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Defining the temporal relationship between the skin microbiome, immune response and skin barrier function during flare and resolution of atopic dermatitis: protocol of a Danish intervention study

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ABSTRACT

Introduction Lesional skin of atopic dermatitis (AD) is often colonised by *Staphylococcus aureus* and the bacterial abundance increases during a flare. However, the role of *S. aureus* and the skin microbiome in the pathogenesis of AD, including its influence on the dysfunctional skin barrier and immune response, remains to be elucidated. In this study, the temporal relationship between alterations in the skin barrier function, inflammation and microbiome is examined in adults with AD.

Methods and analysis This clinical study consists of 81 adult patients with AD, as defined by the Hanifin and Rajka criteria, and 41 age and sex-matched controls. The objectives are to examine alterations in the skin microbiome, skin barrier and immune response during (1) an untreated AD flare, (2) an AD flare treated with topical corticosteroids (TCS), (3) an AD flare treated with systemic dicloxacillin/placebo and TCS or (4) cutaneous exposure to either autologous *S. aureus*, staphylococcal enterotoxin B or a vehicle. Skin biopsies, tape strips, skin and nasal swabs are collected and analysed using RNA sequencing, multiplex immunoassays, liquid chromatography-mass spectrometry and 16S rDNA. Blood samples are analysed for filaggrin gene mutations and leucocyte gene expression.

Ethics and dissemination The scientific Ethical Committee of the Capital Region in Denmark (phases I and II: H-20011047, phases III and IV: H-21079287), the local data protection agency (phases I and II: P-2020-165, phases III and IV: P-2022-250) and the Danish Medicines Agency (phases III and IV: EudraCT 2021-006883-25, ClinicalTrials.gov: NCT05578482) have approved the studies. Participants will give written informed consent prior to study initiation. The study is conducted in accordance with the Helsinki Declaration. Outcomes will be presented at national and international conferences and in international peer-reviewed publications.

Trial registration number NCT05578482, EudraCT 2021-006883-2.

STRENGTHS AND LIMITATIONS OF THIS STUDY

⇒ This is a Danish intervention study investigating temporal changes in the skin microbiome, immune response and skin barrier function during development and resolution of eczema flares in adults with atopic dermatitis (AD).
⇒ Repeated longitudinal objective measurements and sample collection over a 5-day period.
⇒ A randomised, double-blinded study will examine the effects of antibiotics during resolution of AD and the temporal changes in the skin microbiome, immune response and skin barrier function.
⇒ The limited number of participants makes it difficult to investigate smaller differences between groups due to limited statistical power.

INTRODUCTION

Atopic dermatitis (AD) is a common chronic, inflammatory skin disease characterised by dry and itchy eczematous lesions. The disease initiates during the first years of life and is often followed by other atopic diseases, such as food allergy, allergic rhinitis and asthma.[1] AD is associated with a high disease burden where patients experience poor sleep, low self-esteem and reduced quality of life. Further, some patients suffer from mental health issues.[2 3]

The etiopathogenesis of AD is a complex interaction of genetic and environmental factors resulting in immune dysregulation and impaired skin barrier function.[4] Loss-of-function mutations in the filaggrin gene (*FLG*) represent the hitherto strongest genetic risk for AD.[5 6] These mutations reduce the levels of natural moisturising factor (NMF),[7]

leading to dry skin. *FLG* mutations are associated with earlier onset, increased severity, persistent disease and increased risk of allergic comorbidity.[8] Cytokines also play a pertinent role in AD pathogenesis and have been emphasised in recent years. Human monoclonal antibody therapies targeting the interleukin (IL)-4, IL-13 and IL-31 receptors[9 10] as well as Janus kinase (JAK) inhibitors, targeting JAK1 and JAK2 receptors, have shown efficacy in reducing AD severity.[11] Recently, a study investigating the ceramide profile of the stratum corneum (SC) in patients with AD found that the ceramide profiles during AD remission phase are potential biomarkers that may serve as indicators of remission, or for predicting exacerbation of AD.[12] Importantly, *Staphylococcus aureus* colonises a large proportion of patients with AD, especially during flares.[13] Currently, it is unknown whether flare risk increases in association with changes in the AD skin microbiome favouring the presence of *S. aureus*.

This study investigates the temporal changes in skin barrier function, inflammatory response and skin microbiome during development and resolution of AD flares, under the effect of various treatments, and cutaneous exposure to bacteria and toxins.

OBJECTIVES

Primary objective

The primary objective of this study is to describe the temporal relationship between pathogenic changes in the skin microbiome, skin barrier function and immune response during (1) an untreated AD flare, (2) an AD flare treated with topical corticosteroids (TCS), (3) an AD flare treated with systemic dicloxacillin or placebo in combination with TCS and (4) cutaneous exposure to either autologous *S. aureus*, staphylococcal enterotoxin B (SEB) or a vehicle.

Secondary objective

The secondary objective of the study is to characterise the skin microbiome in adults with AD when compared with sex and age-matched controls.

METHODS AND ANALYSIS

Study population and setting

This project is an ongoing intervention study including a total of 81 adults with AD and 41 adult controls. Recruitment began in August 2020 and is expected to end in spring 2023. Inclusion criteria are adults (≥18 years) with European ancestry and an AD diagnosis according to the Hanifin and Rajka criteria.[14] Patients should have had AD for at least 3 years. Patients are excluded if there is evidence of other concomitant inflammatory skin conditions (eg, psoriasis or contact dermatitis), signs of active skin infection that warrants treatment at baseline or history of any condition that may predispose the patient to complications associated with the skin biopsy procedure (eg, tendency to formation of keloid scars). Further, patients cannot participate if pregnant, breast feeding or if AD is only in the face or intimate regions. For phases III and IV, penicillin allergy and bodyweight ≤40 kg will be an exclusion criterion. Controls (≥18 years) with European ancestry are eligible for inclusion if no AD is present in first-generation relatives, no report of current or present chronic skin disease, no report of current or present use of systemic immunosuppressants or biological treatment and no use of systemic antibiotics in the preceding month. Controls are matched according to age (±5 years) and sex. Patients eligible for inclusion are recruited from the Department of Dermatology at Bispebjerg Hospital, the Department of Dermatology and Allergy at Gentofte Hospital, private dermatology clinics in Copenhagen and through advertising on web pages and local media in Denmark.

All patients with AD enter a washout period prior to phases I and III where AD treatment is paused. Patients are instructed to pause topical treatment such as TCS or topical calcineurin inhibitors (TCI) at least 1 week prior to study start, systemic treatment (eg, methotrexate, azathioprine, cyclosporine, mycophenolate mofetil, baricitinib, prednisolone, other systemic anti-inflammatory medication) or systemic antibiotics 4 weeks prior to study start, dupilumab for 13 weeks and ultraviolet therapy 3 weeks prior to study start. Use of disinfectants, bleach and potassium permanganate baths should be paused for at least 2 weeks before study start. After the washout period, an exacerbation of AD is expected to occur in most patients with AD.

Study design

The project consists of four independent study phases (I–IV). An overview is provided in figures 1–5. Phases I and II are designed as prospective cohort studies including 41 patients with AD and 41 controls. Phase III of the study is a randomised, double-blinded, two-arm controlled study including 40 patients with AD. Phase IV is designed as a partly randomised, three-arm controlled study where the patients with AD from phase III are re-examined. All four phases are running over five consecutive visits. If a patient drops out before completion during one of the phases a new patient is recruited for that particular phase.

Study phases

In phase I, the AD flare is allowed to progress due to the patients not receiving any active treatment. The patients attend clinical examinations, sample collection and complete questionnaires for five continuous days (figure 1).

In phase II, remission of AD is monitored during five consecutive study visits. On the day before phase II, patients begin treatment with their regular topical treatment (TCS and/or TCI). Patients apply their regular treatment (TCS and/or TCI) daily with the intention of reducing AD lesions to a minimum. Patients are examined, samples are collected and questionnaires are completed each day (figure 2). Age- and sex-matched

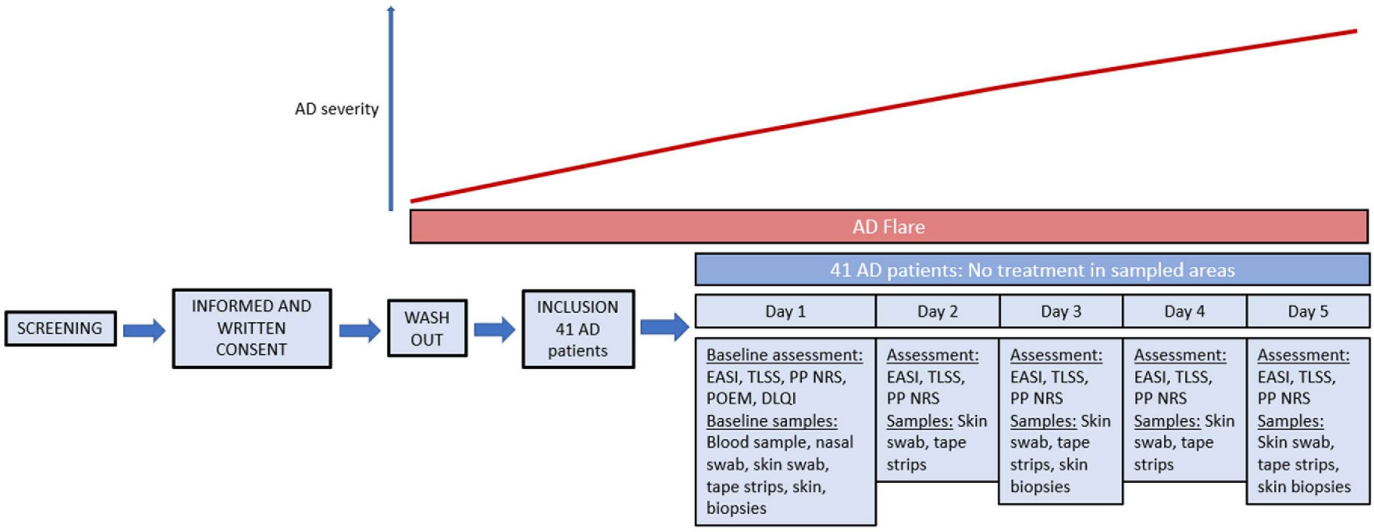

Figure 1 Trial overview of phase I during a flare of atopic dermatitis (AD) and no treatment. DLQI, Dermatology Life Quality Index; EASI, Eczema Area and Severity Index; POEM, Patient-Oriented Eczema Measure; PP NRS, Peak Pruritus Numeric Rating Scale; TLSS, Target Lesion Severity Score.

controls eligible for inclusion attend once during phase II for sample collection and completion of a questionnaire (figure 3).

Prior to phase III, patients with AD enter a washout period as described. Skin swabs are collected at baseline for cultivation of autologous *S. aureus* for use in phase IV (figure 4). Additional baseline samples are collected from lesional and non-lesional skin and a questionnaire regarding bath and hygiene routines is completed. Patients with AD are then randomised to receive either TCS (mometasone furoate 0.1%)+systemic dicloxacillin (1000 mg × 3 times a day) or TCS (mometasone furoate 0.1%)+placebo (3 times a day). Participants are monitored daily for 5 days. Patients attend daily clinical examination, samples are collected and a questionnaire is completed (figure 4). After five continuous days of treatment, patients are instructed to continue application of TCS for one additional week.

Phase IV is initiated when the patients' skin expresses minimal disease activity (Eczema Area and Severity Index (EASI) ≤4). Fifteen of the patients having *S. aureus* isolated from their skin are randomly selected for autologous *S. aureus* application. The remaining patients are randomised to either exposure to SEB from *S. aureus*

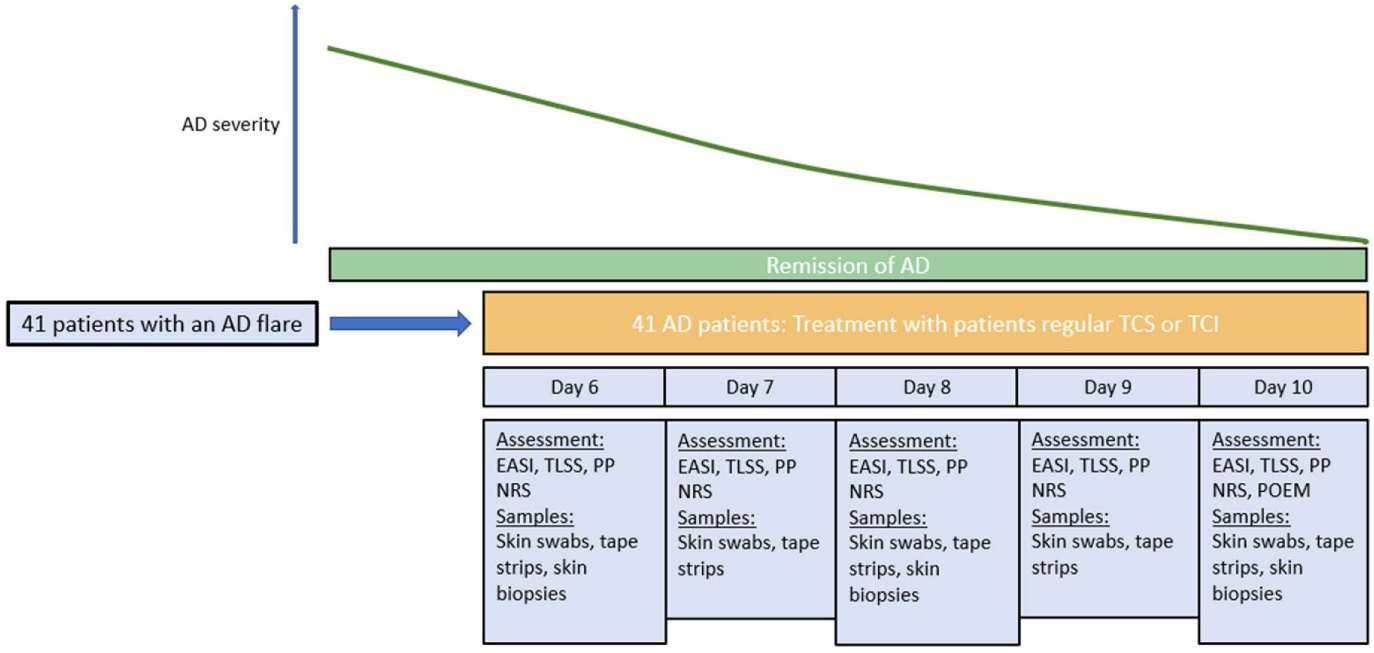

Figure 2 Trial overview of phase II during treatment of an atopic dermatitis (AD) flare with topical corticosteroids (TCS) and topical calcineurin inhibitors (TCI). EASI, Eczema Area and Severity Index; POEM, Patient-Oriented Eczema Measure; PP NRS, Peak Pruritus Numeric Rating Scale; TLSS, Target Lesion Severity Score.

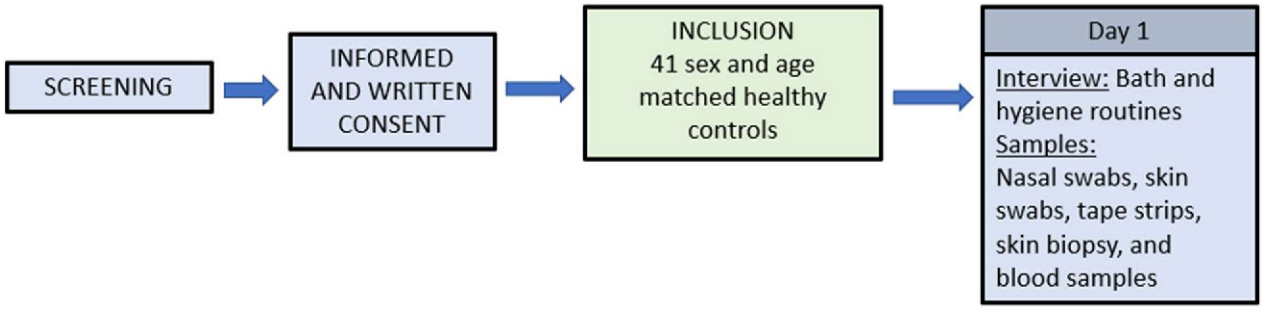

Figure 3 Trial overview: controls.

(Merck, Sigma-Aldrich, Saint Louis, Missouri, USA) (n=15) or vehicle control (n=10). Patients then attend daily clinical examinations and sample collection for five consecutive days (figure 5). Participants are withdrawn from the study if resignation is required or if unacceptable side effects appear from the biopsy extraction, the Dicillin treatment, the autologous *S. aureus* or SEB application.

Baseline interview

All participants complete interviews at baseline prior to study start to obtain information about basic characteristics, bath and hygiene routines. Additional information is collected from patients with AD regarding AD treatment, quality of life, itch, sleep, pain and AD severity.

Study interview

During each visit, patients with AD complete the validated questionnaires; the Peak Pruritus Numeric Rating Scale[15] for the last 24 hours, the Pain Numeric Rating Scale[16] for peak pain for the last 24 hours and the Sleep Disturbance Numeric Rating Scale[17] for the last 24 hours. At day 1 and 5 in each of the four phases, the Patient-Oriented Eczema Measure is used as a subjective measure for AD severity in the past week and the Dermatology Life Quality Index is used to estimate quality of life in the past week.

AD assessment

AD severity is assessed at each visit using the EASI score.[18] Further, the severity of the lesional collection site is assessed using the Target Lesion Severity Score (TLSS).[19]

SKIN MEASUREMENTS

All tape strips, skin swabs and skin biopsies are collected from lesional and non-lesional areas of the skin in patients with AD and at a corresponding area from the controls. Samples from lesional skin are collected in the following priority (eg, if no lesional skin in elbow flexure, move to next skin site): elbow flexure, volar forearm, knee flexure, thigh, abdomen, flanks, back, buttocks and neck. Skin sampling from non-lesional skin is done from the same region, taking moist, oily or dry skin into account.

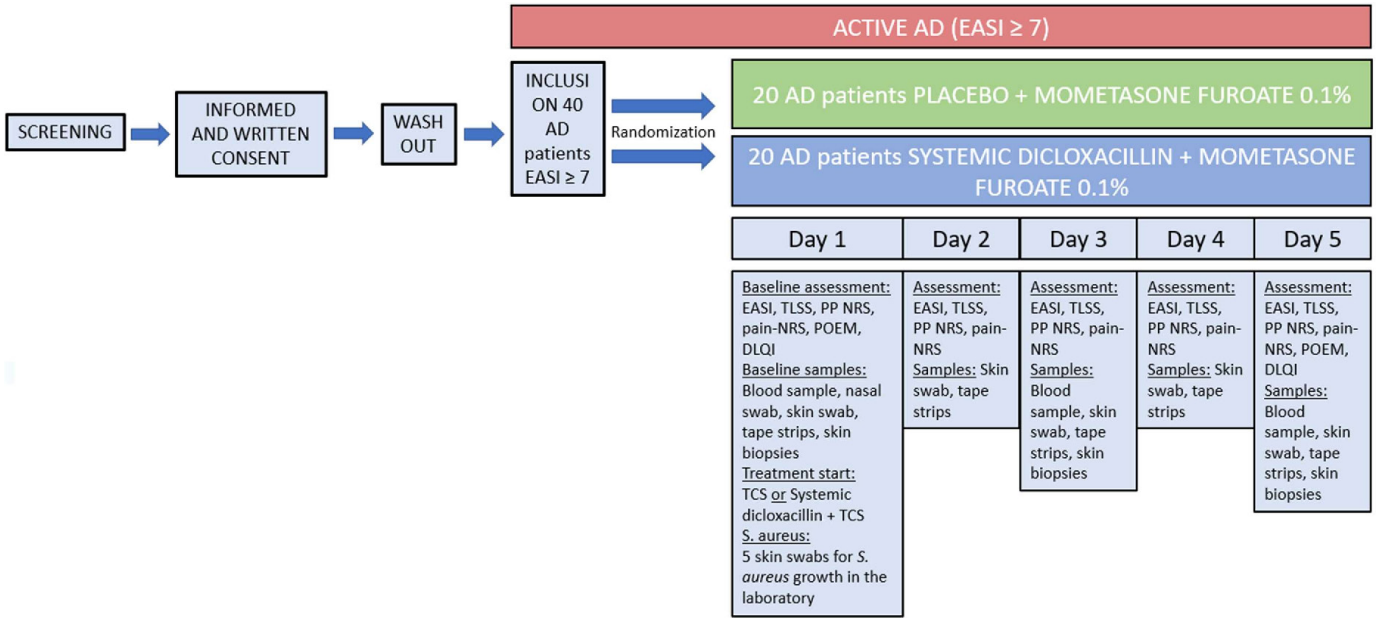

Figure 4 Trial overview of phase III during active atopic dermatitis (AD) and treatment with either topical corticosteroids (TCS) and systemic antibiotics or placebo and TCS. DLQI, Dermatology Life Quality Index; EASI, Eczema Area and Severity Index; pain-NRS, Pain Numeric Rating Scale; POEM, Patient-Oriented Eczema Measure; PP NRS, Peak Pruritus Numeric Rating Scale; TLSS, Target Lesion Severity Score.

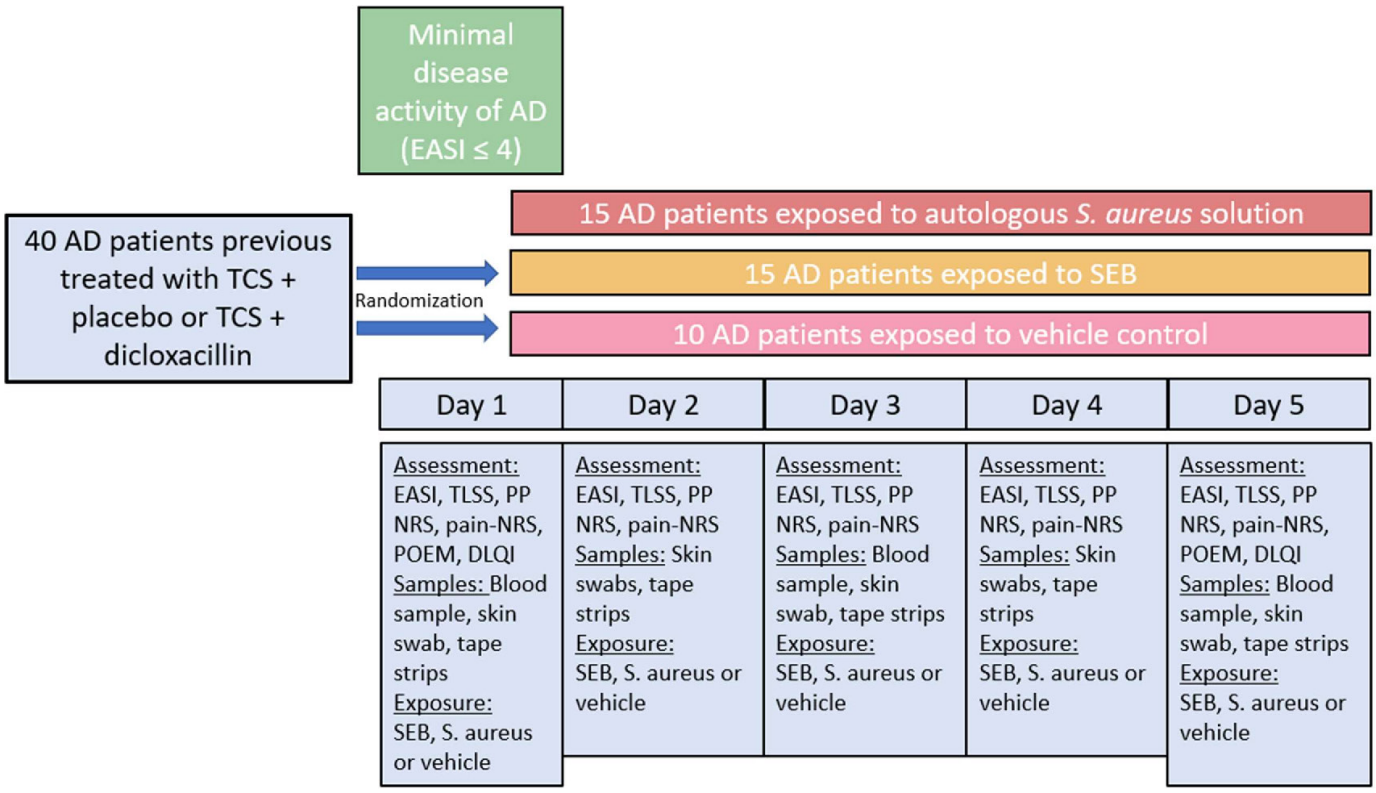

Figure 5 Trial overview of phase IV when patients are exposed to three solutions of respectively *Staphylococcus aureus*, staphylococcal enterotoxin B (SEB) and vehicle control. AD, atopic dermatitis; DLQI, Dermatology Life Quality Index; EASI, Eczema Area and Severity Index; pain-NRS, Pain Numeric Rating Scale; POEM, Patient-Oriented Eczema Measure; PP NRS, Peak Pruritus Numeric Rating Scale; TCS, topical corticosteroid; TLSS, Target Lesion Severity Score.

Non-lesional samples must be at least 10 cm from lesional skin. The chosen sampling site must be large enough to accommodate skin swabs, tape strips and a 2 mm skin biopsy. Skin swab and skin tape strip collection occurs prior to harvesting the skin biopsy. Samples from controls are collected from identical body sites as the matched patient with AD. The location of the sampled skin area is documented to compare the samples.

Superficial SC sampling

Skin tape stripping is used to collect SC.[20 21] Twenty consecutive tape strip discs (22 mm, D-Squame; CuDerm, Dallas, Texas, USA) are applied to lesional and non-lesional skin and a standardised pressure is applied by a D-Squame pressure application pen for 10 s. Swabs are gently removed with tweezers and stored at −80°C. Tape strips are analysed for levels of NMF using a liquid chromatography,[20] cytokine levels using multiplex immunoassays,[22] levels of lipids (eg, glucosylceramide, sphinganine, sphingosine, sphingosine-related ceramides) by extraction and analysing with liquid chromatography-tandem mass spectrometry[23] and corneocyte surface morphology using atomic force microscopy.[24]

Skin and nasal swabs

Bacteria for culturing and microbiome analyses are collected from lesional and non-lesional skin at each study visit with moistened ESwab regular flocked (Copan Diagnostics). Swabs are moistened with sterile saline and gently rubbed against the skin or the nasal mucosa for 60 s to collect the sample. In phases I–IV, one swab is taken from lesional and non-lesional skin at each study visit. An additional skin swab is collected during phase III at baseline to identify *S. aureus* carriers. Further, one nasal swab is collected from each participant at baseline and used as a control for the skin microbiome analyses.

Skin and nasal swabs are analysed by 16S rDNA amplicon high throughput sequencing (HTS) allowing to investigate the bacterial communities and by cultivation to isolate homologous *S. aureus*. Swabs for cultivation and 16S rDNA HTS are stored at 4°C and −80°C, respectively, until further processing. For cultivation, swabs are streaked for isolation on aerobic agar plates selective for *Staphylococcus* spp. Isolated colonies are characterised using MALDI-TOF Biotyper (Bruker). Bacterial isolates are stored at −80°C.

Skin biopsies

We will collect 2 mm skin biopsy following local anaesthesia injection with lidocaine 10 mg/epinephrine 5 µg. Skin biopsies for dual RNA sequencing are collected from both lesional and non-lesional skin during phases I–III for a total of six biopsies per patient with AD in each phase. One biopsy is collected from the controls. In phase IV, no biopsies are taken. Skin biopsies from controls are

collected from the same body site as the matched patient with AD. Biopsies are stored in RNA later at 4°C for 1–7 days. RNA later is then removed, and the biopsies are stored at −80°C until processing.

Blood samples

Blood collection is performed with standard venous blood test equipment. PAXgene Blood RNA tubes and regular blood sample glasses are used for collection. Blood samples are analysed for common *FLG* mutations at the Department of Clinical Biochemistry, Gentofte Hospital, and at the Department of Clinical Biochemistry, Bispebjerg Hospital. Samples are also analysed for blood concentrations of AD immune biomarkers as well as leucocyte gene expression by dual RNA sequencing.

In phase I, blood samples are collected at baseline. In phase II, no blood samples are collected. In phases III and IV blood samples are collected at baseline and at visit days 1, 3 and 5. One blood sample is collected from controls at their visit.

Gene expression and microbiome analysis

Dual RNA sequencing and 16S rDNA amplicon sequencing will quantify gene expression levels (for human and bacterial cells) and abundance/composition of bacterial communities, respectively. Normalised gene expression levels among lesional and non-lesional skin and healthy controls are modelled with DEseq2 to identify human and/or bacterial genes that show significant changes between healthy and lesional skin. Longitudinal changes in lesional skin over time are also examined. Changes in bacterial community composition and abundance along these same parameters are modelled with regression analyses and permutational multivariate analysis of variance. This analysis can detect how the composition of bacterial species presents changes among lesional and healthy skin from patients with AD as well as from healthy controls. To assess longitudinal interactions between bacterial communities and host gene expression, interactions between host gene expression and specific microbial taxa are analysed with a machine learning-based framework.

Sample size estimation

Published data suggest an effect size of 0.71 for comparing alpha diversity between healthy and AD skin.[25] Therefore, a sample size of 32 would be sufficient to detect changes in alpha diversity at a power of 0.8 and alpha level of 0.05. For gene expression data, these sample sizes will allow for the sensitive detection of human and bacterial genes with fold changes >1.5 and >1.75, respectively.

In our previous study,[26] the composition (measured as operational taxonomic unit richness) significantly differed one SD between skin compartments in healthy skin. Therefore, we estimate that a minimum of 30 patients is required to achieve statistical power of 0.9 and alpha level of 0.01. To further improve statistical power, an additional 10 patients were included in each group, resulting in 40 participants in each group (AD group and matched controls). The number of participants needed for phases III and IV is based on newly collected data from the 41 patients with AD from phases I and II treated with TCS once daily for a week. A minimum of 30 patients with AD is needed to detect a difference of 1.00 (SD, 0.98) in mean TLSS score between two groups with a power of 0.8 and alpha level of 0.05. We therefore aim to include 40 patients with moderate to severe AD in phases III and IV.

Data management

Data collection is performed in the online program Research Electronic Data Capture (REDCap) database, which is hosted by the Capital Region of Denmark. Sensitive data (such as RNA sequencing data) are stored at the secured General Data Protection Regulation (GDPR) compliant server for personal data at the Faculty of Health and Medical Sciences, University of Copenhagen, Denmark.

Patient and public involvement

During study design, patients and the public were not involved. All participants will be acknowledged and thanked for their contribution in future publications. Further, participants will receive economic compensation corresponding to the amount of time spent and samples collected. Participation will not affect further course of treatment.

STRENGTHS AND LIMITATIONS

A major strength of this study is the repeated visits with close clinical assessment and sample collection each day in the four phases. This longitudinal design allows identification of key drivers through AD flares and resolutions in concert with the observed changes in severity of signs and symptoms of AD over time. Further, it contributes to the safety of the study, as patients are closely monitored daily. The washout period initiated prior to study start is meant to equalise the patients' microbiome no matter the composition when recruited. The examination of the skin microbiome through all four phases of the study will promote the understanding of the human gene expression and skin microbiome alterations at different points in disease development.

In phases III and IV, only patients with moderate to severe AD are included. In addition, we will use international definitions to diagnose AD and assess disease severity.[14 18] Overall, the microbiome cohort will cover a wide range of potential parameters that will illuminate the relationship between the use of TCS, systemic antibiotics, *S. aureus*, its toxins and the skin microbiome in AD flares and resolution.

The limited number of participants makes it difficult to investigate smaller differences between the groups. All participants have the same ethnicity, which makes the cohort uniform. Nevertheless, a putative role of ethnicity cannot be investigated. Participants' bathing habits prior

to study start are not controlled but only registered at the baseline visit.

ETHICS AND DISSEMINATION

The study is approved by the scientific Ethical Committee of the Capital Region in Denmark (phases I and II: H-20011047, phases III and IV: H-21079287) and the local data protection agency (ID No: phases I and II: P-2020-165, phases III and IV: P-2022-250). Further, phases III and IV are approved by the Danish Medicines Agency (EudraCT 2021-006883-25, ClinicalTrials.gov: NCT05578482). Written informed consent will be given to all included patients and gathered on all participating patients. The project is conducted in accordance with the Declaration of Helsinki. Further, all investigators are trained in Good Clinical Practice and/or Good Laboratory Practice.[27] Outcomes will be presented at national and international conferences and in international peer-reviewed publications.

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Contributors ATMR, IFR, TB and JT have developed the theory, participated in designing the overall study set-up from idea to creating the study protocol and obtained the relevant approvals for the study. LB, A-SH-S and SFT have participated in designing the overall study set-up. ATMR, IFR, TB, JT and SFT planned the experiment days. ATMR, IFR and LB will carry out the study phases. ATMR, LB and A-SH-S wrote the manuscript with support from JT and TB. BGF, LB, RL, IJ and SK have supported how to handle the samples before analysis, verified the analytical methods and will be analysing the samples. All authors have discussed the study set-up, provided critical feedback and contributed to the final manuscript.

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Competing interests Outside of this study, JT has been an advisor for AbbVie, Almirall, Arena Pharmaceuticals, Coloplast, OM Pharma, Aslan Pharmaceuticals, Union Therapeutics, Eli Lilly & Co, LEO Pharma, Pfizer, Regeneron and Sanofi-Genzyme; a speaker for AbbVie, Almirall, Eli Lilly & Co, LEO Pharma, Pfizer, Regeneron and Sanofi-Genzyme; and received research grants from Pfizer, Regeneron and Sanofi-Genzyme. TB has been an advisor for SoftOx Solutions outside the submitted work. SFT has been a speaker or advisor for Sanofi, AbbVie, LEO Pharma, Pfizer, Eli Lilly, Novartis, UCB Pharma, Almirall and Janssen Pharmaceuticals; has received research support from Sanofi, AbbVie, LEO Pharma, Novartis, UCB Pharma and Janssen Pharmaceuticals, outside the submitted work. LB has received research support from the Leo Foundation. A-SH-S has received a speaker honorarium from LEO Pharma and honoraria as a consultant from Coloplast.

Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Patient consent for publication Not applicable.

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