relate to specific disease mechanisms and have not yet given us new insights in the underlying pathology of disease. It has become increasingly clear that AD is not only heterogeneous based on clinical characteristics, but that different underlying pathophysiological processes are found in different subgroups of patients.[11, 12] The primary goals of our study were firstly to test whether AD is heterogeneous at the level of the serum biomarker profile and then to describe this heterogeneity and its relationship to clinical presentation.

To the best of our knowledge, this is the first study that classifies AD patients on serum biomarker based clusters. We used a purely data-driven approach to cluster serum biomarker profiles in a population of moderate to severe AD patients. This approach revealed four distinct clusters of AD patients, showing that adult AD truly is a heterogeneous disorder. Each cluster has a distinct profile of soluble mediators that together may indicate these clusters are driven by distinct, as yet unidentified, underlying pathways. Classification based on subgroups defined by distinct functional or pathophysiological mechanisms is termed endotyping.[13, 14] The four distinct patient clusters that we identified in this study could represent endotypes that have unique biology driving the pathogenesis of disease. Further biological studies and interventional trials will be needed to confirm the endotype of each cluster. However, it is hoped that endotypes could be used to target specific therapies in future clinical trials and daily practice.

Each cluster in our analysis is characterised by certain serum biomarkers and clinical characteristics which are significantly differentially expressed. Two of the clusters, 1 and 3 (SASSADs of 35 and 32), were relatively more severe than clusters 2 and 4 (SASSADs of 25 and 26). Of the more severe clusters, cluster 1 was delineated from the others by having the highest levels of PARC, TARC, TIMP-1 and sCD14. In contrast, cluster 3 was defined by the lowest levels of host-defence associated molecules including IFN-β and MIG, IL-1 family members IL-1α and IL-1β, Th2 cytokines IL-4 and IL-9, as well as the key immune initiating epithelial cytokines, IL-25, IL-33 and TSLP. Of the less severe clusters, cluster 2 was defined by having the lowest levels of TIMP-1, VEGF and IFN-α whereas cluster 4 was defined by being female predominant (68%) and having the highest levels of multiple inflammatory cytokines including the IL-1 family, Th2 cytokines IL-4, IL-9, IL-13 and the epithelial cytokine TSLP.

Moving beyond individual cluster differentiating patterns of mediator expression there were also

interesting commonalities across the clusters (Figure 1C). Patients in cluster 1 and 4, representing 48% of patients, showed particularly high Th2-cytokine levels and an erythematous skin phenotype, and would hypothetically represent ideal patients for Th2 targeting drugs that are currently being tested, including the anti-IL-4Rα antibody dupilumab, and anti-IL-13 antibodies Lebrikizumab and Tralokinumab (NCT02755649, NCT02340234, NCT02347176).[15-17] Moreover, given the recent success of dupilumab in AD trials, the biomarker expression of these two clusters could directly relate to the underlying pathophysiological processes in these patients.

Clusters 2 and 3, representing 52% of the patients, share a Th2-cytokine low and pauci-inflammatory mediator state (analogous to that seen in Th2-low asthma)[18] as well as presenting with a greater frequency of lichenified skin phenotype. These clusters would hypothetically represent patient groups that are not ideal for Th2 targeting drugs and that a more comprehensive biological characterisation of disease mechanisms in these patients will be required to allow generation of more tailored therapeutics.

The statistically non-significant association of an erythematous skin with a strong epithelial cytokine and Th2 phenotype, and the association of a lichenified skin with a weak epithelial cytokine and Th2 phenotype, is intriguing and could imply a mechanistic link (Figure 1C). Whether this mechanistic link is real or simply represents biomarkers of an as yet unidentified biological mechanism remains to be elucidated. Similarly, understanding how the underlying biology of patients in clusters 2 and 3 could drive the lichenification process needs further investigation.

Taken together the clusters found in our study, approximately 48% "Th2 high" and 52% "Th2 low", are consistent with recent phase 3 data from dupilumab in AD[5] demonstrating that 62% patients did not achieve the primary endpoint of reduction in IGA and would support the use of biomarkers to identify optimal dupilumab responders in future trials. Whilst, both clusters 1 and 4 have high Th2 cytokines, they can be clearly differentiated from each other through high expression of PARC and TSLP respectively. This might indicate a pathophysiological process in cluster 4 in which the higher levels of TSLP could drive a more pruritic phenotype.[19] Thus, patients in cluster 4 could represent those patients that would respond best to new anti-TSLP biologics currently being tested.

The identification of phenotypes, leading to hypothetical endotypes, based on cluster analyses of immune mediators has been demonstrated in other diseases including asthma,[7, 20, 21] COPD[7] and chronic rhinosinusitis.[22] If such endotyping can be confirmed with interventional studies it will prove useful with the introduction of new targeted therapies, since patients with different endotypes may respond differently to the same treatment. Sub-grouping patients based on a serum biomarker and blood eosinophils has already been proven to be useful in asthma, where anti-IL-13 therapy appears to be most effective in the specific subgroup of patients with high serum levels of periostin.[23, 24] Similarly, quantifying blood eosinophils

in asthmatics has facilitated the identification of those patients that best respond to IL-5 axis targeting drugs, such as Benralizumab.[25] The ability to endotype AD patients may contribute to precision medicine by allowing treatment to be tailored for individual patients.[14] This would not only be beneficial for patients, but would also reduce health-care costs.[26] We expect that biomarker analyses may become essential in all clinical trials, to enable a better selection of patient populations and optimize therapy.

Data presented in this study also confirms that there is systemic inflammation in AD patients, supporting the hypothesis that AD is a systemic disorder. The high expression of many inflammatory cytokines and chemokines in the serum of AD patients resulted in clear differences in biomarker expression profiles between AD patients and healthy controls, as shown in Table S4 and S5. The concept of AD as a systemic disorder was recently highlighted in a meta-analysis of data from the AD transcriptome.[27] Given that three population-based studies have shown an association between adult AD, cardiovascular disease, and increased heart attacks[28] systemic inflammation in AD may, as in psoriasis, contribute to cardiovascular disease.[29] The results of this study thus warrant further investigation. One limitation of this study is the inability to identify a clear association between the four clusters and clinical characteristics other than severity scores such as the presence of allergic asthma or rhinitis, and age of onset of the disease. The patients in this study were selected solely on disease severity (SASSAD), sex and age, no other clinical characteristics peculiar to AD were considered for selecting patients. Additionally detailed clinical characterisation was not available for every patient. Therefore, the absence of an association between the clusters and other clinical characteristics may be due to missing data or the consequence of the unbalanced presence of these clinical characteristics across the hypothetical endotypes. It also highlights the significant challenges in identifying endotypes with underlying pathophysiological mechanisms based on clinical features such as age of onset or the presence of other atopic diseases. The fact that we could not find any clear differences in sensitization patterns based on 150 specific IgE levels between the patient clusters strengthens this hypothesis. This would also be in accordance with the findings of dupilumab trials in which treatment effects were found to be similar in both intrinsic and extrinsic AD patient groups.[3, 4]

Additionally, the present study is retrospective, and its findings will need further validation of the utility of serum biomarkers for clustering AD. The clusters that were identified in this study should be confirmed in an independent patient population in a prospective study. Prospective studies including more biomarkers may, we anticipate, reveal more subgroups of patients and give more insight into the association with clinical characteristics. Also, a longitudinal study may provide us additional insights into the stability of biomarkers in a patient over time. The current study focused on serum biomarkers, which may just represent the down-stream effects of certain underlying pathways. Interventional clinical trials will help to confirm whether the serum biomarkers associated with each cluster directly relate to a pathological mechanism

and thus confirm clusters as specific endotypes. In order to more precisely endotype patients and identify underlying pathways, future studies should follow a multi-layer approach, integrating high throughput data from genomics, transcriptomics, lipomics, and proteomics with extensive clinical data.

In summary, in a population of moderate to severe AD patients, we have identified four clearly differentiated clusters, illustrating the heterogeneity of AD, at the molecular and phenotypic level. Each cluster is characterised by a specific serum biomarker profile, implying that each of these clusters is driven by a distinct underlying pathway and may represent endotypes. The identification of these endotypes through future interventional studies with specific pathway neutralising agents could enable more specific targeting of the underlying disease pathways and contribute to more personalized treatment strategies for AD patients in the future.

SUPPLEMENTARY DATA

See http://www.dermatologyutrecht.nl/index.php/9-public/115-thijs

REFERENCES

1. Eyerich K, Novak N. Immunology of atopic eczema: overcoming the Th1/Th2 paradigm. Allergy 2013; 68:974-82.
2. Bieber T. Atopic dermatitis 2.0: from the clinical phenotype to the molecular taxonomy and stratified medicine. Allergy 2012; 67:1475-82.
3. Beck LA, Thaci D, Hamilton JD, Graham NM, Bieber T, Rocklin R, et al. Dupilumab treatment in adults with moderate-to-severe atopic dermatitis. N Engl J Med 2014; 371:130-9.
4. Thaci D, Simpson EL, Beck LA, Bieber T, Blauvelt A, Papp K, et al. Efficacy and safety of dupilumab in adults with moderate-to-severe atopic dermatitis inadequately controlled by topical treatments: a randomised, placebo-controlled, dose-ranging phase 2b trial. Lancet 2016; 387:40-52.
5. Simpson EL, Bieber T, Guttman-Yassky E, Beck LA, Blauvelt A, Cork MJ, et al. Two Phase 3 Trials of Dupilumab versus Placebo in Atopic Dermatitis. N Engl J Med 2016.
6. Hatcher L. A Step-by-Step Approach to Using the SAS® System for Factor Analysis and Structural Equation Modeling. Cary, North Carolina: SAS Publishing; 1994.
7. Ghebre MA, Bafadhel M, Desai D, Cohen SE, Newbold P, Rapley L, et al. Biological clustering supports both "Dutch" and "British" hypotheses of asthma and chronic obstructive pulmonary disease. J Allergy Clin Immunol 2015; 135:63-72.
8. Berth-Jones J. Six area, six sign atopic dermatitis (SASSAD) severity score: a simple system for monitoring disease activity in atopic dermatitis. Br J Dermatol 1996; 135 Suppl 48:25-30.
9. Thijs JL, Nierkens S, Herath A, Bruijnzeel-Koomen CA, Knol EF, Giovannone B, et al. A panel of biomarkers for disease severity in atopic dermatitis. Clin Exp Allergy 2015; 45:698-701.
10. Hanifin JM, Rajka G. Diagnostic Features of Atopic-Dermatitis. Acta Derm Venereol 1980:44-7.
11. Suarez-Farinas M, Dhingra N, Gittler J, Shemer A, Cardinale I, de Guzman Strong C, et al. Intrinsic atopic dermatitis shows similar TH2 and higher TH17 immune activation compared with extrinsic atopic dermatitis. J Allergy Clin Immunol 2013; 132:361-70.
12. Noda S, Suarez-Farinas M, Ungar B, Kim SJ, de Guzman Strong C, Xu H, et al. The Asian atopic dermatitis phenotype combines features of atopic dermatitis and psoriasis with increased TH17 polarization. J Allergy Clin Immunol 2015; 136:1254-64.
13. Lotvall J, Akdis CA, Bacharier LB, Bjermer L, Casale TB, Custovic A, et al. Asthma endotypes: a new approach to classification of disease entities within the asthma syndrome. J Allergy Clin Immunol 2011; 127:355-60.

14. Muraro A, Lemanske RF, Jr., Hellings PW, Akdis CA, Bieber T, Casale TB, et al. Precision medicine in patients with allergic diseases: Airway diseases and atopic dermatitis-PRACTALL document of the European Academy of Allergy and Clinical Immunology and the American Academy of Allergy, Asthma & Immunology. J Allergy Clin Immunol 2016; 137:1347-58.

15. A Study to Assess the Efficacy and Safety of Dupilumab in Patients With Severe Atopic Dermatitis (AD) That Are Not Controlled With Oral Cyclosporine A (CSA) or for Those Who Cannot Take Oral CSA Because it is Not Medically Advisable.] Available from https://www.clinicaltrials.gov/ct2/show/NCT02755649.

16. A Study of Lebrikizumab in Patients With Persistent Moderate to Severe Atopic Dermatitis.] Available from https://www.clinicaltrials.gov/ct2/show/NCT02340234.

17. Phase 2 Study to Evaluate the Efficacy and Safety of Tralokinumab in Adults With Atopic Dermatitis (D2213C00001).] Available from https://www.clinicaltrials.gov/ct2/show/NCT02347176.

18. Fahy JV. Type 2 inflammation in asthma--present in most, absent in many. Nat Rev Immunol 2015; 15:57-65.

19. Wilson SR, The L, Batia LM, Beattie K, Katibah GE, McClain SP, et al. The epithelial cell-derived atopic dermatitis cytokine TSLP activates neurons to induce itch. Cell 2013; 155:285-95.

20. Wu W, Bleecker E, Moore W, Busse WW, Castro M, Chung KF, et al. Unsupervised phenotyping of Severe Asthma Research Program participants using expanded lung data. J Allergy Clin Immunol 2014; 133:1280-8.

21. Hinks TS, Brown T, Lau LC, Rupani H, Barber C, Elliott S, et al. Multidimensional endotyping in patients with severe asthma reveals inflammatory heterogeneity in matrix metalloproteinases and chitinase 3-like protein 1. J Allergy Clin Immunol 2016; 138:61-75.

22. Tomassen P, Vandeplas G, Van Zele T, Cardell LO, Arebro J, Olze H, et al. Inflammatory endotypes of chronic rhinosinusitis based on cluster analysis of biomarkers. J Allergy Clin Immunol 2016; 137:1449-56 e4.

23. Corren J, Lemanske RF, Hanania NA, Korenblat PE, Parsey MV, Arron JR, et al. Lebrikizumab treatment in adults with asthma. N Engl J Med 2011; 365:1088-98.

24. Brightling CE, Chanez P, Leigh R, O'Byrne PM, Korn S, She D, et al. Efficacy and safety of tralokinumab in patients with severe uncontrolled asthma: a randomised, double-blind, placebo-controlled, phase 2b trial. Lancet Respir Med 2015; 3:692-701.

25. Castro M, Wenzel SE, Bleecker ER, Pizzichini E, Kuna P, Busse WW, et al. Benralizumab, an anti-interleukin 5 receptor alpha monoclonal antibody, versus placebo for uncontrolled eosinophilic asthma: a phase 2b randomised dose-ranging study. Lancet Respir Med 2014; 2:879-90.

26. Jameson JL, Longo DL. Precision medicine--personalized, problematic, and promising. N Engl J Med 2015; 372:2229-34.

27. Ewald DA, Malajian D, Krueger JG, Workman CT, Wang T, Tian S, et al. Meta-analysis derived atopic dermatitis (MADAD) transcriptome defines a robust AD signature highlighting the involvement of atherosclerosis and lipid metabolism pathways. BMC Med Genomics 2015; 8:60.

28. Silverberg JI. Association between adult atopic dermatitis, cardiovascular disease, and increased heart attacks in three population-based studies. Allergy 2015; 70:1300-8.

29. Rose S, Sheth NH, Baker JF, Ogdie A, Raper A, Saboury B, et al. A comparison of vascular inflammation in psoriasis, rheumatoid arthritis, and healthy subjects by FDG-PET/CT: a pilot study. Am J Cardiovasc Dis 2013; 3:273-8.

Predicting therapy response to Mycophenolic acid using UGT1A9 genotyping: Towards personalized medicine in atopic dermatitis

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ABSTRACT

Atopic dermatitis (AD) is a very common chronic inflammatory skin disease requiring long-term treatment. Mycophenolic acid (MPA) is used off-label in treatment of patients with severe AD failing cyclosporin A treatment, however clinical efficacy is observed in only half of the AD patients. In blood, MPA levels are known to have a large inter individual variability. Low MPA exposure and increased enzyme activity correlates with the presence of UGT1A9 polymorphisms. In this retrospective study, 65 adult AD patients treated with MPA were classified as responder or non-responder to MPA treatment. UGT1A9 polymorphisms were determined using PCR. A significantly higher number of UGT1A9 polymorphisms was found in the group that did not respond to MPA treatment. Of the patients that carried a UGT1A9 polymorphism, 85.7% was non-responder to MPA treatment. This implies that non-responsiveness in AD patients is more likely to occur in carriers of a UGT1A9 polymorphism. In a binary logistic regression analysis the odds ratio was 8.65 (95% confidence interval: 0.93 – 80.17). Our results show that UGT1A9 polymorphisms can be used to identify patients with non-responsiveness to MPA. Patients with UGT1A9 polymorphisms might benefit from higher MPA dosage.

INTRODUCTION

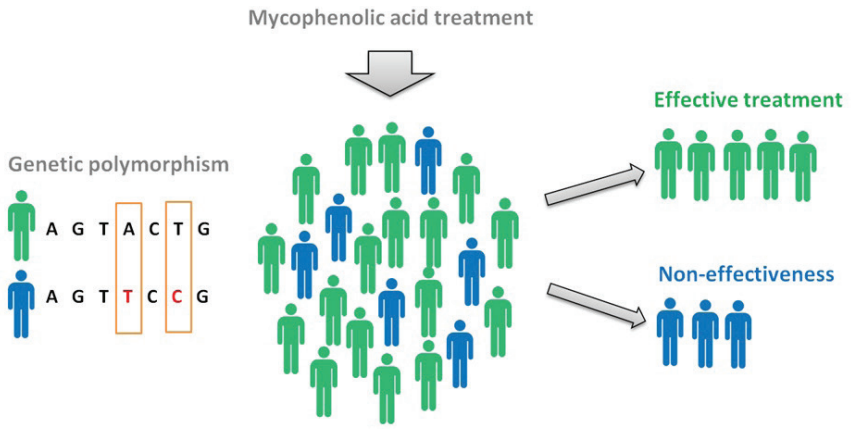
Atopic dermatitis (AD) is one of the most common chronic inflammatory skin diseases worldwide. The lifetime prevalence of AD is estimated between 15-30% in children and 2-10% in adults.[1, 2] AD is characterized by intense itching and follows a relapsing and remitting course.[3] The pathogenesis of AD is multifactorial and involves genetic, immunologic and environmental factors.[4] In the management of AD, stabilization of the disease by prevention of exacerbations is the major treatment goal; therefore long-term treatment is often indicated.

Although the majority of the AD patients can be adequately treated with topical treatment and/or UV-light therapy, there is a large group of patients in which oral immunosuppressive drugs are indicated. Various immunosuppressive drugs are used in AD, including cyclosporin A (CsA), mycophenolic acid (MPA), methotrexate, azathioprine, and oral corticosteroids.[5] In many countries, CsA is the only registered oral immunosuppressive drug for AD and therefore often first choice of treatment in severe AD.[5]

MPA is used off-label in patients with severe AD who have failed CsA treatment.[6] MPA inhibits the de novo purine synthesis by arresting the cell cycle at the G0/G1 to the S transition phase,[7, 8] which results in selective inhibition of cell proliferation of B- and T cells.[6] Since MPA does not influence the survival of activated B and T cells, it has a delayed clinical response of approximately two to three months.[9] The efficacy of MPA in AD has been proven in clinical studies.[6, 9] However, in clinical practice, MPA is ineffective in nearly half of the AD patients.[10, 11] To date, response to treatment with MPA has been very difficult to predict. Delayed clinical response and the difficulties to adequately predict MPA response may result in the fact that some patients are treated with MPA for several months without any clinical benefit.

In blood, MPA levels are known to have a large interindividual variability. This has been observed in kidney transplant recipients, in whom a lower level of MPA exposure is closely associated with lower efficacy of drug therapy and acute rejection of the transplanted organ.[12, 13] Low MPA exposure and increased enzyme activity of the metabolizing enzyme uridine diphosphate-glucuronosyltransferase 1A9 (UGT1A9) correlate to the presence of single nucleotide polymorphisms (SNPs) in the gene promotor region (275T>A and/or 2152C>T).[12, 13] MPA is predominantly metabolized by UGT1A9 to mainly the inactive phenolic-glucuronide metabolite. Therefore, increased UGT1A9 activity due to SNPs would result in lower MPA exposure in serum. We hypothesized that also for AD patients, low MPA exposure due to the presence of UGT1A9 polymorphisms might contribute to the inefficacy during MPA treatment. This would enable prediction of non-response based on UGT1A9 polymorphisms in the future (Figure 1). In this study, we evaluated the difference in frequency of UGT1A9 polymorphisms between responders and non-responders in AD patients treated with MPA, and found a positive association between the presence of the polymorphisms and non-responsiveness.

Figure 1. Pharmacogenomics in AD patients treated with MPA.



Low MPA exposure and increased enzyme activity correlate to the presence of UGT1A9 polymorphisms. It is likely that these UGT1A9 polymorphisms occur in AD patients treated with MPA. We hypothesized that low MPA exposure due to the presence of UGT1A9 polymorphisms might contribute to the inefficacy during MPA treatment. This would enable prediction of non-response based on UGT1A9 polymorphisms in the future.

MATERIALS & METHODS

Study population

In a retrospective cohort study, 65 patients with severe AD treated with MPA 1440mg/day at the University Medical Center Utrecht between January 1st 2004 and January 31st 2016 were included. Data were collected on March 15th 2016. Patients were diagnosed with AD according to the criteria of Hanifin and Rajka.[14] The response to MPA treatment was assessed based on a 6-point Investigators' Global Assessment (IGA).[15] Patients were classified as responders in case the IGA decreased at least two points after minimal three months of treatment, and as non-responder in case the decrease in IGA was less than two points.

The study was approved by the Institutional Review Board of the University Medical Center Utrecht, adhering to the Declaration of Helsinki Principles.

Genotyping

The MagNA Pure LC system (Roche Diagnostics, Mannheim, Germany) was used to isolate genomic DNA was isolated from 1 ml EDTA serum. UGT1A9 genotyping was performed using Taqman allelic discrimination

assays on an ABI prism 7000 sequence detection system (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). For UGT1A9-275T>A polymorphism, PCR was performed in a volume of 12.5 µl, containing assay-specific primers, allele specific probes, TaqMan Universal PCR Master Mix, and genomic DNA (12.5 ng). Genotypes were scored by measuring allelic-specific fluorescence using SDS 1.2.3 software (Applied Biosystems). For UGT1A9-2152C>T polymorphism, genomic DNA (12.5 ng) was amplified in a volume of 50 µl, PCR buffer II (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands), 1.75 mmol/l MgCl2, 0.2 mmol/l deoxynucleotide triphosphates (Roche Diagnostics, Mannheim, Germany), 1.25U AmpliTaq Gold (PerkinElmer), and 40 pmol of each primer. PCR products were incubated with 10 U TruI for two hours. Fragments were separated by electrophoresis on a 3% agarose gel with ethidium bromide staining.

Statistical analysis

Statistical analysis was performed using SPSS (for Windows, version 21.0, SPSS Inc). Pearson's Chi square test or Fisher exact test was used to compare categorical data between groups (e.g. responder and non-responder to MPA treatment). The strength of the association between UGT1A9-275/-2152 genotype and response to MPA treatment was calculated using binary logistic regression. Odds ratios (OR) were stated with 95% confidence intervals (95% CI). Probability levels of 0.05 and below were considered to indicate statistical significance.

RESULTS

Out of 65 patients, 33 were classified as MPA responder and 32 were classified as MPA non-responder. Patient characteristics are shown in table 1. The UGT1A9-275T>A and UGT1A9-2152C>T SNPs were found in seven patients as heterozygous, with all seven heterozygous patients having both SNPs. This prevalence is similar to those previously described in the Caucasian population.[12] The presence of UGT1A9 polymorphisms was significantly higher in the patient group that did not respond to MPA treatment compared to the group that did respond to MPA treatment (Table 1). One out of seven (14.28%) UGT1A9 polymorphism carriers was a responder, and six out of seven (85.71%) patients were non-responders to MPA treatment. This implies that non-responsiveness in AD patients is more likely to occur in carriers of a UGT1A9 polymorphism. In a binary logistic regression analysis, adjusting for age and gender, the odds ratio was 8.65 (95% confidence interval: 0.93 - 80.17) (Table 2). Significance was not reached, probably due to the small number of patients.

Table 1. Baseline table of patient characteristics

	Total (n = 65)	Responders (n = 33)	Non-responders (n = 32)	P-value
Male, n(%)	40 (62.5)	23 (69.7)	17 (53.8)	0.226
Age in years (mean ±)	42.5 (14.0)	39.9 (14.4)	45.2 (13.1)	0.132
Duration of MPA treatment in days (mean ±)	534.94 (616.7)	763.1 (753.6)	292.0 (277.5)	0.063
UGT1A9-275T>A and -2152C>T heterozygote patients, n (%)	7 (10.7)	1 (3.0)	6 (18.8)	0.033*

*$p < 0.05$

Table 2. Binary logistic regression analysis of non-response to MPA treatment, adjusting for age and gender

	OR	95% confidence interval	P-value
Crude			
UGT1A9-275T>A/-2152C>T	7.92	0.90 – 70.01	0.063
Adjusted			
UGT1A9-275T>A/-2152C>T	8.65	0.93 – 80.17	0.058
Age (years)	1.03	0.99 – 1.07	0.100
Gender (male)	0.39	0.15 – 1.33	0.094

DISCUSSION

Pharmacogenomics are increasingly used in the in the management of transplantation patients, however data in chronic inflammatory diseases, such as AD are scarce.

To the best of our knowledge, this is the first study to evaluate the effect of UGT1A9 polymorphisms on MPA therapy responsiveness in AD. A significant higher number of UGT1A9 polymorphisms was found in patients that did not respond to MPA treatment compared to patients that did respond to MPA treatment.

Since previous studies reported an association between the presence of UGT1A9 polymorphisms and low MPA exposure, the non-responsiveness to MPA in patients with UGT1A9 polymorphism is presumably due to low MPA exposure.[13] The therapeutic strategy in non-responsive UGT1A9 polymorphism carriers would have been to prescribe a higher dosage of MPA and thereby increasing MPA exposure.

Our findings are consistent with previous studies investigating UGT1A9 polymorphisms in renal

transplant patients, showing a statistically significantly higher rate of acute rejection of the graft in UGT1A9 heterozygous renal transplant recipients.[13, 16]

Remarkably, one out of seven patients with UGT1A9 polymorphisms, did respond adequately to MPA treatment. A possible explanation is that this patient has an extra genetic polymorphism in UGT1A9, resulting in an overall decrease of UGT1A9 activity, which would increase MPA exposure in contrary to expectations based on the -275 SNP, as this is described before in other studies.[17, 18]

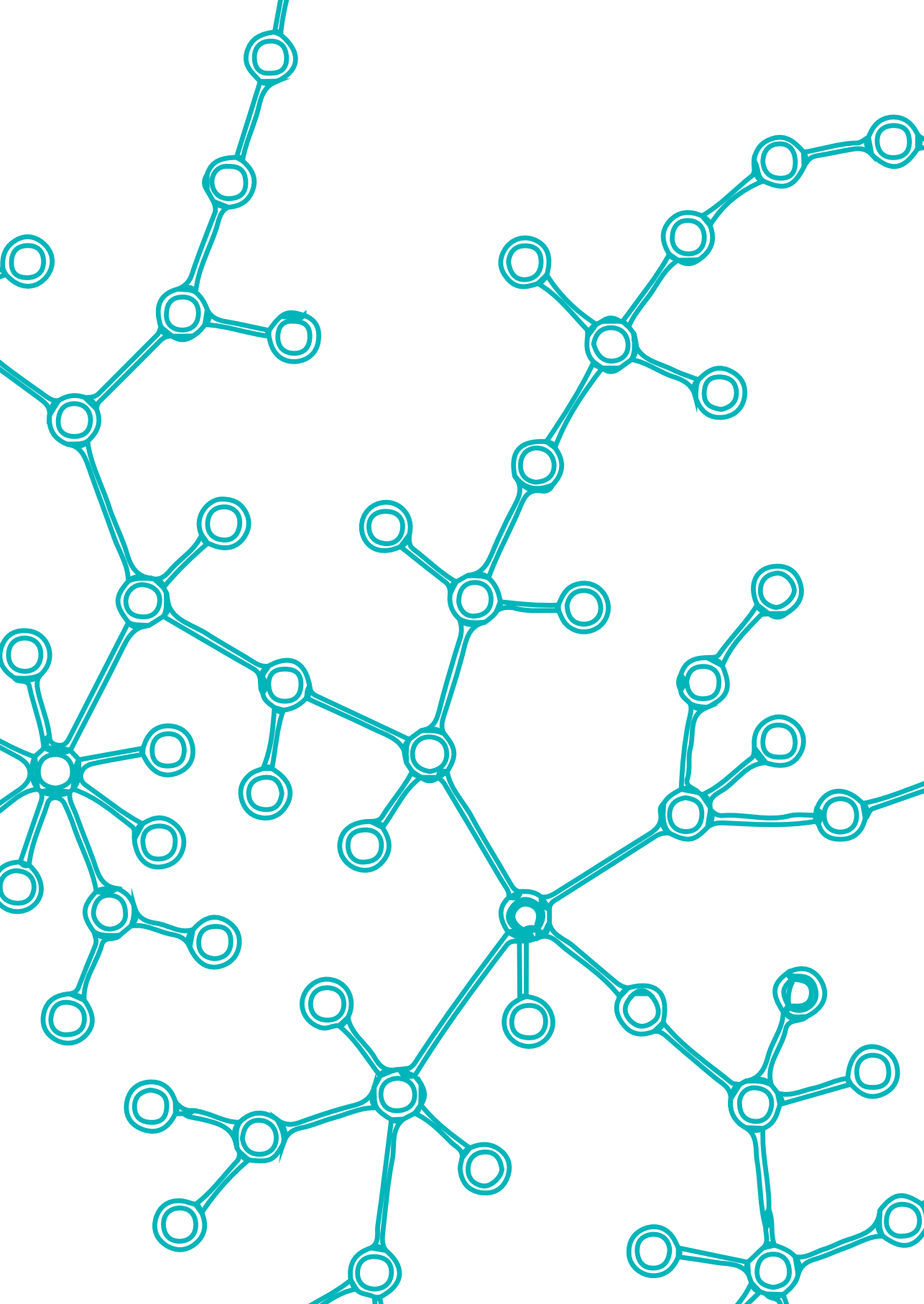
Treatment options in patients with severe AD are very limited at this moment. CsA is often the drug of first choice in these patients, however nearly half of the patients have to discontinue treatment due to side effects and/or inefficacy.[19] MPA is an interesting second choice treatment option in severe AD patients, as the side effect profile seems to be better than most other oral immunosuppressive drugs used in AD.[11, 20] However, previous studies have shown that around half of the AD patients do not respond to MPA.[10, 11] Prediction of treatment response to MPA in AD is very valuable, since treatment response can only be determined after three to four months of treatment.

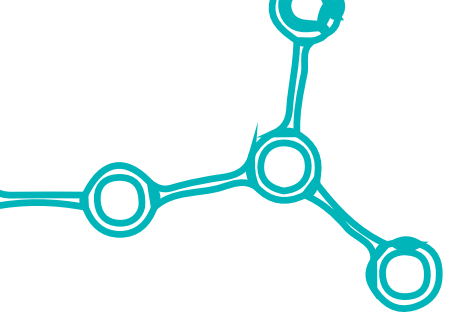
In conclusion, this study found an association between UGT1A9 polymorphisms and non-responsiveness to MPA treatment in severe AD patients. In the non-responder group, six out of 32 patients carried a UGT1A9 polymorphism. This means that pre-treatment screening for UGT1A9 polymorphisms could have identified 19% of the patients with non-responsiveness to MPA treatment. Although this is a relatively small percentage, it does show the potential benefits of pharmacogenetic screening for UGT1A9 in AD. Future research is needed to identify other SNPs and further optimize MPA treatment to ensure maximal efficacy with minimal side effects. Although pharmacogenomics are scarcely used in dermatological research, it enables "personalized medicine" by prescribing drugs based on the genetic makeup of an individual.

REFERENCES

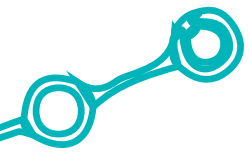
1. Bieber T. Atopic dermatitis. Ann Dermatol 2010; 22:125-37.
2. Asher MI, Montefort S, Bjorksten B, Lai CK, Strachan DP, Weiland SK, et al. Worldwide time trends in the prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and eczema in childhood: ISAAC Phases One and Three repeat multicountry cross-sectional surveys. Lancet 2006; 368:733-43.
3. Garmhausen D, Hagemann T, Bieber T, Dimitriou I, Fimmers R, Diepgen T, et al. Characterization of different courses of atopic dermatitis in adolescent and adult patients. Allergy 2013; 68:498-506.
4. Eyerich K, Novak N. Immunology of atopic eczema: overcoming the Th1/Th2 paradigm. Allergy 2013; 68:974-82.
5. Ring J, Alomar A, Bieber T, Deleuran M, Fink-Wagner A, Gelmetti C, et al. Guidelines for treatment of atopic eczema (atopic dermatitis) Part II. J Eur Acad Dermatol Venereol 2012; 26:1176-93.
6. Sidbury R, Davis DM, Cohen DE, Cordoro KM, Berger TG, Bergman JN, et al. Guidelines of care for the management of atopic dermatitis: section 3. Management and treatment with phototherapy and systemic agents. J Am Acad Dermatol 2014; 71:327-49.
7. Cohn RG, Mirkovich A, Dunlap B, Burton P, Chiu SH, Eugui E, et al. Mycophenolic acid increases apoptosis, lysosomes and lipid droplets in human lymphoid and monocytic cell lines. Transplantation 1999; 68:411-8.
8. Laliberte J, Yee A, Xiong Y, Mitchell BS. Effects of guanine nucleotide depletion on cell cycle progression in human T lymphocytes. Blood 1998; 91:2896-904.
9. Haeck IM, Knol MJ, Ten Berge O, van Velsen SG, de Bruin-Weller MS, Bruijnzeel-Koomen CA. Enteric-coated mycophenolate sodium versus cyclosporin A as long-term treatment in adult patients with severe atopic dermatitis: a randomized controlled trial. J Am Acad Dermatol 2011; 64:1074-84.
10. Garritsen FM, Roekevisch E, van der Schaft J, Deinum J, Spuls PI, de Bruin-Weller MS. Ten years experience with oral immunosuppressive treatment in adult patients with atopic dermatitis in two academic centres. J Eur Acad Dermatol Venereol 2015; 29:1905-12.
11. van der Schaft J, Politiek K, van den Reek JM, Kievit W, de Jong EM, Bruijnzeel-Koomen CA, et al. Drug survival for azathioprine and enteric-coated mycophenolate sodium in a long-term daily practice cohort of adult patients with atopic dermatitis. Br J Dermatol 2016.
12. Kuypers DR, Naesens M, Vermeire S, Vanrenterghem Y. The impact of uridine diphosphate-glucuronosyltransferase 1A9 (UGT1A9) gene promoter region single-nucleotide polymorphisms T-275A and C-2152T on early mycophenolic acid dose-interval exposure in de novo renal allograft recipients. Clin Pharmacol Ther 2005; 78:351-61.
13. van Schaik RH, van Agteren M, de Fijter JW, Hartmann A, Schmidt J, Budde K, et al. UGT1A9 -275T>A/-2152C>T polymorphisms correlate with low MPA exposure and acute rejection in MMF/tacrolimus-treated kidney transplant patients. Clin Pharmacol Ther 2009; 86:319-27.

14. Hanifin JM, Rajka G. Diagnostic Features of Atopic-Dermatitis. Acta Derm Venereol 1980:44-7.
15. Eichenfield LF, Lucky AW, Boguniewicz M, Langley RG, Cherill R, Marshall K, et al. Safety and efficacy of pimecrolimus (ASM 981) cream 1% in the treatment of mild and moderate atopic dermatitis in children and adolescents. J Am Acad Dermatol 2002; 46:495-504.
16. Kuypers DR, de Jonge H, Naesens M, de Loor H, Halewijck E, Dekens M, et al. Current target ranges of mycophenolic acid exposure and drug-related adverse events: a 5-year, open-label, prospective, clinical follow-up study in renal allograft recipients. Clin Ther 2008; 30:673-83.
17. Levesque E, Delage R, Benoit-Biancamano MO, Caron P, Bernard O, Couture F, et al. The impact of UGT1A8, UGT1A9, and UGT2B7 genetic polymorphisms on the pharmacokinetic profile of mycophenolic acid after a single oral dose in healthy volunteers. Clin Pharmacol Ther 2007; 81:392-400.
18. Fukuda T, Goebel J, Cox S, Maseck D, Zhang K, Sherbotie JR, et al. UGT1A9, UGT2B7, and MRP2 genotypes can predict mycophenolic acid pharmacokinetic variability in pediatric kidney transplant recipients. Ther Drug Monit 2012; 34:671-9.
19. van der Schaft J, Politiek K, van den Reek JM, Christoffers WA, Kievit W, de Jong EM, et al. Drug survival for ciclosporin A in a long-term daily practice cohort of adult patients with atopic dermatitis. Br J Dermatol 2015; 172:1621-7.
20. Politiek K, van der Schaft J, Coenraads PJ, de Bruin-Weller MS, Schuttelaar ML. Drug survival for methotrexate in a daily practice cohort of adult patients with severe atopic dermatitis. Br J Dermatol 2016; 174:201-3.

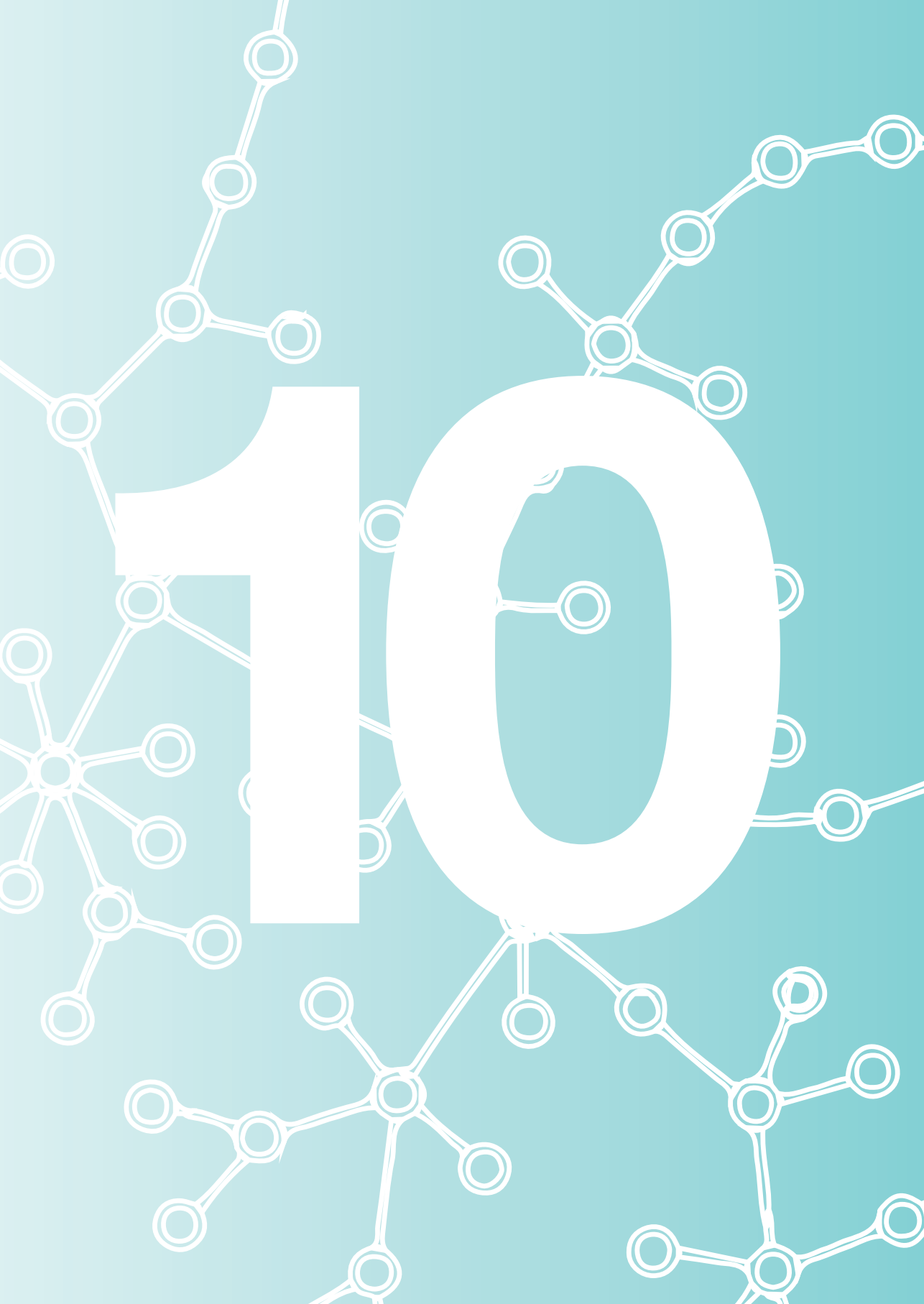




PART III



IMPROVING PRACTICAL ASPECTS OF BIOMARKER MEASUREMENT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New developments in biomarkers for atopic dermatitis

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ABSTRACT

The application of biomarkers in medicine is evolving. Biomarkers do not only give us a better understanding of pathogenesis, but also increase treatment efficacy and safety, further enabling more precise clinical care. This paper focuses on the current use of biomarkers in atopic dermatitis, new developments and future perspectives. Biomarkers can be used for many different purposes, including the objective determination of disease severity, confirmation of clinical diagnosis, and to predict response to treatment. In atopic dermatitis, many biomarkers have been investigated as a marker for disease severity. Currently serum thymus and activation-regulated chemokine (TARC) is the superior biomarker for assessing disease severity. However, we have recently shown that the use of a panel of serum biomarkers is more suitable for assessing disease severity than an individual biomarker. In this overview, we will discuss alternative sources for biomarkers, such as saliva and capillary blood, which can increase the user friendliness of biomarkers in atopic dermatitis. Both methods offer simple, non-invasive and cost effective alternatives to venous blood. This provides great translational and clinical potential. Biomarkers will play an increasingly important role in AD research and personalized medicine. The use of biomarkers will enhance the efficacy of AD treatment by facilitating the individualization of therapy targeting the patients' specific biological signature and also by providing tools for predicting and monitoring of therapeutic response.

INTRODUCTION

The World Health Organization has defined a biomarker as "any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease".[1] Biomarkers have been used in clinical practice for many years. For example, prostate-specific antigen (PSA) is a commonly used biomarker and is used for the follow-up in prostate cancer, as well as serum creatinine levels for diagnosing renal insufficiency.[2, 3] Additionally, in the past decade rapid advances in genomic and proteomic technologies have generated a plethora of candidate biomarkers ranging from antibodies, microbes, DNA, RNA, lipids, metabolites to proteins.[4]

The application of biomarkers in medicine is evolving. Biomarkers do not only give us a better understanding of pathogenesis, but also increase treatment efficacy and safety further enabling more precise clinical care.

Biomarkers can be categorized in different types depending on their specific characteristics. They can be used to identify the risk of developing a disease (screening biomarkers), to identify a disease (diagnostic biomarker), predict disease progression (prognostic biomarker), mark a particular pharmacological response (pharmacodynamic biomarkers), and for monitoring disease activity and clinical response to an intervention or as a surrogate endpoint in clinical trials (severity biomarker).[5] Moreover, biomarker based stratification may identify clinically relevant subgroups and help to provide effective targeted therapies (predictive biomarker).[6]

This paper focuses on the current use of biomarkers in atopic dermatitis (AD), new developments and future perspectives.

BIOMARKERS IN ATOPIC DERMATITIS

Diagnostic biomarker

Atopic dermatitis remains a clinical diagnosis without an objective and reliable biomarker to confirm the diagnosis. Classically AD has been divided into two subtypes, intrinsic versus extrinsic. Patients with an intrinsic form show normal total IgE levels, without specific IgE and have also been termed non-atopic or non-allergic dermatitis. Comparatively, patients with an extrinsic form of AD show high total IgE levels and are often sensitized to multiple allergens.[7] Since total serum IgE levels are not increased in about 20% of AD patients, total serum IgE cannot be used as a diagnostic biomarker for all AD patients.[7] Moreover, total serum IgE levels are also increased in patients with other atopic diseases such as allergic rhinoconjunctivitis and allergic asthma.[8] Using a genomic profiling approach, Suárez-Farinas et al., recently showed common

disease-defining features in patients with intrinsic and extrinsic AD.[9] Interestingly, they found similar Th2 type immune activation in intrinsic and extrinsic AD patients, suggesting that Th2 is not the only cause of high IgE levels in patients with extrinsic AD.

Severity Biomarker

Patients with severe disease tend to have higher IgE levels, but there are also patients with severe eczema that do not show increased IgE levels. Total serum IgE levels are therefore not a reliable biomarker for disease severity.[10] Other frequently reported serum biomarkers for disease severity in AD include eosinophilic cationic protein (ECP),[11] sIL-2R,[12] and thymus and activation-regulated chemokine (TARC/CCL17).[13] In a systemic review on serum biomarkers for disease severity in AD we found that serum TARC levels showed the best correlation to disease severity, with weighted mean r-values of 0.51 and 0.63 in longitudinal and cross-sectional studies, respectively.[14] However, we have shown that patients with severe AD can have serum TARC levels in the normal range, and on the other hand patients with mild to moderate disease may express high TARC levels.[15] This might be explained by the large number of biological pathways involved in the pathogenesis of AD and the clinical heterogeneity.

Thus, we hypothesized that a combination of biomarkers can overcome these problems, by providing more information on different biological pathways and would be applicable to different phenotypical subtypes and thereby correlate better to disease severity. We recently demonstrated that indeed a multivariate signature including four serum biomarkers showed a correlation coefficient of 0.86 to disease severity measured by the six area, six sign atopic dermatitis severity score (SASSAD).[16] Although this was only a pilot study in 17 patients, it showed that using a panel of biomarkers may be necessary in a multifactorial, complex disease such as AD.[17]

Currently, there is no gold standard for measurement of disease severity in AD and more than 20 different composite indices have been described.[18] The Harmonizing Outcome Measurements in Eczema (HOME) initiative is working on a core set of outcome measures but currently there is no consensus (www.homeforeczema.org). The ultimate goal from the perspective of evidence-based medicine is to achieve worldwide consensus to consistently apply a single valid, reliable, and feasible instrument to measure disease severity for AD. We suggest that the use of a panel of biomarkers can add important information or even substitute clinical endpoints, and can be used in daily practice to track changes in disease activity and adapt treatment accordingly. Moreover, the use of biomarkers as a surrogate endpoint in clinical trials will improve comparisons across trials and facilitate meta analyses. With the recent introduction of the first biological for AD[19] further studies are needed to explore the use of a panel of biomarkers as a disease severity measure and possibly treatment predictive tool.

Predictive Biomarker

The introduction of novel agents and "targeted" therapies also drives the need for predictive biomarkers. Because of disease heterogeneity, stratification of subgroups is essential in the development of such a predictive tool. Biomarkers can be used to identify subgroups of patients with shared "biological" characteristics, which are more likely to respond favorably to a given therapy. Using stratification, targeted therapies can be assigned to different subgroups, making personalized medicine possible.

ALTERNATIVE WAYS TO MEASURE BIOMARKERS

Blood is the most commonly used body fluid for biomarker measurements.[20] However, collection of blood is invasive and less suitable for use in the field because of the need for trained personnel. It is also less favored in pediatric medicine, especially since atopic dermatitis usually presents in childhood. The same is true for gene expression profiles determined in skin biopsies. Studies have identified specific molecular signatures for AD patients,[21] and changing expression profiles during therapy.[19, 22] Although tissue biomarker expression patterns may also be extremely helpful in stratification and the identification of new pathways involved in the pathogenesis of AD, they require trained personnel and specialized labs.

We therefore explored alternatives for use in daily practice and longitudinal studies. Specifically dried blood spots (DBS) and saliva as potential alternatives. DBS have been used for decades in screening for inherited metabolic diseases in newborns [23] and can be obtained using a simple, minimally invasive, nearly painless procedure that can be done by the patients themselves. Secondly, saliva is a mirror of the body's health as a wide spectrum of biomolecules is transported from the blood capillaries through the epithelium of salivary glands.[24] Salivary cortisol levels are for instance routinely used as a biomarker of psychological stress. We have preliminary data showing that several inflammatory biomarkers can be measured in saliva samples from AD patients. DBS and saliva may be used as an accurate non-invasive alternative to serum measurements; both methods will subsequently be discussed.

Dried blood spots (DBS)

DBS are samples from drops of capillary whole blood collected from a finger stick and dried on filter paper.[25] Filter paper was first used as a scientific tool in 1815 by the Swedish chemist Jöns Berzelius. Robert Guthrie is widely credited as being the first to use blood dried on filter paper (so-called Guthrie cards) to diagnose phenylketonuria in neonates in 1963.[26] Since that time, filter paper has become a commonly used method

of storing and transporting diverse specimens. Different aspects of the use of DBSs have been reviewed; DBS used for newborn screening assays, for epidemiological studies, human immunodeficiency virus (HIV) detection and monitoring, virology and drug assays.[27, 28]

Collection of a DBS is a relatively simple and minimally invasive, nearly painless procedure. The participant's finger is first wiped with alcohol. A sterile lancet is then used to puncture the skin. The first drop of blood is dabbed, the subsequent four drops of blood are applied to filter paper. The paper is left to dry for a few hours and then stored at room temperature or refrigerated before shipment to the laboratory.[25, 26] Most analytes remain stable for more than a week in filter paper when stored at room temperature.[29]

Sample processing is relatively easy and the requirements are minimal. To extract the analytes from DBS, small discs are punched from the filter paper and eluted with a buffer. Current immunological essay technologies, like multiplex bead-based immunoassay and mass spectrometry, require only small volumes, which provides the ability to measure multiple proteins and peptides from a single DBS sample.[26] Chambers et al. demonstrated that a panel of 40 proteins could be quantitatively extracted from DBS using highly-multiplexed mass spectrometry.[30]

The ease of sample collection, minimal training requirements, and self-applicability by the patient at home are great advantages of DBS sampling. The minimal volume requirements, the stability of analytes for months to years and the ease of sample processing offers practical and financial advantages, making DBS also very suitable for storage in biobanks.[31] Additionally, the low burden and minimally invasive procedure is better suited for pediatric studies and also allows collection of multiple samples over time in longitudinal study designs.[31]

Saliva

In humans, three major glands produce saliva: the parotid, submandibular and sublingual gland. Together they produce over 90% of the total amount of saliva. Saliva is also produced by hundreds of small salivary glands, spread throughout the oral cavity.[32] Epithelial cells generate saliva and it is secreted via salivary ducts into the oral cavity.

Salivary glands are highly permeable and are surrounded by capillaries. These characteristics enable free exchange of molecules from blood to the acinus and therefore it is thought that biomarkers, circulating in blood, can diffuse from blood to the acinar cells and eventually secreted in saliva [33]. This makes saliva an interesting source for the measurement of biomarkers using epigenetic, transcriptomic, proteomic and metabolomic approaches[34] Over the last decade, numerous studies have investigated biomarkers in saliva related to specific diseases. This includes studies on Sjögren's syndrome, rheumatic-, cardiovascular-, and periodontal diseases.[33, 35-37] However, most of these studies need validation before clinical implementation.

The main advantage of saliva over blood sampling is the non-invasive nature of collection, which is particularly interesting for studies in children. Saliva sampling also facilitates and eases the collection of multiple subsequent samples for disease monitoring and longitudinal study designs. Collection and handling of saliva samples is relatively easy, does not require trained personnel and compared to blood, saliva samples cannot clot. In addition, saliva is safer compared to blood regarding the risk of transmission of viruses, such as HIV.[33]

Although saliva seems to be the ideal source for measuring biomarkers, there are many variables regarding the collection and handling of samples.[38] Moreover, the composition and protein concentrations in saliva are influenced by many factors such as age, gender, hydration status, flow rate, time of sampling and diet.[39]

In conclusion, saliva collection is simple and non-invasive and offers great opportunities. However, sta dardization of the methods for collection and handling of saliva samples is required before introduction in daily practice.

CONCLUSIONS

In recent years, biomarkers have been a growing field of study in medicine. Technical advancements now enable the measurement of many biomarkers in small volumes and have been illustrated to be extremely useful in medicine. The application of biomarkers in AD offers great opportunities, both in daily clinical practice as well as for research purposes.

Biomarkers offer reliable and objective outcome measures. Although serum TARC levels provide an excellent adjunct to daily practice and clinical trials, there are limitations to the use of a single biomarker in a complex disease such as AD. We are convinced that a panel of biomarkers will replace traditional outcome measurements in the near future. This will result in clinical trials being more 'comparable', which will prove to be essential with the introduction of biologicals in the treatment of AD.

The heterogeneous character of the disease and lack of clinical stratifiers makes AD highly suitable for a biomarker based stratification. A panel of biomarkers can assess multiple molecular entities, and will be more suitable for assessing disease severity in AD compared to an individual biomarker. Biomarker stratification will identify subgroups of patients that will respond to treatment and enable tailoring of drugs to the biological signature of individual patients, accelerating the translation of new medicine from bench to bedside and can revolutionize AD therapy.

Alternative sources for biomarkers such as saliva and capillary blood can increase the user friendliness of biomarkers in AD. Moreover, it will increase our understanding of the disease as they allow early sampling, for example during an exacerbation. Both DBS and saliva offer simple, non-invasive and cost effective alternatives to venipuncture.[23, 40] The patients can complete collection procedures themselves and handling procedures are relatively simple. This offers great translational and clinical potential, not only for adults but also in pediatric populations. However, both methods need further validation.

In conclusion, biomarkers will play an increasingly important role in AD research and personalized medicine. The use of biomarkers will enhance the efficacy of AD treatment by facilitating the individualization of therapy targeting the patient's specific biological signature and also by providing tools for predicting and monitoring of therapeutic response.

REFERENCES

1. WHO International Programme on Chemical Safety Biomarkers in Risk Assessment: Validity and Validation. http://www.inchem.org/documents/ehc/ehc/ehc222.htm; 2001.].
2. Levey AS, Coresh J, Balk E, Kausz AT, Levin A, Steffes MW, et al. National Kidney Foundation practice guidelines for chronic kidney disease: evaluation, classification, and stratification. Ann Intern Med 2003; 139:137-47.
3. Catalona WJ, Smith DS, Ratliff TL, Dodds KM, Coplen DE, Yuan JJ, et al. Measurement of prostate-specific antigen in serum as a screening test for prostate cancer. N Engl J Med 1991; 324:1156-61.
4. Blumenberg M. Skinomics: past, present and future for diagnostic microarray studies in dermatology. Expert Rev Mol Diagn 2013; 13:885-94.
5. Bieber T, Cork M, Reitamo S. Atopic dermatitis: a candidate for disease-modifying strategy. Allergy 2012; 67:969-75.
6. Frank R, Hargreaves R. Clinical biomarkers in drug discovery and development. Nat Rev Drug Discov 2003; 2:566-80.
7. Akdis CA, Akdis M. Immunological differences between intrinsic and extrinsic types of atopic dermatitis. Clin Exp Allergy 2003; 33:1618-21.
8. Levin AM, Mathias RA, Huang L, Roth LA, Daley D, Myers RA, et al. A meta-analysis of genome-wide association studies for serum total IgE in diverse study populations. J Allergy Clin Immunol 2013; 131:1176-84.
9. Suarez-Farinas M, Dhingra N, Gittler J, Shemer A, Cardinale I, de Guzman Strong C, et al. Intrinsic atopic dermatitis shows similar TH2 and higher TH17 immune activation compared with extrinsic atopic dermatitis. J Allergy Clin Immunol 2013; 132:361-70.
10. Eichenfield LF, Tom WL, Chamlin SL, Feldman SR, Hanifin JM, Simpson EL, et al. Guidelines of care for the management of atopic dermatitis: section 1. Diagnosis and assessment of atopic dermatitis. J Am Acad Dermatol 2014; 70:338-51.
11. Czech W, Krutmann J, Schopf E, Kapp A. Serum eosinophil cationic protein (ECP) is a sensitive measure for disease activity in atopic dermatitis. Br J Dermatol 1992; 126:351-5.
12. Kagi MK, Joller-Jemelka H, Wuthrich B. Correlation of eosinophils, eosinophil cationic protein and soluble interleukin-2 receptor with the clinical activity of atopic dermatitis. Dermatology 1992; 185:88-92.
13. Kakinuma T, Sugaya M, Nakamura K, Kaneko F, Wakugawa M, Matsushima K, et al. Thymus and activation-regulated chemokine (TARC/CCL17) in mycosis fungoides: serum TARC levels reflect the disease activity of mycosis fungoides. J Am Acad Dermatol 2003; 48:23-30.
14. Thijs J, Krastev T, Weidinger S, Buckens CF, de Bruin-Weller M, Bruijnzeel-Koomen C, et al. Biomarkers for atopic dermatitis: a systematic review and meta-analysis. Curr Opin Allergy Clin Immunol 2015; 15:453-60.
15. Landheer J, de Bruin-Weller M, Boonacker C, Hijnen D, Bruijnzeel-Koomen C, Rockmann H. Utility of serum thymus and activation-regulated chemokine as a biomarker for monitoring of atopic dermatitis severity. J Am Acad Dermatol 2014; 71:1160-6.

16. Charman CR, Venn AJ, Williams HC. Reliability testing of the Six Area, Six Sign Atopic Dermatitis severity score. Br J Dermatol 2002; 146:1057-60.

17. Thijs JL NS, Herath A, Bruijnzeel-Koomen CAFM, Giovanonne B, Knol E, de Bruin-Weller MS, Hijnen DJ. A panel of biomarkers for disease severity in atopic dermatitis Clinical Experimental Allergy, accepted for publication.

18. Schmitt J, Langan S, Williams HC. What are the best outcome measurements for atopic eczema? A systematic review. J Allergy Clin Immunol 2007; 120:1389-98.

19. Beck LA, Thaci D, Hamilton JD, Graham NM, Bieber T, Rocklin R, et al. Dupilumab treatment in adults with moderate-to-severe atopic dermatitis. N Engl J Med 2014; 371:130-9.

20. Schulz BL, Cooper-White J, Punyadeera CK. Saliva proteome research: current status and future outlook. Crit Rev Biotechnol 2013; 33:246-59.

21. Quaranta M, Knapp B, Garzorz N, Mattii M, Pullabhatla V, Pennino D, et al. Intraindividual genome expression analysis reveals a specific molecular signature of psoriasis and eczema. Sci Transl Med 2014; 6:244ra90.

22. Hamilton JD, Suarez-Farinas M, Dhingra N, Cardinale I, Li X, Kostic A, et al. Dupilumab improves the molecular signature in skin of patients with moderate-to-severe atopic dermatitis. J Allergy Clin Immunol 2014; 134:1293-300.

23. Li W LM. Dried Blood Spots: Applications and Techniques: Wiley; 2014.

24. Topkas E, Keith P, Dimeski G, Cooper-White J, Punyadeera C. Evaluation of saliva collection devices for the analysis of proteins. Clin Chim Acta 2012; 413:1066-70.

25. McDade TW. Development and validation of assay protocols for use with dried blood spot samples. Am J Hum Biol 2014; 26:1-9.

26. Lehmann S, Delaby C, Vialaret J, Ducos J, Hirtz C. Current and future use of "dried blood spot" analyses in clinical chemistry. Clin Chem Lab Med 2013; 51:1897-909.

27. Smit PW, Elliott I, Peeling RW, Mabey D, Newton PN. An overview of the clinical use of filter paper in the diagnosis of tropical diseases. Am J Trop Med Hyg 2014; 90:195-210.

28. McCann SJ, Gillingwater S, Keevil BG, Cooper DP, Morris MR. Measurement of total homocysteine in plasma and blood spots using liquid chromatography-tandem mass spectrometry: comparison with the plasma Abbott IMx method. Ann Clin Biochem 2003; 40:161-5.

29. Ostler MW, Porter JH, Buxton OM. Dried blood spot collection of health biomarkers to maximize participation in population studies. J Vis Exp 2014:e50973.

30. Chambers AG, Percy AJ, Yang J, Camenzind AG, Borchers CH. Multiplexed quantitation of endogenous proteins in dried blood spots by multiple reaction monitoring-mass spectrometry. Mol Cell Proteomics 2013; 12:781-91.

31. McDade TW, Williams S, Snodgrass JJ. What a drop can do: dried blood spots as a minimally invasive method for integrating biomarkers into population-based research. Demography 2007; 44:899-925.

32. Moore K DA, Agur A. . In: Clinically Oriented Anatomy: Lippincott Williams & Wilkins 2009.

33. Yoshizawa JM, Schafer CA, Schafer JJ, Farrell JJ, Paster BJ, Wong DT. Salivary biomarkers: toward future clinical and diagnostic utilities. Clin Microbiol Rev 2013; 26:781-91.

34. Bonne NJ, Wong DT. Salivary biomarker development using genomic, proteomic and metabolomic approaches. Genome Med 2012; 4:82.

35. Tandon M, Gallo A, Jang SI, Illei GG, Alevizos I. Deep sequencing of short RNAs reveals novel microRNAs in minor salivary glands of patients with Sjogren's syndrome. Oral Dis 2012; 18:127-31.

36. Nagler RM, Salameh F, Reznick AZ, Livshits V, Nahir AM. Salivary gland involvement in rheumatoid arthritis and its relationship to induced oxidative stress. Rheumatology (Oxford) 2003; 42:1234-41.

37. Mirzaii-Dizgah I, Agha-Hosseini F. Unstimulated whole saliva parathyroid hormone in postmenopausal women with xerostomia. J Contemp Dent Pract 2011; 12:196-9.

38. Williamson S, Munro C, Pickler R, Grap MJ, Elswick RK, Jr. Comparison of biomarkers in blood and saliva in healthy adults. Nurs Res Pract 2012; 2012:246178.

39. Quintana M, Palicki O, Lucchi G, Ducoroy P, Chambon C, Salles C, et al. Inter-individual variability of protein patterns in saliva of healthy adults. J Proteomics 2009; 72:822-30.

40. Liu J, Duan Y. Saliva: a potential media for disease diagnostics and monitoring. Oral Oncol 2012; 48:569-77.


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Biomarkers detected in dried blood spots from atopic dermatitis patients strongly correlate with disease severity

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ABSTRACT

Background:

Serum biomarkers offer an objective outcome measure for disease severity in atopic dermatitis (AD). A disadvantage of serum biomarkers is the need for a venipuncture. Dried blood spots (DBS) offer a convenient alternative to venipuncture. DBS can be obtained using a minimally invasive finger prick performed by the patients themselves. The aim of this study was to investigate if biomarkers determined in DBS offer a reliable tool for the assessment of disease severity in AD.

Methods:

Sixty-five AD patients were treated with topical steroids and followed-up for 2 months. Additionally, 14 psoriasis patients and 26 non-atopic controls were included. Disease severity was assessed by EASI, POEM and VAS pruritis. Biomarkers IL-18, IL-22, IL-31, TARC, PARC, MDC sIL-2R, sE-selectin, SDF-1α and I309 were measured in serum and DBS. Stability of TARC in DBS was studied during storage at room temperature, -20℃ and after freeze/thawing.

Results:

Levels of I-309, TARC, PARC, and MDC in DBS were significantly higher in AD than in psoriasis and healthy controls. TARC showed the strongest correlation with disease severity (r=0.68). Interestingly, levels of TARC measured in DBS showed a stronger correlation with EASI than TARC levels measured in serum (r=0.58). Linear mixed models showed that TARC, I-309, PARC, and MDC measured in DBS significantly decrease during effective treatment. Storage at room temperature for 7 days, long-term storage at -80˚, and freeze/thaw cycles did not influence TARC levels in DBS.

Discussion:

In this study we show that TARC level measured in DBS from AD patients highly correlate with disease severity and significantly decrease during effective treatment with topical steroids.

INTRODUCTION

Atopic dermatitis (AD) is a chronic inflammatory skin disease, with a prevalence of 10-20% worldwide.[1] AD is characterized by itch and a relapsing and remitting pattern, and greatly affects patients quality of life. Assessment of disease severity in AD is essential for monitoring treatment effects and for comparison of clinical trials investigating new compounds for AD treatment. Unfortunately, AD disease severity is assessed by an array of time consuming clinical scoring measures that lack harmonization between trials and centers . A systematic review of AD severity measurement tools found that 91% of AD clinical trials used a severity measure, but less than a third of these scales had been previously published.[2] In addition, the validity and reliability of the 20 most commonly used severity measures for AD was assessed by Schmitt et al., and found that only three of the measures performed adequately.[3] To address these shortcomings in AD severity reporting, experts in the field have established the Harmonizing Outcome Measurements in Eczema (HOME) initiative, an attempt to ensure that investigators employ a core set of outcome measures to enhance comparability between studies.[4] We suggest that in addition to improvements in clinical outcome reporting, an objective biomarker for disease severity would be of great value for clinical research in AD.

New potential biomarkers are generated by the discovery of novel cytokines and chemokines. A large number of these serum biomarkers have been found to correlate with disease severity in AD. In a meta-analysis on serum biomarkers for disease severity we found that serum TARC is the most reliable biomarker currently available.[5] Due to the complex pathogenesis of AD, a combination of several biomarkers from different biological pathways may better correlate to disease severity than a single biomarker. Indeed, we recently demonstrated that a multivariate signature consisting of TARC, IL-22, and sIL-2R shows a strong correlation with disease severity in AD patients.[6, 7]

A disadvantage of the use of serum biomarkers is the need for a venipuncture that can only be performed by trained personnel. Furthermore, this blood needs to be processed in a lab and must be centrifuged within four hours of collection, which is costly, not practical and difficult to harmonise between different centers in clinical trials. Dried blood spots (DBS) are a convenient alternative to venipuncture. DBS can be obtained using a simple, minimally invasive, nearly painless procedure that can be done by the patients themselves. DBS have been used for decades in screening for inherited metabolic diseases in newborns.[8] Measurement of cytokines and other inflammatory markers in DBS has been reported in several studies.[9-11] However, no studies measured biomarkers in DBS during follow-up in AD patients or investigated their correlation with disease severity in AD. DBS may offer a simple and minimally invasive tool for objective measurement of disease severity in AD, which can improve monitoring of patients and the comparison of clinical trials. The aim of this study was to investigate if biomarkers determined in DBS offer a reliable tool for the assessment of disease severity in AD.

METHODS

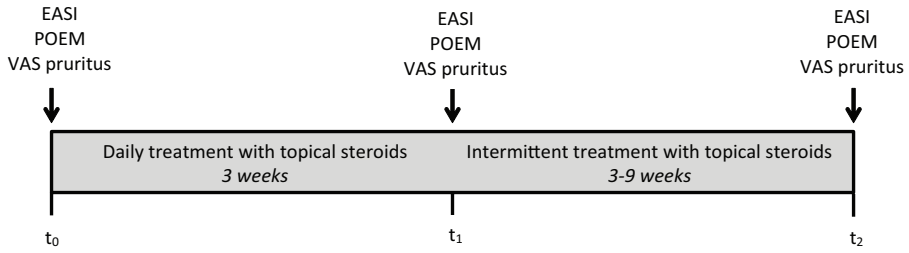
Patients and controls

A cohort of 65 adult AD patients, as defined by the criteria of Hanifin and Raijka,[12] was recruited at the outpatient clinic of the University Medical Center Utrecht. Patients were followed for approximately two months. Sample size was calculated using correlation coefficients from a previously conducted study,[6] applying a Fisher's z-test for Pearson correlation in SAS version 9.2 (SAS Institute, Cary, NC, USA) with the following parameters: one-sided, α=0.05, and power=0.8, and resulted in 60 patients.

During the first visit (t_0) serum and DBS were collected and all patients started with daily application of potent topical steroids (European classification class III: fluticasone furoate, mometasone furoate or betamethasone dipropionate) according to the fingertip unit.[13] During a second visit (t_1) after three weeks, serum and DBS were collected, and patients were instructed to taper the frequency of topical corticosteroid application. Collection of serum and DBS was repeated after 6 to 12 weeks (t_2) of treatment. Patients using oral immunosuppressive drugs or UV-therapy within three months before baseline were excluded from the study. The outline of the study protocol is summarized in Figure 1.

Fourteen psoriasis patients were included as disease controls during a visit to the outpatient clinic and were all treated with topical steroids only. Serum and DBS were collected on a single time point. A second control group consisted of 26 non-atopic healthy controls. The healthy controls were age and sex matched to the AD patients. The protocols of this study were approved by the Institutional Review Board of the University Medical Center Utrecht (Utrecht, The Netherlands), adhering to the Declaration of Helsinki Principles.

Figure 1. Study design.



Patients were included during their first visit to the outpatient clinic (t_0). After t_0 all patients were treated with daily potent topical steroids (European classification class III). After three weeks (t_1) the use of topical corticosteroids was tapered. Disease severity was assessed by EASI, POEM and VAS pruritis at all three timepoints (t_0, t_1, t_2); DBS were collected at the same timepoints. EASI; Eczema Area and Severity Index, POEM; Patient-Oriented Eczema Measure, VAS; visual analogue scale.

Disease severity

The HOME initiative recommends to use the eczema area and severity index (EASI)[14] and the patient oriented eczema measure (POEM)[15] in AD clinical trials. Therefore, we choose to use the EASI as a measurement tool for the follow-up of disease severity and complemented this with the patient reported outcome measures POEM and a pruritis Visual Analogue Scales (VAS).[16] Disease severity in psoriasis patients was assessed by the Psoriasis Area Severity Index (PASI),[17] the self-administered psoriasis area, and severity index (SAPASI),[18] and VAS pruritis.

Dried blood spots

Capillary blood was obtained by a fingerprick lancet (Super Blade, 1.5mm blade/ penetration depth 1.6 mm, Sarstedt) from middle or ring finger and spotted onto filter paper to create DBS (following the protocol published by Ostler et al.).[19] Spotted filter papers were dried overnight at room temperature and subsequently stored with desiccant in individual air tight polyethylene bags at -80°C under constant monitoring of humidity levels until analysis.

For elution of DBS two 3.2 mm disks (containing approximately 3µl of whole blood each) were punched from the central part of each spot into a filter plate well (Merck Chemicals) and 100 µl elution buffer was added per well (PBS containing 5mL/L Tween-20, 10g/L bovine serum albumin and complete protease inhibitor cocktail with EDTA (Roche, one tablet per 25mL buffer)). Plates were sealed and placed overnight at 4°C on a microshaker set to 600 rpm to extract the analytes. Finally, plates were spun down at 2100 g for 2 minutes.

Stability of biomarkers in DBS

After collection, DBS cards are dried for 2-3 hours at room temperature. After drying, DBS can be transported at ambient temperature. To investigate whether biomarkers in DBS stay stable during this transport period, we measured biomarker levels in DBS, stored at room temperature, from 5 AD patients after 1, 3, and 7 days of collection. To study long term stability in DBS, we included DBS from 17 AD patients that were stored at -80°C. Biomarker levels were measured after collection and after 34 months of storage. The influence of freeze thaw cycles on biomarker levels in DBS were also studied. DBS from 5 AD patients were thawed and frozen 3 times, biomarker levels were measured after each cycle.

Measurement of analytes in serum and DBS

A recently published study showed that the biomarkers TARC, MDC, PARC, IL-22, sE-selectin, sIL-2R, IL-18, I-309, SDF-1α showed good correlation with disease severity when measured in serum.[7] We therefore measured these biomarkers both in serum and in the eluted DBS using a Luminex platform. Multiplex immunoassays were performed at the MultiPlex Core Facility of the Laboratory of Translational Immunology (UMC Utrecht, The Netherlands) using an in-house validated panel of analytes as previously described.[20]

Statistical analysis

Patient demographics and sample characteristics were evaluated by an unpaired t-test. Analyte measurements that were above or below the assay limits of detection were given values equivalent to the upper and lower limits respectively. Biomarker concentrations of TARC, PARC, MDC and sE-selectin measured in serum and DBS were normalized by log-transformation. Correlation coefficients were tested using a Pearson correlation test. Levels of biomarkers between groups were compared with ANOVA. Linear mixed models were used to model the change over time of biomarkers after treatment initiation. This kind of model generalizes regression models by taking into account correlation between measurements in longitudinal data. We investigated the effects of the different biomarkers on EASI scores using a piecewise linear mixed model. For all AD patients, two slopes were considered: one for the first treatment period (between t_0 and t_1) and one for second treatment period (between t_1 and t_2). The time for the slope's change (t = 22 days) was based on the median duration of the first treatment period. Correlation between individual baseline values and the subsequent slopes was handled through the unstructured covariance matrix of random effects. Models were adjusted for age and gender. To test if a combination of biomarkers improves prediction of EASI compared to a single biomarker, an EASI prediction model was built using the Akaike Information Criteria (the lower the better) which quantifies the quality of the fit to the data. The criteria were used to identify the model that best described the behaviour of EASI within the AD patients. Differences in biomarker levels in the stability tests were compared by a t-test in case of two measurements and by repeated measures ANOVA in case of more than two measurements. P-values < 0.05 were considered statistically significant. All statistical analyses were conducted using SAS version 9.4. (SAS Institute, Cary, North Carolina, USA) and Prism (version 6; Graphpad).

RESULTS

Study patients

A cohort of 65 AD patients, 14 psoriasis patients and 26 healthy controls were included. AD patients had a median age of 32 years (interquartile range (IQR) 23-47) and disease severity ranged from mild to severe (median EASI score 17.4, IQR 10.0-25.7)(Table 1). Patients started treatment with daily application of potent topical steroids after inclusion (t_0) in the study. Median duration of treatment with daily topical steroids was 22 days (IQR 13-42). The use of topical corticosteroids was tapered to what we consider a safe maintenance scheme at t_1. Patients were followed-up for median 57 days (IQR 35-107). After treatment initiation disease severity decreased to a median EASI score of 3.3 (IQR 1.2- 5.6) at t_1, and remained stable until t_2 (median EASI score 2.7, IQR 1.0-4.6). POEM score and VAS pruritis showed a similar significant decrease during the first treatment period and also remained stable during the second treatment period (Figure 2). Psoriasis patients had a median age of 39 years (IQR 28-68) and a median PASI score of 39.4 (IQR 27.6-67.5). Non-atopic healthy controls had a median age of 37 years (IQR 30-54)(Table 1).

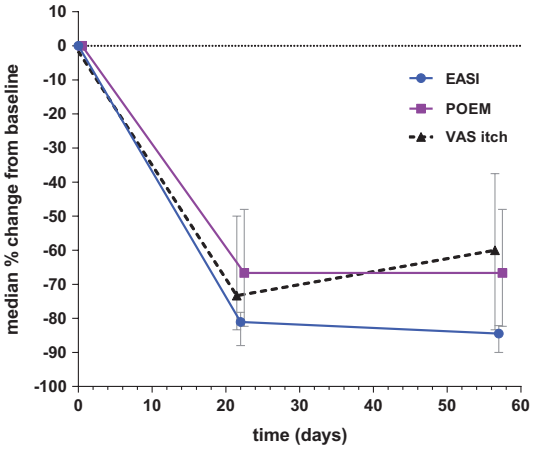
Table 1. Baseline characteristics

	AD patients (n=65)	Psoriasis patients (n=14)	Healthy controls (n=26)
Age, yrs	31.6 (22.6-46.7)	39.4 (27.6-67.5)	36.5 (29.5-54.3)
Male, n (%)	31 (47)	8 (67)	13 (50)
EASI	17.4 (10.0-25.7)	N/A	N/A
POEM	21.0 (17.0-25.0)	N/A	N/A
PASI	N/A	4.2 (2.3-7.5)	N/A
SAPASI	N/A	7.8 (4.8-17.6)	N/A
VAS pruritis	7.5 (6.0-8.0)	5.0 (0.0-7.0)	N/A

Categorical variables are presented as counts and percentages; continues variables are presented as median (InterQuartileRange). ANOVA testing revealed no significant differences in age or gender between the groups. N/A; not available.

Figure 2. Change in EASI, POEM and VAS pruritis in AD patients during treatment.

A.



B.

Severity scores at baseline and during treatment			
Timepoint	**Variable**	**N**	**Median (IQR)**
Baseline (t_0)	EASI	64	17.4 (10.0-25.7)
	POEM	63	21.0 (17.0-25.0)
	VAS pruritis	51	7.5 (6.0-8.0)
t_1	Time since t_0 (days)	63	22.0 (13.0-42.0)
	EASI	62	3.3 (1.2-5.6)
	POEM	62	7.0 (3.0-13.0)
	VAS pruritis	51	2.0 (1.0-4.0)
t_2	Time since t_0 (days)	43	57.0 (35.0-107.0)
	EASI	43	2.7 (1.0-4.6)
	POEM	43	7.0 (3.0-13.0)
	VAS pruritis	35	3.0 (1.0-5.0)

A. median percentage change from baseline for EASI, POEM and VAS pruritis in AD patients during treatment with local topical steroids. B. Median and InterQuartileRanges of EASI, POEM, VAS pruritis, and time of follow-up. EASI; Eczema Area and Severity Index, POEM; Patient-Oriented Eczema Measure, VAS; visual analogue scale.

Baseline DBS biomarkers

Several biomarkers that were measured in DBS at t_0 (baseline) in AD patients showed significant correlations with disease severity measured by EASI (Table 2). TARC showed the strongest correlation with disease severity ($r=0.68$, $p<0.0001$), followed by I-309 ($r=0.52$ $p<0.001$), MDC ($r=0.51$ $p<0.001$), and PARC ($r=0.38$ $p<0.01$).

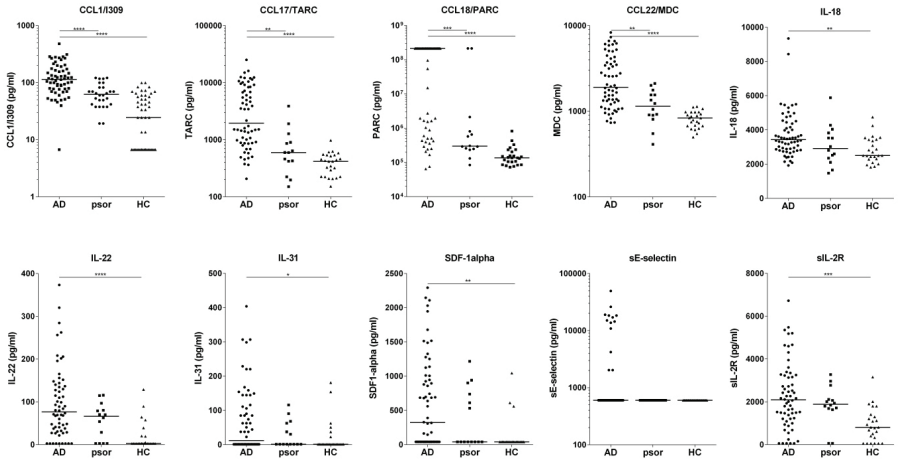
Levels of I-309, TARC, PARC, and MDC in DBS at t_0 were significantly upregulated in AD patients compared psoriasis patients and healthy controls ($p<0.01$) (Figure 3). IL-18, IL-22, IL-31, sIL-2R, and SDF-1α levels measured in DBS from AD patients were significantly higher than in healthy controls ($p<0.05$), but no significant difference was seen between AD and psoriasis patients (Figure 3). No difference was seen between sE-selectin levels measured in DBS from AD patients, psoriasis patients or healthy controls.

Table 2. Correlation between disease severity and biomarkers.

	EASI vs. serum biomarker	EASI vs. DBS biomarker	serum vs. DBS biomarker
I-309	0,41***	0,52****	0,86****
log TARC	0,58****	0,68****	0,90****
log PARC	0,40**	0,38**	0,50****
log MDC	0,41***	0,51****	0,84****
IL-18	0,01	0,13	-0,04
IL-22	0,34**	0,34**	0,27*
IL-31	0,06	0,13	0,30*
SDF-1α	-0,08	0,17	0,22
log sE-selectin	0,39**	0,23	0,32*
sIL-2R	0,08	0,20	0,28*

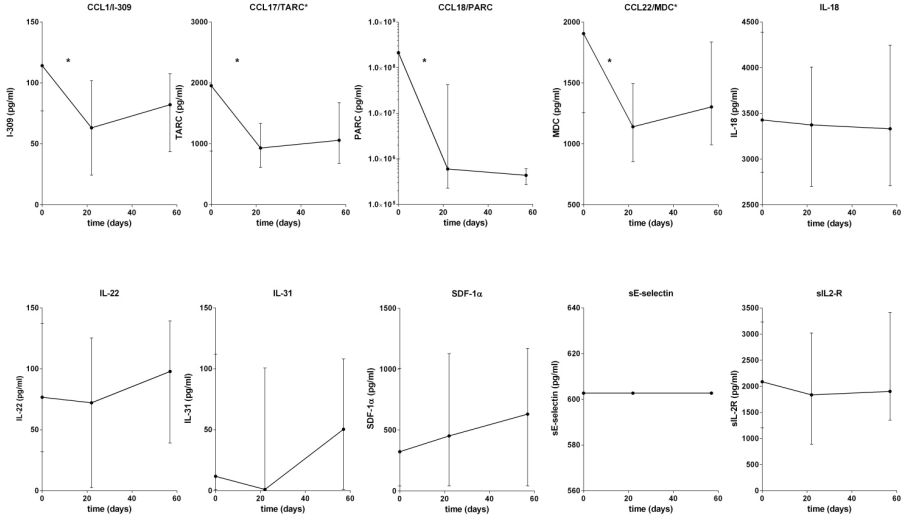
Pearson correlations between disease severity measured by EASI and biomarkers measured in serum and DBS from 64 AD patients. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

Figure 3. Biomarker levels in DBS from AD patients, psoriasis patients and healthy controls.



Differences in biomarker levels between AD patients, psoriasis patients and healthy controls were analysed by ANOVA testing. Horizontal bars represent median biomarkers levels. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. AD; atopic dermatitis; psor; psoriasis; HC; healthy controls. The biomarkers I-309, TARC, PARC, and MDC are significantly higher in DBS from AD patients compared to psoriasis patients and healthy controls. IL-18, IL-22, IL-31, SDF-1α, and sIL-2R levels in DBS are significantly higher in AD patients than in healthy controls, but no difference was found between AD and psoriasis patients. sE-selectin levels measured in DBS from AD patients, psoriasis patients an healthy controls did not differ.

Figure 4. Biomarker levels measured in DBS during treatment of AD patients.



Median biomarker levels measured in DBS during treatment of AD patients. Error bars represent 95% confidence intervals. Linear mixed models were used to model the changes over time of biomarkers after treatment initiation with topical steroids. The biomarkers I-309, TARC, PARC, and MDC showed a significant decrease (*<.0001) during daily treatment with topical steroids (day 0 until day 22) and then remained at stable levels during intermittent treatment with topical steroids (after day 22). P-values are shown in Table 3.

Comparison of DBS and serum biomarkers

To investigate the relationship between biomarker levels measured in serum and DBS, blood was simultaneously drawn when DBS were collected. TARC levels measured in DBS showed a correlation of 0.90 with TARC levels measured in serum ($p<0.0001$)(Table2). I-309 and MDC levels in DBS also highly correlated with levels in serum ($r=0.86$ and $r=0.84$, $p<0.0001$). Biomarkers that showed significant, but less strong, correlations between DBS and serum included PARC, IL-22, IL-31, sE-selectin and sIL-2R. Interestingly, levels of TARC measured in DBS showed a stronger correlation with EASI than TARC levels measured in serum ($r=0.68$ vs. $r=0.58$ respectively)(Table 2).

DBS biomarker changes after treatment initiation

Linear mixed models were used to model the changes over time of DBS biomarkers after treatment initiation with topical steroids. The biomarkers I-309, TARC, PARC, and MDC showed a significant decrease during daily treatment with topical steroids (between t_0 and t_1) and then remained at stable levels during intermittent treatment with topical steroids (between t_1 and t_2) (Figure 4, Table 3). The biomarkers IL-18, IL-22, IL-31, sIL-2R, SDF-1α did not change during daily or intermittent treatment with topical steroids. After adjusting for gender and age, these results remained unchanged.

Multivariate prediction model for EASI scores over time

We then aimed to model EASI score changes over time by a combination of biomarkers using a linear mixed model approach. We included DBS biomarkers that showed a significant decrease during treatment (I-309, TARC, PARC, and MDC). Backward selection of the biomarkers, based on Akaike Information Criteria (see methods), showed that only including TARC resulted in the best model for prediction of EASI scores, both at baseline (i.e. before treatment initiation) and after treatment initiation. Adding other biomarkers to the model did not improve the prediction model.

Stability of TARC in DBS

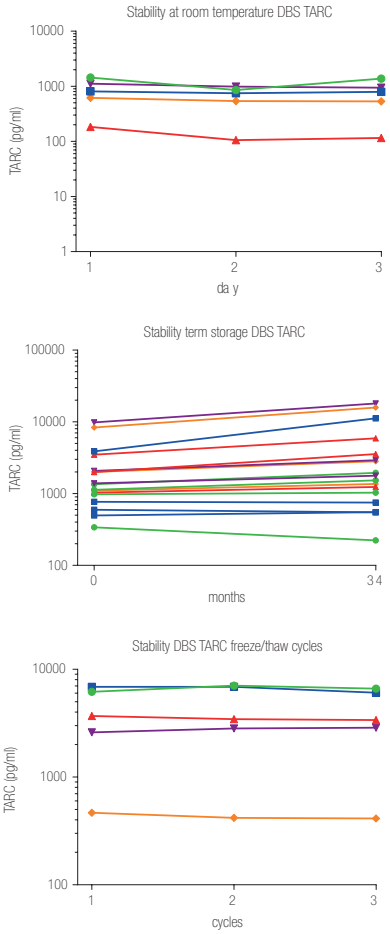
Since our study shows that TARC level measured in DBS is the most promising biomarker for measurement of disease severity in AD, we investigated the stability of TARC levels in DBS. TARC levels did show now statistical differences during storage at room temperature for 7 days (Figure 5A), or during storage at -80°C for 34 months (Figure 5B). TARC levels in DBS did not show any statistical differences during three freeze/thaw cycles. (Figure 5C).

Table 3. Changes over time of biomarkers measured in DBS after treatment initiation.

Biomarker	Estimate (95%CI)	P-value
Baseline		
I-309 (pg/ml)	130.17 (110.00, 150.35)	<.0001
Log TARC (pg/ml)	3.37 (3.24, 3.49)	<.0001
Log PARC (pg/ml)	7.31 (7.00, 7.63)	<.0001
IL-18 (pg/ml)	3674.08 (3337.49, 4010.67)	<.0001
IL-22 (pg/ml)	93.60 (73.17, 114.02)	<.0001
IL-31 (pg/ml)	66.35 (43.36, 89.34)	<.0001
Log MDC (pg/ml)	3.33 (3.25, 3.40)	<.0001
sIL-2R (pg/ml)	2316.19 (1920.67, 2711.71)	<.0001
Log sE-selectin (pg/ml)	3.05 (2.91, 3.19)	<.0001
SDF-1α (pg/ml)	628.21 (460.86, 795.57)	<.0001
Change/week (between t_0 and t_1)		
I-309 (pg/ml)	-19.49 (-26.69, -12.29)	<.0001
Log TARC (pg/ml)	-0.13 (-0.17, -0.10)	<.0001
Log PARC (pg/ml)	-0.35(-0.48, -0.23)	<.0001
IL-18 (pg/ml)	-72.15 (-161.57, 17.28)	0.11
IL-22 (pg/ml)	-4.63 (-12.83, 3.57)	0.26
IL-31 (pg/ml)	-0.09 (-9.37, 9.20)	0.99
Log MDC (pg/ml)	-0.08 (-0.10, -0.06)	<.0001
sIL-2R (pg/ml)	-94.48 (-211.77, 22.80)	0.11
Log sE-selectin (pg/ml)	-0.05 (-0.11, 0.01)	0.08
SDF-1α (pg/ml)	-22.21 (-82.38, 37.96)	0.4632
Change/week (between t_1 and t_2)		
I-309 (pg/ml)	0.52 (-1.28, 2.32)	0.56
Log TARC (pg/ml)	0.004 (-0.005, 0.01)	0.39
Log PARC (pg/ml)	0.02 (-0.02, 0.05)	0.37
IL-18 (pg/ml)	15.41 (-12.025, 42.85)	0.26
IL-22 (pg/ml)	1.18 (-0.94, 3.30)	0.27
IL-31 (pg/ml)	-0.29 (-2.85, 2.28)	0.82
Log MDC (pg/ml)	0.001 (-0.004, 0.007)	0.61
sIL-2R (pg/ml)	13.88 (-19.54, 47.30)	0.41
Log sE-selectin (pg/ml)	0.01 (0.002, 0.03)	0.03
SDF-1α (pg/ml)	11.53 (-5.96, 29.02)	0.19

Linear mixed models were used to model the changes over time of biomarkers measured in DBS after treatment initiation with topical steroids. The biomarkers I-309, TARC, PARC, and MDC showed a significant decrease during daily treatment with topical steroids (between t_0 and t_1) and then remained at stable levels during intermittent treatment with topical steroids (between t_1 and t_2).

Figure 5. Stability of TARC in DBS from atopic dermatitis patients.



A. TARC levels in DBS from 5 AD patients stay stable during 1, 3 and 7 days after collection when stored at room temperature. Repeated measures ANOVA did not show statistical differences between TARC levels after 1,3 or 7 days of storage.

B. TARC levels measured in DBS from 17 AD patients stay stable during 34 months when stored at -80°C.

C. A t-test did not show differences between TARC levels at 0 months and 34 months. C. TARC levels measured in DBS from 5 AD patients are not influenced by freeze thaw cycles. Repeated measures ANOVA did not show statistical differences between TARC levels after 1,2 or 3 cycles.

DISCUSSION

Biomarkers offer an objective alternative for the classically used physician assessed outcome measures which are subject to inter- and intra-observer differences. Disadvantages of the use of serum biomarkers are the need for a venipuncture, the processing in a lab and the challenges in harmonizing the logistics between different labs. DBS offer a simple and minimally invasive alternative to venipuncture. In this study we show that TARC levels measured in DBS from AD patients highly correlates with disease severity and significantly decreases during effective treatment with topical steroids.

Robert Guthrie is widely credited as being the first to use blood dried on filter paper (so-called Guthrie cards) to diagnose phenylketonuria in neonates in 1963.[21] Since then, the use of filter paper for storing and transporting diverse specimens has become a common method. In 2005, Skogstrand et al.[11] were the first to measure cytokines in DBS using a multiplex immunoassay technology. Measurement of cytokines in DBS by multiplex has been repeated in several publications in different fields of study.[22-25] The current study is the first to measure cytokines and chemokines in DBS from AD patients by multiplex immunoassay technology.

An advantage of DBS over venipuncture is the simple, minimally-invasive and nearly painless procedure. Trained personnel are needed for blood collection by venipuncture, whereas DBS can be performed by patients themselves. The majority of adult patients prefers a small finger-prick over intravenous blood draws.[26, 27] DBS also require a very small amount of blood (±10 µL), which is advantageous in studies that include newborns and infants. DBS are not only easier to collect, but also offer a huge simplification in storage and transport, which makes harmonizing logistics between centers within a clinical trial much easier. After collection, DBS cards are dried horizontally in a card rack at 2-3 hours at room temperature. After drying, DBS can be transported at ambient temperature by regular mail, which reduces sample transportation and storage costs. We have now shown that TARC levels in DBS stay stable when stored at room temperature up to 7 days after collection. Moreover, when DBS are stored at -80°C TARC, levels remain stable up to 34 months and are not influenced by freezing and thawing of the DBS. Earlier reports have shown that cytokine levels in DBS remain stable up to 23 years when DBS are stored at -24°C.[11] Finally, DBS are safer to transport and considered as non-regulated and exempt material, which allows shipping of DBS through mail or courier.[28, 29] This makes TARC measured in DBS a very reliable and cost-effective biomarker for disease severity in AD.

Another advantage of DBS is the possibility to measure of drug concentrations, which is possible in a range of drugs,[30] including cyclosporin A,[31-34] mycophenolic acid,[35] and tacrolimus,[34, 36, 37] drugs which are frequently used in AD treatment.[38] DBS can also be used to investigate the pharmacokinetics of new biologic agents.[39] Combining measurement of drug concentrations in DBS with disease severity biomarkers would

be ideal in clinical trials. Measurement of creatinine levels in DBS has also been described.[37] Since decrease in kidney function is one of the most common adverse effects in cyclosporin A treatment, measurement of creatinine levels in DBS would be of great interest in the follow-up of AD patients treated with cyclosporin A. Combining measurement of severity, CsA blood levels and creatinine levels in DBS can facilitate home follow-up of patients through email or video consultations, replacing face-to-face consultations which saves costs and time for patients.

In the current study TARC levels measured in DBS showed the strongest correlation with disease severity measured by EASI. This is in accordance with literature on biomarkers measured in serum. A recent meta-analysis found that serum TARC is the most reliable biomarker for disease severity in AD currently available.[5] In the current study, the biomarkers I-309, PARC and MDC in DBS also correlated with disease severity and significantly decreased during effective treatment with topical steroids. However, including these biomarkers in a linear mixed model analysis for prediction of EASI scores did not improve the model. This is in contrast with results from studies on serum biomarkers, in which a panel including multiple biomarkers better correlates with disease severity than a single biomarker.[6, 7] We noticed that TARC levels measured in DBS highly correlated with I-309, PARC and MDC levels measured in DBS (data not shown). These correlations may explain why adding I-309, PARC or MDC into a prediction model adds no value to a model that includes TARC.

Interestingly, TARC shows a stronger correlation with disease severity when measured in DBS compared to serum. This may be explained by higher recovery levels in DBS than in serum due to measurement of intracellular components in DBS, as was previously shown.[40] The stronger correlation of TARC in DBS might also be caused by different biomarker concentrations in capillary blood compared to venous blood, which may lead to different biomarker concentrations when measured in finger-prick spotted DBS (capillary blood) compared with traditional venous sampling.

A limitation of our study is the fact that we only measured a small amount of biomarkers in DBS, which were selected based on their correlation with disease severity in serum. There might be other biomarkers that we did not measure in this study, but that do correlate with disease severity when measured in DBS.

In conclusion, we have shown that in a population of mild to severe AD patients TARC levels measured in DBS highly correlate with disease severity and significantly decrease during effective treatment. DBS offer a simple and minimally invasive method for measurement of biomarkers and provide a tool for objective severity outcome measurements in AD that can easily be applied in clinical trials and daily practice.

REFERENCES

1. Deckers IA, McLean S, Linssen S, Mommers M, van Schayck CP, Sheikh A. Investigating international time trends in the incidence and prevalence of atopic eczema 1990-2010: a systematic review of epidemiological studies. PLoS One 2012; 7:e39803.
2. Charman C, Chambers C, Williams H. Measuring atopic dermatitis severity in randomized controlled clinical trials: what exactly are we measuring? J Invest Dermatol 2003; 120:932-41.
3. Schmitt J, Langan S, Williams HC, European Dermato-Epidemiology N. What are the best outcome measurements for atopic eczema? A systematic review. J Allergy Clin Immunol 2007; 120:1389-98.
4. Schmitt J, Langan S, Stamm T, Williams HC, Harmonizing Outcome Measurements in Eczema Delphi p. Core outcome domains for controlled trials and clinical recordkeeping in eczema: international multiperspective Delphi consensus process. J Invest Dermatol 2011; 131:623-30.
5. Thijs J, Krastev T, Weidinger S, Buckens CF, de Bruin-Weller M, Bruijnzeel-Koomen C, et al. Biomarkers for atopic dermatitis: a systematic review and meta-analysis. Curr Opin Allergy Clin Immunol 2015; 15:453-60.
6. Thijs JL, Nierkens S, Herath A, Bruijnzeel-Koomen CA, Knol EF, Giovannone B, et al. A panel of biomarkers for disease severity in atopic dermatitis. Clin Exp Allergy 2015; 45:698-701.
7. Thijs JL DJ, Fiechter R, Strickland I, Sleeman MA, Herath A, May RD, Bruijnzeel-Koomen CAFM, Knol EF, Giovannone B, de Bruin-Weller MS, Nierkens S, Hijnen DJ. EASI p-EASI: Utilising a combination of serum biomarkers offers an objective measurement tool for disease severity in atopic dermatitis patients. Journal of Allergy and Clinical Immunology Under review.
8. Sood BG, Madan A, Saha S, Schendel D, Thorsen P, Skogstrand K, et al. Perinatal systemic inflammatory response syndrome and retinopathy of prematurity. Pediatr Res 2010; 67:394-400.
9. Miller EM, McDade TW. A highly sensitive immunoassay for interleukin-6 in dried blood spots. Am J Hum Biol 2012; 24:863-5.
10. Aabye MG, Eugen-Olsen J, Werlinrud AM, Holm LL, Tuuminen T, Ravn P, et al. A simple method to quantitate IP-10 in dried blood and plasma spots. PLoS One 2012; 7:e39228.
11. Skogstrand K, Thorsen P, Norgaard-Pedersen B, Schendel DE, Sorensen LC, Hougaard DM. Simultaneous measurement of 25 inflammatory markers and neurotrophins in neonatal dried blood spots by immunoassay with xMAP technology. Clin Chem 2005; 51:1854-66.
12. Hanifin JM, Rajka G. Diagnostic Features of Atopic-Dermatitis. Acta Derm Venereol 1980:44-7.
13. Long CC, Finlay AY. The finger-tip unit--a new practical measure. Clin Exp Dermatol 1991; 16:444-7.
14. Hanifin JM, Thurston M, Omoto M, Cherill R, Tofte SJ, Graeber M. The eczema area and severity index (EASI): assessment of reliability in atopic dermatitis. EASI Evaluator Group. Exp Dermatol 2001; 10:11-8.

15. Charman CR, Venn AJ, Williams HC. The patient-oriented eczema measure: development and initial validation of a new tool for measuring atopic eczema severity from the patients' perspective. Arch Dermatol 2004; 140:1513-9.

16. Phan NQ, Blome C, Fritz F, Gerss J, Reich A, Ebata T, et al. Assessment of pruritus intensity: prospective study on validity and reliability of the visual analogue scale, numerical rating scale and verbal rating scale in 471 patients with chronic pruritus. Acta Derm Venereol 2012; 92:502-7.

17. Fredriksson T, Pettersson U. Severe psoriasis--oral therapy with a new retinoid. Dermatologica 1978; 157:238-44.

18. Feldman SR, Fleischer AB, Jr., Reboussin DM, Rapp SR, Exum ML, Clark AR, et al. The self-administered psoriasis area and severity index is valid and reliable. J Invest Dermatol 1996; 106:183-6.

19. Ostler MW, Porter JH, Buxton OM. Dried blood spot collection of health biomarkers to maximize participation in population studies. J Vis Exp 2014:e50973.

20. Thijs JL SI, Bruijnzeel-Koomen CAF, Nierkens S, Giovannone B, Csomor E, Sellman BR, Mustelin T, Sleeman MA, de Bruin-Weller MS, Herath A, Drylewicz J, May RD, Hijnen DJ. Moving towards endotypes in atopic dermatitis: identification of patient clusters based on serum biomarker analysis. Journal of Allergy and Clinical Immunology in press.

21. Lehmann S, Delaby C, Vialaret J, Ducos J, Hirtz C. Current and future use of "dried blood spot" analyses in clinical chemistry. Clin Chem Lab Med 2013; 51:1897-909.

22. Sood BG, Shankaran S, Schelonka RL, Saha S, Benjamin DK, Jr., Sanchez PJ, et al. Cytokine profiles of preterm neonates with fungal and bacterial sepsis. Pediatr Res 2012; 72:212-20.

23. Andersen NJ, Mondal TK, Preissler MT, Freed BM, Stockinger S, Bell E, et al. Detection of immunoglobulin isotypes from dried blood spots. J Immunol Methods 2014; 404:24-32.

24. Nielsen PR, Agerbo E, Skogstrand K, Hougaard DM, Meyer U, Mortensen PB. Neonatal levels of inflammatory markers and later risk of schizophrenia. Biol Psychiatry 2015; 77:548-55.

25. Broadney MM, Chahal N, Michels KA, McLain AC, Ghassabian A, Lawrence DA, et al. Impact of parental obesity on neonatal markers of inflammation and immune response. Int J Obes (Lond) 2016.

26. Merton G, Jones K, Lee M, Johnston A, Holt DW. Accuracy of cyclosporin measurements made in capillary blood samples obtained by skin puncture. Ther Drug Monit 2000; 22:594-8.

27. Woods K, Douketis JD, Schnurr T, Kinnon K, Powers P, Crowther MA. Patient preferences for capillary vs. venous INR determination in an anticoagulation clinic: a randomized controlled trial. Thromb Res 2004; 114:161-5.

28. Federal Register. 49 CFR part 173 Shippers General Requirements for Shipments and packaging. Section 173.134(b) 2011.] Available from https://www.gpo.gov/fdsys/pkg/CFR-2011-title49-vol2/pdf/CFR-2011-title49-vol2-sec173-134.pdf.

29. Guidance on regulations for the Transport of Infectious Substances 2015–2016.] Available from http://apps.who.int/iris/bitstream/10665/149288/1/WHO_HSE_GCR_2015.2_eng.pdf?ua=1.

30. Sharma A, Jaiswal S, Shukla M, Lal J. Dried blood spots: concepts, present status, and future perspectives in bioanalysis.

Drug Test Anal 2014; 6:399-414.

31. Lampe D, Scholz D, Prumke HJ, Blank W, Huller H. Capillary blood, dried on filter paper, as sample for monitoring cyclosporin A concentrations. Clin Chem 1987; 33:1643-4.

32. Mohamed R, Mercolini L, Cuennet-Cosandey S, Chavent J, Raggi MA, Peyrou M. Validation of a dried blood spot LC-MS/MS approach for cyclosporin A in cat blood: Comparison with a classical sample preparation. Journal of Pharmaceutical and Biomedical Analysis 2012; 66:298-305.

33. Hinchliffe E, Adaway JE, Keevil BG. Simultaneous measurement of cyclosporin A and tacrolimus from dried blood spots by ultra high performance liquid chromatography tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 2012; 883-884:102-7.

34. Koster RA, Alffenaar JWC, Greijdanus B, Uges DRA. Fast LC-MS/MS analysis of tacrolimus, sirolimus, everolimus and cyclosporin A in dried blood spots and the influence of the hematocrit and immunosuppressant concentration on recovery. Talanta 2013; 115:47-54.

35. Heinig K, Bucheli F, Hartenbach R, Gajate-Perez A. Determination of mycophenolic acid and its phenyl glucuronide in human plasma, ultrafiltrate, blood, DBS and dried plasma spots. Bioanalysis 2010; 2:1423-35.

36. Hoogtanders K, van der Heijden J, Christiaans M, Edelbroek P, van Hooff JP, Stolk LM. Therapeutic drug monitoring of tacrolimus with the dried blood spot method. J Pharm Biomed Anal 2007; 44:658-64.

37. Koop DR, Bleyle LA, Munar M, Cherala G, Al-Uzri A. Analysis of tacrolimus and creatinine from a single dried blood spot using liquid chromatography tandem mass spectrometry. Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences 2013; 926:54-61.

38. Ring J, Alomar A, Bieber T, Deleuran M, Fink-Wagner A, Gelmetti C, et al. Guidelines for treatment of atopic eczema (atopic dermatitis) Part II. J Eur Acad Dermatol Venereol 2012; 26:1176-93.

39. Burns D, Brunner L, Rajendran S, Johnson B, Ma M, Wang J. Validation of a Ligand Binding Assay Using Dried Blood Spot Sampling. Aaps Journal 2013; 15:123-31.

40. Pennings JL, Siljee JE, Imholz S, Kuc S, de Vries A, Schielen PC, et al. Comparison of different blood collection, sample matrix, and immunoassay methods in a prenatal screening setting. Dis Markers 2014; 2014:509821.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General discussion

MAIN FINDINGS OF THIS THESIS

Biomarkers for disease severity in atopic dermatitis

- A meta-analysis including 222 studies showed that serum TARC level is the best biomarker for disease severity currently available (chapter 2).
- Immunoglobulin free light chains have been shown to correlate with disease severity in paediatric AD. However, they do not correlate with disease severity in adult AD (chapter 6).
- A pilot study in 17 AD patients showed that a combination of serum biomarkers is better in assessing disease severity than a single biomarker (chapter 3).
- In a prospective cohort of 200 patients it was shown that combining serum TARC, IL-22 and sIL-2R levels in an algorithm accurately predicts clinically measured disease severity (predicted EASI) in 90% of AD patient treated with topical steroids (chapter 4).
- The p-EASI also predicts disease severity in patients treated with cyclosporin A (chapter 5).
- The p-EASI offers an objective outcome measure for disease severity in prospective AD studies (chapter 4 and 5).

Biomarkers enabling precision medicine in atopic dermatitis

- High expression levels of circulating inflammatory biomarkers suggest that AD is a systemic disease. Recently described comorbidities may be the result of this systemic inflammation, and emphasize the need for a multidisciplinary approach for optimal management of AD and its comorbidities (chapter 9).
- AD is a heterogeneous disease both clinically and biologically. We have identified four clusters of AD patients based on specific serum biomarker profiles, implying that each of these clusters is driven by a distinct underlying pathway and these clusters may represent different endotypes (chapter 7).
- UGT1A9 polymorphisms can be used to identify patients with non-responsiveness to mycophenolic acid therapy, thereby showing the potential of pharmacodynamic biomarkers in AD (chapter 8).

Improving practical aspects of biomarker measur ement

- The biomarkers studied in this thesis are measured in serum. However, multiple other sources for biomarker measurement exist, for instance saliva and dried blood spots (chapter 10).
- A disadvantage of the use of serum biomarkers is the need for a venipuncture. TARC levels measured in dried blood spots also highly correlate with disease severity and significantly decrease during effective treatment. Therefore, dried blood spots may offer a simple and minimally invasive method for measurement of biomarkers in AD (chapter 11).

GENERAL DISCUSSION

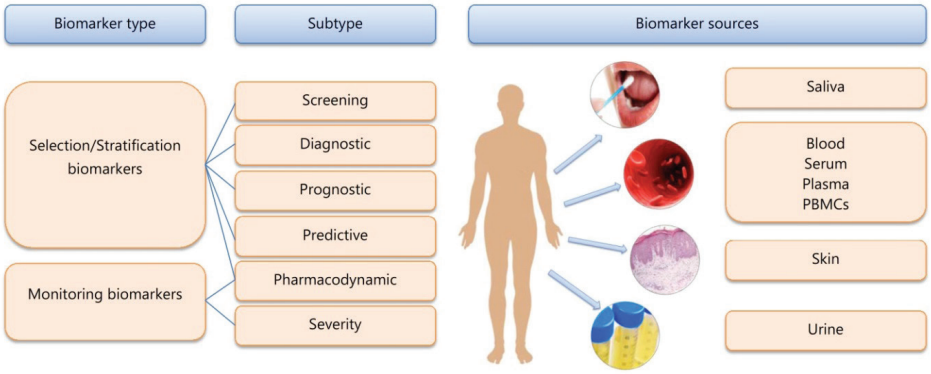
Atopic dermatitis (AD) is one of the most common chronic inflammatory skin diseases worldwide. Living with AD has a great impact on the quality of life of patients. The pathophysiology of AD is complex, and thought to be the result of both genetic and environmental factors, resulting in immunologic and barrier dysfunctions.[1] Many attempts have been made to define subsets of patients based on clinical characteristics. However, the current characterization of AD patients might not adequately reflect the pathophysiologic diversity within patients with AD.

Most patients with mild disease can be effectively treated with topical steroids, however, there is a high unmet need in the treatment of patients with moderate to severe disease. In recent years, the number of clinical trials evaluating biological agents targeting specific cytokines or cytokine receptors has steadily increased, and may provide new, highly effective treatment options for AD in the near future.[2] With the increasing number of clinical trials evaluating these new drugs, comparability of study outcomes is of major importance.

Technological advances now allow us to determine large numbers of biomarkers in small volumes of body fluids. The figure below contains an overview of the different biomarker types, their clinical applications and sources. The increased use of biomarkers in AD research will result in objective outcome measures that will allow better comparison of current and new treatments, furthermore biomarkers may enable better characterization and stratification of AD patients, and a better understanding of the underlying pathomechanisms, enabling precision medicine.

The research presented in this thesis focuses on biomarkers for monitoring clinical disease severity and biomarkers for stratification of pathophysiological mechanisms, measured in serum and dried blood spots of patients with AD.

Figure 1. Biomarker types and sources. This figure summarizes the different types of biomarkers and potential biomarker sources.



Biomarkers can broadly be separated into two categories. The first category comprises biomarkers that are used to identify persons at risk to develop a disease, patients with active disease, and populations of patients that are most likely to benefit from a given therapy. The second category includes biomarkers for monitoring treatment effects. Biomarkers can be obtained from biological fluids like blood, saliva, and urine, or from tissue samples. PBMCs: peripheral blood mononuclear cells.

BIOMARKERS FOR DISEASE SEVERITY IN ATOPIC DERMATITIS

Atopic dermatitis (AD) is one of the most common chronic inflammatory skin disease worldwide with a high burden of disease, and socio-economic impact.[3-6] Although most AD patients with mild disease can be effectively treated with topical steroids, there is a high unmet need in the treatment of patients with moderate to severe disease. In recent years, the number of clinical trials evaluating biological agents targeting specific pathways and mechanisms in AD has steadily increased and may provide new treatment options for AD in the near future that could considerably improve patients' quality of life.[2] With the increasing number of clinical trials evaluating these new biological agents, the comparability of study outcomes is of major importance.

Over 50 different clinical outcome measures for disease severity have been described in AD.[7] A systematic review on validity and reliability of the 20 most commonly used clinician rated severity measures for AD showed that only three severity measures performed adequately.[8] To address these shortcomings in reporting, experts in the field have established the Harmonizing Outcome Measurements in Eczema (HOME) initiative, an attempt to ensure that investigators employ a core set of clinical outcome measures to enhance comparability between studies. The HOME initiative recently recommended the use of the Eczema Area and Severity Index (EASI) and the Patient Oriented Eczema Measure (POEM) as core outcome instruments for measuring disease severity and symptoms in AD.[9, 10] A shortcoming of clinician rated severity measures is the subjective judgment of categories and domains rated by the physician. Therefore, subjectivity of clinician rated severity measures is inevitable, which leads to inter- and intra-observer variations as was confirmed by several studies.[8, 11, 12] We hypothesized that the use of objective serum biomarkers may replace clinician rated severity measures. We think that a combination of objective serum biomarkers and patient-reported outcomes, will highly improve outcome measures in AD and comparability of studies.

In a meta-analysis examining the correlation of biomarkers with disease severity in AD patients, we included and critically appraised a total of 222 articles, reporting on 115 different biomarkers in a total of 30.063 patients (chapter 2). We found that serum thymus and activation-regulated chemokine (TARC/CCL17) levels currently perform best as objective biomarker for disease severity. TARC is a member of the CC chemokine group and is mainly produced by dendritic cells, although some have suggested that endothelial cells and keratinocytes may also produce TARC.[13, 14] TARC is a ligand for CCR4 and plays a role in the recruitment of CCR4 expressing T cells into the skin.[15]

The meta-analysis in chapter 2 also taught us that severity biomarkers should not be studied on a single time point only, but also longitudinally. The reason for that is demonstrated by serum total IgE, which is the most commonly measured biomarker in AD trials. Meta-analysis of studies investigating the correlation of total IgE with disease severity showed a moderate correlation when measured on a cross-sectional time

point. However, there is no correlation of total IgE with disease severity in longitudinal cohort studies. Thus, when studying biomarkers for disease severity, a longitudinal study design that includes at least two time points is essential.

A biomarker of potential interest for disease severity in AD is immunoglobulin free light chain (Ig-FLC) level measured in serum. Ig-FLC was not included as a biomarker in the meta-analysis in chapter 2, due to the fact that it's measured in only two studies. Both studies found increased levels of kappa Ig-FLCs in the serum of infants with severe AD compared to infants without AD.[16, 17] Moreover, levels of Ig-FLCs correlated with disease activity in children with severe AD.[16] To study Ig-FLCs in adult AD, we measured serum Ig-FLC levels in 82 moderate to severe AD patients and 49 non-atopic controls (chapter 6). No differences in serum kappa Ig-FLC levels between adult AD patients and non-atopic controls were found. Serum kappa Ig-FLC levels did not correlate with disease severity determined by he Six Area Six Sign Atopic Dermatitis (SASSAD) score or by serum TARC levels. Out of 82 patients, 32 were treated during a clinical admission, in this subpopulation a second blood sample was taken after two weeks of treatment. These patients showed a significant reduction in disease severity, but did not show a decrease in serum kappa Ig-FLC levels. This makes serum kappa Ig-FLC levels not suitable as a biomarker for disease severity in adult patient with AD.

The absence of a correlation between serum Ig-FLC levels and disease severity, does not rule out a role for Ig-FLCs in the pathogenesis of AD. It has been shown that mast cell-bound Ig-FLC are capable to bind antigen in vitro, and that antigen-specific Ig-FLCs play a role in eliciting inflammatory responses leading to contact sensitivity, asthma, IBD, and food allergy in mice.[17-20] Although serum Ig-FLC levels are not elevated in adult AD, they theoretically may play a role in antigen presentation in AD.

IgE responses were thought to be of major importance in the pathogenesis of AD. However, elevated serum IgE levels may also be an epi-phenomenon that reflects the underlying atopic constitution which is present in the majority of AD patients. This would mean that IgE responses are not directly connected to the pathophysiology of the chronic and relapsing course of AD, explaining our observations that both total IgE and kappa Ig-FLC serum levels do not reflect disease severity in AD during follow-up.

Combining multiple biomarkers for the assessment of disease severity in atopic dermatitis

In chapter 2 we showed that serum TARC levels currently performs best as an objective biomarker for measuring disease severity in AD. The majority of studies included in the meta-analysis that measured serum TARC levels were cross-sectional cohort studies, only a few studies had a longitudinal design. From experience in daily practice we know that serum TARC levels show a strong correlation with disease severity in the follow-up of individual patients.[21] However, patients with similar disease severity scores, can show varying serum TARC levels in cross-sectional cohorts of patients, consequently showing a relatively low correlation

between serum TARC and disease severity.[21] Although serum TARC level performs relatively good, and is currently the best performing biomarker available for assessing disease severity in AD, the correlation with disease severity is not strong enough to replace clinical outcome scores.

Since AD is a complex disease, with a large number of biologic pathways involved in its pathogenesis, we hypothesized that the use of a combination of biomarkers from different biologic pathways would better reflect disease activity than a single biomarker.

To test this hypothesis, we performed a pilot study with 17 AD patients with an exacerbation of AD (chapter 3). All patients were effectively treated with potent topical steroids only, resulting in a significant decrease of disease severity measured by SASSAD, which was our standard for disease severity measurement at time of this pilot study. Based on the findings in our meta-analysis on biomarkers for disease severity in AD (chapter 2), a selection of serum biomarkers was measured at admission to our inpatient clinic and after two weeks of treatment at discharge. Of 31 markers studied, seven of these showed a statistically significant decrease after treatment. These included TARC, macrophage-derived chemokine (MDC/CCL22), IL-22, pulmonary and activation-regulated chemokine (PARC/CCL18), sIL-2R, sE-selectin and IL-16. To assess whether a combination of markers showed a stronger correlation with disease severity, we performed a stepwise regression analysis with leave one out cross validation. Since the study comprised of 17 randomly selected patients, the demographic characteristics gender and age were not well balanced and therefore included as variables in the analysis. This led to the construction of a five-parameter multivariate signature containing demographic characteristics of gender (sex), and the four serum biomarkers TARC, PARC, IL-22, sIL-2R. This combination of biomarkers showed a correlation coefficient of 0.86, whereas the correlation coefficient to disease severity for the individual biomarkers ranged from 0.42 to 0.74. This confirmed our hypothesis that a combination of biomarkers shows a better correlation with disease severity compared to individual biomarkers.

Creating a new gold standard for outcome measures in atopic dermatitis

In chapter 3, we learned that a combination of biomarkers performs better than a single biomarker as outcome measure for disease severity in AD. As a follow-up of these findings we conducted a larger and prospective study to validate our findings, and create a new (objective) gold standard for outcome measured in AD (chapter 4). The study in chapter 4 contained two steps: (i) potential new serum biomarkers that correlated with disease severity were identified in a retrospective cohort of 193 moderate to severe AD patients, next (ii) this combination of severity biomarkers was validated in a prospective cohort of 65 AD patients treated with topical steroids

Step one of the study described in chapter 4 included a retrospective cohort consisting of 193

moderate to severe AD patients, in which 150 serum biomarkers were measured. A significant correlation with SASSAD was found for 32 out of 147 serum biomarkers. Serum TARC levels showed the strongest correlation with disease severity ($r=0.40$, $p<0.01$). Other biomarkers that significantly correlated with disease severity and had correlation coefficients higher than 0.30 (, PARC, IL-22, sIL-2R, IL-18, I-309/CCL1, MDC/CCL22, sE-selectin, and SDF-1α) were included in the next step of the study in which we validated our findings in a prospective AD cohort. The correlation between the biomarkers and disease severity is rather low in comparison with the findings in our meta-analysis (chapter 2), this is probably due to the high number of different assessors that scored disease severity.

Step two of the study conducted in chapter 4 included a prospective cohort consisting of 65 patients, in which disease severity was assessed by EASI, POEM and VAS pruritis, and serum biomarkers were measured at three timepoints during a two-month treatment period with topical steroids only. All 65 patients showed significantly decreasing EASI, POEM and VAS pruritis scores during treatment. Serum biomarkers IL-18, IL-22, I309, MDC, PARC, sE-selectin, sIL-2R and TARC also significantly decreased. Linear mixed model analyses revealed an optimal combination of TARC, IL-22 and sIL-2R as predictor of EASI scores. This model was repeatedly tested in ten randomly selected patients out of the 65 patients in the cohort and showed a correct prediction of EASI scores in 90% of the cases (sensitivity of 100% and specificity of 88.9%) thereby validating our model.

Interestingly, there were several patients in which clinician rated disease severity (EASI) decreased, but TARC levels stayed more or less stable. In these patients, the biomarker IL-22 and/or sIL-2R decreased similarly with EASI. This confirms our hypothesis that a combination of biomarkers that covers multiple pathways involved in the pathogenesis of AD is better for the assessment of disease severity than a single biomarker, since not all pathways seem to be equally important in all patients.

In contrast to our pilot study (chapter 3, n=17 patients), serum PARC levels and gender are not included in the model constructed in chapter 4 (n=65 patients). Although PARC levels highly correlated with disease severity, adding PARC did not improve the model. A possible explanation for this can be the correlation between serum PARC and serum TARC levels ($r=0.43$). Both PARC and TARC are members of the cc chemokine family,[15, 22] and may represent overexpression in the same biological pathway. The presence of gender as an item in the model generated in the pilot study was probably the result of a gender misbalance in the relatively small patient population.

Another variation between the studies in chapter 3 and 4 is the usage of different clinician rated outcome measures. At the time that we conducted the pilot study (chapter 3), SASSAD was used as a standard measure for disease severity of AD in our hospital. Shortly after this study, the HOME initiative recommended the use of the EASI for measuring disease severity in AD. [9] Therefore, the eventual model

for predicting disease severity, as presented in chapter 4, was based on EASI scores. Although the use of different clinician rated outcome measures is not ideal, SASSAD and EASI scores have been shown to be highly correlated to each other (r=0.86).[23]

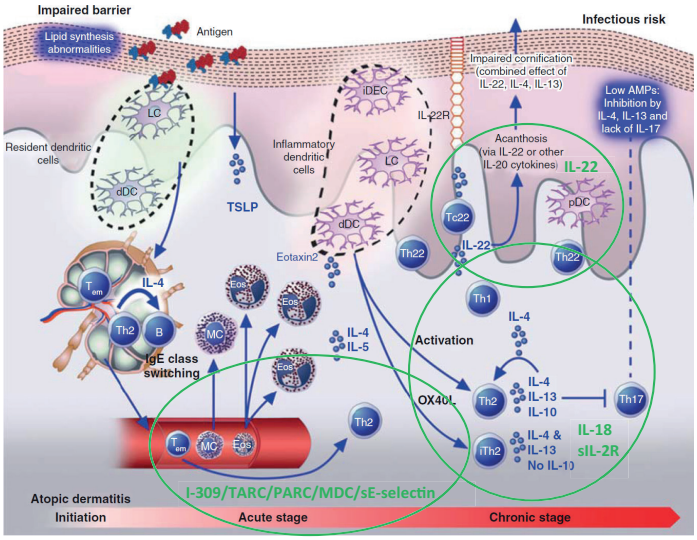
We showed that a combination of biomarkers is more suitable for follow-up of disease severity than a single biomarker, since a combination of biomarkers can cover multiple biological pathways. The biomarkers included in our model (TARC, IL-22 and sIL-2R) are indeed all involved in inflammation and are known to play a role in the pathogenesis of AD. Although the biomarkers I-309, PARC, MDC, IL-18 and s-E-selectin were not included in the model, they did show a significant decrease during treatment, and are also thought to play a role in the pathogenesis of AD. TARC, PARC and MDC are members of the CC chemokine family and involved in the recruitment of T cells into the skin.[15] TARC is mainly produced by dendritic cells in the dermis,[13] whereas PARC is produced by epidermal Langerhans cells and by dendritic cells in the dermis.[22] MDC is also produced by dendritic cells, but $CD3^+$ T lymphocytes in lesional AD skin has also been shown to express MDC.[24]

I-309 is another chemokine that has shown to be upregulated in patients with AD. I-309 is expressed mainly on dendritic cells, mast cells and endothelial cells of the dermis.[25] I-309 is the ligand to CCR8, which promotes the recruitment of T cells and Langerhans cell–like DCs in vitro.[25] sE-selectin, an adhesion molecule expressed on activated endothelial cells, is also involved in the recruitement of immune cells into the skin. sE-selectin promotes migration of $CD4^+$ and $CD8^+$ T cells and is able to recruit neutrophils and monocytes.[26, 27] The infiltrate of immune cells in lesional AD skin mainly consists of T cells.

IL-18, a member of the IL-1 family, is produced by several immune cells, including monocytes,[28] dendritic cells, macrophages,[29] and keratinocytes,[30] and is known to stimulate T cells to produce Th2 cytokines.[31] sIL-2R, synthesized and secreted by T cells, was found to reflect the activation state of the T cells in skin.[32] In our meta-analysis on biomarkers for disease severity (chapter 2) we reported that sIL-2R levels show good correlation to disease severity, although only studied in small numbers of patients. We now confirmed in a higher number of AD patients that sIL-2R is indeed a good biomarker for disease severity. IL-22 has less been studied as biomarker for disease severity, but has been recognized to play a role in the pathogenesis of AD. IL-22 induces keratinocyte proliferation resulting in acanthosis, a histopathological feature of AD. It was recently shown that IL-22 is produced by both CD4+ and CD8+ T cells isolated from lesional skin of AD patients,[33] and that IL-22 is upregulated in AD skin compared with psoriasis lesions and healthy skin.[34] In vivo experiments using cultured human keratinocytes have shown that IL-22 downregulates filaggrin expression contributing to epidermal barrier dysfunction in AD.[35] Barrier dysfunction might result in S. aureus infiltration into the epidermis, which was shown to be correlated with increased expression of IL-22.[36] Mechanistic studies in mice have shown that sIL-22 stimulated keratinocytes produce Th2 cytokines,

downregulate tight junction genes and skin barrier proteins like fillagrin.[37] The role of the biomarkers in the pathogenesis of AD is schematically depicted in figure 2.

Figure 2. Pathogenesis of atopic dermatitis. Schematic drawing of the different pathways involved in the pathogenesis of AD.



Green circles represent the involvement of the different biomarkers in the pathogenesis of AD. Figure adapted from "New Era of Biological Therapeutics in Atopic Dermatitis" by E. Guttman-Yassky, N. Dhingra, D.Y.M. Leung, 2013, Expert Opin Biol Ther., 13(4):549-561.

The influence of therapy on serum biomarkers

The performance of biomarkers for measuring disease severity should be independent of the type of therapy used. Therapies used in AD are topical steroids, topical calcineurin inhibitors, UV-therapy, systemic immunosuppressive drugs like CsA, methotrexate, azathioprine or mycophenolic acid,[38, 39] and newly developed targeted therapies like dupilumab.[40] In a trial comparing CsA and mycophenolic acid for treatment of AD it was shown that sCD30 levels in serum, an activation marker of Th2-cell clones,[41] do reflect disease severity during treatment with CsA, but not during treatment with mycophenolic acid.[42] This demonstrated that the performance of a biomarker can indeed be influenced by the type of therapy used. The current combination of biomarkers was constructed in cohorts of AD patients treated with topical steroids only. We aimed to validate the combination of biomarkers in a cohort of AD patients with systemic therapy, since most of the future 'breakthrough' therapies will probably be systemic compounds. In chapter 5, we therefore tested the combination of biomarkers in a retrospective cohort of 26 severe AD patients treated with cyclosporin A (CsA) (approximately 5 mg/kg/day). The predicted EASI score using the formula that included serum TARC, IL-22

and sIL-2R levels was in agreement with the clinician reported score in 73% of the cases. The combination of biomarkers showed a sensitivity of 82.6% and a specificity of 88.4% and was therefore qualified as a good predictor of disease severity. The lower sensitivity and specificity of the model between patients treated with CsA (chapter 5) compared to patients treated with local topical steroids (chapter 4) is probably due to the different physicians scoring disease severity. In chapter 4, disease severity was assessed by a selected number of trained physicians regularly filling out EASI scores. The validation of the model in patients treated with CsA in chapter 5 was based on data from daily practice, in which over 20 physicians with different levels of experience rated severity using the EASI. This leads to more inter individual variation in EASI scores, consequently resulting in a lower sensitivity and specificity of the biomarker model. This again highlights the shortcomings of physician assessed outcomes that will always be subject to inter rater variability, and emphasizes the need for an objective outcome measures in AD.

Biomarker for itch

In addition to a biomarker model for assessment of disease severity, we sought to identify a biomarker for objective measurement of itch. Itch is an important characteristic of AD and has a great impact on the quality of life of patients. Although several questionnaires for measuring itch have been developed, there are no objective tools available to measure itch. IL-31 has been associated with pruritus in AD, and might be a potential biomarker for itch.[43] To investigate this, we analyzed serum IL-31 levels and measured pruritus using a visual analogue scale (VAS) in the prospective patient cohort as described in chapter 4. Pruritus scores significantly decreased during treatment (Figure 3), but serum IL-31 levels did not show a corresponding decrease. Raap et al. found that serum IL-31 levels correlated to disease severity,[44] however, this was a cross-sectional study that only included 37 AD patients. A longitudinal study by Kyoya et al. showed no significant decrease of serum IL-31 levels during effective treatment of AD with topical steroids.[45] And a recent cross-sectional study of Nygaard et al. that included 163 adults and children with AD, was unable to confirm the correlation between serum IL-31 and disease severity.[46] Kim et al. found that IL-31 mRNA expression is higher in biopsies from AD patients with high pruritus scores compared to patients with low pruritus scores,[47] suggesting that measurement of IL-31 in skin may be a more relevant measure for itch. Although IL-31 expression patterns in skin biopsies may also be extremely helpful in stratification, skin biopsies are invasive, require trained personnel and specialized labs. Measurement of IL-31 in skin is therefore not suitable as an objective marker for itch in AD.

Disease severity biomarkers in children with atopic dermatitis

Since the prevalence of AD is highest during childhood, applicability of the biomarker signature in the

pediatric AD population is of great interest. A clear difference between childhood and adult AD is the clinical presentation. In newborns, AD presents generally acute, with facial lesions and lesions the extensor surfaces of the limbs. From age 1–2 years onwards, skin lesions are more polymorphous and particularly seen in the flexural folds. Lesions in adults are often more lichenified and excoriated, and can be present all over the body. Adults can also have solitary chronic hand eczema or present with prurigo-like lesions.[48] The differences in clinical presentations during life, raises the question whether the biomarker signature for the assessment of disease severity in AD, as presented in chapter 3, 4 and 5, that was based on data from adult AD patients, is applicable in children with AD. Future studies to investigate the performance of our biomarker signature consisting of TARC, sIL-2R and IL-22 are needed to answer this question, however, one can speculate on the possible outcome of such studies based on what is known on the performance of the individual biomarkers in pediatric AD in literature.

In 2003, Leung et al. were the first to show that serum TARC does not only correlate with disease severity in adult AD, but also correlates with disease severity in children with AD with (age ranging from 0.6 to 4.2 years).[49] These findings were confirmed by Song et al. in a big cross-sectional study that included 260 children with both intrinsic and extrinsic AD (mean age 4.9 years).[50] That serum TARC does not only correlate in cross-sectional cohorts of children with AD, but also decreases during effective treatment was shown by Fujisawa et al. in 2009.[51] Interestingly, both Song et al. and Fujisawa et al. showed that serum TARC levels are negatively correlated with age.[50, 51] This means that TARC levels are higher in very young children, which is probably due to a higher body surface area/blood volume rate in young children. In chapter 4, we have shown that biomarker levels in adult AD patients are not influenced by age.

Serum sIL-2R levels were shown to be upregulated in children with AD compared to healthy controls by Matsumota et al.[52] Just as serum TARC levels, serum sIL-2R levels were significantly lower in children aged 10-15 years compared to children that were 1-5 years.[52] Serum sIL-2R was shown to correlate with disease severity in children with AD by two independent studies. Frezzolini et al. studied serum sIL-2R in 25 children with AD, aged 2-8 years, and showed a positive correlation with disease severity measured by SCORing Atopic Dermatitis (SCORAD).[53] Halmerbauer et al. studied serum sIL-2R levels in 20 children with AD, with a mean age of 13.7 years, and showed a correlation with disease severity, but also showed that serum sIL-2R decreases during treatment with prednisolone therapy.[54]

There have been no studies on serum IL-22 levels in children with AD. However, the differences between T-cell subsets in blood of paediatric and adult AD patients was recently studied by Czarnowicki et al. in 19 children with AD, with a mean age of 25 months, and 42 adult AD patients.[55] AD onset in children was found to be Th2-dominated, while adult AD extended to additional Th subsets, particularly Th22.[55] The relative absence of Th22 subset in paediatric AD might be explained by the difference in clinical presentation

between children and adults with AD. While children have more acute lesions with erythema and dryness, adults with long-standing disease have marked thickening or lichenification of lesions. IL-22 is known to induce epidermal hyperplasia,[56] which supports a role for IL-22 in disease chronicity and not in disease initiation. Therefore, the performance of serum IL-22 as a biomarker for disease severity might not be as good in children as in adults.

A biomarker signature for assessment of disease severity in children with AD would be of great value. However, findings from literature suggest that that are differences in serum biomarker levels between adults and children. While serum TARC and sIL-2R are shown to be suitable as a biomarker in pediatric AD, they were also found to be age dependent. Whether serum IL-22 can function as a biomarker in pediatric AD, especially in young children, needs to be further elucidated. Future studies that study multiple biomarkers and incorporate the variable age into a biomarker signature for assessment of disease severity in paediatric AD are needed.

In summary, we showed that a combination of serum biomarkers TARC, IL-22 and sIL-2R offers an objective and reliable outcome measure in adult AD.

Valorisation

To make a combination of biomarkers suitable for routine use, simplicity and ease of interpretation is key. We think that serum TARC, IL-22 and sIL-2R levels should not be interpreted separately by the clinician because it is the combination of these biomarkers that reflects the disease activity. Combining levels of biomarkers in an algorithm results in a single value that is comparable to EASI and therefore easy to interpret. Since the algorithm was developed to predict EASI scores, we named this outcome measure the predicted-EASI (p-EASI). In contrast to EASI, the p-EASI provides an objective outcome measure that is not subjected to intra- and inter-rater variability.

Currently the HOME initiative recommends to use of a core outcome set to measure disease severity in AD clinical trials. This core outcome set comprises of four domains: 1. Clinician reported signs, 2. Patient reported symptoms, 3. Quality of Life, and 4. Long term control. We believe that replacing clinician reported signs with an algorithm based on a serum biomarker combination, like the p-EASI, in this core outcome set will highly improve outcome measures in AD. With the increasing number of clinical trials evaluating new biological agents for the treatment of AD, the comparability of study outcomes is of major importance.

The development of a commercially available assay kit for measuring of TARC, sIL-2R and IL-22, coupled to the p-EASI algorithm, would enhance the use of the p-EASI in future clinical trials. Such a kit will provide a convenient and simple tool for objective outcome measures that can be applied in future clinical trials.

Future studies

In our studies we mainly included Caucasian patients. Recently it was shown that skin of Asian AD patients express higher levels of IL-22 than Caucasian patients. If the p-EASI is also valid for use in Asian patients needs further investigation.

Ideally, disease severity should be rated by a limited number of trained clinicians, to minimize inter- and intra-variability of the EASI scores. Secondly, the p-EASI should be validated in a cohort of patients treated with new biologics. The recently published phase II and III trials testing the IL-4Rα targeting antibody dupilumab have shown promising results. Both EASI scores and serum TARC levels showed a significant reduction in AD patients treated with dupilumab. The patient cohorts from these studies would offer an ideal population for validation of the p-EASI. This validation should also include comparison of the performance of TARC alone with the performance of the p-EASI. After validation in these cohorts, the p-EASI is ready for application in future clinical trials testing new biological drugs for AD. A final step would be the application of the p-EASI in daily practice, eventually replacing clinician reported outcomes. Separate studies following the same steps are needed to develop and validate a biomarker signature for disease severity in paediatric AD.

ATOPIC DERMATITIS IS A SYSTEMIC DISEASE

In chapter 9 we showed that levels of many inflammatory cytokines and chemokines in the serum of AD patients are highly increased compared to healthy controls, resulting in clear differences in biomarker expression profiles. The presence of inflammatory cytokines in the circulation of AD patients, may have effect on distant organs and contribute to other systemic diseases. Recent studies have also shown associations between AD and alopecia areata, neuropsychiatric and cardiovascular diseases (CVD).[57]

The association between adult AD and CVD, would be consistent with a recent study that showed the occurrence of severe CVD and metabolic abnormalities in a mouse model of severe dermatitis resulting from persistent release of IL-1 family cytokines from the skin.[58] Interestingly, these pathologies were ameliorated by combination treatment with anti–IL-1α- and anti–IL-1β neutralizing antibodies. In chapter 7 we are the first to show that serum IL-1Rα, IL-1β and IL-1R1 levels are significantly different in AD compared to healthy controls. This supports the concept of an inflammatory skin march,[58, 59] in which spilled-over IL-1s from inflamed skin end up into the circulation, thereby affecting distant organs and causing cardiovascular changes. Whether serum levels of IL-1s decrease during treatment, and the subsequent effect on cardiovascular risk factors, is unknown and has yet to be studied.

In chapter 9 we also show that IL-7 serum levels in AD patients are significantly higher than levels in controls. IL-7 is produced by keratinocytes[60] and is essential for T cell development, survival and proliferation of memory and naive T cells and Th17 cells.[61] Transgenic mice constitutively expressing IL-7 develop severe dermatitis, characterized by massive infiltration of γδ T cells in the dermal lesion.[61] In addition, a polymorphism in the IL-7R gene that lead to enhanced IL-7 bioactivity, was genetically associated to susceptibility to AD.[62-64] The same IL-7R polymorphism is associated with higher risk of type I diabetes,[65] rheumatoid arthritis,[66] sarcoidosis,[67] multiple sclerosis[68] and asthma.[69] These findings make the IL-7 axis interesting as a therapeutic target in AD, but also highlight the link between systemic diseases and AD.

Taken together, our study confirms the presence of systemic inflammation in AD patients and supports the hypothesis that AD is a systemic disorder.

Valorisation

It is known that patients with AD have a higher incidence of allergic asthma, rhinitis and food allergy. Evidence for a higher incidence of other comorbidities in AD, like rheumatoid arthritis and cardiovascular diseases, is raising. This emphasizes the need for a multidisciplinary approach for optimal management of AD and its comorbidities. Examination of AD patients should not be limited to the skin, but should include screening for comorbidities like asthma, allergic rhinitis, eye involvement, and food allergy. This might need to be ex-

tended with screening for rheumatoid arthritis, inflammatory bowel diseases, and cardiovascular risk factors and diseases. Early detection of comorbid conditions or even identification of patients at risk of developing comorbidities, can reduce or prevent burden of disease and save future costs of care.

Future studies

The presence of an association between AD and cardiovascular diseases have been shown in epidemiologic studies, however, this does not prove causality. Mechanistic studies are needed to reveal mechanisms of disease associations. Research on the concept of an inflammatory skin march should not be limited to mouse models, but should also include humans to determine if shedding of IL-1s from inflamed skin really are biologically active and indeed affects distant organs. A first step would be to investigate if circulating IL-1 levels decrease after effective treatment of skin inflammation.

It has been often hypothesized that impairment of the epidermal barrier is the main pathologic condition responsible for the atopic march. It has however become more and more clear that immunologic abnormalities are of major importance in the development of AD. It would be interesting to study if intervention of these immunologic abnormalities (e.g. with dupilumab) on a very young age can halt the inflammatory skin march and prevent the development of comorbidities by resetting the immune system.

ATOPIC DERMATITIS: A HETEROGENEOUS DISEASE

AD is recognized to be a complex and highly heterogeneous disease, characterized by a diverse clinical manifestation.[1] Classically, clinical characteristics such as age of onset, persistence of disease after childhood, and the presence of atopy and presence of other atopic diseases such as allergic rhinitis and asthma are used to divide AD into different disease phenotypes.[70] However, these phenotypical characteristics do not seem to relate to specific disease mechanisms and have not yet given us new insights in the underlying pathology of disease.

It has become increasingly clear that AD is not only heterogeneous based on clinical characteristics, but that different underlying pathophysiological processes can be seen in different subgroups of patients. Serum total IgE was one of the first immunological parameters used for subgrouping of AD, dividing the disease into non-IgE-associated or 'intrinsic' and IgE-associated or 'extrinsic' AD. Other immunological parameters that can subgroup AD are the presence of specific IgE (sIgE) and eosinophilia.[71] The strongest genetic association with AD has been shown for loss-of-function mutations in the filaggrin gene (FLG). However, only about one third of the AD patients are carrier of a FLG mutation.[72]

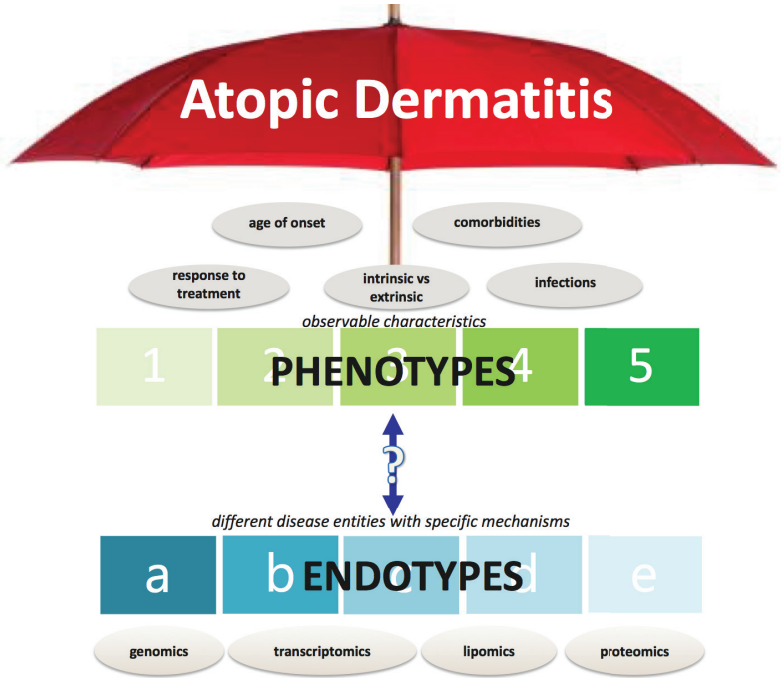
Due to this heterogeneity, it is unlikely that newly developed biological drugs that target specific components of the immune system will be effective in all AD patients. To be able to provide the right drug to the right patient, it is essential to gain better insight into the heterogeneous character of the disease. One as yet unexplored approach to this would be to classify patients by endotypes. Endotypes are defined as "a subtype of a condition, which is defined by a distinct functional or pathophysiological mechanism".[73] Instead of stratifying patients into clinical phenotypes, we propose, to stratify patients into endotypes based on distinct functional or pathophysiological mechanisms (Fig. 3). We suggest that endotyping AD patients will result in better understanding of the underlying pathomechanisms thereby enabling more targeted, and therefore more efficient, treatments for individual patients, a step towards precision medicine.

Serum biomarker based patient clusters: a first step towards endotypes in AD

In chapter 7 we showed that by analyzing 150 serum biomarkers in an unbiased, data-driven approach, patients could be assigned to four distinct clusters. Each cluster was characterised by a specific serum.

Biomarker profile, implying that each of these clusters is driven by a distinct underlying immunopathological pathway and may represent endotypes with different biological mechanisms. Interestingly, patients in cluster 1 and 4, representing about half of the patients with moderate to severe AD that were included in this study, showed particularly high Th2-cytokine levels (including IL-4, IL-5, and IL-13). These patients would hypothetically represent ideal patients for Th2 targeting drugs that are currently being tested, including the anti-IL-4Rα antibody dupilumab, and anti-IL-13 antibodies lebrikizumab and tralokinumab.[74-76] Whilst, both clusters 1 and 4 have high Th2 cytokines compared to clusters 2 and 3, the elevation of Th2 cytokines is most pronounced in cluster 4. Furthermore, cluster 1 can be differentiated from each cluster by a very high expression of PARC . PARC is a chemokine secreted by monocytes and dendritic cells and is involved in the recruitment of CC chemokine receptor 4 expressing memory T cells.[77-79] CCR4 is preferentially expressed on CLA+CD4+ T cells, which are known to dominate the cell infiltrate in lesional skin of AD patients.[80]

Figure 3. Schematic representation of the umbrella term 'atopic dermatitis'.



Key clinical features such as age of onset, response to treatment, severity, skin infections, the presence of (atopic) comorbidities, can be used to classify AD patients into associated phenotypes. Whereas a phenotype is based on observable clinical characteristics, an endotype is a subtype of AD defined by a distinct pathophysiological mechanism. In order to endotype patients and identify underlying pathways, studies should follow a multi-layer approach, integrating high-throughput data from genomics, transcriptomics, lipomics, and proteomics. Linking of endotypes to clinical phenotypes and to endotype specific biomarkers are crucial, because phenotypes and biomarkers are more accessible to clinicians than endotypes are.

The high expression of PARC makes patients in cluster 1 an interesting population for treatment with mogamulizumab, a humanized immunoglobulin monoclonal antibody that targets CCR4. Mogamulizumab was originally developed for treatment of T-cell leukemia-lymphoma and cutaneous T-cell lymphoma, and is currently being tested in phase I and II clinical trials.[81] Interestingly, cluster 4 shows high expression of TSLP compared to cluster 1, this might indicate a pathophysiological mechanism in cluster 4 in which TSLP activates dendritic cells that prime naive T cells towards a Th2 phenotype.[82] Thus, patients in cluster 4 could represent those patients that that would respond to new anti-TSLP biologics currently being tested.

The other two clusters shared a relatively low Th2-cytokine profile and were characterized by a relatively pauci-inflammatory mediator state (analogous to that seen in Th2-low asthma).[83] These clusters would hypothetically represent patient groups that are not ideal for Th2 targeting drugs. A more comprehensive biological characterisation of disease mechanisms in these patients will be required to allow generation of more tailored therapeutics. The findings from our study are consistent with recent phase 3 data from the Th2 targeting drug dupilumab in AD, demonstrating that around half of the patients treated with dupilumab did not achieve the primary endpoint of reduction in IGA.[84] This emphasizes the need for biomarkers to identify those patients that would most optimally respond to this treatment and even more important those that may not.

To investigate whether the expression of biomarkers in the four clusters could be linked to specific immunological pathways, we used a gene set enrichment analysis. This analysis is normally used for gene expression data. Since we investigated serum biomarker levels and not gene expression in our study, we coded the serum biomarkers by their gene names. Although we found clear differences in serum biomarkers expression levels between clusters, we did not find pathways that were specifically expressed in certain clusters. The pathway analysis showed that leukocyte- and dendritic cell migration pathways and chemotaxis are expressed in all four clusters. Based on these findings, we concluded that certain pathways may be more important in driving AD in certain patient clusters, leading to differences in serum biomarker expression, but that there is no black and white difference between the pathways involved between the four clusters.

A Th2 cytokine that could be of therapeutic interest is IL-5. IL-5 is upregulated in cluster 1, and particularly in cluster 4. It is a potent cytokine responsible for growth and differentiation of eosinophils in the bone marrow,[85] and stimulates the emigration from bone marrow into peripheral blood.[86] A humanized monoclonal antibody that recognizes IL-5 (mepolizumab) in AD, explored in a previous study, failed to demonstrate efficacy.[87] In that study, eosinophil counts in blood significantly decreased, but no clinical success was reached and the influx of tissue eosinophil numbers in the atopy patch test was not inhibited. However, four out of 18 patients treated with mepolizumab, did show a significant decrease in disease severity. Moreover, only a minority of the patients had a peripheral blood eosinophilia at baseline. And maybe even of

more important, patients received only two doses of mepolizumab in two weeks, which might not be sufficient for a significant reduction of tissue eosinophils. Initial trials with mepolizumab for the treatment of asthma also failed to demonstrate efficacy.[88, 89] However, subsequent studies in patients with severe asthma with frequent exacerbations and sputum or peripheral blood eosinophilia showed that mepolizumab significantly reduced the number of exacerbations compared to a placebo treatment.[90, 91] Subsequent studies showed that peripheral blood eosinophilia can indeed be used as a biomarker for prediction of response to mepolizumab in asthma.[92, 93] Recently, the Food and Drug Administration (FDA) approved the use of mepolizumab for patients with severe uncontrolled eosinophilic asthma. Although we did not measure eosinophilic blood counts in our study, we speculate that high IL-5 serum levels might correlate with blood and tissue eosinophilia, just as in asthma.[94] It is likely that this precision medicine approach in AD, in which patients are selected based on either serum IL-5 levels or eosinophilia, will also contribute to treatment success.

Serum IL-31 levels in atopic dermatitis

IL-31 is a cytokine that is thought to play a key role in the induction of pruritis in AD patients.[95-99] Nemolizumab is a humanized anti-human IL-31 receptor A antibody that was recently tested in phase I and II trials.[100, 101] Although treatment with nemolizumab resulted in a large decrease of pruritus in both trials, exacerbations of AD were reported in around half of the patients treated with nemolizumab in the phase I trial, and exacerbations were more common in the nemolizumab groups than in the placebo group in the phase II trial. These results suggest that not all patients respond optimally to nemolizumab treatment, and that nemolizumab might be more effective in a selected group of patients. In chapter 7, we report serum IL-31 levels from 193 AD patients and 30 controls. Interestingly, we found an almost black and white difference in IL-31 expression levels in serum. In AD patients, IL-31 levels were either high; higher than in normal controls, or undetectable (Fig. 4). This suggests that IL-31 indeed might only play a role in a subgroup of patients. It will be interesting to see whether there is indeed a difference in response to nemolizumab between patients with or without detectable serum IL-31 levels.

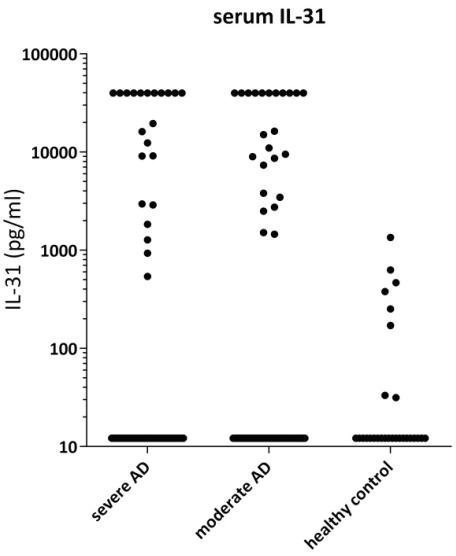


Figure 4. serum IL-31 is only detectable in a subset of AD patients (n=193) and healthy controls (n=30).

The link between phenotypes and endotypes

Patients with extrinsic AD have high serum IgE levels. Allergen penetration into the skin has been suggested to result in IgE mediated mast cell activation and release of pro-inflammatory mediators such as histamine and Th2 cytokines IL-4 and IL-13.[102] Other important cell types in the skin that bear IgE receptors and can contribute to IgE-mediated immune response are epidermal Langerhans cells and inflammatory dendritic epidermal cells.[103] Through IgE binding, these cells can take up allergens and activate Th2 cells to produce IL-4 and IL-13.[103] Therefore, extrinsic AD has been related to Th2 cytokine pathway activation.[102] Remarkably, we did not find any differences in total IgE levels between the four clusters as described in chapter 7. Neither did we find a relationship between total serum IgE levels and levels of Th2 cytokines, or differences between the clusters and biomarkers related to severity including TARC, IL-22 and sIL-2R. We also investigated the presence of other atopic comorbidities. Although, cluster 1 and 3 showed a slightly higher prevalence of allergic asthma and rhinitis, we could not find any clear differences in sensitization patterns based on 150 specific IgE levels between the patient clusters. In conclusion, the Th2 high and low clusters found in our study do not seem to be related to atopic constitution. These findings are in concordance with a recent study that showed similar mRNA levels of Th2 cytokines IL-4, IL-5, and IL-13 in lesional skin of both intrinsic and extrinsic AD patients.[104] Moreover, treatment effects in dupilumab trials were found to be similar in both intrinsic and extrinsic AD patient groups, and while disease severity significantly decreased within two weeks of treatment, total serum IgE only started to decrease after 12 weeks of treatment with dupilumab.[40, 105] The results from these studies suggest that the link between Th2 type inflammation and atopic constitution that is classically made, might not be as strong as has been assumed so far. The high levels of total IgE in serum as seen in AD patients might just be an epiphenomenon reflecting strong Th2 activation in skin.

There have been many attempts to classify AD based on phenotypical characteristics like the presence of atopic comorbidities.[70] However, this has not yet lead to better understanding in the underlying pathology of disease. We also investigated the relationship between phenotypical characteristics and the different patient clusters found. Next to the classically used characteristics like the presence of atopic comorbidities and age of onset of the disease, we stratified patients into a subset with predominantly chronic lichenified eczema, and another subset with a predominantly erythematous eczema. This classification is based on our observations in the clinic from which we got the impression that there are two main subsets of patients; patients that have a predominantly chronic lichenified eczema, and a second group of patients that present with a predominantly erythematous eczema. Whilst not reaching statistical significance, cluster 1 and 4, the most Th2 skewed clusters, tended to have more erythematous skin, while cluster 2 and 3 tended to have a more chronic lichenified skin. Thus, the implication is that a Th2 driven AD may result in a more erythematous skin phenotype. Whether Th2 cytokines directly drive this erythematous phenotype or simply represent biomark-

ers of an as yet unidentified biological mechanism remains to be elucidated. Similarly, understanding how the underlying biology of patients in clusters 2 and 3 drives the lichenification process needs further investigation. Interestingly, the keratinocyte proliferation inducing cytokine IL-22 was significantly higher in cluster 2 than in the other clusters.[106] Induced keratinocyte proliferation microscopically results in acanthosis, and may lead to a more lichenified eczema phenotype.

Heterogeneity in children with atopic dermatitis

The four clusters described in chapter 7 were identified in adult patients with moderate to severe AD. There have been no studies investigating endotypes of children with AD. Whether the endotypes that we found in adult patients will also be present in children with AD is questionable. The clinical presentation of AD in childhood and adults is clearly different, as was described previously. These clinical differences, may be the result of different endotypes presenting at different ages.

Another reason to consider the presence of different or additional endotypes in childhood, is the fact that AD is transient in the majority of children with AD. Birth cohort studies have suggested that, in up to 70% of cases, AD greatly improves or resolves until late childhood.[107, 108] One could speculate that resolving childhood AD may represent an independent endotype of AD. However, several birth cohort studies have shown that severity is one of the strongest risk factor for a non resolving AD.[107, 108] Therefore, it could also be the case that endotypes do not differ between adults and children with AD, but that children with resolving disease suffer from a milder form of AD, irrespective the endotype they belong to. This theory would be in agreement with our findings in chapter 7, in which we showed that disease severity does not influence cluster membership of adult AD patients.

In chapter 7, we also showed that patients in two out of four clusters have particularly high levels of Th2 cytokines (IL-4, IL-5, IL-13). Czarnowicki et al. recently showed that circulating skin-homing T cells in children with AD (mean age 25 months) are predominantly Th2 type cells, in contrast with adults who also showed increased Th1 and Th22 populations.[55] In a subsequent study by the same group using the same patient population, disproportionally higher T-cell than B-cell counts were found in skin lesions of pediatric patients compared to adult patients with AD.[109] Data from these studies suggest that Th2 activation plays a more important role in children with AD, and that Th1 and Th22 activation may be influenced by immune development and disease chronicity. This may suggest that the presence of Th2 high clusters might be even more pronounced in paediatric AD than in adult AD.

Valorisation

Endotypes may prove useful with the introduction of new targeted therapies, since patients with different endotypes may respond differently to the same treatment. Sub-grouping patients based on a serum biomarker and blood eosinophils has already been proven to be useful in asthma, where anti-IL-13 therapy appears to be most effective in the specific subgroup of patients with high serum levels of periostin.[110, 111] Similarly, quantifying blood eosinophils in asthmatics has facilitated the identification of those patients that may best respond to IL-5 axis targeting drugs, such as mepolizumab and benralizumab.[93, 112] The ability to endotype AD patients may contribute to precision medicine by allowing treatment to be tailored for individual patients.[113] This would not only be beneficial for patients, but would also reduce health-care costs.[114]

Future studies

The patient clusters identified in our study need confirmation in an independent patient population in a prospective study. The current study included 150 serum biomarkers. To avoid selection bias all analytes that were available in our MultiPlex Facility were included in the study. However, research in our laboratory focusses on chronic inflammatory diseases, which may have resulted in a bias to inflammation related biomarkers available for multiplex analysis. Advances in high-scale proteomics have led to the possibility of measuring over 1000 analytes in small amounts of serum.[115] Including these techniques in future studies may identify new biomarkers for endotyping AD. We also did not study gene expression in our patient population. It is known that AD has a genetic predisposition, the strongest genetic risk factor for AD is a loss of function mutation in the skin barrier gene encoding filaggrin (FLG).[72] However, over half of the patient with AD do not carry this mutation, and 60% of the carriers of a FLG mutation do not develop atopic disease.[72] This highlights the heterogeneous character of AD, making genetic profiling very interesting to include in future studies.

We were unable to identify a clear association between the four clusters and other clinical characteristics like the presence of allergic asthma or rhinitis, or age of onset of the disease. Patients in our study were selected solely on disease severity (SASSAD), sex and age, no other clinical characteristics peculiar to AD were considered for selecting patients. Therefore, the absence of an association between the clusters and other clinical characteristics may be the consequence of the unbalanced presence of these clinical characteristics across the hypothetical endotypes. However, it also highlights the significant challenges in identifying endotypes with underlying pathophysiological mechanisms based on clinical features such as age of onset or the presence of other atopic diseases, and suggests that the Th2 high and Th2 low clusters found in our study do not seem to be related to atopic constitution. The fact that we could not find any clear differences in sensitization patterns based on 150 specific IgE levels between the patient clusters strengthens this hypothesis. Future studies including detailed clinical data may be able to link clinical characteristics to specific

endotypes.

Also, a longitudinal study may provide us additional insights into the stability of biomarkers in a patient over time. Since AD follows a relapsing and remitting course, it might be possible that biomarker profiles and corresponding endotypes vary over time, highlighting the need for a prospective study. To answer the question whether paediatric endotypes show overlap with adult endotypes of AD, longitudinal studies with follow-up during childhood and adult life are needed.

Interventional clinical trials will be the final step in the confirmation of the different endotypes. Interventional clinical trials with drugs targeting a specific pathway will help to confirm whether the serum biomarkers associated with each cluster directly relate to a pathological mechanism and thus confirm clusters as specific endotypes.

IMPROVING THE PERFORMANCE OF IMMUNOSUPPRESSIVE DRUGS USING PHARMACOGENOMIC BIOMARKERS

The introduction of biologicals will provide new treatment options in AD that could considerably improve patients' quality of life.[2] However, possible long-term side effects of these biologics are unknown,[116] and biologics still are relatively expensive. Clinicians will have to evaluate the risks, benefits, and costs accordingly. We expect that even after the introduction of biologics in AD, classic immunosuppressive drugs will still be used. Firstly, because the high costs of biologics will lead to strict reimbursement criteria. In the Netherlands, the requirement for reimbursement for biologics in AD will probably include prior treatment with at least one oral immunosuppressive drug such as CsA. Secondly, we know that in other chronic inflammatory diseases, like psoriasis, Crohn's disease and rheumatoid arthritis, immunosuppressive drugs are still regularly prescribed although many biologics are available.[117-119]

Cyclosporin A is currently the only registered drug for the treatment of AD in the Netherlands. Other drugs that are frequently prescribed (off-label) are azathioprine (AZA), methotrexate (MTX), tacrolimus, and mycophenolic acid (MPA). Pharmacogenetic biomarkers may play a role in optimising the performance of these drugs. In chapter 8 we studied the relationship between the presence of enzyme uridine diphosphate-glucuronosyltransferase 1A9 (UGT1A9) polymorphisms and response to MPA. The efficacy of MPA in AD has been proven in clinical studies.[120] However, in clinical practice, MPA is ineffective in nearly half of the AD patients.[121, 122] In blood, MPA levels are known to have a large inter-individual variability. Low MPA exposure and increased enzyme activity has been shown to correlate with the presence of UGT1A9 polymorphisms in kidney transplant patients, leading to rejection of the transplanted kidney. We hypothesized that also for AD patients, low MPA exposure due to the presence of UGT1A9 polymorphisms might contribute to the inefficacy during MPA treatment. Indeed, in chapter 8 we retrospectively showed that pre-treatment screening for UGT1A9 polymorphisms could have identified 19% of the patients with non-responsiveness to MPA treatment. The therapeutic strategy in non-responsive UGT1A9 polymorphism carriers would have been to prescribe a higher dosage of MPA, thereby increasing MPA exposure and possibly lead to better treatment effect. This study shows the potential benefit of pharmacogenetic screening for UGT1A9 in AD. Prediction of treatment response to MPA in AD is very valuable, since treatment response can only be determined after three to four months of treatment.

Pharmacogenetic biomarkers can also be used to optimize the performance of other immunosuppressive drugs in the treatment of AD. Bioavailability and systemic clearance of the calcineurin inhibitor CsA is also mainly controlled by the isoenzymes CYP3A4 and CYP3A5.[123] Studies in renal transplant patients showed that the presence of SNPs in CYP3A4 and CYP3A5 influences blood levels of CsA.[124] Pharmacoge-

netic testing of SNPs in CYP3A4 and CYP3A5 may also be of use in the treatment of patients with CsA and needs further investigation.

The performance of tacrolimus, another (off label) calcineurin inhibitor for treatment of AD, can also be personalized and optimized by pharmacogenetic testing.[125] Tacrolimus is extensively metabolized by CYP3A4 and CYP3A5. The presence of a C>T SNP in intron 6 of CYP3A4 is associated with intermediate or poor tacrolimus metabolism, resulting in high tacrolimus blood levels. In addition, the carriage of CYP3A5*3 (slow metabolism) and CYP3A5*1 (fast metabolism) alleles also affects tacrolimus metabolism. Expression analysis of SNPs in the genes for CYP3A4 and CYP3A5 enables classification of AD patients into poor, intermediate or extensive metabolizers of tacrolimus.[121] This CYP3A4/CYP3A5 genotype cluster classification can be used to tailor dosing of tacrolimus to the individual patient, and to avoid adverse effects.

Azathioprine is another second line drug that is prescribed off-label for treatment of AD. Just as MPA, AZA shows a good clinical performance in only half of the patients.[122] The performance of AZA is mainly limited by discontinuation of treatment due to side effects, like gastro-intestinal symptoms and increased transaminases.[122] Studies in inflammatory bowel disease have shown that genetic polymorphisms in thiopurine methyltransferase (TPMT) influences the metabolism of AZA.[126] Genotyping of TPMT prior to the start of AZA treatment allows identification of those at increased risk for adverse events.[126] During treatment, monitoring of the AZA metabolites 6-thioguanine nucleotides (6-TGN) and 6-methylmercaptopurine ribonucleotides (6-MMPN) can be used for the risk assessment of myelotoxicity and liver toxicity.[126] Azathioprine is another second line drug that is prescribed off-label for treatment of AD. Just as MPA, AZA shows a good clinical performance in only half of the patients.[122] The performance of AZA is mainly limited by discontinuation of treatment due to side effects, like gastoro-intestinal symptoms and increased transaminases.[122] During treatment, monitoring of the AZA metabolites 6-thioguanine nucleotides (6-TGN) and 6-methylmercaptopurine ribonucleotides (6-MMPN) can be used for the risk assessment of myelotoxicity and liver toxicity.[126]

Valorisation

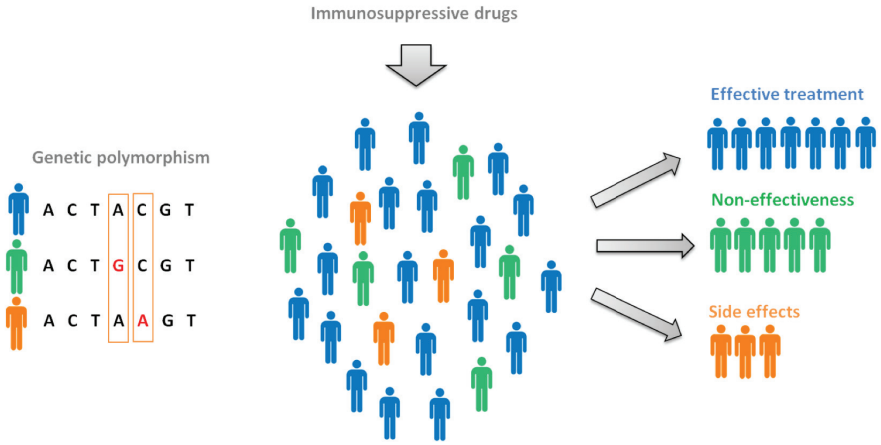
Although pharmacogenomics are currently scarcely used in dermatologic treatment, it also contributes to more “personalized medicine” by enabling prescription of drugs based on the genetic makeup of an individual. Pharmacogenetic profiling of patients prior to the start of oral immunosuppressive therapy can highly improve therapy outcome by preventing adverse effects and non-responsiveness to treatment (Figure 4). This will not only lead to a decrease in the burden of patients, but will also decrease costs.

Future studies

To further optimize MPA treatment future research is needed to identify other SNPs and to prospectively study the relationship between UGT1A9 polymorphisms, MPA blood levels and treatment effect to ensure maximal efficacy with minimal side effects.

The relationship between CYP3A4 and CYP3A5 and response to CsA therapy has not yet been studied in AD patients. Although non-effectiveness is less relevant in patients treated with CsA (±20%), identification of poor metabolizers of CsA would be of interest, since side effects are the major cause for discontinuation of CsA treatment in AD patients (±30%).[127, 128] In AD patients treated with AZA, evaluation of the TPMT genotype status and monitoring of 6-TGN and 6-MMPN is of great interest. Pharmacogenomic profiling may help identifying those patients to identify at risk of developing severe side effects such as suppression of the bone marrow. Studies that monitor 6-TGN during AZA treatment will give us more insight in the therapeutic range that is needed for effective treatment of AD.

Figure 5. Pharmacogenomics in atopic dermatitis.



Pharmacogenomics have been defined as the study of variability in drug response caused by heredity. Pharmacogenomic research explores the effect of pharmacokinetics, pharmacodynamics, efficacy, and safety of drug treatments in relation to genome variations. Genetic variations, like single-nucleotide polymorphisms (SNPs), genetic copy number variations, and genomic insertions and deletions can influence the response of a patient to a specific drug and can be used as biomarkers.

IMPROVING PRACTICAL ASPECTS OF BIOMARKER MEASUREMENT

Biomarkers measured in serum of AD patients are the main focus of the studies presented in this thesis. A disadvantage of serum biomarkers in the need for a venipuncture that can only be performed by trained personnel. Furthermore, this blood needs to be processed in a lab and must be centrifuged within four hours of collection, which is costly and not practical. Serum biomarkers are also less favored in pediatric medicine, especially since AD usually presents in childhood. We explored alternatives sources for biomarker measurement that can be used in both research and daily practice.

Dried blood spots (DBS) might represent a convenient alternative to venipuncture. Dried blood spots can be obtained using a simple, minimally invasive, nearly painless procedure that can be done by the patients themselves. DBS can be easily stored and shipped at room temperature until storage in a freezer. Dried blood spots have been used for decades in screening for inherited metabolic diseases in newborns.[129] In chapter 11, we investigated if biomarkers determined in DBS offer a reliable tool for the assessment of disease severity in AD. Comparison of biomarkers measured in DBS and serum, revealed a very strong correlation between concentrations of TARC (CCL17), I-309 (CCL1) and MDC (CCL22) levels in DBS and serum. Interestingly, TARC showed a stronger correlation with disease severity when measured in DBS compared to serum ($r=0.68$ vs. $r= 0.58$ respectively).

A higher recovery level of biomarkers in DBS than in serum has previously been shown.[130] The higher recovery levels in DBS are probably due to different biomarker concentrations in capillary blood compared to venous blood. Dried blood spots are composed of capillary (whole) blood and contain all blood cells and platelets, which hold many inflammatory analytes, while serum is the fluid remaining after blood coagulation and contains no blood cells or platelets. It has been shown that platelets from patients with AD contain high levels of TARC.[131] Platelets probably lyse when spotted on filter paper, consequently releasing their content, which can be measured in eluted samples. This may contribute to the strong correlation between DBS TARC and disease severity.

In serum, we showed that a combination of the biomarkers TARC, sIL-2R and IL-22 performs better in assessing disease severity than a single biomarker (chapter 3 and 4). Levels of sIL-2R and IL-22 measured in DBS only weakly correlated with disease severity and did not decrease during effective treatment with topical steroids. Therefore, sIL-2R and IL-22 measured in DBS are not suitable as a biomarker for disease severity. The differences between sIL-2R and IL-22 in serum and DBS may be explained by the effect that spotting onto filter paper could have on analytes; analytes might bind to the paper or undergo structural changes during DBS formation.[72, 132] We did find other biomarkers, besides TARC, in DBS that correlated with disease severity and significantly decreases during treatment: I-309, PARC and MDC. However,

including these biomarkers in a linear mixed model analysis for prediction of EASI scores did not improve the model. We noticed that TARC levels measured in DBS highly correlated with I-309, PARC and MDC levels measured in DBS. These correlations may explain why adding I-309, PARC or MDC into a prediction model adds no value to a model that includes TARC.

In chapter 11, the stability of biomarkers in DBS was also demonstrated. Levels of TARC measured in DBS stayed stable irrespective of the amount of days (1, 3 or 7 days) left at room temperature before storing at -20°C. TARC levels in DBS stored over two years at -80°C remained stable as well. A previous study showed that cytokine levels in DBS remain stable up to 23 years when stored at -24°C.[133] Moreover, we showed that TARC levels in DBS are detectable in equal concentrations regardless of repeated freeze/thaw cycles.

The easy collection and stability of DBS offer a huge simplification in storage and transport. The stability of DBS at room temperature reduces sample transportation and storage costs. Dried blood spots are safer to transport and considered as non-regulated and exempt material, which allows shipping of DBS through mail or courier.[134] The long term stability of biomarkers in DBS and small volume, makes DBS ideal for biobanking and multi-center trials.

In conclusion, DBS offer a offer a simple and minimally invasive and low-cost method for measurement of a selection of biomarkers and provide a tool for objective severity outcome measurements in AD that can easily be applied in clinical trials and daily practice.

Valorisation

We showed that TARC measured in DBS offers an objective assessment of disease severity in AD. Combining TARC measurement in DBS with a patient oriented outcome measure like POEM, offers a reliable tool for monitoring disease activity and burden of disease in AD.

Disease severity assessment by physicians during consultations in the hospital, can be replaced by DBS biomarkers and patient assessed outcomes. This facilitates distant monitoring of patients, reducing the number of face-to-face consultations and saving costs and time for patients and society.

Patients treated with oral immunosuppressive drugs regularly visit the hospital for to monitoring of side effects and laboratory testing. The frequency of these visits can be reduced by the use of DBS. Measurement of drug concentrations in DBS is possible in a range of drugs,[135] including CsA,[136-139] mycophenolic acid,[140] and tacrolimus,[139, 141, 142] drugs which are frequently prescribed in AD treatment.[143] Measurement of creatinine levels in DBS has also been described.[142] Since decrease in kidney function is one of the most common adverse effects in CsA treatment, measurement of creatinine levels in DBS can be very interesting for the follow-up of AD patients treated with CsA. Combining measurement of drug concentrations in DBS

with disease severity biomarkers would be ideal for home follow-up and can improve patient care by individually adjusting dosages, but would also be of great interest in clinical trials.

Future studies

Dried blood spots offer an interesting tool for studying early changes in biomarker profile during a flare of AD. If a patient experiences a flare, it happens quite often that by the time the patient attends for an appointment, the flare has subsided or have passed into a chronic severe state of disease. If patients are instructed to collect DBS in the first days of a flare, a lag period between flare commencement and time to be able to consult primary care physician can be avoided. Biomarker profiles measured in DBS during early flare stages might identification novel pathways that contribute to disease pathogenesis and flaring of AD.

FUTURE PERSPECTIVES

We think that the increased application of biomarkers in AD research will result in better characterization and stratification of patients, and will give a better understanding of the underlying pathomechanisms in AD. Furthermore, it will result in objective outcome measures that will allow better comparison of current and new treatments. We hypothesize that in the near future, patients with AD will be stratified based on biomarker expression levels in body fluids (blood/saliva/tears), and tissue (biopsies/skin strips), on genetic variants, or combined biomarker expression patterns (composite biomarker scores). With many new targeted therapies currently investigated in phase I to III clinical trials, biomarkers will lead to better identification of patients that can benefit from these highly specific, but expensive new treatments. In addition to a role for biomarkers in new treatments, the use of pharmacogenomic biomarkers, can highly improve effectivity of currently used oral immunosuppressive drugs in AD. Measurement of biomarkers in alternative sources , such as DBS, facilitates distant monitoring, which is more convenient for patients and reduces healthcare costs. We believe that the application of biomarkers in AD is essential in moving forward to predictive, personalized, preventive, and participatory medicine.

REFERENCES

1. Eyerich K, Novak N. Immunology of atopic eczema: overcoming the Th1/Th2 paradigm. Allergy 2013; 68:974-82.
2. Boyman O, Kaegi C, Akdis M, Bavbek S, Bossios A, Chatzipetrou A, et al. EAACI IG Biologicals task force paper on the use of biologic agents in allergic disorders. Allergy 2015; 70:727-54.
3. Williams H, Stewart A, von Mutius E, Cookson W, Anderson HR. Is eczema really on the increase worldwide? J Allergy Clin Immunol 2008; 121:947-54 e15.
4. Deckers IA, McLean S, Linssen S, Mommers M, van Schayck CP, Sheikh A. Investigating international time trends in the incidence and prevalence of atopic eczema 1990-2010: a systematic review of epidemiological studies. PLoS One 2012; 7:e39803.
5. Zuberbier T, Lotvall J, Simoens S, Subramanian SV, Church MK. Economic burden of inadequate management of allergic diseases in the European Union: a GA(2) LEN review. Allergy 2014; 69:1275-9.
6. Simpson EL, Bieber T, Eckert L, Wu R, Ardeleanu M, Graham NM, et al. Patient burden of moderate to severe atopic dermatitis (AD): Insights from a phase 2b clinical trial of dupilumab in adults. J Am Acad Dermatol 2016; 74:491-8.
7. Charman C, Chambers C, Williams H. Measuring atopic dermatitis severity in randomized controlled clinical trials: what exactly are we measuring? J Invest Dermatol 2003; 120:932-41.
8. Schmitt J, Langan S, Williams HC. What are the best outcome measurements for atopic eczema? A systematic review. J Allergy Clin Immunol 2007; 120:1389-98.
9. Schmitt J, Spuls PI, Thomas KS, Simpson E, Furue M, Deckert S, et al. The Harmonising Outcome Measures for Eczema (HOME) statement to assess clinical signs of atopic eczema in trials. J Allergy Clin Immunol 2014; 134:800-7.
10. Chalmers JR, Simpson E, Apfelbacher CJ, Thomas KS, von Kobyletzki L, Schmitt J, et al. Report from the fourth international consensus meeting to harmonize core outcome measures for atopic eczema/dermatitis clinical trials (HOME initiative). Br J Dermatol 2016; 175:69-79.
11. Charman CR, Venn AJ, Williams HC. Measurement of body surface area involvement in atopic eczema: an impossible task? Br J Dermatol 1999; 140:109-11.
12. Zhao CY, Tran AQ, Lazo-Dizon JP, Kim J, Daniel BS, Venugopal SS, et al. A pilot comparison study of four clinician-rated atopic dermatitis severity scales. Br J Dermatol 2015; 173:488-97.
13. Kakinuma T, Nakamura K, Wakugawa M, Mitsui H, Tada Y, Saeki H, et al. Thymus and activation-regulated chemokine in atopic dermatitis: Serum thymus and activation-regulated chemokine level is closely related with disease activity. J Allergy Clin Immunol 2001; 107:535-41.
14. Zheng X, Nakamura K, Furukawa H, Nishibu A, Takahashi M, Tojo M, et al. Demonstration of TARC and CCR4 mRNA expression and distribution using in situ RT-PCR in the lesional skin of atopic dermatitis. J Dermatol 2003; 30:26-32.
15. Imai T, Nagira M, Takagi S, Kakizaki M, Nishimura M, Wang J, et al. Selective recruitment of CCR4-bearing Th2 cells toward

antigen-presenting cells by the CC chemokines thymus and activation-regulated chemokine and macrophage-derived chemokine. Int Immunol 1999; 11:81-8.

16. Kayserova J, Capkova S, Skalicka A, Vernerova E, Polouckova A, Malinova V, et al. Serum immunoglobulin free light chains in severe forms of atopic dermatitis. Scand J Immunol 2010; 71:312-6.

17. Schouten B, van Esch BC, van Thuijl AO, Blokhuis BR, Groot Kormelink T, Hofman GA, et al. Contribution of IgE and immunoglobulin free light chain in the allergic reaction to cow's milk proteins. J Allergy Clin Immunol 2010; 125:1308-14.

18. Rijnierse A, Redegeld FA, Blokhuis BR, Van der Heijden MW, Te Velde AA, Pronk I, et al. Ig-free light chains play a crucial role in murine mast cell-dependent colitis and are associated with human inflammatory bowel diseases. J Immunol 2010; 185:653-9.

19. Kraneveld AD, Kool M, van Houwelingen AH, Roholl P, Solomon A, Postma DS, et al. Elicitation of allergic asthma by immunoglobulin free light chains. Proc Natl Acad Sci U S A 2005; 102:1578-83.

20. Redegeld FA, van der Heijden MW, Kool M, Heijdra BM, Garssen J, Kraneveld AD, et al. Immunoglobulin-free light chains elicit immediate hypersensitivity-like responses. Nat Med 2002; 8:694-701.

21. Landheer J, de Bruin-Weller M, Boonacker C, Hijnen D, Bruijnzeel-Koomen C, Rockmann H. Utility of serum thymus and activation-regulated chemokine as a biomarker for monitoring of atopic dermatitis severity. J Am Acad Dermatol 2014; 71:1160-6.

22. Pivarcsi A, Gombert M, Dieu-Nosjean MC, Lauerma A, Kubitza R, Meller S, et al. CC chemokine ligand 18, an atopic dermatitis-associated and dendritic cell-derived chemokine, is regulated by staphylococcal products and allergen exposure. J Immunol 2004; 173:5810-7.

23. Yang HJ, Jeon YH, Pyun BY. Evaluation of patient's subjective severity using various scoring system in Korean children with atopic dermatitis. Asian Pac J Allergy Immunol 2010; 28:130-5.

24. Galli G, Chantry D, Annunziato F, Romagnani P, Cosmi L, Lazzeri E, et al. Macrophage-derived chemokine production by activated human T cells in vitro and in vivo: preferential association with the production of type 2 cytokines. Eur J Immunol 2000; 30:204-10.

25. Gombert M, Dieu-Nosjean MC, Winterberg F, Bunemann E, Kubitza RC, Da Cunha L, et al. CCL1-CCR8 interactions: an axis mediating the recruitment of T cells and Langerhans-type dendritic cells to sites of atopic skin inflammation. J Immunol 2005; 174:5082-91.

26. Morita H, Kitano Y, Kawasaki N. Elevation of serum-soluble E-selectin in atopic dermatitis. J Dermatol Sci 1995; 10:145-50.

27. Wolkerstorfer A, Laan MP, Savelkoul HF, Neijens HJ, Mulder PG, Oudesluys-Murphy AM, et al. Soluble E-selectin, other markers of inflammation and disease severity in children with atopic dermatitis. Br J Dermatol 1998; 138:431-5.

28. Ushio S, Namba M, Okura T, Hattori K, Nukada Y, Akita K, et al. Cloning of the cDNA for human IFN-gamma-inducing factor, expression in Escherichia coli, and studies on the biologic activities of the protein. J Immunol 1996; 156:4274-9.

29. Stoll S, Jonuleit H, Schmitt E, Muller G, Yamauchi H, Kurimoto M, et al. Production of functional IL-18 by different subtypes of murine and human dendritic cells (DC): DC-derived IL-18 enhances IL-12-dependent Th1 development. Eur J Immunol 1998; 28:3231-9.

30. Stoll S, Muller G, Kurimoto M, Saloga J, Tanimoto T, Yamauchi H, et al. Production of IL-18 (IFN-gamma-inducing factor) messenger RNA and functional protein by murine keratinocytes. J Immunol 1997; 159:298-302.

31. Hoshino T, Yagita H, Ortaldo JR, Wiltrout RH, Young HA. In vivo administration of IL-18 can induce IgE production through Th2 cytokine induction and up-regulation of CD40 ligand (CD154) expression on CD4(+) T cells. European Journal of Immunology 2000; 30:1998-2006.

32. Walker C, Kagi MK, Ingold P, Braun P, Blaser K, Bruijnzeel-Koomen CA, et al. Atopic dermatitis: correlation of peripheral blood T cell activation, eosinophilia and serum factors with clinical severity. Clin Exp Allergy 1993; 23:145-53.

33. Hijnen D, Knol EF, Gent YY, Giovannone B, Beijn SJ, Kupper TS, et al. CD8(+) T cells in the lesional skin of atopic dermatitis and psoriasis patients are an important source of IFN-gamma, IL-13, IL-17, and IL-22. J Invest Dermatol 2013; 133:973-9.

34. Nograles KE, Zaba LC, Shemer A, Fuentes-Duculan J, Cardinale I, Kikuchi T, et al. IL-22-producing "T22" T cells account for upregulated IL-22 in atopic dermatitis despite reduced IL-17-producing TH17 T cells. J Allergy Clin Immunol 2009; 123:1244-52 e2.

35. Gutowska-Owsiak D, Schaupp AL, Salimi M, Taylor S, Ogg GS. Interleukin-22 downregulates filaggrin expression and affects expression of profilaggrin processing enzymes. Br J Dermatol 2011; 165:492-8.

36. Nakatsuji T, Chen TH, Two AM, Chun KA, Narala S, Geha RS, et al. Staphylococcus aureus Exploits Epidermal Barrier Defects in Atopic Dermatitis to Trigger Cytokine Expression. J Invest Dermatol 2016; 136:2192-200.

37. Lou H, Lu J, Choi EB, Oh MH, Jeong M, Barmettler S, et al. Expression of IL-22 in the Skin Causes Th2-Biased Immunity, Epidermal Barrier Dysfunction, and Pruritus via Stimulating Epithelial Th2 Cytokines and the GRP Pathway. J Immunol 2017.

38. Ring J, Alomar A, Bieber T, Deleuran M, Fink-Wagner A, Gelmetti C, et al. Guidelines for treatment of atopic eczema (atopic dermatitis) Part II. J Eur Acad Dermatol Venereol 2012; 26:1176-93.

39. Ring J, Alomar A, Bieber T, Deleuran M, Fink-Wagner A, Gelmetti C, et al. Guidelines for treatment of atopic eczema (atopic dermatitis) part I. J Eur Acad Dermatol Venereol 2012; 26:1045-60.

40. Beck LA, Thaci D, Hamilton JD, Graham NM, Bieber T, Rocklin R, et al. Dupilumab treatment in adults with moderate-to-severe atopic dermatitis. N Engl J Med 2014; 371:130-9.

41. DElios MM, Romagnani P, Scaletti C, Annunziato F, Manghetti M, Mavilia C, et al. In vivo CD30 expression in human diseases with predominant activation of Th2-like T cells. Journal of Leukocyte Biology 1997; 61:539-44.

42. Kwakkel-van Erp JM, Haeck IM, Paantjens AW, van de Graaf EA, van Ginkel WG, Knol MJ, et al. Differential usefulness of biomarkers thymus and activation-regulated chemokine and soluble CD30 during enteric coated mycophenolate sodium

and cyclosporine therapy in atopic dermatitis. J Am Acad Dermatol 2010; 63:e70-2.

43. Mollanazar NK, Smith PK, Yosipovitch G. Mediators of Chronic Pruritus in Atopic Dermatitis: Getting the Itch Out? Clin Rev Allergy Immunol 2016; 51:263-92.

44. Raap U, Wichmann K, Bruder M, Stander S, Wedi B, Kapp A, et al. Correlation of IL-31 serum levels with severity of atopic dermatitis. J Allergy Clin Immunol 2008; 122:421-3.

45. Kyoya M, Kawakami T, Soma Y. Serum thymus and activation-regulated chemokine (TARC) and interleukin-31 levels as biomarkers for monitoring in adult atopic dermatitis. J Dermatol Sci 2014; 75:204-7.

46. Nygaard U, Hvid M, Johansen C, Buchner M, Folster-Holst R, Deleuran M, et al. TSLP, IL-31, IL-33 and sST2 are new biomarkers in endophenotypic profiling of adult and childhood atopic dermatitis. J Eur Acad Dermatol Venereol 2016; 30:1930-8.

47. Kim S, Kim HJ, Yang HS, Kim E, Huh IS, Yang JM. IL-31 Serum Protein and Tissue mRNA Levels in Patients with Atopic Dermatitis. Ann Dermatol 2011; 23:468-73.

48. Weidinger S, Novak N. Atopic dermatitis. Lancet 2016; 387:1109-22.

49. Leung TF, Ma KC, Hon KL, Lam CW, Wan H, Li CY, et al. Serum concentration of macrophage-derived chemokine may be a useful inflammatory marker for assessing severity of atopic dermatitis in infants and young children. Pediatr Allergy Immunol 2003; 14:296-301.

50. Song TW, Sohn MH, Kim ES, Kim KW, Kim KE. Increased serum thymus and activation-regulated chemokine and cutaneous T cell-attracting chemokine levels in children with atopic dermatitis. Clin Exp Allergy 2006; 36:346-51.

51. Fujisawa T, Nagao M, Hiraguchi Y, Katsumata H, Nishimori H, Iguchi K, et al. Serum measurement of thymus and activation-regulated chemokine/CCL17 in children with atopic dermatitis: elevated normal levels in infancy and age-specific analysis in atopic dermatitis. Pediatr Allergy Immunol 2009; 20:633-41.

52. Matsumoto T, Miike T, Yamaguchi K, Murakami M, Kawabe T, Yodoi J. Serum levels of soluble IL-2 receptor, IL-4 and IgE-binding factors in childhood allergic diseases. Clin Exp Immunol 1991; 85:288-92.

53. Frezzolini A, Paradisi M, Ruffelli M, Cadoni S, De Pita O. Soluble CD30 in pediatric patients with atopic dermatitis. Allergy 1997; 52:106-9.

54. Halmerbauer G, Frischer T, Koller DY. Monitoring of disease activity by measurement of inflammatory markers in atopic dermatitis in childhood. Allergy 1997; 52:765-9.

55. Czarnowicki T, Esaki H, Gonzalez J, Malajian D, Shemer A, Noda S, et al. Early pediatric atopic dermatitis shows only a cutaneous lymphocyte antigen (CLA) 1 T(H)2/T(H)1 cell imbalance, whereas adults acquire CLA1(+) T(H)22/T(C)22 cell subsets. Journal of Allergy and Clinical Immunology 2015; 136:941-+.

56. Boniface K, Bernard FX, Garcia M, Gurney AL, Lecron JC, Morel F. IL-22 inhibits epidermal differentiation and induces proinflammatory gene expression and migration of human keratinocytes. J Immunol 2005; 174:3695-702.

57. Brunner PM, Silverberg JI, Guttman-Yassky E, Paller AS, Kabashima K, Amagai M, et al. Increasing Comorbidities Suggest

that Atopic Dermatitis Is a Systemic Disorder. Journal of Investigative Dermatology 2017; 137:18-25.

58. Yamanaka K, Nakanishi T, Saito H, Maruyama J, Isoda K, Yokochi A, et al. Persistent release of IL-1s from skin is associated with systemic cardio-vascular disease, emaciation and systemic amyloidosis: the potential of anti-IL-1 therapy for systemic inflammatory diseases. PLoS One 2014; 9:e104479.

59. Boehncke WH, Boehncke S, Tobin AM, Kirby B. The 'psoriatic march': a concept of how severe psoriasis may drive cardiovascular comorbidity. Exp Dermatol 2011; 20:303-7.

60. Heufler C, Topar G, Grasseger A, Stanzl U, Koch F, Romani N, et al. Interleukin-7 Is Produced by Murine and Human Keratinocytes. Journal of Experimental Medicine 1993; 178:1109-14.

61. Mazzucchelli RI, Riva A, Durum SK. The human IL-7 receptor gene: deletions, polymorphisms and mutations. Semin Immunol 2012; 24:225-30.

62. Hoffjan S, Beygo J, Akkad DA, Parwez Q, Petrasch-Parwez E, Epplen JT. Analysis of variation in the IL7RA and IL2RA genes in atopic dermatitis. Journal of Dermatological Science 2009; 55:138-40.

63. Paternoster L, Standl M, Waage J, Baurecht H, Hotze M, Strachan DP, et al. Multi-ancestry genome-wide association study of 21,000 cases and 95,000 controls identifies new risk loci for atopic dermatitis. Nature Genetics 2015; 47:1449-+.

64. Lundstrom W, Highfill S, Walsh ST, Beq S, Morse E, Kockum I, et al. Soluble IL7Ralpha potentiates IL-7 bioactivity and promotes autoimmunity. Proc Natl Acad Sci U S A 2013; 110:E1761-70.

65. Todd JA, Walker NM, Cooper JD, Smyth DJ, Downes K, Plagnol V, et al. Robust associations of four new chromosome regions from genome-wide analyses of type 1 diabetes. Nat Genet 2007; 39:857-64.

66. O'Doherty C, Alloza I, Rooney M, Vandenbroeck K. IL7RA polymorphisms and chronic inflammatory arthropathies. Tissue Antigens 2009; 74:429-31.

67. Heron M, Grutters JC, Van Moorsel CH, Ruven HJ, Kazemier KM, Claessen AM, et al. Effect of variation in ITGAE on risk of sarcoidosis, CD103 expression, and chest radiography. Clin Immunol 2009; 133:117-25.

68. Gregory SG, Schmidt S, Seth P, Oksenberg JR, Hart J, Prokop A, et al. Interleukin 7 receptor alpha chain (IL7R) shows allelic and functional association with multiple sclerosis. Nat Genet 2007; 39:1083-91.

69. Kurz T, Hoffjan S, Hayes MG, Schneider D, Nicolae R, Heinzmann A, et al. Fine mapping and positional candidate studies on chromosome 5p13 identify multiple asthma susceptibility loci. J Allergy Clin Immunol 2006; 118:396-402.

70. Bieber T. Atopic dermatitis 2.0: from the clinical phenotype to the molecular taxonomy and stratified medicine. Allergy 2012; 67:1475-82.

71. Bieber T, Cork M, Reitamo S. Atopic dermatitis: a candidate for disease-modifying strategy. Allergy 2012; 67:969-75.

72. Irvine AD, McLean WH, Leung DY. Filaggrin mutations associated with skin and allergic diseases. N Engl J Med 2011; 365:1315-27.

73. Lotvall J, Akdis CA, Bacharier LB, Bjermer L, Casale TB, Custovic A, et al. Asthma endotypes: A new approach to classification of disease entities within the asthma syndrome. Journal of Allergy and Clinical Immunology 2011; 127:355-60.

74. A Study to Assess the Efficacy and Safety of Dupilumab in Patients With Severe Atopic Dermatitis (AD) That Are Not Controlled With Oral Cyclosporine A (CSA) or for Those Who Cannot Take Oral CSA Because it is Not Medically Advisable.] Available from https://www.clinicaltrials.gov/ct2/show/NCT02755649.

75. A Study to Evaluate the Safety of Lebrikizumab Compared to Topical Corticosteroids in Adult Patients With Atopic Dermatitis.] Available from https://clinicaltrials.gov/ct2/show/NCT02465606: Hoffmann-La Roche.

76. Phase 2 Study to Evaluate the Efficacy and Safety of Tralokinumab in Adults With Atopic Dermatitis (D2213C00001).] Available from https://www.clinicaltrials.gov/ct2/show/NCT02347176.

77. Adema GJ, Hartgers F, Verstraten R, deVries E, Marland G, Menon S, et al. A dendritic-cell-derived C-C chemokine that preferentially attracts naive T cells. Nature 1997; 387:713-7.

78. Gunther C, Bello-Fernandez C, Kopp T, Kund J, Carballido-Perrig N, Hinteregger S, et al. CCL18 is expressed in atopic dermatitis and mediates skin homing of human memory T cells. J Immunol 2005; 174:1723-8.

79. Chenivesse C, Chang Y, Azzaoui I, Ait Yahia S, Morales O, Ple C, et al. Pulmonary CCL18 recruits human regulatory T cells. J Immunol 2012; 189:128-37.

80. Wakugawa M, Nakamura K, Kakinuma T, Onai N, Matsushima K, Tamaki K. CC chemokine receptor 4 expression on peripheral blood CD4(+) T cells reflects disease activity of atopic dermatitis. Journal of Investigative Dermatology 2001; 117:188-96.

81. Ogura M, Ishida T, Hatake K, Taniwaki M, Ando K, Tobinai K, et al. Multicenter Phase II Study of Mogamulizumab (KW-0761), a Defucosylated Anti-CC Chemokine Receptor 4 Antibody, in Patients With Relapsed Peripheral T-Cell Lymphoma and Cutaneous T-Cell Lymphoma. Journal of Clinical Oncology 2014; 32:1157-+.

82. Soumelis V, Reche PA, Kanzler H, Yuan W, Edward G, Homey B, et al. Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. Nat Immunol 2002; 3:673-80.

83. Fahy JV. Type 2 inflammation in asthma--present in most, absent in many. Nat Rev Immunol 2015; 15:57-65.

84. Simpson EL, Bieber T, Guttman-Yassky E, Beck LA, Blauvelt A, Cork MJ, et al. Two Phase 3 Trials of Dupilumab versus Placebo in Atopic Dermatitis. N Engl J Med 2016.

85. Sanderson CJ. Interleukin-5, eosinophils, and disease. Blood 1992; 79:3101-9.

86. Collins PD, Marleau S, Griffiths-Johnson DA, Jose PJ, Williams TJ. Cooperation between interleukin-5 and the chemokine eotaxin to induce eosinophil accumulation in vivo. J Exp Med 1995; 182:1169-74.

87. Oldhoff JM, Darsow U, Werfel T, Katzer K, Wulf A, Laifaoui J, et al. Anti-IL-5 recombinant humanized monoclonal antibody (mepolizumab) for the treatment of atopic dermatitis. Allergy 2005; 60:693-6.

88. Flood-Page P, Swenson C, Faiferman I, Matthews J, Williams M, Brannick L, et al. A study to evaluate safety and efficacy of mepolizumab in patients with moderate persistent asthma. Am J Respir Crit Care Med 2007; 176:1062-71.

89. Leckie MJ, ten Brinke A, Khan J, Diamant Z, O'Connor BJ, Walls CM, et al. Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyper-responsiveness, and the late asthmatic response. Lancet 2000; 356:2144-8.

90. Nair P, Pizzichini MM, Kjarsgaard M, Inman MD, Efthimiadis A, Pizzichini E, et al. Mepolizumab for prednisone-dependent asthma with sputum eosinophilia. N Engl J Med 2009; 360:985-93.

91. Ortega H, Chupp G, Bardin P, Bourdin A, Garcia G, Hartley B, et al. The role of mepolizumab in atopic and nonatopic severe asthma with persistent eosinophilia. Eur Respir J 2014; 44:239-41.

92. Ortega HG, Liu MC, Pavord ID, Brusselle GG, FitzGerald JM, Chetta A, et al. Mepolizumab treatment in patients with severe eosinophilic asthma. N Engl J Med 2014; 371:1198-207.

93. Pavord ID, Korn S, Howarth P, Bleecker ER, Buhl R, Keene ON, et al. Mepolizumab for severe eosinophilic asthma (DREAM): a multicentre, double-blind, placebo-controlled trial. Lancet 2012; 380:651-9.

94. Walker C, Bode E, Boer L, Hansel TT, Blaser K, Virchow JC. Allergic and Nonallergic Asthmatics Have Distinct Patterns of T-Cell Activation and Cytokine Production in Peripheral-Blood and Bronchoalveolar Lavage. American Review of Respiratory Disease 1992; 146:109-15.

95. Bilsborough J, Leung DYM, Maurer M, Howell M, Boguniewcz M, Yao L, et al. IL-31 is associated with cutaneous lymphocyte antigen-positive skin homing T cells in patients with atopic dermatitis. Journal of Allergy and Clinical Immunology 2006; 117:418-25.

96. Cevikbas F, Wang XD, Akiyama T, Kempkes C, Savinko T, Antal A, et al. A sensory neuron-expressed IL-31 receptor mediates T helper cell-dependent itch: Involvement of TRPV1 and TRPA1. Journal of Allergy and Clinical Immunology 2014; 133:448-+.

97. Nobbe S, Dziunycz P, Muhleisen B, Bilsborough J, Dillon SR, French LE, et al. IL-31 Expression by Inflammatory Cells is Preferentially Elevated in Atopic Dermatitis. Acta Dermato-Venereologica 2012; 92:24-8.

98. Sonkoly E, Muller A, Lauerma AI, Pivarcsi A, Soto H, Kemeny L, et al. IL-31: A new link between T cells and pruritus in atopic skin inflammation. Journal of Allergy and Clinical Immunology 2006; 117:411-7.

99. Szegedi K, Kremer AE, Kezic S, Teunissen MBM, Bos JD, Luiten RM, et al. Increased frequencies of IL-31-producing T cells are found in chronic atopic dermatitis skin. Experimental Dermatology 2012; 21:431-6.

100. Nemoto O, Furue M, Nakagawa H, Shiramoto M, Hanada R, Matsuki S, et al. The first trial of CIM331, a humanized antihuman interleukin-31 receptor A antibody, in healthy volunteers and patients with atopic dermatitis to evaluate safety, tolerability and pharmacokinetics of a single dose in a randomized, double-blind, placebo-controlled study. Br J Dermatol 2016; 174:296-304.

101. Ruzicka T, Hanifin JM, Furue M, Pulka G, Mlynarczyk I, Wollenberg A, et al. Anti-Interleukin-31 Receptor A Antibody for Atopic Dermatitis. N Engl J Med 2017; 376:826-35.

102. Leung DY, Guttman-Yassky E. Deciphering the complexities of atopic dermatitis: shifting paradigms in treatment approaches. J Allergy Clin Immunol 2014; 134:769-79.

103. Novak N, Valenta R, Bohle B, Laffer S, Haberstok J, Kraft S, et al. FcepsilonRI engagement of Langerhans cell-like dendritic cells and inflammatory dendritic epidermal cell-like dendritic cells induces chemotactic signals and different T-cell pheno-

types in vitro. J Allergy Clin Immunol 2004; 113:949-57.

104. Suarez-Farinas M, Dhingra N, Gittler J, Shemer A, Cardinale I, de Guzman Strong C, et al. Intrinsic atopic dermatitis shows similar TH2 and higher TH17 immune activation compared with extrinsic atopic dermatitis. J Allergy Clin Immunol 2013; 132:361-70.

105. Thaci D, Simpson EL, Beck LA, Bieber T, Blauvelt A, Papp K, et al. Efficacy and safety of dupilumab in adults with moderate-to-severe atopic dermatitis inadequately controlled by topical treatments: a randomised, placebo-controlled, dose-ranging phase 2b trial. Lancet 2016; 387:40-52.

106. Zheng Y, Danilenko DM, Valdez P, Kasman I, Eastham-Anderson J, Wu J, et al. Interleukin-22, a T(H)17 cytokine, mediates IL-23-induced dermal inflammation and acanthosis. Nature 2007; 445:648-51.

107. Hua TC, Hwang CY, Chen YJ, Chu SY, Chen CC, Lee DD, et al. The natural course of early-onset atopic dermatitis in Taiwan: a population-based cohort study. British Journal of Dermatology 2014; 170:130-5.

108. Illi S, von Mutius E, Lau S, Nickel R, Gruber C, Niggemann B, et al. The natural course of atopic dermatitis from birth to age 7 years and the association with asthma. Journal of Allergy and Clinical Immunology 2004; 113:925-31.

109. Czarnowicki T, Esaki H, Gonzalez J, Renert-Yuval Y, Brunner P, Oliva M, et al. Alterations in B-cell subsets in pediatric patients with early atopic dermatitis. J Allergy Clin Immunol 2016.

110. Corren J, Lemanske RF, Hanania NA, Korenblat PE, Parsey MV, Arron JR, et al. Lebrikizumab treatment in adults with asthma. N Engl J Med 2011; 365:1088-98.

111. Brightling CE, Chanez P, Leigh R, O'Byrne PM, Korn S, She D, et al. Efficacy and safety of tralokinumab in patients with severe uncontrolled asthma: a randomised, double-blind, placebo-controlled, phase 2b trial. Lancet Respir Med 2015; 3:692-701.

112. Castro M, Wenzel SE, Bleecker ER, Pizzichini E, Kuna P, Busse WW, et al. Benralizumab, an anti-interleukin 5 receptor alpha monoclonal antibody, versus placebo for uncontrolled eosinophilic asthma: a phase 2b randomised dose-ranging study. Lancet Respir Med 2014; 2:879-90.

113. Muraro A, Lemanske RF, Jr., Hellings PW, Akdis CA, Bieber T, Casale TB, et al. Precision medicine in patients with allergic diseases: Airway diseases and atopic dermatitis-PRACTALL document of the European Academy of Allergy and Clinical Immunology and the American Academy of Allergy, Asthma & Immunology. J Allergy Clin Immunol 2016; 137:1347-58.

114. Jameson JL, Longo DL. Precision medicine--personalized, problematic, and promising. N Engl J Med 2015; 372:2229-34.

115. Gold L, Walker JJ, Wilcox SK, Williams S. Advances in human proteomics at high scale with the SOMAscan proteomics platform. New Biotechnology 2012; 29:543-9.

116. Bremmer MS, Bremmer SF, Baig-Lewis S, Simpson EL. Are biologics safe in the treatment of atopic dermatitis? A review with a focus on immediate hypersensitivity reactions. J Am Acad Dermatol 2009; 61:666-76.

117. van der Valk ME, van Oijen MG, Ammerlaan M, Siersema PD, Oldenburg B. Crohn's disease patients treated with adalim-

umab benefit from co-treatment with immunomodulators. Gut 2012; 61:324-5.

118. Gomollon F, Dignass A, Annese V, Tilg H, Van Assche G, Lindsay JO, et al. 3rd European Evidence-based Consensus on the Diagnosis and Management of Crohn's Disease 2016: Part 1: Diagnosis and Medical Management. J Crohns Colitis 2017; 11:3-25.

119. Singh JA, Saag KG, Bridges SL, Jr., Akl EA, Bannuru RR, Sullivan MC, et al. 2015 American College of Rheumatology Guideline for the Treatment of Rheumatoid Arthritis. Arthritis Rheumatol 2016; 68:1-26.

120. Sidbury R, Davis DM, Cohen DE, Cordoro KM, Berger TG, Bergman JN, et al. Guidelines of care for the management of atopic dermatitis: section 3. Management and treatment with phototherapy and systemic agents. J Am Acad Dermatol 2014; 71:327-49.

121. Garritsen FM, Roekevisch E, van der Schaft J, Deinum J, Spuls PI, de Bruin-Weller MS. Ten years experience with oral immunosuppressive treatment in adult patients with atopic dermatitis in two academic centres. J Eur Acad Dermatol Venereol 2015; 29:1905-12.

122. van der Schaft J, Politiek K, van den Reek JM, Kievit W, de Jong EM, Bruijnzeel-Koomen CA, et al. Drug survival for azathioprine and enteric-coated mycophenolate sodium in a long-term daily practice cohort of adult patients with atopic dermatitis. Br J Dermatol 2016.

123. de Jonge H, Kuypers DR. Pharmacogenetics in solid organ transplantation: current status and future directions. Transplant Rev (Orlando) 2008; 22:6-20.

124. Elens L, Hesselink DA, Bouamar R, Budde K, de Fijter JW, De Meyer M, et al. Impact of POR*28 on the pharmacokinetics of tacrolimus and cyclosporine A in renal transplant patients. Ther Drug Monit 2014; 36:71-9.

125. van der Schaft J, van Schaik RH, van Zuilen AD, Hijnen DJ, Berg MT, van den Broek MP, et al. First experience with extended release tacrolimus in the treatment of adult patients with severe, difficult to treat atopic dermatitis: Clinical efficacy, safety and dose finding. J Dermatol Sci 2016; 81:66-8.

126. Bloomfeld RS BS, Levine ME, Carroll S. Thiopurine methyltransferase activity is correlated with azathioprine metabolite levels in inflammatory bowel disease patients in clinical gastroenterology practice. Journal of Applied Research 2004; 6:5.

127. van der Schaft J, Politiek K, van den Reek JM, Christoffers WA, Kievit W, de Jong EM, et al. Drug survival for ciclosporin A in a long-term daily practice cohort of adult patients with atopic dermatitis. Br J Dermatol 2015; 172:1621-7.

128. Hijnen DJ, ten Berge O, Timmer-de Mik L, Bruijnzeel-Koomen CA, de Bruin-Weller MS. Efficacy and safety of long-term treatment with cyclosporin A for atopic dermatitis. J Eur Acad Dermatol Venereol 2007; 21:85-9.

129. Sood BG, Madan A, Saha S, Schendel D, Thorsen P, Skogstrand K, et al. Perinatal Systemic Inflammatory Response Syndrome and Retinopathy of Prematurity. Pediatric Research 2010; 67:394-400.

130. Pennings JL, Siljee JE, Imholz S, Kuc S, de Vries A, Schielen PC, et al. Comparison of different blood collection, sample matrix, and immunoassay methods in a prenatal screening setting. Dis Markers 2014; 2014:509821.

131. Fujisawa T, Fujisawa R, Kato Y, Nakayama T, Morita A, Katsumata H, et al. Presence of high contents of thymus and

activation-regulated chemokine in platelets and elevated plasma levels of thymus and activation-regulated chemokine and macrophage-derived chemokine in patients with atopic dermatitis. J Allergy Clin Immunol 2002; 110:139-46.

132. Koster RA, Alffenaar JW, Greijdanus B, Uges DR. Fast LC-MS/MS analysis of tacrolimus, sirolimus, everolimus and cyclosporin A in dried blood spots and the influence of the hematocrit and immunosuppressant concentration on recovery. Talanta 2013; 115:47-54.

133. Skogstrand K, Thorsen P, Norgaard-Pedersen B, Schendel DE, Sorensen LC, Hougaard DM. Simultaneous measurement of 25 inflammatory markers and neurotrophins in neonatal dried blood spots by immunoassay with xMAP technology. Clin Chem 2005; 51:1854-66.

134. Federal Register. 49 CFR part 173 Shippers General Requirements for Shipments and packaging. Section 173.134(b) 2011.] Available from https://www.gpo.gov/fdsys/pkg/CFR-2011-title49-vol2/pdf/CFR-2011-title49-vol2-sec173-134.pdf.

135. Sharma A, Jaiswal S, Shukla M, Lal J. Dried blood spots: concepts, present status, and future perspectives in bioanalysis. Drug Test Anal 2014; 6:399-414.

136. Lampe D, Scholz D, Prumke HJ, Blank W, Huller H. Capillary blood, dried on filter paper, as sample for monitoring cyclosporin A concentrations. Clin Chem 1987; 33:1643-4.

137. Mohamed R, Mercolini L, Cuennet-Cosandey S, Chavent J, Raggi MA, Peyrou M. Validation of a dried blood spot LC-MS/MS approach for cyclosporin A in cat blood: Comparison with a classical sample preparation. Journal of Pharmaceutical and Biomedical Analysis 2012; 66:298-305.

138. Hinchliffe E, Adaway JE, Keevil BG. Simultaneous measurement of cyclosporin A and tacrolimus from dried blood spots by ultra high performance liquid chromatography tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 2012; 883-884:102-7.

139. Koster RA, Alffenaar JWC, Greijdanus B, Uges DRA. Fast LC-MS/MS analysis of tacrolimus, sirolimus, everolimus and cyclosporin A in dried blood spots and the influence of the hematocrit and immunosuppressant concentration on recovery. Talanta 2013; 115:47-54.

140. Heinig K, Bucheli F, Hartenbach R, Gajate-Perez A. Determination of mycophenolic acid and its phenyl glucuronide in human plasma, ultrafiltrate, blood, DBS and dried plasma spots. Bioanalysis 2010; 2:1423-35.

141. Hoogtanders K, van der Heijden J, Christiaans M, Edelbroek P, van Hooff JP, Stolk LM. Therapeutic drug monitoring of tacrolimus with the dried blood spot method. J Pharm Biomed Anal 2007; 44:658-64.

142. Koop DR, Bleyle LA, Munar M, Cherala G, Al-Uzri A. Analysis of tacrolimus and creatinine from a single dried blood spot using liquid chromatography tandem mass spectrometry. Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences 2013; 926:54-61.

143. Ring J, Alomar A, Bieber T, Deleuran M, Fink-Wagner A, Gelmetti C, et al. Guidelines for treatment of atopic eczema (atopic dermatitis) Part II. J Eur Acad Dermatol Venereol 2012; 26:1176-93.


13

Nederlandse samenvatting

BELANGRIJKSTE BEVINDINGEN VAN DIT PROEFSCHRIFT

Biomarkers for ziekte ernst in constitutioneel eczeem

- Een meta-analyse van 222 studies laat zien dat serum TARC concentratie momenteel de beste biomarker is voor het vervolgen van ziekte ernst (hoofdstuk 2).
- Eerdere studies lieten zien dat immunoglobuline vrije lichte ketens gemeten in bloed van kinderen met constitutioneel eczeem (CE) correleren met ziekte ernst. Bij volwassen met CE correleren deze echter niet met ziekte ernst (hoofdstuk 6).
- Een pilot studie met 17 patiënten liet zien dat een combinatie van meerdere biomarkers gemeten in bloed een betere weerspiegeling geeft van ziekte ernst dan de meting van een enkele biomarker (hoofdstuk 3).
- In een daaropvolgende studie waarin 65 patiënten gedurende twee maanden werden behandeld met lokale steroïden, werd aangetoond dat een formule met daarin een combinatie van biomarkers (de predicted-EASI of p-EASI genaamd), te weten TARC, IL-22 en sIL-2R een correcte voorspelling van ziekte ernst geeft in 90% van de gevallen (hoofdstuk 4).
- De p-EASI weerspiegelt ziekte ernst (EASI) ook juist in patiënten die werden behandeld met ciclosporine A (hoofdstuk 5).
- De p-EASI biedt een objectieve uitkomst maat voor de ernst van constitutioneel eczeem in prospectieve studies (hoofdstuk 4 en 5).

Biomarkers voor het stratificeren van patiënten met constitutioneel eczeem

- CE is zowel klinisch als biologisch gezien erg heterogeen. Op basis van biomarkers gemeten in bloed kunnen vier clusters van patiënten worden geïdentificeerd, welke elk een specifiek biomarker profiel laten zien. Dit impliceert dat deze clusters verschillende onderliggende ziekteprocessen hebben, ook wel endotypes genoemd (hoofdstuk 7). Patienten uit verschillende endotypes reageren mogelijk verschillend op nieuwe 'targeted therapies', onze bevindingen kunnen daardoor bijdragen aan personalized medicine.
- De aanwezigheid van mutaties in het UGT1A9 gen kan worden gebruikt om CE patiënten te identificeren die niet goed reageren op therapie met mycophenol zuur. Dit laat de potentie van farmacodynamische biomarkers in de behandeling van CE zien (hoofdstuk 8).
- Biomarker profielen gemeten in bloed suggereren dat CE een systemische ziekte is die niet beperkt is tot afwijkingen in de huid (hoofdstuk 9).

Alternatieve bronnen voor het meten van biomarkers

- De meeste biomarkers worden gemeten in veneus bloed. Er bestaan echter nog andere interessante bronnen waarin biomarkers gemeten kunnen worden, zoals speeksel of dried blood spots (hoofdstuk 10).
- Een nadeel van het meten van biomarkers in veneus bloed is de noodzaak voor een venapunctie. TARC waardes gemeten in dried blood spots geven een goede weerspiegeling van ziekte ernst. Dried blood spots bieden een simpel en minimaal invasief alternatief voor het meten van biomarkers in CE (hoofdstuk 11).

NEDERLANDSE SAMENVATTING

Constitutioneel eczeem (CE) is een van de meest voorkomende chronische ontstekingsziekten van de huid wereldwijd. Het hebben van CE heeft een grote impact op de kwaliteit van leven van patiënten. In het complexe ontstaansmechanisme van CE spelen zowel genetische- als omgevingsfactoren een rol, welke uiteindelijk leiden tot afwijkingen in het afweersysteem en een verminderde huid barrière. Er zijn veel pogingen gedaan om CE patiënten te karakteriseren op basis van klinische kenmerken, zoals bijvoorbeeld de leeftijd waarop de ziekte is ontstaan, of de aanwezigheid van andere atopische ziekten zoals astma en hooikoorts. Deze indeling geeft echter geen goede weerspiegeling van de verschillende onderliggende processen die ten grondslag liggen aan CE.

Het merendeel van de patiënten kan goed worden behandeld met hormoonzalven, echter, er is een groot tekort aan behandelopties voor patiënten met matig tot ernstig CE. In de afgelopen jaren zijn veel nieuwe medicijnen ontwikkeld die aangrijpen of specifieke cytokines of cytokine receptoren die een rol spelen in de pathogenese van CE. De resultaten van de eerste studies met deze medicijnen zijn veelbelovend en bieden hopelijk goede behandelopties voor patiënten met matig en ernstig CE in de nabije toekomst.

Dit proefschrift focust op biomarkers voor CE. Biomarkers zijn karakteristieke biologische eigenschappen die gemeten kunnen worden in het menselijk lichaam (bijvoorbeeld in het bloed of in de urine). Biomarkers kunnen onder andere van belang kunnen zijn bij het diagnosticeren van ziektes, het volgen van het ziekte activiteit of het voorspellen van het effect van behandelingen. Voorbeelden van bekende biomarker zijn lichaamstemperatuur voor het vaststellen van koorts, en CRP gemeten in bloed voor het vervolgen van infectie.

In dit proefschrift staat de zoektocht naar biomarkers voor het vervolgen van ziekte ernst, en biomarkers voor het groeperen van patiënten op basis van onderliggende ziekte processen bij CE centraal.

Biomarkers voor het vervolgen van ziekte ernst in constitutioneel eczeem

In de dagelijkse dermatologische praktijk wordt de ernst van CE meestal “gemeten” doordat er in het hoofd van de behandelaar een integratie is van het klinische beeld en de anamnese (jeuk, zichtbaarheid, effect op kwaliteit van leven, etc.). Op basis van de integratie van die gegevens worden in samenspraak met de patiënt beslissingen genomen over de behandeling. In wetenschappelijke studies is het echter wenselijk een kwantitatieve maat te hebben, zodat het effect van behandeling kan worden onderzocht op basis van harde getallen. In het eczeemonderzoek wordt historisch gezien de ernst van CE gemeten op basis van scoresystemen zoals SCORAD, SASSAD, of EASI, die worden ingevuld door de behandelend arts of verpleegkundige. Deze score systemen houden rekening met de mate van roodheid, schilfering, lichenificatie, krabeffecten en

het oppervlakte van de aangedane huid. Omdat dit soort scoresystemen allerlei beperkingen hebben zijn er in de loop der tijd steeds nieuwe systemen ontwikkeld. Er bestaan momenteel meer dan 50 verschillende score systemen, wat de onderlinge vergelijking van studies naar therapieën voor CE bemoeilijkt. Bovendien zijn al deze scoresystemen subjectief en hebben ze daardoor als nadeel dat er grote inter- en intra variabiliteit ontstaat.

In tegenstelling tot de subjectieve score systemen bieden biomarkers een objectieve uitkomstmaat voor het meten van ziekte ernst. In de afgelopen decennia zijn eczeem onderzoekers op zoek geweest naar een geschikte objectieve biomarker. Dit heeft geleid tot publicaties over meer dan 100 verschillende biomarkers die mogelijk correleren met ziekte ernst bij eczeem. Onze meta-analyse van 222 studies laat zien dat serum TARC concentratie momenteel de beste biomarker is voor het vervolgen van ziekte ernst (hoofdstuk 2). Deze meta-analyse leerde ons ook dat biomarkers voor ziekte ernst niet op één, maar op meerdere tijdstippen gedurende een behandeling moeten worden onderzocht. De reden hiervan kan worden gedemonstreerd aan de hand van de totaal immunoglobuline E (IgE) waarde in bloed, de meest frequent gemeten biomarker in studies naar behandelingen voor CE. De meta-analyse uit hoofdstuk 2 laat zien dat totaal IgE goed correleert met ziekte ernst op een enkel tijdstip. Echter, wanneer het CE verbetert of verslechtert, blijft de totaal IgE waarde stabiel.

Tijdens de synthese van immunoglobulines worden zware en lichte ketens geproduceerd. In eerdere studies is aangetoond dat de waarde van deze vrije ketens gemeten in bloed van kinderen met CE een weerspiegeling geeft van ziekte ernst. In hoofdstuk 6 is onderzocht of deze lichte ketens ook geschikt zijn als biomarker voor ziekte ernst bij volwassenen met CE. Immunoglobuline lichte ketens werden zowel voor als na behandeling gemeten in het bloed van 82 CE patiënten. De waardes van Immunoglobuline lichte ketens van CE patiënten verschilden niet van gezonde personen en namen ook niet af tijdens effectieve behandeling van CE. Dit maakt dat Immunoglobuline lichte ketens gemeten in bloed geen geschikte biomarker is voor het vervolgen van ziekte ernst van CE.

Een combinatie van biomarkers voor het vervolgen van ziekte ernst in constitutioneel eczeem

De meta-analyse in hoofdstuk 2 laat zien dat TARC momenteel de beste biomarker is voor het meten van ziekte ernst van CE. Vanuit onze ervaring in de dagelijkse praktijk weten we dat TARC inderdaad een goede biomarker is voor het vervolgen van ziekte ernst in een individuele patiënt. Echter, we weten ook dat TARC waardes erg kunnen variëren tussen patiënten met dezelfde ziekte ernst, waardoor TARC ongeschikt is voor vervanging van klinische score systemen.

Zoals eerder beschreven is CE een complex ziektebeeld, waarin verschillende onderliggende ziekte processen een rol spelen. Wij stelden daarom de hypothese dat een combinatie van biomarkers, welke

verschillende processen in de pathogenese representeren, een betere weerspiegeling geeft van ziekte ernst dan een enkele biomarker. Om dit te onderzoeken is in hoofdstuk 3 een pilot studie uitgevoerd waarin werd aangetoond dat een combinatie van de biomarkers TARC, PARC, sIL-2R en IL-22 gemeten in bloed de ziekte ernst inderdaad een stuk beter weerspiegelt dan de individuele biomarkers (correlatie van 0,9 t.o.v. 0,4-0,7).

Omdat dit een studie van slechts 17 patiënten betrof, is bevestiging van onze bevindingen in een grotere populatie CE patiënten gewenst. In hoofdstuk 4 zijn 65 CE patiënten gedurende twee maanden behandeld met 'hormoonzalven', waarbij ziekte ernst zowel op basis van de klinische score 'Eczema Area and Severity Index' (EASI) als op basis van biomarkers gemeten in bloed werd bepaald. Uit deze studie bleek dat de combinatie van TARC, IL-22 en sIL-2R het meest optimaal is voor het vervolgen van ziekte ernst in patiënten met CE. Omdat de biomarker combinatie is gebaseerd op EASI scores, noemen we deze uitkomstmaat de 'predicted EASI' (p-EASI).

De p-EASI is ontwikkeld met behulp van gegevens van patiënten behandeld met 'hormoonzalven'. In hoofdstuk 5 is daarom onderzocht of de p-EASI ook kan worden toegepast in patiënten met CE die worden behandeld met ciclosporine A, het enige geregistreerde orale immunosuppressivum voor behandeling van CE .

Concluderend tonen de studies in hoofdstuk 4 en 5 dat de p-EASI een goede weerspiegeling geeft van ziekte ernst in CE patiënten in prospectieve studies. In tegenstelling tot de klassiek gebruikte subjectieve uitkomstmaten voor het meten van ernst van CE, biedt de p-EASI een objectieve uitkomstmaat. Het gebruik van een objectieve uitkomstmaat, zoals de p-EASI, is van essentieel belang voor de vergelijkbaarheid van toekomstige klinische studies naar nieuwe medicijnen voor CE.

Constitutioneel eczeem is een systemische ziekte

CE is geassocieerd met andere atopische aandoeningen zoals astma, allergische rhinitis en voedselallergie. Recente studies hebben ook associaties tussen CE en ander aandoeningen, zoals alopecia areata, neuropsychiatrisch en cardiovasculaire ziekten, aangetoond. Op basis van deze bevindingen wordt gehypothetiseerd dat systematische inflammatie in CE bijdraagt aan de ontwikkeling van deze comorbiditeiten.

In hoofdstuk 9 van dit proefschrift werden 150 verschillende biomarkers in het bloed van 193 CE patiënten en 30 gezonde personen gemeten. Het grootse deel van deze biomarkers was sterk verhoogd in CE patiënten in vergelijking met gezonde personen. Dit suggereert dat CE niet alleen een ziekte van de huid is, maar dat er inderdaad sprake is van een systemische afwijking in het afweersysteem, wat mogelijk bijdraagt aan de ontwikkeling van comorbiditeiten.

Biomarkers om de prestatie van toekomstige therapieën te verbeteren

CE is, zoals eerder beschreven, een complexe en heterogene aandoening. Het indelen van patiënten op basis van klinische eigenschappen, zoals het wel of niet hebben van astma, of de leeftijd waarop het eczeem zich presenteerde, heeft tot nu toe niet geleid tot verbetering in de therapie van CE. Met het oog op nieuwe 'targeted therapies' is het belangrijk om beter inzicht te krijgen in de heterogeniteit van de ziekteprocessen die ten grondslag liggen aan CE om zo de juiste patiënt met de juiste therapie te kunnen behandelen.

In hoofdstuk 7 van dit proefschrift is onderzocht of op basis van biomarkers gemeten in bloed, onafhankelijk van de klinische kenmerken, verschillende patiënt groepen geïdentificeerd kunnen worden. In een groep van 193 CE patiënten tonen we aan dat er op basis van 150 serum biomarkers vier duidelijk verschillende patiënt clusters te onderscheiden zijn. De vier patiënt clusters hebben allen een specifiek biomarker profiel, wat er op zou kunnen wijzen dat deze vier clusters verschillende onderliggende ziekteprocessen weerspiegelen, dit worden endotypes genoemd.

Een interessante bevinding in deze studie was het verschil in waardes van Th2 biomarkers tussen de groepen. Zowel de waardes van IL-4 en IL-13 zijn significant hoger in twee van de vier patiënt groepen. Theoretisch gezien zouden de patiënten uit de groepen met hoge Th2 biomarkers, ideale kandidaten zijn voor behandelingen waarbij Th2 inflammatie wordt geremd. Momenteel worden diverse therapieën die gericht zijn tegen Th2 inflammatie getest. Een van deze veelbelovende behandelingen is dupilumab, een anti-IL-4Rα blokker: dit middel blokkeert de werking van de hierboven genoemde cytokines IL-4 en IL-13. Fase 3 studies hebben laten zien dat behandeling met dupilumab in ongeveer de helft van de CE patiënten zeer effectief is. Deze resultaten komen overeen met onze bevinding dat ongeveer de helft van de CE patiënten uitgesproken hoge Th2 biomarker waardes hebben. Naast de verschillen in IL-4 en IL-13 biomarkers, verschillen de clusters ook in andere biomarkers die gerelateerd kunnen worden aan specifieke therapieën, zoals TSLP, IL-5 en IL-31.

Samenvattend laat hoofdstuk 7 zien dat CE niet alleen op basis van klinische kenmerken erg heterogeen is, maar ook op basis van biomarker profielen gemeten in bloed. Deze biomarker profielen kunnen een weerspiegeling van onderliggende ziekte processen zijn, en zijn daarom mogelijk van belang bij het selecteren van de juiste (nieuwe) therapie voor de juiste patiënt.

Farmacogenetische biomarkers om de prestatie van huidige therapieën te verbeteren

Farmacogenetica is de studie naar genetische verschillen in de opname en afbraak van medicijnen. Genmutaties kunnen afwijkingen veroorzaken in de mate waarin bepaalde enzymen medicijnen afbreken. Als een genmutatie een verhoogde afbraak van een bepaald medicijn veroorzaakt, is de bloedspiegel van dit medicijn lager en het medicijn daardoor minder effectief.

In hoofdstuk 9 van dit proefschrift werd retrospectief onderzocht of mutaties in het UGT1A9 gen de effectiviteit van mycofenolzuur voor de behandeling van CE kunnen voorspellen. Van de patiënten waarbij mycofenolzuur geen effect had op het CE, bleek 19% te kunnen worden geïdentificeerd aan de hand van een UGT1A9 mutatie. In patiënten met een UGT1A9 mutatie kan mycofenolzuur hoger worden gedoseerd, om zo toch een therapeutische spiegel en effectiviteit te behalen. UGT1A9 genmutaties kunnen worden ingezet als farmacodynamische biomarker om de prestatie van mycofenolzuur voor de behandeling van CE te verbeteren.

Verbetering van praktische aspecten van biomarker metingen

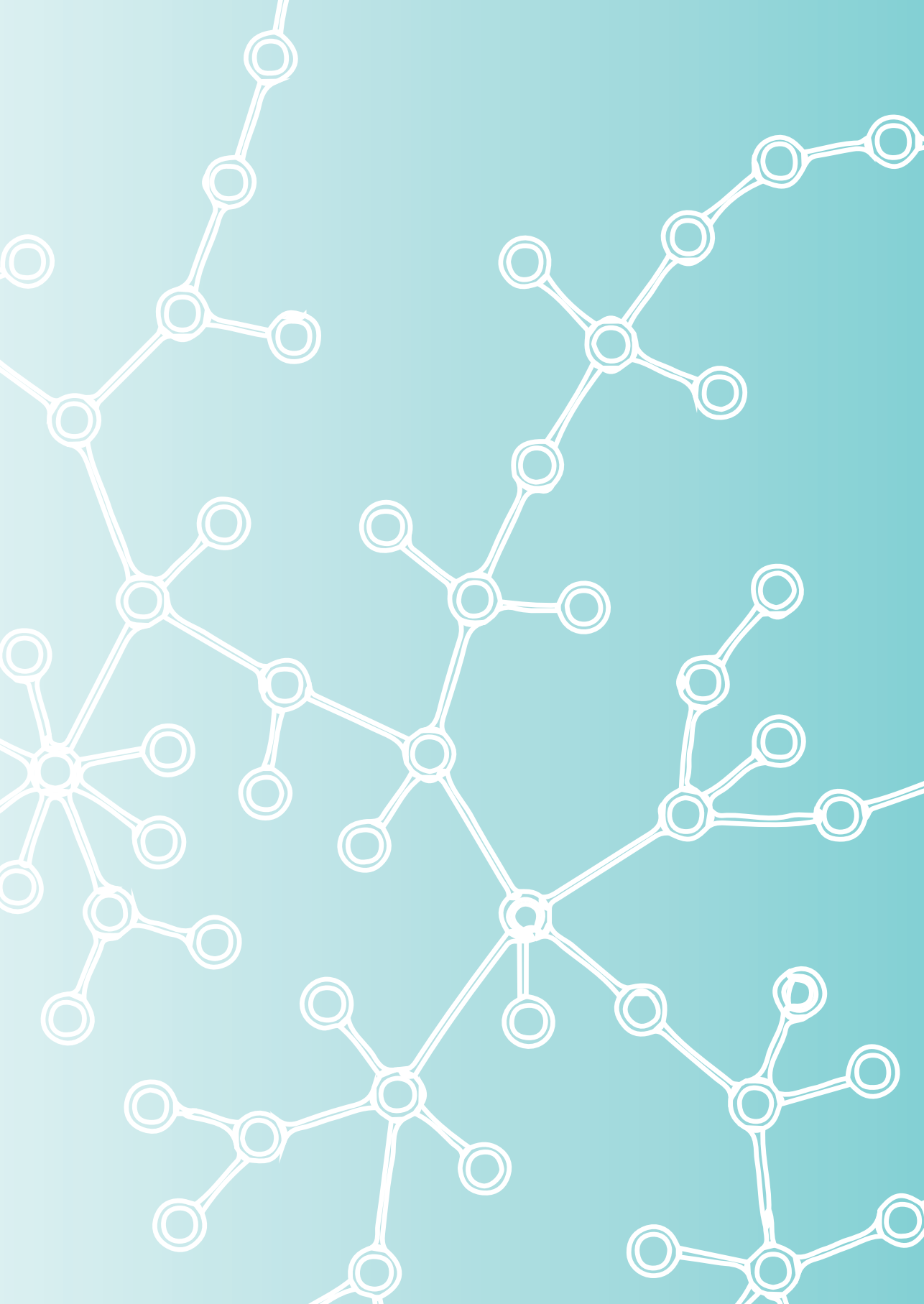
De studies in dit proefschrift richten zich met name op biomarkers gemeten in veneus bloed. Een nadeel van bloed is de noodzaak van een veneuze bloedafname, welke alleen door getraind personeel kan worden uitgevoerd. Ook moet het bloed binnen een uur worden verwerkt in het laboratorium, wat kostbaar en niet in alle situaties praktisch is.

Dried blood spots (DBS) bieden een gebruiksvriendelijk alternatief voor veneuze bloedafname. Een bekende toepassing van DBS is de hielprik bij pasgeborenen ter opsporing van metabole ziekten. Deze vindt plaats in de thuissituatie en het verzamelde bloed kan via reguliere post worden verstuurd naar een laboratorium voor analyse. Voor het verzamelen van DBS is slechts een kleine vingerprik nodig, welke door de patiënt zelf kan worden uitgevoerd. DBS kunnen gemakkelijk worden opgeslagen en worden verstuurd op kamertemperatuur. In hoofdstuk 11 hebben we aangetoond dat de biomarkers I-309, TARC, PARC en MDC gemeten in DBS een goede weerspiegeling van ziekte ernst geven tijdens effectieve behandeling van CE. Een interessante bevinding is dat TARC gemeten in DBS zelfs een betere weerspiegeling van ziekte ernst geeft dan TARC gemeten in serum. In hoofdstuk 11 werd ook de stabiliteit van TARC gemeten in DBS onderzocht: zowel vries/dooi cycli, opslag op kamertemperatuur en langdurige opslag bij -20℃ beïnvloedt de TARC waarden gemeten in DBS niet.

Concluderend bieden DBS een praktisch en minimaal invasief alternatief voor het meten van biomarkers in patiënten met CE.

Toekomstperspectief

De toepassing van biomarkers in CE maakt het objectief meten van ziekte ernst mogelijk, waardoor bestaande en toekomstige therapieën beter met elkaar vergeleken kunnen worden. Tevens zullen biomarkers in toenemende mate een rol gaan spelen in het karakteriseren en stratificeren van CE patiënten. Dit kan in belangrijke mate bijdragen aan het behandelen van de juiste patiënt met het juiste geneesmiddel en het betaalbaar houden van de medische zorg. Voorts kunnen farmacodynamische biomarkers het therapeutische effect van klassieke immunosuppressieve medicijnen flink gaan verbeteren. Het meten van biomarkers in DBS maakt het mogelijk om patiënten van een afstand te 'monitoren' in combinatie met eHealth applicaties, wat de kwaliteit van zorg ten goede kan komen en kosten kan besparen. Concluderend kan worden gesteld dat biomarkers essentieel zijn voor de verdere ontwikkeling, verbetering en personalisering van de behandeling van CE.



Appendices

Abbrevations

Contributing authors

Acknowledgements

List of publications

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LIST OF ABBREVATIONS

AD	Atopic dermatitis
AIC	Akaike Information Criteria
AZA	Azathioprine
BSA	Body surface area
CI	Confidence interval
CNV	Genetic copynumber variation
CsA	Cyclosporin A
CTACK	Cutaneous T-cell-attracting chemokine
DBS	Dried blood spot
EASI	Eczema Area Severity Index
ECP	Eosinophil Cationic Protein
HC	Healthy control
HOME	Harmonizing Outcome Measurements in Eczema
IGA	Investigators' Global Assessment
Ig-FLC	Immunoglobulin free light chain
IL	Interleukin
IQR	InterQuartileRange
I-309	T Lymphocyte-Secreted Protein I-309
LDH	Lactate DeHydrogenase
LLOQ	Lower limit of quantification
MDC	Macrophage-derived chemokine

6-MMP	6-methylmercaptopurine ribonucleotides
MPA	Mycophenolic acid
OR	Odds ratio
PARC	Pulmonary and activation-regulated chemokine
PASI	Psoriasis Area Severity Index
p-EASI	Predicted-EASI
POEM	Patient-Oriented Eczema Measure
PC	Principal component
PCA	Principal component analysis
PRISMA	Preferred Reporting Items of Systematic Reviews and Meta-Analyses
SA-PASI	Self-administered psoriasis area and severity index
SASSAD	Six Area Six Sign Atopic Dermatitis
SCORAD	SCORing of Atopic Dermatitis
SDF-1α	Stromal cell-derived factor-1α
SNP	Single-nucleotide polymorphism
6-TGN	6-thioguanine nucleotides
UGT1A9	Uridine diphosphate-glucuronosyltransferase 1A9
ULOQ	Upper limit of quantification
VAS	Visual analogue scale
TARC	Thymus and activation-regulated chemokine
Th	T helper
TSLP	Thymic stromal lymphopoietin

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LIST OF PUBLICATIONS

This thesis

Thijs JL, Nierkens S, Herath A, Bruijnzeel-Koomen CAFM, Knol EF, Giovannone B, de Bruin-Weller MS, Hijnen DJ. A panel of biomarkers for disease severity in atopic dermatitis. Clin Exp Allergy. 2015 Mar;45(3):698-701.

Thijs JL, van Seggelen WO, Bruijnzeel-Koomen CAFM, de Bruin-Weller MS, Hijnen DJ. New Developments in Biomarkers for Atopic Dermatitis. J Clin Med. 2015 Mar 16;4(3):479-87.

Thijs J, Krastev T, Weidinger S, Buckens CF, de Bruin-Weller M, Bruijnzeel-Koomen C, Flohr C, Hijnen DJ. Biomarkers for atopic dermatitis: a systematic review and meta-analysis. Curr Opin Allergy Clin Immunol. 2015 Oct;15(5):453-60.

Thijs JL, Van Der Geest BA, Van Der Schaft J, Van Den Broek MP, Van Seggelen WO, Bruijnzeel-Koomen CAFM, Hijnen DJ, Van Schaik RH, De Bruin-Weller MS. Predicting therapy response to mycophenolic acid using UGT1A9 genotyping: towards personalized medicine in atopic dermatitis. J Dermatolog Treat. 2016 Sep 13:1-4.

Thijs JL, Knipping K, Bruijnzeel-Koomen CAFM, Garssen J, de Bruin-Weller MS, Hijnen DJ. Immunoglobulin free light chains in adult atopic dermatitis patients do not correlate with disease severity. Clin Transl Allergy. 2016 Dec 6;6:44.

Thijs JL, Drylewicz J, Fiechter R, Strickland I, Sleeman MA, Herath A, May RD, Bruijnzeel-Koomen CAFM, Knol EF, Giovannone B, de Bruin-Weller MS, Nierkens S, Hijnen DJ. EASI p-EASI: Utilising a combination of serum biomarkers offers an objective measurement tool for disease severity in atopic dermatitis patients. J Allergy Clin Immunol 2017. Accepted.

Thijs JL, Strickland I, Bruijnzeel-Koomen CAFM, Nierkens S, Giovannone B, Knol EF, Csomor E, Sellman BR, Mustelin T, Sleeman MA, de Bruin-Weller MS, Herath A, Drylewicz J, May RD, Hijnen DJ. Moving towards endotypes in atopic dermatitis: identification of patient clusters based on serum biomarker analysis. J Allergy Clin Immunol 2017. Accepted.

Thijs JL, Strickland I, Bruijnzeel-Koomen CAFM, Nierkens S, Giovannone B, Knol EF, Csomor E, Sellman BR, Mustelin T, Sleeman MA, de Bruin-Weller MS, Herath A, Drylewicz J, May RD, Hijnen DJ. Serum biomarker profiles suggest that atopic dermatitis is a systemic disease. Under review.

Thijs JL, Drylewicz J, Bruijnzeel-Koomen CAFM, Giovannone B, Knol EF, de Bruin-Weller MS, Nierkens S, Hijnen DJ. Levels of serum TARC, IL-22 and sIL-2R predict disease severity in atopic dermatitis patients treated with Cyclosporin A. Under review.

Other publications

Thijs JL, Damoiseaux RA, Lucassen P, Pasmans SG, de Bruin-Weller M, Bruijnzeel-Koomen CAFM. Allergy testing in atopic dermatitis: often unnecessary. Ned Tijdschr Geneeskd. 2013;157(12):A5652.

Thijs JL, Toonstra J, Hijnen DJ. Twee gevallen van acute gelokaliseerde exanthemateuze pustulose (ALEP). Nederlands tijdschrift voor dermatologie en venereologie. 2014;4:195-199.

Garritsen FM, Thijs JL, Hijnen DJ, Bruijnzeel-Koomen CAFM, de Bruin-Weller MS. Constitutioneel eczeem bij kinderen; Pathofysiologie, diagnostiek en behandeling. Praktische pediatrie. 2014;3:129-135.

Urbach C, Gordon NC, Strickland I, Lowne D, Joberty-Candotti C, May R, Herath A, Hijnen D, Thijs JL, Bruijnzeel-Koomen CA, Minter RR, Hollfelder F, Jermutus L. Combinatorial Screening Identifies Novel Promiscuous Matrix Metalloproteinase Activities that Lead to Inhibition of the Therapeutic Target IL-13. Chem Biol. 2015 Nov 19;22(11):1442-52.

Thijs JL, de Bruin-Weller MS, Hijnen DJ. Current and Future Biomarkers in Atopic Dermatitis. Immunol Allergy Clin North Am. 2017 Feb;37(1):51-61.

Thijs JL, Herath A, de Bruin-Weller MS, Hijnen D. Multiplex platform technology and bioinformatics are essential for development of biomarkers in atopic dermatitis. J Allergy Clin Immunol. 2017 Mar;139(3):1065.

CURRICULUM VITAE

Judith werd geboren op 5 maart 1988 te Eindhoven. Na het behalen van haar atheneum diploma aan het Christiaan Huygens College te Eindhoven in 2006, begon zij in datzelfde jaar met de studie rechtsgeleerdheid aan de Universiteit van Utrecht. Na het succesvol afronden van haar propedeuse rechtsgeleerdheid werd zij ingeloot voor de studie geneeskunde aan dezelfde universiteit. In jaar vier van haar studie heeft ze haar coschap gynaecologie gelopen aan de University of Malaya in Kuala Lumpur, Maleisië. In de laatste twee jaar van de opleiding heeft zij onder begeleiding van DirkJan Hijnen en Carla Bruijnzeel-Koomen onderzoek gedaan naar constitutioneel eczeem op de afdeling Dermatologie/Allergologie. Na het behalen van haar arts-examen in 2013, is zij aansluitend aangenomen als arts-onderzoeker op deze afdeling. De bevindingen van haar onderzoek naar constitutioneel eczeem hebben geleid tot dit proefschrift. In 2017 is zij begonnen met de opleiding tot dermatoloog in het UMC Utrecht onder leiding van Vigfús Sigurdsson.