THE MICROBIOME AND THE SKIN BARRIER IN ATOPIC DERMATITIS

Minke M.F. van Mierlo

TOWARDS PERSONALIZED TREATMENT

The microbiome and the skin barrier in atopic dermatitis

Towards personalized treatment

Minke M.F. van Mierlo

Layout and printed by: Optima Grafische Communicatie (www.ogc.nl)
ISBN: 978-94-6361-549-5

Financial support for the publication of this thesis was generously provided by:
BAP-medical
ChipSoft
DermaCura
Eucerin
Galderma
La Roche Posay
Leo Pharma
Louis Widmer
Merz Pharma
Micreos
Olmed
Piere Fabre
UCB
VMCE

The microbiome and the skin barrier in atopic dermatitis
Towards personalized treatment

Het microbioom en de huidbarrière bij constitutioneel eczeem
Op zoek naar aangrijpingspunten voor gepersonaliseerde behandeling

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de rector magnificus
Prof. dr. F.A. van der Duijn Schouten
en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
Dinsdag 22 juni 2021 om 10.30 uur.

door

Minke Maria Francisca van Mierlo
geboren te Nijmegen

Erasmus University Rotterdam

PROMOTIECOMMISSIE

Promotor	Prof. dr. S.G.M.A. Pasmans
Overige leden	Dr. S. Kezic
	Prof. dr. E.P. Prens
	Prof. dr. M.C. Vos
Copromotor	Dr. L.M. Pardo

CONTENTS

Chapter 1

General introduction and aims of the thesis

ATOPIC DERMATITIS – GENERAL BACKGROUND OF THE DISEASE

Atopic dermatitis (AD) is the most common inflammatory skin disease, which mainly affects children. The prevalence of childhood AD is up to 25% compared to 2-10% in adults.[1-3] The hallmarks of AD are intense itching and a dry skin with recurrent heterogeneous eczematous lesions, including erythema with scaling, exudation, blistering, crusting, fissuring and/or lichenification.[4, 5] The eczematous lesions can be present at any part of the body, but mostly show age related distribution that can be roughly subdivided in three stages: infancy, childhood and adolescence/adulthood.[6] Bacterial and viral infections are common complications in AD skin.[7] Especially more severe AD is associated with diseases of the atopic syndrome that include food allergy, asthma and allergic rhinitis.[8, 9] Furthermore, AD has a significant impact on the quality of life, particularly due to effects on social functioning and the psychological well-being.[10, 11]

Both genetic and environmental factors are implicated in the development of AD. As AD is highly heritable (approximately 75% in a twin study), the strongest risk factor is a positive family history for atopic diseases, especially for AD.[12, 13] In past genome-wide association studies (GWAS), 34 risk loci associated with AD were identified.[14] These loci account for less than 20% of the heritability and are mainly involved in the skin barrier function and immune response pathways.[14] The inherited susceptibility is triggered by environmental and lifestyle-related factors. Important exposures associated with AD include climate, socio-economic status, housing conditions, rural or urban living, diet and microbial exposure.[15, 16]

The diagnosis of AD is primarily based on clinical features.[17] To create more uniformity in this diagnosis, several diagnostic criteria for the use in clinical practice and population based studies have been developed.[18-20] Particularly in non-responders to therapy, skin biopsy, laboratory testing, patch testing and/or genetic testing are used to rule out other disorders presenting with a dermatitis.[17]

ATOPIC DERMATITIS – PATHOGENESIS

The etiology of AD is multifactorial and not completely understood, with complex interactions between the skin microbiome, the skin barrier and the immune system as highlighted in figure 1.[21]

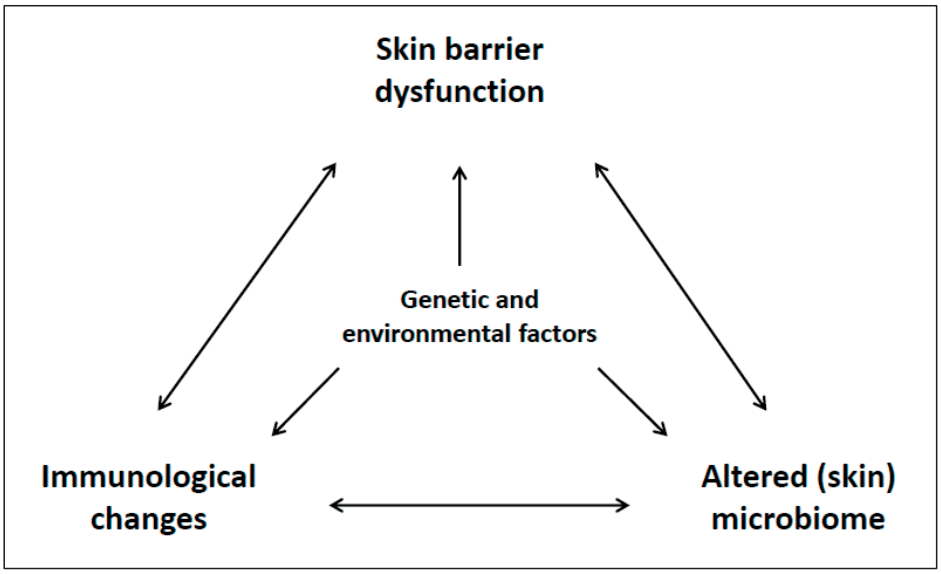

Figure 1. Disease model of AD (illustration J.E.E. Totte[22])

Altered (skin) microbiome

The human microbiome is the collection of all microbiota that reside on or within human tissues. Since next generation sequencing has become more available for microbiome analysis, our knowledge on microbial communities and even functional properties has grown. It was shown to have an important impact on the physiology and metabolism in humans.[23] Both endogenous and exogenous factors (i.e., sex, age, climate and lifestyle) may modulate the microbiome, leading to a large interpersonal variation and variation over the lifetime of an individual.[24, 25] Changes in the microbiome are associated with a wide array of diseases, including skin diseases as AD.

The skin microbiome is thought to play a role in both the pathogenesis and severity of the symptoms in AD.[26] It has been proposed that there is a window in neonatal life in which exposure to certain microbes affects the development of both the innate and adaptive immune response and the tolerance against certain species, which plays a role in the susceptibility to diseases.[27] This idea has arisen from studies showing a reduced microbial diversity and the presence of certain gut bacteria in the first days of life in individuals developing atopy.[28, 29] There is also recent evidence from mice studies for an effect of skin microbes in shaping the immune system.[27, 30] These mice studies suggest that skin colonization with commensal bacterium in neonatal mice leads to less inflammation to these microbes upon re-challenging.[27, 30] In addition, several studies in humans suggest

that alterations in the skin microbiome in the early life of individuals are associated with an increased risk to develop AD.[31, 32]

Later in life, the skin microbiome contributes to the course of the disease. Especially the *S. aureus* bacterium is associated with AD. Both the lesional and nonlesional skin of patients with AD are highly colonized by *S. aureus* and show reduced bacterial diversity.[26, 33] Analysis of the skin microbiome shows a positive correlation between *S. aureus* colonization and disease severity, with increased *S. aureus* abundance during disease flares.[33-36] A meta-analysis including 95 observational studies showed that colonization rates for *S. aureus* on lesional skin, nonlesional skin and in the nose were 70%, 39% and 62%, respectively.[37] Although current data are not sufficiently robust, there is also evidence of other microbes involved in AD with reduced levels of the *Cutibacterium* and *Malassezia* species.[38, 39]

Although multiple studies have shown the correlation between an increase in *S. aureus* abundance and disease severity, it is not entirely known how this bacteria can lead to the AD characteristic inflammation.[40, 41] The *S. aureus* bacterium can be present in humans both as a commensal organism and as a pathogen responsible for disease.[42] *S. aureus* secretes a variety of enzymes, cytotoxines and exoproteins to convert host tissues into nutrients for bacterial growth and to inhibit host immune responses.[43] Different strains of the bacterium carry a different combination of virulence factors and therefore it is possible that certain strains of this bacterium are more prone to cause AD.[41] For a better understanding of the role for *S. aureus* in the pathogenesis of AD, it is crucial to gain more knowledge on the genetic background and behavior of this bacterium in patients with AD. In addition, exploring endogenous and exogenous factors that affect the skin microbiome might help in the development of future treatment strategies.

Skin barrier dysfunction

The main function of the skin is the defense against environmental influences. The stratum corneum (SC) is the outermost layer of the skin and plays the most important role in the skin barrier function by preventing extensive water loss and penetration of environmental compounds (i.e., allergens, irritants, pathogens).[44] Genetic and acquired defects in structural proteins, lipids and tight junctions (TJ) of the skin lead to an impairment in the skin barrier function as seen in AD.[45]

Mutations in the gene encoding the SC structural protein filaggrin (*FLG*) have shown to be the most important risk factor in the development of AD.[46] *FLG* is located within the epidermal differentiation complex (EDC) on chromosome 1q12 and encodes profilaggrin, the precursor of the structural protein filaggrin.[47] *FLG* consists of 3 exons of which the third is one of the largest exomes (>12kilobases) in the genome, including 10-12 repeat units

which are highly homologous.[47] Filaggrin is essential for the construction and maintenance of the skin barrier function. This protein facilitates the binding and collapsing of keratin filaments, which helps in the mechanical strength of the SC.[47, 48] Subsequently, filaggrin is degraded by proteases into hygroscopic amino acids and forms, together with specific salts and sugars, the largest part of the natural moisturizing factor (NMF). NMF plays an important role in skin hydration, maintenance of the skin pH and ultra-violet (UV) protection.[49, 50] It was suggested that the concentrations of NMF in the SC of AD patients can be used as a proxy for the presence of *FLG* null mutations.[50, 51]

In AD patients, the prevalence of a mutation in *FLG* is up to 40%.[47, 52] Distinct sets of *FLG* mutations are present in each population.[53] For Western-European populations, 2282del4, R2447*, R501* and S3247* are commonly described.[54] Carriers of a heterozygous loss-of-function mutation in *FLG* have a 50% reduction in filaggrin expression, compared to a total loss of protein in homozygous and compound heterozygous carriers.[55] Large longitudinal studies have shown that AD patients with a mutation in *FLG* have a more severe disease trajectory compared to wild-type patients.[48] This group is characterized by early onset disease with persistence into adulthood with an increased risk to develop secondary allergic conditions (i.e., food allergies, asthma, and allergic rhinitis).[56, 57] There is also recent evidence suggesting that patients with a primary skin barrier defect might have less benefit from systemic immunomodulatory treatment.[58, 59] Therefore, determining the presence of a mutation in *FLG* could be useful for early identification of the patients who might develop a more severe phenotype and for personalization of treatment. However, genotyping is time consuming, expensive and DNA collection poses ethical considerations and is therefore not used in daily practice.[46, 60] As AD mostly affects children, non-invasive measurement of NMF concentrations in the SC as biomarker for *FLG* mutation status is of added value.

Immunological changes

The immune response in AD is characterized by altered T helper (Th) cell activation. Three different immune phenotypes have been described for AD, including the nonlesional skin, disease flares (lesional skin) and the chronic AD skin.[61] The nonlesional skin of AD patients shows an increased infiltrate of inflammatory T-cells compared to normal skin, which is mostly characterized by increased numbers of Th2, Th22 and Th17.[61, 62] The combination of this pro-inflammatory state in nonlesional skin with the impaired skin barrier allows the penetration of environmental stimuli into deeper skin layers. This triggers the expression of pattern recognition receptors (PRRs) by keratinocyte and antigen-presenting cells (APCs) in the skin.[6, 63] PRRs are innate immune receptors which form the first line of defense mechanism to prevent microbial invasion and further tissue injury.[63] PRRs recognize pathogen-associated molecular patterns (PAMPs) and in response to microbes or tissue

damage, PRRs release a broad range of inflammatory mediators, including cytokines, chemokines and anti-microbial peptides (AMPs).[64] Keratinocyte-derived cytokines interleukin (IL)-25, IL-33 and Thymic stromal lymphopoietin (TSLP), promote Th2 cytokine responses either directly by Th2 cytokine expressing cells or indirectly via dendritic cell (DC) polarization.[65] TSLP expression is positively correlated with disease severity in AD, which results in increased Th2 cell infiltration with the production of multiple pro-inflammatory cytokines, including IL-4, 13 and 31 in lesional skin.[65] The chronic inflammation in AD skin is marked by a shift towards Th1 cell infiltration. This shift is supported by DCs secreting large amounts of IL-12 under the influence of Th2 cytokines or by the binding of *S. aureus*-derived lipoteichoic acid to DCs.[66] In turn, Th1 cells produce Interferon gamma (IFN-γ), which inhibits keratinocyte differentiation resulting in epidermal hyperplasia as seen in chronic AD lesions.[67]

Another important immunological feature in AD is the humoral immune response, characterized by the presence of immunoglobulin (Ig)E. Upon penetration of allergens into the impaired skin barrier, the Th2-driven response leads to Ig-class switching in B cells to IgE.[68] The presence of increased IgE levels against inhalant and food allergens are common among AD patients and even an increased IgE response against micro-organisms can be observed.[69-71]

Interactions between the microbiome, skin barrier and immune system

Several models have been suggested to explain the etiology of AD, mostly referred to as the 'inside-out' (altered inflammation triggers barrier dysfunction, enabling the penetration of environmental stimuli) and 'outside-in' theory (skin barrier dysfunction allows penetration of stimuli to induce immune responses).[72] Recently, both models have been proven of importance in AD, underlining the multidirectional interactions between the microbiome, skin barrier and immune response.[73]

The impaired skin barrier in patients with AD leads to an increased penetration of microbes. This association has been mostly investigated for *FLG* mutation status in relation to *S. aureus* colonization. The AD skin is characterized by decreased skin hydration, increased skin pH, altered keratinocyte adhesion properties and the activation of a number of serine proteases, leading to desquamation.[74] The increased adhesion sites and the altered milieu on the skin is thought to enable the growth of different microbes than present on the healthy skin, leading to increased presence of *S. aureus*.[74-77] In turn, the penetration of *S. aureus* into the epidermidis leads to the secretion of a number of molecules further enabling *S. aureus* colonization and skin barrier damage through direct effects or stimulation of the immune system.[74, 78] The direct skin barrier damage is caused by the cytolytic activity from *S. aureus* exotoxins and the ability of *S. aureus* to stimulate keratinocytes

to produce endogenous serine proteases.[79, 80] *S. aureus* secreted enterotoxins acting as superantigens (Sags) can directly stimulate T-cell proliferation and recruitment to the skin.[43] Activated Th2 cytokines IL-4 and IL-13 downregulate filaggrin expression, decrease sphingomyelinase (normally protecting against α-toxin induced keratinocyte damage), induce fibronectin synthesis and suppress the expression of AMPs.[81] Furthermore, the expression of Th2 cytokine IL-31 leads to increased pruritus, resulting in further skin barrier dysfunction and exposing extracellular matrix (ECM) adhesins for *S. aureus* binding.[82]

Less is known about the effect of *FLG* mutations on the complete microbiome composition and the immune cell numbers in patients with AD. Recently, one study investigated the skin microbiome in adult AD patients with *FLG* mutations and showed a different microbiome on nonlesional skin compared to wild-type patients.[75] Current evidence for the association between *FLG* mutations and the immune cell composition come from a small number of AD case series, mice studies and one skin equivalent study.[83-87] These studies suggested an increase in circulating Th2, Th17 and γδT17 cells as a result of *FLG* mutations.[83-87]

Complex interactions between the skin microbiome, skin barrier and immune system contribute to the inflammation in AD. It is likely that the role of each element differs per patient and even within a patient over time. Knowledge of the complex bidirectional interactions in the pathogenesis is important for a better understanding of this complex disease and to further develop targeted treatment.

ATOPIC DERMATITIS – TREATMENT TARGETING THE MICROBIOME

The aim of treatment in AD is to improve symptoms and gain long-term disease control. Cornerstones in the treatment of AD are the use of emollients and trigger avoidance (i.e., irritants, allergens and stress).[88] The mainstay of anti-inflammatory treatment includes the use of topical corticosteroids (TCS) and topical calcineurin inhibitors (TCI), but in severe/refractory cases the use of systemic immune suppressive treatment can be indicated.[89, 90]

Due to the association between *S. aureus* and AD, therapeutic strategies targeting the microbiome are being investigated. In current guidelines, short term use of oral antibiotics against *S. aureus* is indicated in clinically infected AD.[91, 92] In recurrent infected AD, the use of topical antiseptics (i.e., povidone-iodine and chlorhexidine) are advised.[92] The role of *S. aureus* is less clear in clinically non-infected AD and a Cochrane review found insufficient evidence on the benefit of anti-staphylococcal agents in these cases.[93] However, this Cochrane review did not include long-term follow-up studies and as regrowth of *S. aureus* might occur quickly in patients with AD, long-term treatment might be beneficial.[94] Since

continued use of anti-microbial treatment also affects commensal micro-organisms and might induce bacterial resistance, alternative treatments targeting the microbiome should be evaluated.[95, 96] In this context, studying the effect of bacteriophages and bacteriophage lysins in eradicating *S. aureus* could be helpful. Bacteriophages are viruses that infect and replicate within their host and produce endolysins that digest the bacterial cell wall and selectively kill specific bacteria.[97] It is important to gain more knowledge on the impact of currently available and novel treatments on the skin microbiome. This will lead to insights into the role of the microbiome in the pathogenesis and disease course of AD and improve current treatment strategies.

AIMS OF THIS THESIS

The pathophysiology of AD is highly complex with a heterogeneous clinical presentation. Recent knowledge emphasizes that the skin microbiome, skin barrier and immune system are all interacting and together reinforce the process of inflammation in AD. However, not all interactions are thoroughly investigated and research on the impact of the impaired skin barrier, caused by *FLG* mutations, on the microbiome and immune cells is lacking and needs to be validated. Furthermore, most studies investigating the skin microbiome in patients with AD have used methods that are unable to classify microbes up to species level or to identify genetically distinct strains. Therefore, knowledge is lacking on how the *S. aureus* bacterium is able to cause symptoms of AD. Knowledge on the multidirectional interactions of the pathophysiological aspects and the role of *S. aureus* as aggravating factor will lead to a better understanding of this disease and help develop new personalized treatment strategies. In this thesis we aimed to fill some gaps in the current knowledge on the pathophysiology of AD. The specific aims are listed below:

- Gain more knowledge on the role of *S. aureus* in the pathogenesis of AD by investigating which strains of this bacterium are present over time in AD patients and interact with disease severity (**Chapter 2**).
- Further investigate the bidirectional interactions between the skin microbiome, skin barrier and immune system by evaluating if mutations in *FLG* shape both the microbiome in AD (**Chapter 3.1)** and circulating immune cells (**Chapter 3.2**) in the general population and AD patients.
- Validate the use of measuring NMF content in the SC to stratify patients based on *FLG* mutations status to enable the identification of patients at high risk for a more severe phenotype and to enable personalized treatment (**Chapter 4**).
- Study the effect of treatment interfering with the skin microbiome using alpine climate treatment (**Chapter 5.1**) and a targeted intervention against *S. aureus* (**Chapter 5.2**) in

patients with AD to further elucidate the role of the microbiome in the pathogenesis of AD.

STUDY DESIGN

The research in this thesis was based on four study cohorts, including the DAVOS, MAAS, KinderHaven and Generation R cohort. The DAVOS cohort included 79 children with difficult to treat AD between the age of 8 and 18 years.[98] This randomized controlled trial (RCT) was designed to investigate the short and long-term effectiveness of inpatient alpine climate treatment in comparison to an outpatient treatment setting in moderate maritime climate. Clinical data, microbial and DNA samples from this cohort were used to evaluate the effect of alpine climate treatment on the composition of the skin microbiome on lesional and nonlesional skin. Furthermore, these data were used to evaluate the association between a mutation in *FLG* and the microbiome. The MAAS cohort included 100 adult patients with clinically non-infected, moderate to severe AD who were participating in a RCT to evaluate the effect of a targeted intervention against *S. aureus* with topical endolysin treatment (Staphefekt SA.100) on the usage of TCS.[99] The *S. aureus* cultures that were collected during this trial also enabled us to determine the *S. aureus* genotypes present in AD patients, by characterizing *S. aureus* isolates with *spa*-typing. The KinderHaven cohort included 837 children (0-18 year) with AD that consulted the dermatologist in a pediatric multidisciplinary atopic expert center between 2016 and 2019. During their visit, the NMF content in the SC was measured using Raman Spectroscopy. In addition, DNA samples (using buccal swabs) were collected from 101 children to detect mutations in *FLG.* With these data we could determine the value of NMF as a biomarker for *FLG* mutation status. Finally, the Generation R study is a population-based prospective birth cohort study following children from their early fetal life onwards.[100] Data from 523 children in this cohort (DNA samples and immune cell numbers) were used to determine the effect of *FLG* mutations on the B -and T-cell subsets (at the age of 10 years) in children of the general population and in a subgroup of 102 children with AD.

REFERENCES

1. Odhiambo JA, Williams HC, Clayton TO, Robertson CF, Asher MI, Group IPTS. Global variations in prevalence of eczema symptoms in children from ISAAC Phase Three. J Allergy Clin Immunol. 2009;124(6):1251-8 e23.
2. 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(7):e39803.
3. Abuabara K, Yu AM, Okhovat JP, Allen IE, Langan SM. The prevalence of atopic dermatitis beyond childhood: A systematic review and meta-analysis of longitudinal studies. Allergy. 2018;73(3):696-704.
4. Weidinger S, Beck LA, Bieber T, Kabashima K, Irvine AD. Atopic dermatitis. Nat Rev Dis Primers. 2018;4(1):1.
5. Weidinger S, Novak N. Atopic dermatitis. Lancet. 2016;387(10023):1109-22.
6. Bieber T. Atopic dermatitis. N Engl J Med. 2008;358(14):1483-94.
7. Alexander H, Paller AS, Traidl-Hoffmann C, Beck LA, De Benedetto A, Dhar S, et al. The role of bacterial skin infections in atopic dermatitis: expert statement and review from the International Eczema Council Skin Infection Group. Br J Dermatol. 2019.
8. Kapoor R, Menon C, Hoffstad O, Bilker W, Leclerc P, Margolis DJ. The prevalence of atopic triad in children with physician-confirmed atopic dermatitis. J Am Acad Dermatol. 2008;58(1):68-73.
9. Wuthrich B. Clinical aspects, epidemiology, and prognosis of atopic dermatitis. Ann Allergy Asthma Immunol. 1999;83(5):464-70.
10. Blome C, Radtke MA, Eissing L, Augustin M. Quality of Life in Patients with Atopic Dermatitis: Disease Burden, Measurement, and Treatment Benefit. Am J Clin Dermatol. 2016;17(2):163-9.
11. Kiebert G, Sorensen SV, Revicki D, Fagan SC, Doyle JJ, Cohen J, et al. Atopic dermatitis is associated with a decrement in health-related quality of life. Int J Dermatol. 2002;41(3):151-8.
12. Thomsen SF, Ulrik CS, Kyvik KO, Hjelmborg J, Skadhauge LR, Steffensen I, et al. Importance of genetic factors in the etiology of atopic dermatitis: a twin study. Allergy Asthma Proc. 2007;28(5):535-9.
13. Edfors-Lubs ML. Allergy in 7000 twin pairs. Acta Allergol. 1971;26(4):249-85.
14. Bin L, Leung DY. Genetic and epigenetic studies of atopic dermatitis. Allergy Asthma Clin Immunol. 2016;12:52.
15. Flohr C, Mann J. New insights into the epidemiology of childhood atopic dermatitis. Allergy. 2014;69(1):3-16.
16. Flohr C, Yeo L. Atopic dermatitis and the hygiene hypothesis revisited. Curr Probl Dermatol. 2011;41:1-34.
17. 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(2):338-51.
18. Thomsen SF. Atopic dermatitis: natural history, diagnosis, and treatment. ISRN Allergy. 2014;2014:354250.
19. Hanifin JM, Cooper KD, Ho VC, Kang S, Krafchik BR, Margolis DJ, et al. Guidelines of care for atopic dermatitis, developed in accordance with the American Academy of Dermatology (AAD)/American Academy of Dermatology Association "Administrative Regulations for Evidence-Based Clinical Practice Guidelines". J Am Acad Dermatol. 2004;50(3):391-404.
20. Williams HC, Burney PG, Hay RJ, Archer CB, Shipley MJ, Hunter JJ, et al. The U.K. Working Party's Diagnostic Criteria for Atopic Dermatitis. I. Derivation of a minimum set of discriminators for atopic dermatitis. Br J Dermatol. 1994;131(3):383-96.

21. Weidinger S, Beck LA, Bieber T, Kabashima K, Irvine AD. Atopic dermatitis. Nature Reviews Disease Primers. 2018;4(1):1.
22. Totté JEE. The Microbiome and the Human Immune Response in Atopic Dermatitis : Exploring Microbial Targets for Personalized Treatment: Erasmus University Rotterdam; 2018.
23. Sender R, Fuchs S, Milo R. Are We Really Vastly Outnumbered? Revisiting the Ratio of Bacterial to Host Cells in Humans. Cell. 2016;164(3):337-40.
24. Gilbert JA, Blaser MJ, Caporaso JG, Jansson JK, Lynch SV, Knight R. Current understanding of the human microbiome. Nat Med. 2018;24(4):392-400.
25. Grice EA, Segre JA. The skin microbiome. Nat Rev Microbiol. 2011;9(4):244-53.
26. Paller AS, Kong HH, Seed P, Naik S, Scharschmidt TC, Gallo RL, et al. The microbiome in patients with atopic dermatitis. J Allergy Clin Immunol. 2019;143(1):26-35.
27. Scharschmidt TC, Vasquez KS, Truong HA, Gearty SV, Pauli ML, Nosbaum A, et al. A Wave of Regulatory T Cells into Neonatal Skin Mediates Tolerance to Commensal Microbes. Immunity. 2015;43(5):1011-21.
28. Fujimura KE, Sitarik AR, Havstad S, Lin DL, Levan S, Fadrosh D, et al. Neonatal gut microbiota associates with childhood multisensitized atopy and T cell differentiation. Nat Med. 2016;22(10):1187-91.
29. Arrieta MC, Stiemsma LT, Dimitriu PA, Thorson L, Russell S, Yurist-Doutsch S, et al. Early infancy microbial and metabolic alterations affect risk of childhood asthma. Sci Transl Med. 2015;7(307):307ra152.
30. Scharschmidt TC, Vasquez KS, Pauli ML, Leitner EG, Chu K, Truong HA, et al. Commensal Microbes and Hair Follicle Morphogenesis Coordinately Drive Treg Migration into Neonatal Skin. Cell Host Microbe. 2017;21(4):467-77 e5.
31. Meylan P, Lang C, Mermoud S, Johannsen A, Norrenberg S, Hohl D, et al. Skin Colonization by Staphylococcus aureus Precedes the Clinical Diagnosis of Atopic Dermatitis in Infancy. J Invest Dermatol. 2017;137(12):2497-504.
32. Kennedy EA, Connolly J, Hourihane JO, Fallon PG, McLean WH, Murray D, et al. Skin microbiome before development of atopic dermatitis: Early colonization with commensal staphylococci at 2 months is associated with a lower risk of atopic dermatitis at 1 year. J Allergy Clin Immunol. 2017;139(1):166-72.
33. Kong HH, Oh J, Deming C, Conlan S, Grice EA, Beatson MA, et al. Temporal shifts in the skin microbiome associated with disease flares and treatment in children with atopic dermatitis. Genome Res. 2012;22(5):850-9.
34. Bunikowski R, Mielke ME, Skarabis H, Worm M, Anagnostopoulos I, Kolde G, et al. Evidence for a disease-promoting effect of Staphylococcus aureus-derived exotoxins in atopic dermatitis. J Allergy Clin Immunol. 2000;105(4):814-9.
35. Tauber M, Balica S, Hsu CY, Jean-Decoster C, Lauze C, Redoules D, et al. Staphylococcus aureus density on lesional and nonlesional skin is strongly associated with disease severity in atopic dermatitis. J Allergy Clin Immunol. 2016;137(4):1272-4 e3.
36. Higaki S, Morohashi M, Yamagishi T, Hasegawa Y. Comparative study of staphylococci from the skin of atopic dermatitis patients and from healthy subjects. Int J Dermatol. 1999;38(4):265-9.
37. Totte JE, van der Feltz WT, Hennekam M, van Belkum A, van Zuuren EJ, Pasmans SG. Prevalence and odds of Staphylococcus aureus carriage in atopic dermatitis: a systematic review and meta-analysis. Br J Dermatol. 2016;175(4):687-95.
38. Bjerre RD, Bandier J, Skov L, Engstrand L, Johansen JD. The role of the skin microbiome in atopic dermatitis: a systematic review. Br J Dermatol. 2017;177(5):1272-8.

39. Totte JEE, Pardo LM, Fieten KB, Vos MC, van den Broek TJ, Schuren FHJ, et al. Nasal and skin microbiomes are associated with disease severity in paediatric atopic dermatitis. Br J Dermatol. 2019;181(4):796-804.
40. van Belkum A, Melles DC. Not all Staphylococcus aureus strains are equally pathogenic. Discov Med. 2005;5(26):148-52.
41. Feil EJ, Cooper JE, Grundmann H, Robinson DA, Enright MC, Berendt T, et al. How clonal is Staphylococcus aureus? J Bacteriol. 2003;185(11):3307-16.
42. Wertheim HF, Melles DC, Vos MC, van Leeuwen W, van Belkum A, Verbrugh HA, et al. The role of nasal carriage in Staphylococcus aureus infections. Lancet Infect Dis. 2005;5(12):751-62.
43. Dinges MM, Orwin PM, Schlievert PM. Exotoxins of Staphylococcus aureus. Clin Microbiol Rev. 2000;13(1):16-34, table of contents.
44. Bouwstra JA, Ponec M. The skin barrier in healthy and diseased state. Biochim Biophys Acta. 2006;1758(12):2080-95.
45. Kezic S, Novak N, Jakasa I, Jungersted JM, Simon M, Brandner JM, et al. Skin barrier in atopic dermatitis. Front Biosci (Landmark Ed). 2014;19:542-56.
46. Irvine AD, McLean WH. Breaking the (un)sound barrier: filaggrin is a major gene for atopic dermatitis. J Invest Dermatol. 2006;126(6):1200-2.
47. Irvine AD, McLean WH, Leung DY. Filaggrin mutations associated with skin and allergic diseases. N Engl J Med. 2011;365(14):1315-27.
48. Drislane C, Irvine AD. The role of filaggrin in atopic dermatitis and allergic disease. Ann Allergy Asthma Immunol. 2019.
49. Sandilands A, Sutherland C, Irvine AD, McLean WH. Filaggrin in the frontline: role in skin barrier function and disease. J Cell Sci. 2009;122(Pt 9):1285-94.
50. Kezic S, Kammeyer A, Calkoen F, Fluhr JW, Bos JD. Natural moisturizing factor components in the stratum corneum as biomarkers of filaggrin genotype: evaluation of minimally invasive methods. Br J Dermatol. 2009;161(5):1098-104.
51. Kezic S, Kemperman PM, Koster ES, de Jongh CM, Thio HB, Campbell LE, et al. Loss-of-function mutations in the filaggrin gene lead to reduced level of natural moisturizing factor in the stratum corneum. J Invest Dermatol. 2008;128(8):2117-9.
52. Palmer CN, Irvine AD, Terron-Kwiatkowski A, Zhao Y, Liao H, Lee SP, et al. Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. Nat Genet. 2006;38(4):441-6.
53. Akiyama M. *FLG* mutations in ichthyosis vulgaris and atopic eczema: spectrum of mutations and population genetics. Br J Dermatol. 2010;162(3):472-7.
54. Brown SJ, McLean WH. One remarkable molecule: filaggrin. J Invest Dermatol. 2012;132(3 Pt 2):751-62.
55. Sandilands A, Terron-Kwiatkowski A, Hull PR, O'Regan GM, Clayton TH, Watson RM, et al. Comprehensive analysis of the gene encoding filaggrin uncovers prevalent and rare mutations in ichthyosis vulgaris and atopic eczema. Nat Genet. 2007;39(5):650-4.
56. Barker JN, Palmer CN, Zhao Y, Liao H, Hull PR, Lee SP, et al. Null mutations in the filaggrin gene (*FLG*) determine major susceptibility to early-onset atopic dermatitis that persists into adulthood. J Invest Dermatol. 2007;127(3):564-7.
57. McAleer MA, Irvine AD. The multifunctional role of filaggrin in allergic skin disease. J Allergy Clin Immunol. 2013;131(2):280-91.

58. Hotze M, Baurecht H, Rodríguez E, Chapman-Rothe N, Ollert M, Fölster-Holst R, et al. Increased efficacy of omalizumab in atopic dermatitis patients with wild-type filaggrin status and higher serum levels of phosphatidylcholines. Allergy. 2014;69(1):132-5.
59. Roekevisch E, Leeflang MMG, Schram ME, Campbell LE, Irwin McLean WH, Kezic S, et al. Patients with atopic dermatitis with filaggrin loss-of-function mutations show good but lower responses to immunosuppressive treatment. Br J Dermatol. 2017;177(6):1745-6.
60. Botkin JR. Ethical issues in pediatric genetic testing and screening. Curr Opin Pediatr. 2016;28(6):700-4.
61. 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(5):1347-58.
62. Gittler JK, Shemer A, Suarez-Farinas M, Fuentes-Duculan J, Gulewicz KJ, Wang CQ, et al. Progressive activation of T(H)2/T(H)22 cytokines and selective epidermal proteins characterizes acute and chronic atopic dermatitis. J Allergy Clin Immunol. 2012;130(6):1344-54.
63. Kuo IH, Yoshida T, De Benedetto A, Beck LA. The cutaneous innate immune response in patients with atopic dermatitis. J Allergy Clin Immunol. 2013;131(2):266-78.
64. De Benedetto A, Agnihothri R, McGirt LY, Bankova LG, Beck LA. Atopic dermatitis: a disease caused by innate immune defects? J Invest Dermatol. 2009;129(1):14-30.
65. Brandt EB, Sivaprasad U. Th2 Cytokines and Atopic Dermatitis. J Clin Cell Immunol. 2011;2(3):110.
66. Biedermann T, Skabytska Y, Kaesler S, Volz T. Regulation of T Cell Immunity in Atopic Dermatitis by Microbes: The Yin and Yang of Cutaneous Inflammation. Front Immunol. 2015;6:353.
67. Spergel JM, Mizoguchi E, Oettgen H, Bhan AK, Geha RS. Roles of TH1 and TH2 cytokines in a murine model of allergic dermatitis. The Journal of clinical investigation. 1999;103(8):1103-11.
68. Wypych TP, Marzi R, Wu GF, Lanzavecchia A, Sallusto F. Role of B cells in T(H) cell responses in a mouse model of asthma. The Journal of allergy and clinical immunology. 2018;141(4):1395-410.
69. de Benedictis FM, Franceschini F, Hill D, Naspitz C, Simons FE, Wahn U, et al. The allergic sensitization in infants with atopic eczema from different countries. Allergy. 2009;64(2):295-303.
70. Sonesson A, Bartosik J, Christiansen J, Roscher I, Nilsson F, Schmidtchen A, et al. Sensitization to skin-associated microorganisms in adult patients with atopic dermatitis is of importance for disease severity. Acta Derm Venereol. 2013;93(3):340-5.
71. Reginald K, Westritschnig K, Werfel T, Heratizadeh A, Novak N, Focke-Tejkl M, et al. Immunoglobulin E antibody reactivity to bacterial antigens in atopic dermatitis patients. Clin Exp Allergy. 2011;41(3):357-69.
72. Boguniewicz M, Leung DY. Recent insights into atopic dermatitis and implications for management of infectious complications. J Allergy Clin Immunol. 2010;125(1):4-13; quiz 4-5.
73. Patrick GJ, Archer NK, Miller LS. Which Way Do We Go? Complex Interactions in Atopic Dermatitis Pathogenesis. J Invest Dermatol. 2020.
74. Williams MR, Gallo RL. The role of the skin microbiome in atopic dermatitis. Curr Allergy Asthma Rep. 2015;15(11):65.
75. Clausen ML, Agner T, Lilje B, Edslev SM, Johannesen TB, Andersen PS. Association of Disease Severity With Skin Microbiome and Filaggrin Gene Mutations in Adult Atopic Dermatitis. JAMA Dermatol. 2018;154(3):293-300.
76. Miajlovic H, Fallon PG, Irvine AD, Foster TJ. Effect of filaggrin breakdown products on growth of and protein expression by Staphylococcus aureus. J Allergy Clin Immunol. 2010;126(6):1184-90 e3.

77. Feuillie C, Vitry P, McAleer MA, Kezic S, Irvine AD, Geoghegan JA, et al. Adhesion of Staphylococcus aureus to Corneocytes from Atopic Dermatitis Patients Is Controlled by Natural Moisturizing Factor Levels. mBio. 2018;9(4).
78. 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(11):2192-200.
79. Williams MR, Nakatsuji T, Gallo RL. Staphylococcus aureus: Master Manipulator of the Skin. Cell Host Microbe. 2017;22(5):579-81.
80. Williams MR, Nakatsuji T, Sanford JA, Vrbanac AF, Gallo RL. Staphylococcus aureus Induces Increased Serine Protease Activity in Keratinocytes. J Invest Dermatol. 2017;137(2):377-84.
81. Hata TR, Gallo RL. Antimicrobial peptides, skin infections, and atopic dermatitis. Seminars in cutaneous medicine and surgery. 2008;27(2):144-50.
82. Furue M, Yamamura K, Kido-Nakahara M, Nakahara T, Fukui Y. Emerging role of interleukin-31 and interleukin-31 receptor in pruritus in atopic dermatitis. Allergy. 2018;73(1):29-36.
83. Bonefeld CM, Petersen TH, Bandier J, Agerbeck C, Linneberg A, Ross-Hansen K, et al. Epidermal filaggrin deficiency mediates increased systemic T-helper 17 immune response. Br J Dermatol. 2016;175(4):706-12.
84. Moosbrugger-Martinz V, Gruber R, Ladstatter K, Bellutti M, Blunder S, Schmuth M, et al. Filaggrin null mutations are associated with altered circulating Tregs in atopic dermatitis. J Cell Mol Med. 2019;23(2):1288-99.
85. Wallmeyer L, Dietert K, Sochorova M, Gruber AD, Kleuser B, Vavrova K, et al. TSLP is a direct trigger for T cell migration in filaggrin-deficient skin equivalents. Sci Rep. 2017;7(1):774.
86. Favre A. Identification of filaggrin in Hassall's corpuscle by histochemical and immunohistochemical methods. Acta Anat (Basel). 1989;135(1):71-6.
87. Jee MH, Johansen JD, Buus TB, Petersen TH, Gadsboll AO, Woetmann A, et al. Increased Production of IL-17A-Producing gammadelta T Cells in the Thymus of Filaggrin-Deficient Mice. Front Immunol. 2018;9:988.
88. Langan SM, Williams HC. What causes worsening of eczema? A systematic review. Br J Dermatol. 2006;155(3):504-14.
89. 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(8):1045-60.
90. 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(9):1176-93.
91. 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(2):327-49.
92. NVDV. Richtlijn Constitutioneel eczeem 2019. 2019.
93. George SM, Karanovic S, Harrison DA, Rani A, Birnie AJ, Bath-Hextall FJ, et al. Interventions to reduce Staphylococcus aureus in the management of eczema. The Cochrane database of systematic reviews. 2019;2019(10):CD003871.
94. Hepburn L, Hijnen DJ, Sellman BR, Mustelin T, Sleeman MA, May RD, et al. The complex biology and contribution of Staphylococcus aureus in atopic dermatitis, current and future therapies. Br J Dermatol. 2017;177(1):63-71.
95. Niebuhr M, Mai U, Kapp A, Werfel T. Antibiotic treatment of cutaneous infections with Staphylococcus aureus in patients with atopic dermatitis: current antimicrobial resistances and susceptibilities. Exp Dermatol. 2008;17(11):953-7.

96. Gonzalez ME, Schaffer JV, Orlow SJ, Gao Z, Li H, Alekseyenko AV, et al. Cutaneous microbiome effects of fluticasone propionate cream and adjunctive bleach baths in childhood atopic dermatitis. Journal of the American Academy of Dermatology. 2016;75(3):481-93.e8.
97. Pastagia M, Schuch R, Fischetti VA, Huang DB. Lysins: the arrival of pathogen-directed anti-infectives. J Med Microbiol. 2013;62(Pt 10):1506-16.
98. Fieten KB, Zijlstra WT, van Os-Medendorp H, Meijer Y, Venema MU, Rijssenbeek-Nouwens L, et al. Comparing high altitude treatment with current best care in Dutch children with moderate to severe atopic dermatitis (and asthma): study protocol for a pragmatic randomized controlled trial (DAVOS trial). Trials. 2014;15:94.
99. Joan Totté JdW, Luba Pardo, Frank Schuren, Martijn van Doorn and Suzanne Pasmans. Study protocol: Targeted anti-staphylococcal therapy with endolysins in atopic dermatitis and the effect on steroid use, disease severity and the microbiome: study protocol for a randomized controlled trial (MAAS trial). Trials.
100. Kooijman MN, Kruithof CJ, van Duijn CM, Duijts L, Franco OH, van Ijzendoorn MH, et al. The Generation R Study: design and cohort update 2017. European journal of epidemiology. 2016;31(12):1243-64.

Chapter 2

Temporal variation in *Staphylococcus aureus spa*-genotypes from nose and skin in atopic dermatitis patients

M.M.F. van Mierlo
S.G.M.A. Pasmans
J.E.E. Totté
J. de Wit
B.L. Herpers
M.C. Vos
C.H.W. Klaassen
L.M. Pardo

Accepted for publication in Dermatology

ABSTRACT

Background

Staphylococcus (S.) aureus colonization is associated with increased disease severity in patients with atopic dermatitis (AD).

Objective

To investigate temporal variation in *S. aureus spa*-types isolated from the nasal cavity and lesional skin, and the correlation of *spa*-types with disease severity.

Results

This study included 96 adult AD patients who were assessed at baseline (T0) and after a strict two week follow-up period (T1) in which treatment was standardized with topical corticosteroid. Fifty-five different *spa*-types were detected within the nose and skin cultures. Seventy-three patients were colonized with *S. aureus* in the nasal cavity at both time points (persistent carriership) of whom 59 (81%) had identical *spa*-types over time. For skin samples, 42 (75%) of the 56 persistent skin carriers had identical *spa*-types over time. Patients carried the same *spa*-type in nose and skin in 79% and 77% at T0 and T1, respectively. More severe disease was not associated with specific *spa*-types nor with temporal variation in *spa*-type.

Conclusion

S. aureus strains in AD are highly heterogeneous between patients. The majority of patients carries the same *spa*-type in the nose and on the skin without temporal variation, suggesting clonal colonization within individual patients. No predominant *spa*-type nor temporal variation is associated with increased disease severity.

INTRODUCTION

Atopic dermatitis (AD) is the most common inflammatory skin disease with a lifetime prevalence of 15—20% and a significant negative impact on the quality of life.[1] The pathogenesis of AD is multifactorial, involving skin barrier defects and immunological dysregulation.[2] Moreover, strong evidence links the skin microbiome, characterized by an increased abundance of *Staphylococcus (S.) aureus* and less bacterial diversity, to the pathogenesis and disease severity in this patient group.[2-6]

S. aureus is both a human commensal organism and a pathogen.[7] The most frequent carriage site is the nose, which is assumed to be the main reservoir for the spread of *S. aureus* associated with skin infections.[7, 8] Approximately 20% of the general population is a persistent carrier of *S. aureus* in the nose.[9] Cross-sectional data in AD showed that 62% of the patients was colonized in the nasal cavity, compared to 70% on lesional skin.[3]

The association between *S. aureus* colonization and disease severity has been thoroughly investigated, however the underlying mechanisms resulting in AD characteristic inflammation remain unclear.[10] It is challenging to understand which specific strains can cause inflammation as it can carry a wide diversity of virulence factors enabling transmission, virulence and antibiotic resistance.[11] It was proposed that the immunologic response varies depending on the isolated strain.[12] In addition, temporal variation in colonizing *S. aureus* strain might aggravate the symptoms in AD due to a new trigger that is introduced to the immune system.[13, 14] This emphasizes that strain differences may contribute to the complexity of the disease, encouraging a more extensive investigation into the *S. aureus* populations in AD.

Multiple molecular identification methods are available for discriminating among *S. aureus* isolates. *Spa*-typing is a single-locus typing technique which offers a subtyping resolution comparable to more expensive and laborious techniques such as multilocus sequence typing (MLST) and pulsed field gel electrophoresis (PFGE).[15] The *spa*-typing technique targets the polymorphic region of the *S. aureus*-specific staphylococcal protein A (*spa*) gene. The encoding region consists of a variable number of repeats with highly variable nucleotide sequences.[16] Since it is sequence-based, the results produced in different labs are easily comparable.

Literature on the temporal variation of *S. aureus* and the association with disease severity is scarce. Due to low patient numbers with multiple treatment regimens and the absence of a strict follow-up time, results are subject to confounders.[13, 14, 17] This study investigates temporal variation in *S. aureus spa*-types in adult AD patients with a strict follow-up period

of two weeks in which treatment was standardized with topical corticosteroids (TCS), which reduced possible confounding of the comparisons over time. Furthermore, we assess similarities in *spa*-types from nose and skin and its correlation with disease severity. Knowledge of the colonization patterns of *S. aureus* in AD contributes to the development of new strategies to improve inflammation in AD.

MATERIAL AND METHODS

Study design

This was a longitudinal study embedded in the MAAS trial (ClinicalTrials.gov NCT02840955). Details of the clinical trial and primary outcome have been published previously.[18, 19] For our current study we included measurements from time point 0 (T0, baseline) and time point 1 (T1). T1 was scheduled after a two-week run-in period, in which treatment was standardized with triamcinoloneacetonide 0.1% cream according to a corticosteroid dosing regimen. Study procedures were reviewed and approved by the Medical Ethics Committee of the Erasmus MC University Medical Center Rotterdam, the Netherlands (MEC-2016-233). Signed informed consent was obtained from all participants.

Study population

Adult patients (≥18 years) with moderate to severe AD, according to the UK working party diagnostic criteria for AD, defined by an Eczema Area and Severity Index (EASI) score of 7.1 to 50.0, were eligible for participation in the study.[20] Other inclusion criteria were the use of any TCS before enrolment and the ability to read and understand the patient information. Patients were ineligible for participation if they used systemic antibiotics or systemic corticosteroids in the previous two months, oral immunosuppressive agents or ultraviolet light therapy in the previous three months, or topical antibiotics in the previous week. Another exclusion criterion was the presence of another skin condition that could interfere with the assessment of the AD severity. Patients were characterized by demographic data and disease severity was assessed at both time points by the research physician using the EASI score. Based on the continuous EASI score, patients were categorized in almost-clear, mild, moderate or severe disease as described previously.[21]

Sample collection and *S. aureus* typing

Samples for culture analysis were collected using sterile dry Copan 490CE swabs, which were transferred into liquid Amies medium after collection. Skin swabs were collected from the lesional skin, preferably located at the antecubital fold or the popliteal fold at T0. These lesions did not show any signs of impetigo. At T1, the skin swab was collected

from the location chosen at T0. Nose swabs were collected from both the anterior nares at T0 and T1.

Bacterial semi-quantitative cultures were performed using routine diagnostic culture procedures, using blood agar plates and specific *S. aureus* culture plates (ChromID *S. aureus* Elite agar (SAIDE), Biomérieux, France). Plates were incubated at 35°C and growth was assessed after 1 and 2 days of culture. Subsequent species determination by MALDI-TOF (Bruker Daltonics, Bremen, Germany) was performed. For each distinct colony morphology of *S. aureus* in a sample, a representative colony was picked to store the strain at -20°C for future analysis. The semi-quantitative *S. aureus* cultures were reported on a categorical scale ranging from 0 (negative) to 4 (strongly positive).

DNA was extracted from the collected *S. aureus* isolates using a MagNA Pure 96 platform in combination with the MagNA Pure DNA and Viral Nucleic Acid Small Volume Kit (Roche diagnostics, Almere, the Netherlands). PCR reactions were performed in 25 µl reactions using 1 µL of isolated DNA in 1x FastStart PCR Master (Roche) and 0.5 µM of both forward primer (5'-AACAACGTAACGGCTTCATCC-3') and reverse primer (5'- GCTTTTGCAATGTCATTTACTG-3'). Thermal cycling consisted of an initial denaturation step for 10 min at 95°C followed by 35 cycles of 30s at 95°C, 30s at 60°C and 1 min at 72°C. After a final extension step of 10 min at 72°C, reactions were cooled to room temperature. Five µl of PCR product was analyzed on agarose gel to confirm amplification. The remainder of the PCR product was treated with 2 µL of ExoSAP-IT (Isogen Life Science, De Meern, the Netherlands) for 15 min at 37°C following inactivation for 15 min at 80°C. Amplicon sequencing was performed by BaseClear (Leiden, the Netherlands) using the forward amplification primer as sequencing primer. Electropherograms were analysed and interpreted using the *spa*-typing plugin in BioNumerics v7.6 software (Applied Maths, Sint-Martens-Latem, Belgium).

Statistical analysis

Descriptive statistics were used to present patient and sample characteristics. Persistent nose and skin carriers were defined as patients with a positive *S. aureus* culture at both time points for nose or skin, respectively. Intermittent carriers were defined as having a positive *S. aureus* culture at only one time point (T0 or T1), either in nose or skin. For the difference in EASI score over time, a Wilcoxon Signed Rank test was used. A Mann-Whitney U test was performed to determine the differences in EASI score for *S. aureus* colonization and temporal variation in *spa*-type. The *S. aureus* load, as determined by semi-quantitative culture, between both time points was tested using a Chi-square test. Statistical analysis was performed in SPSS (version 25). A p-value of ≤0.05 was considered statistically significant.

RESULTS

Population characteristics

A total of 100 patients were eligible for participation in this study. From 96 patients bacterial cultures were available at both time points and therefore included in this study. The median age was 32 years (interquartile range (IQR) 25—47) and 45 patients (47%) were female. The median EASI score at T0 was 13.1 (IQR 9.4—19.0) compared to 8.1 (5.0—13.8) after the two-week run-in period with triamcinolone ($p<0.01$) (Table 1). As shown in Table 1 and Figure 1, we found high colonization rates in both nose and skin and both time points.

Table 1. Patient characteristics

	Total (n=96)
Age, median (IQR)	32.0 (25.0—47.0)
Female, n(%)	45 (46.9)
Race, n (%)	
American or Alaska native	5 (5.2)
Asian	10 (10.4)
Black or African American	7 (7.3)
White	74 (77.1)
EASI[a], median (IQR)	
T0	13.1 (9.4—19.0)
T1	8.1 (5.0—13.8)
Positive for *S. aureus* nose[b], n(%)	
T0	79 (82.3)
T1	78 (81.3)
Positive for *S. aureus* skin[c], n(%)	
T0	71 (74.0)
T1	62 (64.6)

Abbreviations: EASI, Eczema Area and Severity Index; IQR, interquartile range; T0, baseline. T1, two weeks after baseline. [a]EASI score ranges from 0—72. [b]73 patients (76.0%) were colonized in the nasal cavity at both time points. [c]56 patients (58.3%) were colonized on the skin at both time points.

A heterogeneous *S. aureus* among AD patients, but identical *spa*-types in the nose and on the skin

Of the 290 positive *S. aureus* cultures (nose T0: 79; nose T1: 78; skin T0: 71; skin T1: 62), 285 isolates were available for *spa*-typing. All *S. aureus* positive cultures contained only one distinct morphological colony type of *S. aureus*. Fifty-five different *spa*-types were found, with t002 (10%), t091 (8%), t127 (8%), t571 (7%) and t189 (6%) as the most common, accounting for less than 40% of all *spa*-types. Figure 2 shows the prevalence of the 10 most common *spa*-types separated for nose and skin. The *spa*-types that were identi-

fied in our study population were also present in previously investigated AD populations and individuals of the general population (Table S1 and Figure S1).[22-27]

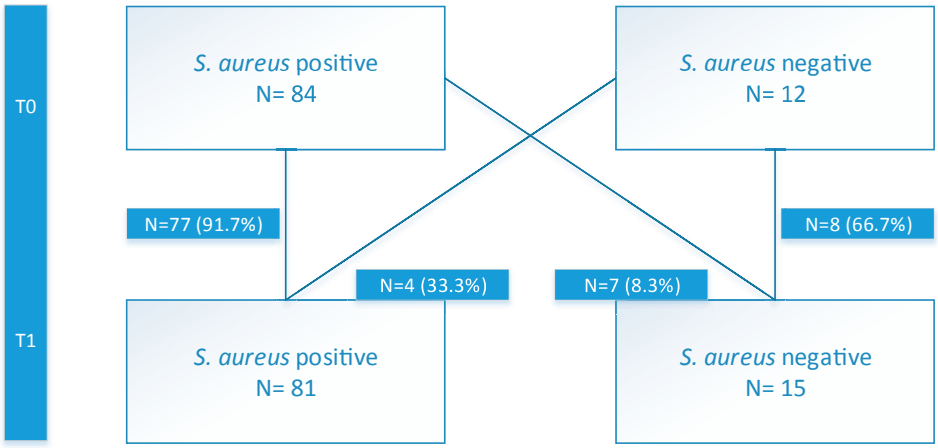

Figure 1. Temporal *S. aureus* colonization
Of the 84 patients positive for *S. aureus* at baseline, 66 patients (79%) were positive in the nasal cavity and skin, 13 patients (15%) were positive only in the nasal cavity and 5 patients (6%) only on skin. Of the 81 patients positive for *S. aureus* at T1, 59 patients (73%) were positive in the nasal cavity and skin, 19 patients (23%) positive only in the nasal cavity and 3 (4%) positive only on skin. *Abbreviations*: n, number; T0, baseline; T1, two weeks after baseline.

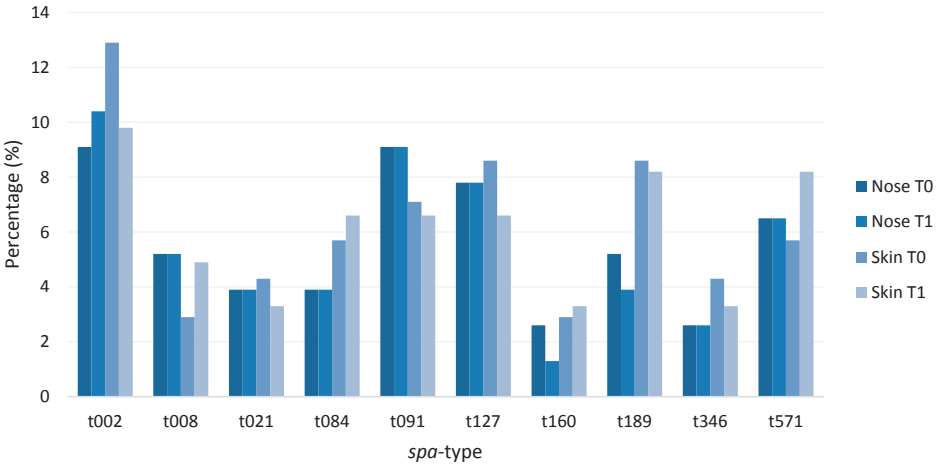

Figure 2. Frequency of the 10 most common *spa*-types per location and time point
The 10 most common *spa*-types in the nose samples at T0, represent 48.1% of the total *spa*-types. For the nose samples at T1, the 10 most common *spa*-types represented 53.8% of the total. For the skin samples at T0 and T1, this was 61.9% and 59.8%, respectively. The overview of all *spa*-types is represented in Figure S1. *Abbreviations*: T0, baseline; T1, two weeks after baseline.

Sixty-six patients had positive *S. aureus* cultures in both nose and skin at baseline, including 79% (52/66) with identical *spa*-types at both locations. At T1, the group of patients positive at both sample locations included 59 patients, of whom 78% (46/59) carried the same *spa*-type.

High prevalence of persistent *S. aureus* carriers in the nose and on the skin with identical *spa*-types

Our study population comprised 76% (73/96) persistent and 11% (11/96) intermittent nose carriers. 80% (59/73) of the persistent nose carriers were colonized with the same *spa*-type during follow-up. On AD skin, 58% (56/96) patients were persistent carrier and 22% (21/96) intermittent carrier. Within the group of persistent skin carriers, 79% (44/56) had identical *spa*-types over time.

Disease severity is not associated with the *spa*-type present in the nose and on the skin

Patients who were positive for *S. aureus* (nose and/or skin) at baseline had a significantly higher EASI score at T0 compared to patients who were negative for *S. aureus* (p=0.02). The median EASI score was 14.2 (IQR 9.9—19.2) and 9.1 (IQR 7.7—14.8), respectively. At T1, after the standardized two-week run-in treatment with triamcinolone, there was no difference in EASI score anymore between patients positive and negative for *S. aureus* (EASI 8.5 (IQR 5.0—14.4) and 8.0 (IQR 4.8—12.0), respectively (p=0.55)). Interestingly, both the EASI score and the *S. aureus* density in nose and skin at T1 was significantly lower compared to T0 (Table 1 and Table S2). Looking into the abundance of the five most common *spa*-types per severity category, we did not find an overrepresentation of a certain *spa*-type (Figure S2). Because of the high number of *spa*-types with low prevalence and the fact that these numbers are accounted for by a few patients, this was not amenable for statistical testing. Between the patients with and without temporal variation in colonizing *spa*-type (nose and/or skin), the change in EASI score from T0 to T1 did not differ significantly (p=0.11, Figure S3).

DISCUSSION

Our study showed a heterogeneous *S. aureus* population between the AD patients, as seen in the general population. *Spa*-types t002, t091, t127, t571 and t189 were the most common and the remaining types were almost unique to one patient. A large proportion of the patients (79% at T0 and 78% at T1) had positive cultures in both the nose and skin and were colonized with only one *spa*-type. 80% (59/73) of the persistent nose carriers were colonized with the same *spa*-type compared to 79% (44/56) of the persistent skin

carriers. Lastly, we showed that disease severity was not affected by the *spa*-type present nor by a change in *spa*-type over time.

Our results are in line with previous publications describing a heterogeneous population structure of *S. aureus* in AD and the absence of a prevailing strain.[14, 22, 23] Moreover, the most common *spa*-types found in our study population are also present in previously investigated AD populations and the general population, although the prevalence point estimates are not the same.[14, 22-28] It could be argued that *spa*-typing does not have enough resolution to identify the genetic diversity between strains in AD. Compared to whole genome sequencing (WGS) techniques, that examines complete genomes, *spa*-typing is based on a single gene.[29] However, WGS remains expensive to conduct in large series of cases and the *spa*-typing technique has been proven successful in settings requiring high levels of discrimination.[29, 30]

The nose is commonly described as the most important reservoir for *S. aureus* and several studies showed identical *S. aureus* strains in the nasal cavity and infected skin regions.[7, 31, 32] Moreover, nasal eradication with the application of an anti-staphylococcal agent leads to decolonization of other body sites and can prevent infection in surgical and dialysis patients, suggesting a causal relation between nasal carriage and infection.[33, 34] Our results show that up to 79% of AD patients carried the same *spa*-type in the nose and on the skin, indicating clonal colonization within individual patients and a possible role for endogenous *S. aureus* transmission from nose to skin and/or vice versa. These data form an important lead for future intervention studies investigating the effect of nasal *S. aureus* eradication on skin colonization patterns and their role in current treatment guidelines

A large proportion of the patients was persistent *S. aureus* carrier in the nose (76%) and on the skin (58%). There is no general definition for persistent and intermittent skin carriers, nor consensus on how many cultures should be taken from a skin site to define these groups.[7, 32] Studies in non-AD patients suggested that persistent carriers are colonized by a single strain in the nasal cavity over time and have a higher *S. aureus* load, with an increased risk of infection.[7] Literature on this subject in AD patients is scarce and include low patient numbers, but seem to confirm these findings.[13, 14, 17] Due to the absence of a general definition on carriership, the comparison between studies investigating *S. aureus* colonization patterns and temporal strain variation is difficult, as different sample frequencies and definitions have been used. Our study, with a strict two-week follow-up and larger sample size, confirmed high rates of colonization by a single strain per individual in persistent carriers. We did not find a difference in disease severity between patients with and without temporal variation in colonizing *S. aureus* strain. However, more studies with larger populations should follow.

We found a significantly higher EASI score at baseline in patients positive for *S. aureus* in comparison to *S. aureus* negative patients, which was not observed anymore after the two-week run-in period with triamcinolone (T1). Furthermore, a significantly lower EASI score and *S. aureus* load was seen at T1 as compared to T0. The discrepancies in these results could be explained by the standardized TCS treatment, which is known to suppress inflammation in AD skin. Our hypothesis is that, due to a suppression of inflammation, the skin can recover, making it less prone to *S. aureus* overgrowth. Thus, the use of TCS disturbs the correlation at this time point. This was also proposed in a previous study, showing a reduction in *S. aureus* load and a correlation with the disease severity after the use of TCS treatment.[35]

A major strength of this follow-up study is that all patients were sampled within the same interval of two weeks. Because treatment was similar in all patients, our results were not subject to differences in treatment regimens between participants. However, it is likely that the standardized use of corticosteroids affected the disease severity and *S. aureus* density.

This study has several limitations. First, due to a lack of a control group, we were not able to compare the presence of certain *spa*-types to a well characterized control population. For this reason, the *spa*-types present in our study population were compared to previous publications describing genetic populations in the general population and data from 'ridom *Spa*Server'.[27] Second, as mentioned before, due to the *spa*-typing technique used we may have not detected changes in other genes that may be important in AD. Last, due to the high heterogeneity and low numbers per *spa*-type, statistical testing for differences between the genetic types was difficult. Larger studies using WGS will help to clarify the role of specific *S. aureus* genes rather than specific strains. Furthermore, the lack of association of specific *spa*-types with disease severity encourages to further investigate the potential role of other virulence factors, beyond the *spa*-gene.

In conclusion, our results show that the colonizing *S. aureus* strains are not specific for AD and might rather be caused by host genetic and environmental factors. The majority of the AD patients is persistently colonized with identical *S. aureus spa*-types in the nose and skin. The results of this study underline the relevance of investigating the clinical benefit of anti-staphylococcal treatment in AD patients that are persistently colonized with *S. aureus*.

REFERENCES

1. Odhiambo JA, Williams HC, Clayton TO, Robertson CF, Asher MI, Group IPTS. Global variations in prevalence of eczema symptoms in children from ISAAC Phase Three. J Allergy Clin Immunol. 2009;124(6):1251-8 e23.
2. Weidinger S, Beck LA, Bieber T, Kabashima K, Irvine AD. Atopic dermatitis. Nature Reviews Disease Primers. 2018;4(1):1.
3. Totte JE, van der Feltz WT, Hennekam M, van Belkum A, van Zuuren EJ, Pasmans SG. Prevalence and odds of Staphylococcus aureus carriage in atopic dermatitis: a systematic review and meta-analysis. Br J Dermatol. 2016;175(4):687-95.
4. Guzik TJ, Bzowska M, Kasprowicz A, Czerniawska-Mysik G, Wojcik K, Szmyd D, et al. Persistent skin colonization with Staphylococcus aureus in atopic dermatitis: relationship to clinical and immunological parameters. Clin Exp Allergy. 2005;35(4):448-55.
5. Tauber M, Balica S, Hsu CY, Jean-Decoster C, Lauze C, Redoules D, et al. Staphylococcus aureus density on lesional and nonlesional skin is strongly associated with disease severity in atopic dermatitis. J Allergy Clin Immunol. 2016;137(4):1272-4 e3.
6. Higaki S, Morohashi M, Yamagishi T, Hasegawa Y. Comparative study of staphylococci from the skin of atopic dermatitis patients and from healthy subjects. Int J Dermatol. 1999;38(4):265-9.
7. Wertheim HF, Melles DC, Vos MC, van Leeuwen W, van Belkum A, Verbrugh HA, et al. The role of nasal carriage in Staphylococcus aureus infections. Lancet Infect Dis. 2005;5(12):751-62.
8. Toshkova K, Annemuller C, Akineden O, Lammler C. The significance of nasal carriage of Staphylococcus aureus as risk factor for human skin infections. FEMS Microbiol Lett. 2001;202(1):17-24.
9. Kluytmans J, van Belkum A, Verbrugh H. Nasal carriage of Staphylococcus aureus: epidemiology, underlying mechanisms, and associated risks. Clin Microbiol Rev. 1997;10(3):505-20.
10. Kong HH, Oh J, Deming C, Conlan S, Grice EA, Beatson MA, et al. Temporal shifts in the skin microbiome associated with disease flares and treatment in children with atopic dermatitis. Genome Res. 2012;22(5):850-9.
11. Kuroda M, Ohta T, Uchiyama I, Baba T, Yuzawa H, Kobayashi I, et al. Whole genome sequencing of meticillin-resistant Staphylococcus aureus. Lancet. 2001;357(9264):1225-40.
12. Byrd AL, Deming C, Cassidy SKB, Harrison OJ, Ng WI, Conlan S, et al. Staphylococcus aureus and Staphylococcus epidermidis strain diversity underlying pediatric atopic dermatitis. Sci Transl Med. 2017;9(397).
13. Lomholt H, Andersen KE, Kilian M. Staphylococcus aureus clonal dynamics and virulence factors in children with atopic dermatitis. J Invest Dermatol. 2005;125(5):977-82.
14. Clausen ML, Edslev SM, Norreslet LB, Sorensen JA, Andersen PS, Agner T. Temporal variation of Staphylococcus aureus clonal complexes in atopic dermatitis: a follow-up study. Br J Dermatol. 2019;180(1):181-6.
15. Narukawa M, Yasuoka A, Note R, Funada H. Sequence-based spa typing as a rapid screening method for the areal and nosocomial outbreaks of MRSA. Tohoku J Exp Med. 2009;218(3):207-13.
16. Hallin M, Friedrich AW, Struelens MJ. spa typing for epidemiological surveillance of Staphylococcus aureus. Methods Mol Biol. 2009;551:189-202.
17. Alsterholm M, Strombeck L, Ljung A, Karami N, Widjestam J, Gillstedt M, et al. Variation in Staphylococcus aureus Colonization in Relation to Disease Severity in Adults with Atopic Dermatitis during a Five-month Follow-up. Acta Derm Venereol. 2017;97(7):802-7.

18. Totte J, de Wit J, Pardo L, Schuren F, van Doorn M, Pasmans S. Targeted anti-staphylococcal therapy with endolysins in atopic dermatitis and the effect on steroid use, disease severity and the microbiome: study protocol for a randomized controlled trial (MAAS trial). Trials. 2017;18(1):404.
19. de Wit J, Totte JEE, van Mierlo MMF, van Veldhuizen J, van Doorn MBA, Schuren FHJ, et al. Endolysin treatment against Staphylococcus aureus in adults with atopic dermatitis: A randomized controlled trial. J Allergy Clin Immunol. 2019.
20. Williams HC, Burney PG, Hay RJ, Archer CB, Shipley MJ, Hunter JJ, et al. The U.K. Working Party's Diagnostic Criteria for Atopic Dermatitis. I. Derivation of a minimum set of discriminators for atopic dermatitis. Br J Dermatol. 1994;131(3):383-96.
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(5):1353-7.
22. Kim DW, Park JY, Park KD, Kim TH, Lee WJ, Lee SJ, et al. Are there predominant strains and toxins of Staphylococcus aureus in atopic dermatitis patients? Genotypic characterization and toxin determination of S. aureus isolated in adolescent and adult patients with atopic dermatitis. J Dermatol. 2009;36(2):75-81.
23. Yeung M, Balma-Mena A, Shear N, Simor A, Pope E, Walsh S, et al. Identification of major clonal complexes and toxin producing strains among Staphylococcus aureus associated with atopic dermatitis. Microbes Infect. 2011;13(2):189-97.
24. Holtfreter S, Grumann D, Balau V, Barwich A, Kolata J, Goehler A, et al. Molecular Epidemiology of Staphylococcus aureus in the General Population in Northeast Germany: Results of the Study of Health in Pomerania (SHIP-TREND-0). J Clin Microbiol. 2016;54(11):2774-85.
25. Sangvik M, Olsen RS, Olsen K, Simonsen GS, Furberg AS, Sollid JU. Age- and gender-associated Staphylococcus aureus spa types found among nasal carriers in a general population: the Tromso Staph and Skin Study. J Clin Microbiol. 2011;49(12):4213-8.
26. Becker K, Schaumburg F, Fegeler C, Friedrich AW, Kock R, Prevalence of Multiresistant Microorganisms PMMS. Staphylococcus aureus from the German general population is highly diverse. Int J Med Microbiol. 2017;307(1):21-7.
27. GmbH R. [updated December 2005. Available from: https://www.spaserver.ridom.de/.
28. Asadollahi P, Farahani NN, Mirzaii M, Khoramrooz SS, van Belkum A, Asadollahi K, et al. Distribution of the Most Prevalent Spa Types among Clinical Isolates of Methicillin-Resistant and -Susceptible Staphylococcus aureus around the World: A Review. Front Microbiol. 2018;9:163.
29. SenGupta DJ, Cummings LA, Hoogestraat DR, Butler-Wu SM, Shendure J, Cookson BT, et al. Whole-genome sequencing for high-resolution investigation of methicillin-resistant Staphylococcus aureus epidemiology and genome plasticity. J Clin Microbiol. 2014;52(8):2787-96.
30. Koreen L, Ramaswamy SV, Graviss EA, Naidich S, Musser JM, Kreiswirth BN. spa typing method for discriminating among Staphylococcus aureus isolates: implications for use of a single marker to detect genetic micro- and macrovariation. J Clin Microbiol. 2004;42(2):792-9.
31. Sivaraman K, Venkataraman N, Cole AM. Staphylococcus aureus nasal carriage and its contributing factors. Future Microbiol. 2009;4(8):999-1008.
32. Sakr A, Bregeon F, Mege JL, Rolain JM, Blin O. Staphylococcus aureus Nasal Colonization: An Update on Mechanisms, Epidemiology, Risk Factors, and Subsequent Infections. Front Microbiol. 2018;9:2419.
33. Kluytmans JA, Wertheim HF. Nasal carriage of Staphylococcus aureus and prevention of nosocomial infections. Infection. 2005;33(1):3-8.

34. Peng HM, Wang LC, Zhai JL, Weng XS, Feng B, Wang W. Effectiveness of preoperative decolonization with nasal povidone iodine in Chinese patients undergoing elective orthopedic surgery: a prospective cross-sectional study. Braz J Med Biol Res. 2017;51(2):e6736.
35. Gonzalez ME, Schaffer JV, Orlow SJ, Gao Z, Li H, Alekseyenko AV, et al. Cutaneous microbiome effects of fluticasone propionate cream and adjunctive bleach baths in childhood atopic dermatitis. J Am Acad Dermatol. 2016;75(3):481-93 e8.

SUPPLEMENTARY MATERIAL

Table S1. Frequency of *spa*-types in nose and skin

Spa-type	All isolates from current study, n (%)	Nose isolates from current study, n (%)	Skin isolates from current study, n (%)	World frequency[1], (%)	Nasal isolates in the general European population, (%)	Skin isolates in previously described AD populations, (%)
Sample missing	5 (1,7)	3 (1,9)	2 (1.5)	-		
t002	30 (10,3)	15 (9,6)	15 (11,3)	6,6		2,7(2) 5,0(3)
t005	4 (1,4)	2 (1.3)	2 (1.5)	0,7	2,8(4)	
t008	13 (4,5)	8 (5,1)	5 (3,8)	5,9	5,1(4)	5,1(3)
t012	2 (0,7)	1 (0.6)	1 (0.8)	1,5	8,4(5) 6,2(4) 5,2(6)	2,2(3)
t015	4 (1,4)	4 (2,5)	-	1,0	4,6(4)	0,1(3)
t019	1 (0,3)	1 (0.6)	-	1,0		
t021	11 (3,8)	6 (3,8)	5 (3,8)	1,1	4,0(4)	
t024	1 (0,3)	-	1 (0.8)	0,7		
t026	5 (1,7)	3 (1,9)	2 (1.5)	0,5		0,1(3)
t050	1 (0,3)	1 (0.6)	-	0,2		
t065	3 (1,0)	2 (1.3)	1 (0.8)	0,5	4.9(5)	6,2(3)
t084	14 (4,8)	6 (3,8)	8 (6,0)	1,7	7.6(5) 7,7(6)	2,7(2) 5,4(3)
t091	23 (7,9)	14 (8,9)	9 (6,8)	1,0	5,2(4) 6,1(6)	1,0(3)
t100	3 (1,0)	2 (1.3)	1 (0.8)	0,0		
t127	22 (7,6)	12 (7,6)	10 (7,5)	2,6		13,9(2) 2,1(3)
t1293	1 (0,3)	1 (0.6)	-	0,0		
t1307	1 (0,3)	-	1 (0.8)	0,0		
t1346	5 (1,7)	3 (1,9)	2 (1.5)	0,0		
t1451	1 (0,3)	1 (0.6)	-	0,2		
t148	5 (1,7)	2 (1.3)	3 (2,3)	0,3		2,7(2)
t1504	2 (0,7)	2 (1.3)	-	0,0		
t160	7 (2,4)	3 (1,9)	4 (3,0)	0,3		
t1601	1 (0,3)	1 (0.6)	-	0,0		
t164	4 (1,4)	2 (1.3)	2 (1.5)	0,2		11,1(2)
t189	18 (6,2)	7 (4,5)	11 (8,3)	0,6		19,4(2) 3,0(3)
t1994	4 (1,4)	2 (1.3)	2 (1.5)	0,0		
t216	3 (1,0)	2 (1.3)	1 (0.8)	0,4		
t2246	3 (1,0)	2 (1.3)	1 (0.8)	0		
t230	6 (2,1)	2 (1.3)	4 (3,0)	0,4		
t275	2 (0,7)	2 (1.3)	-	0,1		
t2973	3 (1,0)	1 (0.6)	2 (1.5)	0,0		
t346	9 (3,1)	4 (2,5)	5 (3,8)	0,3		2,7(2) 1,0(3)

t363	4 (1,4)	2 (1.3)	2 (1.5)	0,1		
t369	1 (0,3)	1 (0.6)	-	0,0		
t398	4 (1,4)	2 (1.3)	2 (1.5)	0,1		
t445	2 (0,7)	2 (1.3)	-	0,0		
t458	2 (0,7)	-	2 (1.5)	0,1		
t491	1 (0,3)	1 (0.6)	-	0,1		1,0(3)
t5078	1 (0,3)	1 (0.6)	-	0,0		
t559	2 (0,7)	1 (0.6)	1 (0.8)	0,0		
t571	19 (6,6)	10 (6,4)	9 (6,8)	0,4		
t6587	1 (0,3)	1 (0.6)	-	0,0		
t686	4 (1,4)	2 (1.3)	2 (1.5)	0,0		
t693	2 (0,7)	1 (0.6)	1 (0.8)	0,1		
t774	1 (0,3)	1 (0.6)	-	0,1		1,0(3)
t803	5 (1,7)	2 (1.3)	3 (2,3)	0,1		
t837	7 (2,4)	4 (2,5	3 (2,3)	0,0		
t854	2 (0,7)	2 (1.3)	-	0,0		
t975	1 (0,3)	1 (0.6)	-	0,0		
t9252	3 (1,0)	2 (1.3)	1 (0.8)	-		
t9253	4 (1,4)	2 (1.3)	2 (1.5)	-		
t9254	1 (0,3)	-	1 (0.8)	-		
t19255	4 (1,4)	2 (1.3)	2 (1.5)	-		
unknown1	1 (0,3)	-	1 (0.8)	-		
unknown2	1 (0,3)	-	1 (0.8)	-		
Total	290 (100,0)	157 (100,0)	133 (100,0)	-		

Column 2, 3 and 4 represent data from this present study and are collected during both time points. The marked cells represent the five most common *spa*-types. Column 5 represent the world's prevalence of *spa*-types as registered by the *ridom spa-server*. Column 6 represents the prevalence of *spa*-types in the nose as described by previous publications on the presence in the general European populations. Column 7 represents the prevalence of *spa*-types on the skin as described by previous publications on the presence in AD populations.

Table S2. *S. aureus* load in nose and skin at both time points

	T0	T1	P-value[a]
Nose n(%)			P<0.03
Load 1	19 (24.4)	21 (27.3)	
Load 2	31 (39.7)	33 (42.9)	
Load 3	26 (33.3)	18 (23.4)	
Load 4	2 (2.6)	5 (6.5)	
Total	78	77	
Skin n(%)			P=0.04
Load 1	22 (31.4)	24 (38.7)	
Load 2	24 (34.3)	30 (48.4)	
Load 3	16 (22.9)	7 (11.3)	
Load 4	8 (11.4)	1 (1.6)	
Total	70	62	

Results on *S. aureus* load were determined using bacterial semi-quantitative cultures and reported on a categorical scale ranging from 0 (negative) to 4 (strongly positive). *Abbreviations*: n, number; T0, baseline. T1, two weeks after baseline. [a]Statistical testing was performed using a Chi-square test.

Figure S1. Prevalence of *spa*-types in nose and skin
Abbreviations: T0, baseline; T1, two weeks after baseline. *unknown *spa*-types in *spa*-server.ridom

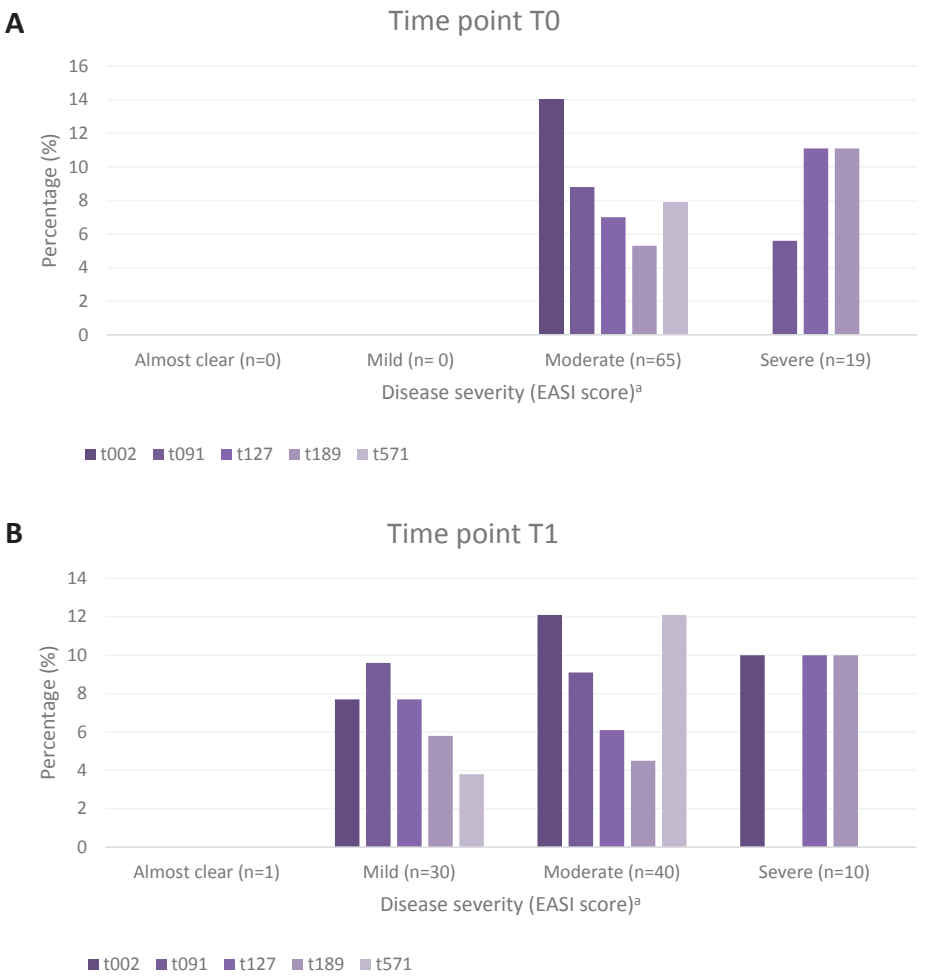

Figure S2. The abundance of the five most common *spa*-types per severity score

The five most common *spa*-types found in nose and skin in relation to the EASI score. A. At baseline (T0) in patients positive for *S. aureus*. B. Two weeks after baseline (T1) in patients positive for *S. aureus*. There was no overrepresentation of a specific *spa*-type per severity category at T0 and T1. *Abbreviations:* EASI, Eczema Area and Severity Index; T0, baseline. T1, two weeks after baseline; n, number. [a]Disease severity represented by EASI categories. Almost clear = EASI score 0.1– 1.0; Mild = EASI score 1.1–7.0; Moderate = EASI score 7.1–21.0; Severe = EASI score 21.1–50.0.

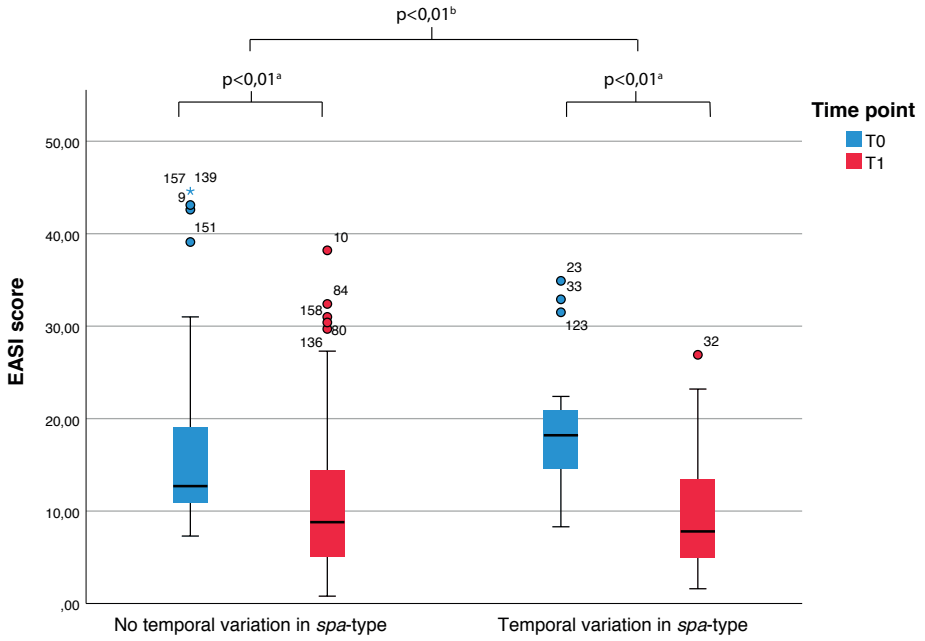

Figure S3. Disease severity separated for patients with and without temporal variation in *spa*-type
The patient group carrying an identical *spa*-type (in nose and skin) at both time points had a reduction in EASI score from a median of 12.7 (IQR 10.7-19.1) to 8.8 (IQR 5.0-14.7). Patients with a temporal variation in *spa*-type (nose and/or skin) had a median EASI score of 18.2 (IQR 14.6-22.0) and 7.8 (IQR 4.8-14.9) at T0 and T1, respectively. The change in EASI score between both groups was not statistically significant. *Abbreviations:* EASI, Eczema Area and Severity Index; T0, baseline; T1, two weeks after baseline. [a]Wilcoxon Signed Rank test. [b]Independent samples t-test.

REFERENCES SUPPLEMENTARY MATERIAL

1. GmbH R. [updated December 2005. Available from: https://www.*spa*server.ridom.de/.
2. Kim DW, Park JY, Park KD, Kim TH, Lee WJ, Lee SJ, et al. Are there predominant strains and toxins of Staphylococcus aureus in atopic dermatitis patients? Genotypic characterization and toxin determination of *S. aureus* isolated in adolescent and adult patients with atopic dermatitis. J Dermatol. 2009;36(2):75-81.
3. Yeung M, Balma-Mena A, Shear N, Simor A, Pope E, Walsh S, et al. Identification of major clonal complexes and toxin producing strains among Staphylococcus aureus associated with atopic dermatitis. Microbes Infect. 2011;13(2):189-97.
4. Holtfreter S, Grumann D, Balau V, Barwich A, Kolata J, Goehler A, et al. Molecular Epidemiology of Staphylococcus aureus in the General Population in Northeast Germany: Results of the Study of Health in Pomerania (SHIP-TREND-0). J Clin Microbiol. 2016;54(11):2774-85.
5. Sangvik M, Olsen RS, Olsen K, Simonsen GS, Furberg AS, Sollid JU. Age- and gender-associated Staphylococcus aureus *spa* types found among nasal carriers in a general population: the Tromso Staph and Skin Study. J Clin Microbiol. 2011;49(12):4213-8.
6. Becker K, Schaumburg F, Fegeler C, Friedrich AW, Kock R, Prevalence of Multiresistant Microorganisms PMMS. Staphylococcus aureus from the German general population is highly diverse. Int J Med Microbiol. 2017;307(1):21-7.

Chapter 3.1

The skin and nose microbiome and its association with filaggrin mutations in pediatric atopic dermatitis

M.M.F. van Mierlo
L.M. Pardo*
K.B. Fieten*
T.J. van den Broek
F.H.J. Schuren
M. van Geel
S.G.M.A. Pasmans
**These authors contributed equally to this work*

Submitted

ABSTRACT

Background

Complex interactions between the skin barrier, immune system and microbiome underlie the development of atopic dermatitis (AD). Our aim was to investigate the skin and nasal microbiome in relation to filaggrin gene (*FLG*) mutations.

Methods

A cross-sectional study including 77 children with difficult to treat AD. The entire encoding region of *FLG* was screened for mutations using single molecule molecular inversion probes (smMIPs) and next-generation sequencing (NGS). Bacterial swabs from the anterior nares, lesional and nonlesional skin were analyzed using 16S rRNA sequencing. For skin samples, additional qPCR was performed for *Staphylococcus (S.) aureus* and *S. epidermidis*.

Results

The prevalence of patients with a mutation in *FLG* was 40%, including a total of 10 different mutations. Analyzing the bacterial swabs from all 3 niches showed a significant effect for both niche and *FLG* mutation status on the overall microbiome composition. Using a subset analysis to test the effect of *FLG* mutation status per niche separately (nose, lesional and nonlesional skin) did not show a significant association with the microbiome. Shannon diversity and *S. aureus* abundance were significantly affected by the niche, but not by the presence of a *FLG* mutation.

Conclusions

Screening the entire encoding *FLG* region improves the sensitivity of mutation detection, resulting in better patient stratification. Our results suggest only a minor role for *FLG* mutation status on the overall microbiome, which is rather caused by differences in the present genera than by microbe richness and evenness.

INTRODUCTION

Atopic dermatitis (AD) is a chronic inflammatory skin disorder primarily present in children. This condition is characterized by a dry, itchy skin with eczematous skin lesions that show an age related distribution.[1] The pathogenesis of AD is complex, including alterations in the skin microbiome, epidermal barrier dysfunction and immunological changes.[2, 3]

The skin microbiome in AD is characterized by a low bacterial diversity, with a high abundance of *Staphylococcus (S.) aureus* and *S. epidermidis* and low abundance of the genera *Propionibacterium, Streptococcus, Acinetobacter, Corynebacterium* and *Prevotella.*[4] The skin microbiome is in constant interaction with the skin barrier and immune system, leading to inflammation characteristic for AD.[5-7] Previous studies showed that skin barrier impairment, characterized by mutations in the gene encoding filaggrin (*FLG*), causes modified fibronectin binding, higher skin pH and an increased serine protease activity, which can lead to altered colonization patterns.[8, 9] Interestingly, skin colonization with *S. aureus* leads to the secretion of virulence factors that increase inflammation and further damage the skin barrier.[8, 10] Furthermore, activated T-helper (Th)2 cells in AD can downregulate the filaggrin expression in the stratum corneum (SC).[11, 12]

Mutations in *FLG* are the strongest genetic risk factors for AD susceptibility.[11, 13] In West-European populations, 2282del4, R2447*, R501* and S3247* are the most common mutations with a prevalence around 40% in AD patients.[11, 14] However, up to 113 different mutations have been described in literature of which the presence depends on ethnic background.[15 16, 17] Due to the complexity of *FLG*, with the third exon encoding between 10 to 12 nearly identical repeats, screening for mutations in this gene has been challenging. Classical Sanger sequencing is frequently used, but this method is too cumbersome to screen the whole encoding gene for mutations.[18] A recent method, using single molecule molecular inversion probes (smMIPs) and next-generation sequencing (NGS) permits comprehensive sequencing of the entire gene.[15, 19] Instead of screening for population-specific mutations only, this technique enables to analyze the entire gene and detect population-specific mutations as well as specific mutations not previously published.

Previous studies investigating whether *S. aureus* colonization is more common in patients with a mutation in *FLG* compared to wild-type patients have shown conflicting results.[10, 20-23] Publications on the relation between mutations in *FLG* and the composition of the entire microbiome in AD are scarce. It was recently shown that the microbiome on nonlesional skin in adult AD patients was associated with *FLG* mutation status.[7] However, this study included a smaller number of AD patients and only screened for the most common muta-

tions in *FLG* which might have led to an underrepresentation of *FLG* mutation carriers. In addition, no other studies are available to validate these results.

The aim of the present study was to profile the entire mutational background of *FLG* using smMIP-NGS technique in children with difficult to treat AD. Second, we describe the microbiome in this population and investigate differences in the microbiome from the nose, lesional and nonlesional skin in AD children with and without a mutation in *FLG*.

METHODS

Study design

This study was performed as part of the DAVOS-trial, a pragmatic randomized controlled trial (RCT) comparing the long-term effectiveness of treatment in alpine climate to treatment in moderate maritime climate in children with difficult to treat AD (ISRCTN88136485). The detailed study protocol and primary outcomes have been published previously.[24, 25] Study procedures were reviewed and approved by the Medical Ethics Committee of the University Medical Center Utrecht, the Netherlands (reference 09-192/K). All patients and, if needed, their parents/guardians have provided written informed consent. The present study involves secondary outcomes from this RCT and included data before the start of the intervention period.

Participants

Dutch children between 8 and 18 years old, with difficult to treat AD, were inclued. Difficult to treat AD was determined before the start of the study and defined as the use of at least a class 3 topical corticosteroid and not being able to step down, current use of systemic immunosuppressive treatment, repeated treatment with potent topical corticosteroids or systemic immunosuppressive treatment, a history of use of systemic treatment, a significant impact of AD on the child's or the families quality of life, or seemingly unresponsive to conventional therapy according to current guidelines.[25] Demographic data were extracted from questionnaires and electronic patient files. Due to the severity of the disease in this patient group, concurrent medication was continued. Disease severity was determined using the Self-Administered Eczema Area and Severity Index (SA- EASI, range (0-96)) and microbiome as well as blood samples were collected. Study assessments were performed in Utrecht, the Netherlands.

Microbial samples

Microbial samples were collected from the anterior nares, lesional and nonlesional skin. Samples taken from the lesional skin were preferably taken from the antecubital fold or

the popliteal fold. Nonlesional skin samples were taken from the volar arm if possible (Table S1).[26] All swabs were collected using sterile cotton swabs soaked in sterile NaCl 0.9% and stored at -80°C until further processing.

DNA isolation, qPCR and sequencing

Extensive description of the procedures have been published previously.[26] Microbiome analysis was performed on bacterial swabs from the nasal cavity, lesional and nonlesional skin with sequencing of the 16S rRNA gene using V4 hypervariable region on the Illumina MiSeq sequencer (Illumina, San Diego, CA). A genus table with raw read counts was generated for downstream analysis. Quantitative (q)PCR was performed on samples from the lesional and nonlesional skin to identify *S. aureus* and *S. epidermidis*. The bacterial DNA concentration are reported as log10 transformed, femtogram per microliter (fg/ul) in this paper.

FLG mutation profiling

FLG mutations were determined on DNA isolated from blood samples. We used smMIPs-NGS strategy to screen the entire encoding region of *FLG* to identify all mutations resulting in premature protein termination. Detailed information about the sample preparation and smMIP-NGS technique have been described previously.[15, 19] Previous use of this technique showed that more than one mutation may be present on the same allele, especially when this includes rare or new mutations. Therefore, patients in whom we found two different mutations in *FLG* were not classified as compound heterozygous but as patients with more than one mutation. '*FLG* mutation status' is used throughout this paper to describe the dichotomous outcome as used in our statistical models: *FLG* wild-type patients (FLG^+) vs. *FLG* mutation carriers (FLG^-, patients with one or more mutations in *FLG*).

Statistical analysis

To determine differences in demographic data and disease severity between patients with and without a mutation in *FLG*, Chi-square, Fisher exact or Mann-Whitney U tests were used when appropriate. Descriptive statistics were used to present the *FLG* mutations in our study population. For analysis of the microbiome, we used non-subsampled genus tables after filtering (Shannon diversity index was calculated on unfiltered data). Filtering was applied by selecting the genera representing less than 97.5% of all counts. Prior to ordination analysis, the filtered genus tables were square-root transformed with subsequent application of Wisconsin double standardization. To visualize the microbial composition for all 3 niches sampled (nose, lesional and nonlesional skin) and *FLG* mutation status (FLG^+ and FLG^-), Bray-Curtis-distance based multidimensional scaling (MDS) was used. Permutational multivariable analysis of variance (PERMANOVA) correcting for repeated measurements was used to determine significant differences in microbial composition while accounting for *FLG* mutation status. We included the covariates 'niche' and 'mutation

status' as interaction terms in this model. When appropriate, PERMANOVA coefficients were obtained to determine the genera that contributed to the significant differences.[27] Second, we performed subset analyses for the skin samples (lesional and nonlesional skin) and the niches individually (nose, lesional and nonlesional skin) to investigate the effect of *FLG* mutation status on the microbial composition within each niche.

In depth analysis was performed to assess the effect of *FLG* mutation status on *S. aureus* and *S. epidermidis* colonization rates using a Chi-square test. Furthermore, the effect on *S. aureus* and *S. epidermidis* abundance in patients with positive bacterial swabs was analyzed with a linear mixed-effect model. This statistical model was also used in analyzing the effect of *FLG* mutation status on the Shannon diversity index.

Statistical analyses were performed in SPSS (version 21) and R software (version 3.5.1). We used the packages 'phyloseq' and 'vegan' for microbiome analyses and accounted for repeated measurements using the 'strata' argument.[28] R package 'ggplot2' was used for visualization.[29] Linear mixed-effect models were performed using 'lme' and 'lme4' package.[30] A two-sided *p*-value of ≤0.05 was considered statistically significant.

RESULTS

Study subjects and sample characteristics

A total of 79 patients were included in the study, of whom 77 patients gave permission for genetic analysis Lesional and nonlesional skin swabs were available for 76 patients (99%) and analyzed using qPCR techniques. Sufficient amount of DNA to perform 16S rRNA sequencing was available in 63 patients (82%) for lesional skin, 55 patients (71%) for nonlesional skin and 73 patients (95%) for the nose (Figure S1). Patient characteristics of missing data did not differ from the study group. Demographic data, comorbidities, medication use and disease severity are noted in Table 1. The median severity score was 37.2 (17.6-52.6) in the FLG^{+} group, compared to 41.4 (26.4-60.6) in the FLG^{-} group (p=0.29).

Screening the entire *FLG* improves the sensitivity of mutation detection

A total of 10 different mutations leading to a premature protein termination were found of which c.5128G>T (p.(Gly1710*)) in repeat 4 and c.7211C>G (p.(Ser2404*)) in repeat 6 have not been described before (Figure 1, Table S2). Compared to the most common mutations in West-European populations (2282del4, R2447*, R501*, and S3247*), the prevalence of patients with a *FLG* mutation increased from 33% (25 of 77) to 40% (31 of 77) screening the entire encoding gene. This included 8 (26%) patients with more than one mutation.

Table 1. Patient characteristics

	Wild type (n=46)	*FLG* mutation carriers[a] (n=31)	p-value[b]
Sex (female), n(%)	20 (43.5)	19 (61.3)	0.13
Age, median (IQR)	13.0 (11.0-15.0)	12.0 (11.0-15.0)	0.64
Age of AD onset < 6 months, n(%)	34 (80.4)	28 (90.3)	0.34
Asthma[c], n(%)	39 (84.8)	26 (83.9)	0.91
Rhinitis[d], n(%)	38 (82.6)	29 (93.5)	0.30
Food allergy[e,f], n(%)	25 (54.3)	26 (83.9)	**<0.01**
SA-EASI[g], median (IQR)	37.2 (17.6-52.6)	41.4 (26.4-60.6)[h]	0.29
Topical corticosteroids previous 7 days[i], n(%)			0.89
None	1 (2.2)	1 (3.2)	
Moderate	2 (4.3)	2 (6.5)	
Potent	40 (87.0)	27 (87.1)	
Very potent	3 (6.5)	1 (3.2)	
Systemic medication, n(%)			1.00
Prednisone	2 (4.3)	1 (3.2)	
Cyclosporine	4 (8.7)	3 (9.7)	
Oral antibiotics	-	1 (3.2)	

Abbreviations: AD, atopic dermatitis; *FLG*, Filaggrin gene; IQR ,interquartile range; n, number; SA-EASI, Self-Administered Eczema Area and Severity Index; SD, standard deviation. [a]Including 23 patients who were heterozygous, 4 patients homozygous and 4 patients with two different mutations. [b]p-values were analyzed using chi-square, fisher exact or Mann-Whitney U test when appropriate. [c] Asthma was diagnosed based on spirometry reversibility testing and Methacholine Challenge Test. [d] Rhinitis was diagnosed based on assessment by a pediatrician. [e] Food allergy was defined as a positive double-blind placebo-controlled food challenge (DBPCFC) or convincing clinical history (a reported Type I allergic reaction with acute symptoms within 2 hours after ingestion of the food) in combination with sensitization to the specific food allergen. [f]Missing n=2 (both groups n=1). [g]Range 0-96 (<17 mild; 18-46 moderate; >47 severe)[1]. [h]missing n=1. [i]UK potency system used.

The microbial composition in children with AD

All sequences belonged to 844 genera of which 81 genera remained after filtering. The genus *Staphylococcus* was predominant in both lesional and nonlesional skin samples, with a median relative abundance (RA) of 78% and 44%, respectively. This was followed by *Streptococcus* (median RA 3% and 4%) and *Corynebacterium* genera (median RA 2% and 5%) (Figure S2). Within the nose, the genera *Corynebacterium* (31%), *Staphylococcus* (11%) and *Dolosigranulum* (9%) were the mostprevalent (Figure S2). The differences in microbial composition between the 3 sampled niches were visualized and tested (Figure S3). All 3 niches harbored a significantly different microbiome (lesional skin vs. nonlesional skin, nose vs. lesional skin, nose vs. nonlesional skin: PERMANOVA R^2=0.027, p<0.01; R^2=0.190, p<0.01, and R^2=0.139, p<0.01, respectively).

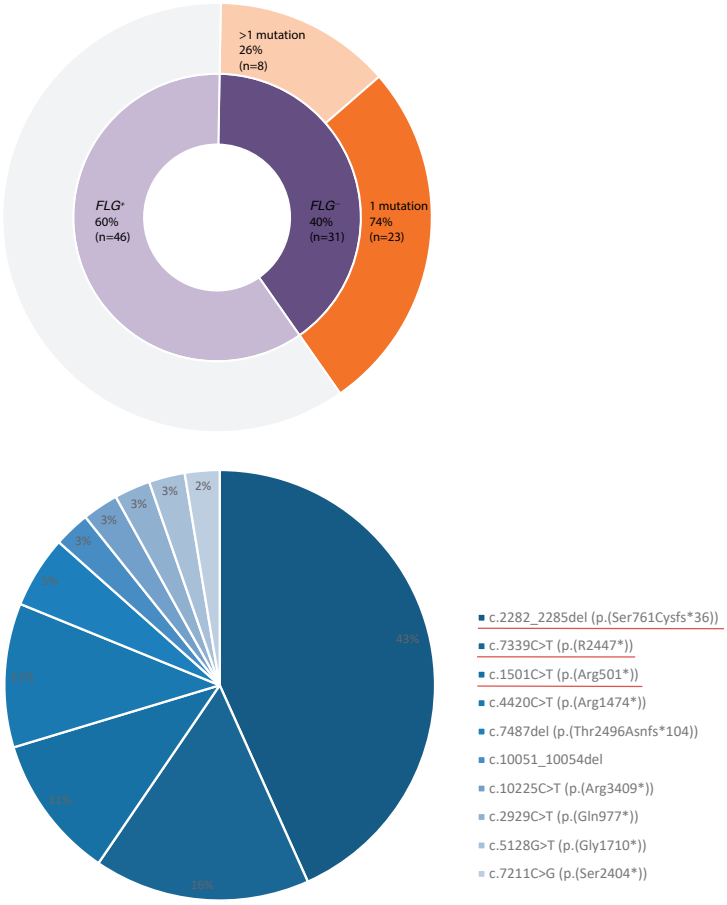

Figure 1. Prevalence of *FLG* mutations using smMIP-NGS
(A) Prevalence of patients carrying *FLG* mutations. 40% (n=31) of the patients had a mutation in *FLG*. Within this group of mutation carriers, more than one mutation was detected in 8 (26%) patients. (B) Prevalence of the 10 different *FLG* mutations in our study population. Underlined mutations represent mutations which are most commonly detected in West-European populations.

The overall microbial composition is affected by *FLG* mutation status

Community composition was visualized considering *FLG* mutation status in Figure 2 (skin samples only in Figure S4). The PERMANOVA analysis showed that both niche and *FLG* mutation status were significantly associated with the overall microbial composition (niche: R^2=0.183, p<0.01; *FLG* status: R^2=0.006, p<0.01; interaction 'niche**FLG* status': R^2=0.008, p=0.47). The association between the microbiome and *FLG* mutation status was mainly driven by the *Corynebacterium*, that was less abundant in *FLG* mutation carriers (Figure S5a). Significant effects for niche and *FLG* mutation status were also observed in the subset analysis including only skin samples (niche: R^2=0.027, p<0.01; *FLG* status: R^2=0.010,

p<0.01; interaction 'niche*_FLG_ status': R^2=0.005, p=0.56; figure S4). In this model, the *Staphylococcus* (less abundant in *FLG*$^-$) and *Acidovorax* genera (more abundant in *FLG*$^-$) mainly contributed to the effect for *FLG* mutation status (Figure S5b). Performing subset analyses to test the effect of *FLG* mutation status within each niche separately (nose, lesional and nonlesional skin), did not yield any significant associations between *FLG* mutation status and the microbiome composition (nose PERMANOVA R^2=0.014, p=0.39; lesional skin PERMANOVA R^2=0.013, p=0.72; nonlesional skin PERMANOVA R^2=0.0185, p=0.42). Alpha diversity, indicated with Shannon diversity index, was significantly affected by niche (p=0.01) but not by *FLG* mutation status (p=0.35, interaction 'niche*_FLG_ status' p=0.85) (Figure 3).

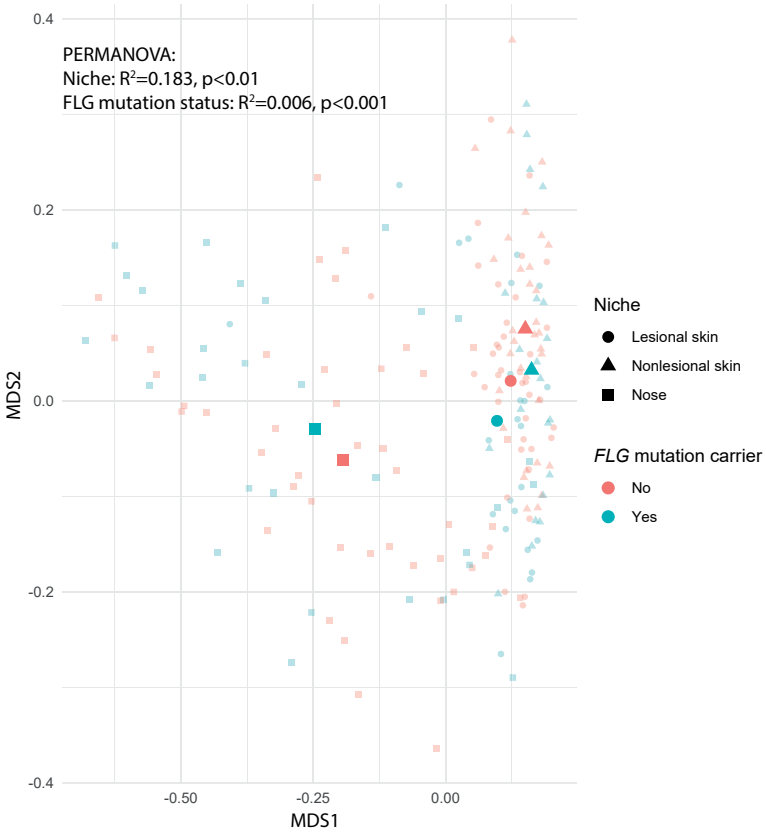

Figure 2. Bray-Curtis-distance based multidimensional scaling plot showing the microbiota for nose and skin in relation to *FLG* mutation status

For ordination analysis, genus tables were standardized using square-root transformation with subsequent application of Wisconsin double standardization. *FLG* mutation status was significantly associated with the overall microbial composition using PERMANOVA analysis (niche: R^2=0.183, p<0.01 ; *FLG* status: R^2=0.006, p<0.01, interaction: R^2=0.008, p=0.47).

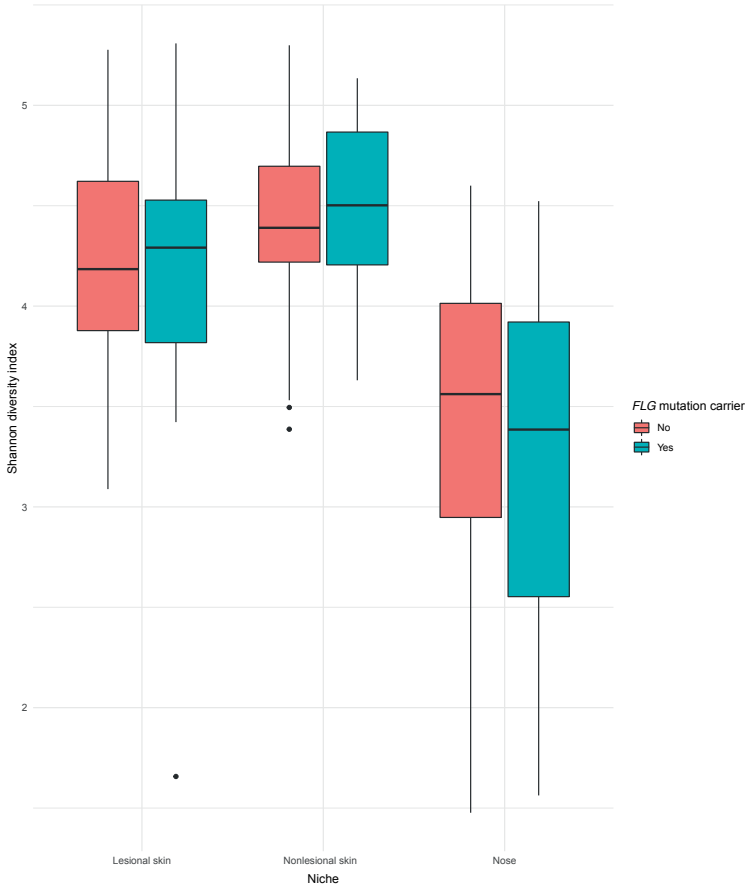

Figure 3. Shannon diversity index in relation to *FLG* mutation status
Shannon diversity index represents the number of different genera (richness) and how even they are distributed (evenness). The boxes represent the 25th percentile, median, and 75th percentile. *FLG* mutation status did not affect the Shannon diversity index in all 3 niches as determined using a Linear Mixed-Effect model (niche p=0.01; *FLG* status p=0.35; 'niche**FLG* status' p=0.85).

Association between *S. aureus* and *S. epidermidis* abundance and *FLG* mutation status

qPCR was performed to gain more information on the species *S. aureus* and *S epidermidis*. In the total study population, 56 patients (72%) were positive for *S. aureus* on lesional skin, with similar proportions in FLG^+ and FLG^- patients (p=0.10) (Table 2). Interestingly, on nonlesional skin, we found a higher proportion of patients colonized with *S. aureus* in the FLG^+ group than in the FLG^- group (57% for FLG^+ vs. 32% *in* FLG^- group; p=0.04) (Table 2). The mixed model analysis showed that niche (lesional, nonlesional) was significantly associated with *S. aureus* load, but not with *FLG* mutation status (niche p<0.01; *FLG* status p=0.10; interaction 'niche**FLG* status' p=0.81) (Figure 4). *S. epidermidis* was detected on lesional skin in 70 participants (91%) and in 69 participants (90%) on nonlesional skin

Table 2. Colonization rates for *S. aureus* and *S. epidermidis*

	Wild type (n=46)	*FLG* mutation carriers (n=31)	p-value[a]
Positive for *S. aureus*			
Lesional skin	37 (80.4)[b]	19 (61.3)[c]	0.10
Nonlesional skin	26 (56.5)[c]	10 (32.3)[c]	**0.04**
Positive for *S. epidermidis*			
Lesional skin	43 (93.5)	28 (90.3)[b]	0.65
Nonlesional skin	43 (93.5)	29 (93.5)[b]	0.54

Abbreviations: *FLG,* Filaggrin gene; n, number. [a]p-values were analyzed using chi-square test. [b]missing n=1; [c]missing n=2.

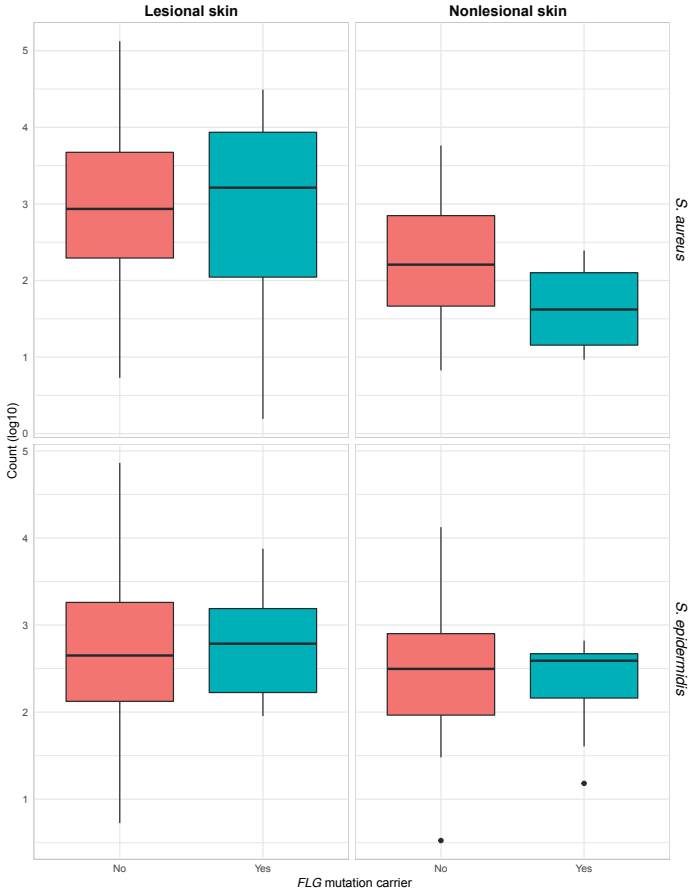

Figure 4. Quantitative PCR results of the skin samples for *S. aureus* and *S. epidermidis* in relation to *FLG* mutation status

The results for *S. aureus* and *S. epidermidis* load are shown using log10 transformed data. The boxes represent the 25th percentile, median, and 75th percentile. *S. aureus* density was significantly associated with the sampled niche, but not with *FLG* mutation status (niche p<0.01; *FLG* status p=0.10, 'niche**FLG* status' p=0.81).

(Table 2). The *S. epidermidis* load was not affected by niche (p=0.17) or by *FLG* mutation status (p=0.08) (Figure 4).

DISCUSSION

To our knowledge, this is the first study investigating the effect of *FLG* mutation status on the microbial composition in children with AD. In this cohort of children with difficult to treat AD, the prevalence of *FLG* mutations was 40% when screening the entire encoding gene. We demonstrated that the nose, lesional and nonlesional skin all harbored a significantly different microbiome. In addition, analyzing all samples together (and skin samples only), *FLG* mutation status was of significant influence on the microbial composition, although the impact on the contribution was rather small. *FLG* mutations did not affect the Shannon diversity index or *S. aureus* load. Using subset analyses per niche, we were not able to confirm these results for the effect of *FLG* mutation status on the microbial composition.

The prevalence of *FLG* mutations was increased with 7% using the smMIP-NGS strategy in comparison to including only the four most common mutations in the West-European population. It has been shown previously that only analyzing population specific mutations gives an underrepresentation of *FLG* mutation prevalence in AD populations.[15-17] Since the C-terminus has a critical role in the processing from profilaggrin to filaggrin and thus the presence of filaggrin in the epidermis,[16, 31] all mutations leading to a premature protein termination (and lack of this C-terminal) contribute to the disease burden in AD.[16, 32] This underlines the relevance of screening the entire coding region of *FLG,* instead of focusing on population specific mutations. Interestingly, the S3247* mutation, which is often described as a common European mutation variant, was not detected in our population. Possible explanations for the absence of S3247* is that mutation distribution can vary in every European country. Second, S3247* is described as a less common variant in comparison to R501* and 2282del4.[16]

A significant effect for *FLG* mutation status on the overall skin microbiome was found when analyzing all samples together (and analyzing lesional and nonlesional skin samples together), but not using subset analysis per individual niche. This might indicate that the effect for *FLG* mutation status was only minor (R^2 of 0.006 and 0.010, respectively) and/or that the number of samples per niche in our study was too small, leading to insufficient power to properly detect significant differences. Only one previous publication, including a smaller number of adult AD patients with less severe disease, screening for 3 mutations in *FLG*, investigated the association between *FLG* mutations and the microbiome.[7] They

found a significant effect of *FLG* mutation status on the microbial composition on nonlesional skin (with *S. caprae* being more prevalent in FLG^-), but not on lesional skin or in the nose.[7] Combining these and our results, a role for *FLG* mutation status on the microbial composition could be suggested. Interestingly, looking into the Shannon diversity index, our results are in line with two previous publications including AD and Ichthyosis Vulgaris (IV) patients, showing that Shannon diversity index was not affected by *FLG* mutation status.[7, 33] The explanation could be that patients with and without a mutation in *FLG* are colonized by different genera and do not differ based on the number of genera (richness) or how even they are distributed (evenness).

We found similar *S. aureus* colonization rates in FLG^+ and FLG^- patients on lesional skin, but significantly higher colonization rates on nonlesional skin for FLG^+ patients. Previous studies on this association have shown conflicting results. In vitro studies showed that the absence of filaggrin breakdown products, natural moisturizing factor (NMF), increases *S. aureus* corneocyte adhesion and growth rate.[20, 34] These findings were supported by clinical data in adult AD patients, with higher colonization rates on lesional skin and in the nose of patients with a mutation in *FLG*.[21] In contrast, other clinical studies did not show reduced filaggrin levels in patients colonized with *S. aureus.*[23, 35, 36] The differences in outcomes between clinical studies can be largely affected by disease severity in the studied AD population. In active AD skin the Th2 response is upregulated along with the production of cytokines interleukin (IL)-4 and IL-13 which are known to downregulate filaggrin expression independent of *FLG* mutation status.[11, 12] Since our study included patients who were difficult to treat and included 74% with severe or very severe disease (based on SA-EASI score), it is possible that due to an upregulation of the immune system, filaggrin expression was suppressed irrespective of *FLG* mutation status and masked the effect for wild-type patients.

A major strength of this study is the comprehensive sequencing of the entire encoding region of *FLG,* enabling to detect all mutations leading to premature protein termination. This technique enables to identify all patients who are at risk to develop a more severe and persistent disease course and are susceptible for the development of other atopic diseases (i.e., asthma and allergic sensitization). Furthermore, it could be useful to stratify patients in cohort studies. The following limitations need to be mentioned. First, since our cohort included children with difficult to treat AD, we did not discontinue concurrent medication. In addition, previous studies have shown that both age (tanner stage) and disease severity may influence the composition of the microbiome.[37, 38] In this explorative study, we did not correct for these effects in our statistical analysis. However, the use of topical and systemic medication, age and disease severity did not differ between patients with and without a mutation in *FLG.* Furthermore, excluding patients with systemic

treatment from our statistical analysis did not yield different results (data not shown). Larger groups of patients with AD might be helpful in correcting for these effects. Second, heterozygous *FLG* mutations lead to 50% reduction in filaggrin expression as compared to a complete absence of filaggrin with bi-allelic mutations and might lead to different effects.[11] This gene-dosage effect was suggested in a previous study including IV patients, with significant differences in the microbial composition between bi-allelic mutation carriers and wild-type patients, but not between bi-allelic and heterozygous mutation carriers.[33] Although our study included a small group of patients with more than one mutation in *FLG* (n=8), we did not find different results for patients with one and more than one mutation in *FLG* (data not shown). Future studies should include larger number of patients, investigating the gene-dosage effect on the microbiome in AD. In addition, by measuring *FLG* breakdown products, we can determine the effect of the Th2 response in active AD. Lastly, as described previously,[26] the impact of the inability to properly detect the *Propionibacterium* or to classify the *Staphylococci* at species level with the use of the V4 hypervariable region, was only minor due to the sampled body regions and the use of qPCR to detect *S. aureus* and *S. epidermidis* species.[39]

In conclusion, sequencing the entire *FLG* for novel, family and population specific mutations, contributing to the disease burden in AD, leads to better patient stratification. Our results suggest that the presence of a mutation in *FLG* influences the microbial composition, but not the Shannon diversity. This indicates that differences in the microbiome between FLG^{+} and FLG^{-} patients is rather caused by the presence of distinct genera than by differences in the alpha-diversity. More research into the interaction of the microbiome with the skin barrier and immune system in large patient groups will lead to a better understanding of the etiology of AD and will help develop new preventive and treatment strategies.

REFERENCES

1. Akdis CA, Akdis M, Bieber T, Bindslev-Jensen C, Boguniewicz M, Eigenmann P, et al. Diagnosis and treatment of atopic dermatitis in children and adults: European Academy of Allergology and Clinical Immunology/American Academy of Allergy, Asthma and Immunology/PRACTALL Consensus Report. J Allergy Clin Immunol. 2006;118(1):152-69.
2. Weidinger S, Beck LA, Bieber T, Kabashima K, Irvine AD. Atopic dermatitis. Nature Reviews Disease Primers. 2018;4(1):1.
3. Bin L, Leung DY. Genetic and epigenetic studies of atopic dermatitis. Allergy Asthma Clin Immunol. 2016;12:52.
4. Bjerre RD, Bandier J, Skov L, Engstrand L, Johansen JD. The role of the skin microbiome in atopic dermatitis: a systematic review. Br J Dermatol. 2017;177(5):1272-8.
5. Geoghegan JA, Irvine AD, Foster TJ. Staphylococcus aureus and Atopic Dermatitis: A Complex and Evolving Relationship. Trends Microbiol. 2017.
6. Travers JB. Toxic interaction between Th2 cytokines and Staphylococcus aureus in atopic dermatitis. J Invest Dermatol. 2014;134(8):2069-71.
7. Clausen ML, Agner T, Lilje B, Edslev SM, Johannesen TB, Andersen PS. Association of Disease Severity With Skin Microbiome and Filaggrin Gene Mutations in Adult Atopic Dermatitis. JAMA Dermatol. 2018;154(3):293-300.
8. Williams MR, Gallo RL. The role of the skin microbiome in atopic dermatitis. Curr Allergy Asthma Rep. 2015;15(11):65.
9. Williams MR, Nakatsuji T, Sanford JA, Vrbanac AF, Gallo RL. Staphylococcus aureus Induces Increased Serine Protease Activity in Keratinocytes. J Invest Dermatol. 2017;137(2):377-84.
10. 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(11):2192-200.
11. Irvine AD, McLean WH, Leung DY. Filaggrin mutations associated with skin and allergic diseases. N Engl J Med. 2011;365(14):1315-27.
12. McAleer MA, Irvine AD. The multifunctional role of filaggrin in allergic skin disease. J Allergy Clin Immunol. 2013;131(2):280-91.
13. Drislane C, Irvine AD. The role of filaggrin in atopic dermatitis and allergic disease. Ann Allergy Asthma Immunol. 2020;124(1):36-43.
14. Palmer CN, Irvine AD, Terron-Kwiatkowski A, Zhao Y, Liao H, Lee SP, et al. Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. Nat Genet. 2006;38(4):441-6.
15. van Leersum FS, Nagtzaam IF, van Oosterhoud CN, Ghesquiere SAI, Brandts R, Gostynski A, et al. Improving the diagnostic yield for filaggrin; concealed mutations in the Dutch population. J Allergy Clin Immunol. 2020;145(6):1704-6 e2.
16. Sandilands A, Terron-Kwiatkowski A, Hull PR, O'Regan GM, Clayton TH, Watson RM, et al. Comprehensive analysis of the gene encoding filaggrin uncovers prevalent and rare mutations in ichthyosis vulgaris and atopic eczema. Nat Genet. 2007;39(5):650-4.
17. Chen H, Common JE, Haines RL, Balakrishnan A, Brown SJ, Goh CS, et al. Wide spectrum of filaggrin-null mutations in atopic dermatitis highlights differences between Singaporean Chinese and European populations. Br J Dermatol. 2011;165(1):106-14.

18. Wong X, Denil S, Foo JN, Chen H, Tay ASL, Haines RL, et al. Array-based sequencing of filaggrin gene for comprehensive detection of disease-associated variants. J Allergy Clin Immunol. 2018;141(2):814-6.
19. Eijkelenboom A, Kamping EJ, Kastner-van Raaij AW, Hendriks-Cornelissen SJ, Neveling K, Kuiper RP, et al. Reliable Next-Generation Sequencing of Formalin-Fixed, Paraffin-Embedded Tissue Using Single Molecule Tags. J Mol Diagn. 2016;18(6):851-63.
20. Miajlovic H, Fallon PG, Irvine AD, Foster TJ. Effect of filaggrin breakdown products on growth of and protein expression by Staphylococcus aureus. J Allergy Clin Immunol. 2010;126(6):1184-90 e3.
21. ML C. Staphylococcus aureus colonization in atopic eczema and its association with filaggrin gene mutations. Br J Dermatol. 2017.
22. Simpson EL, Villarreal M, Jepson B, Rafaels N, David G, Hanifin J, et al. Atopic dermatitis Subjects Colonized with Staphylococcus aureus have a Distinct Phenotype and Endotype. J Invest Dermatol. 2018.
23. Berents TL, Carlsen KC, Mowinckel P, Skjerven HO, Kvenshagen B, Rolfsjord LB, et al. Skin Barrier Function and Staphylococcus aureus Colonization in Vestibulum Nasi and Fauces in Healthy Infants and Infants with Eczema: A Population-Based Cohort Study. PLoS One. 2015;10(6):e0130145.
24. Fieten KB, Zijlstra WT, van Os-Medendorp H, Meijer Y, Venema MU, Rijssenbeek-Nouwens L, et al. Comparing high altitude treatment with current best care in Dutch children with moderate to severe atopic dermatitis (and asthma): study protocol for a pragmatic randomized controlled trial (DAVOS trial). Trials. 2014;15:94.
25. Fieten K, Schappin R, Zijlstra W, Figee L, Beutler J, Raymakers F, et al. Effectiveness of alpine climate treatment for children with difficult to treat atopic dermatitis: results of a pragmatic randomized controlled trial (DAVOS trial). Clin Exp Allergy. 2017.
26. van Mierlo MMF, Totte JEE, Fieten KB, van den Broek TJ, Schuren FHJ, Pardo LM, et al. The influence of treatment in alpine and moderate maritime climate on the composition of the skin microbiome in patients with difficult to treat atopic dermatitis. Clin Exp Allergy. 2019;49(11):1437-45.
27. Gonzáles I DS, Martin P.G.P et al. CCA: An R Package to extend canonical correlation analysis., 23. JoSS.
28. Oksanen J BF, Friendly M et al. The Vegan Package. Community Ecology Package. [28-02-2018]; Available from: https://cran.r-project.org/web/packages/vegan/vegan.pdf.
29. Wickham H. ggplot2: Elegant Graphics for Data Analysis Springer-Verlag. 2009.
30. Harrison XA, Donaldson L, Correa-Cano ME, Evans J, Fisher DN, Goodwin CED, et al. A brief introduction to mixed effects modelling and multi-model inference in ecology. PeerJ. 2018;6:e4794.
31. Sandilands A, Sutherland C, Irvine AD, McLean WH. Filaggrin in the frontline: role in skin barrier function and disease. J Cell Sci. 2009;122(Pt 9):1285-94.
32. Morar N, Cookson WO, Harper JI, Moffatt MF. Filaggrin mutations in children with severe atopic dermatitis. J Invest Dermatol. 2007;127(7):1667-72.
33. Zeeuwen PL, Ederveen TH, van der Krieken DA, Niehues H, Boekhorst J, Kezic S, et al. Gram-positive anaerobe cocci are underrepresented in the microbiome of filaggrin-deficient human skin. J Allergy Clin Immunol. 2017;139(4):1368-71.
34. Feuillie C, Vitry P, McAleer MA, Kezic S, Irvine AD, Geoghegan JA, et al. Adhesion of Staphylococcus aureus to Corneocytes from Atopic Dermatitis Patients Is Controlled by Natural Moisturizing Factor Levels. mBio. 2018;9(4).
35. Broccardo CJ, Mahaffey S, Schwarz J, Wruck L, David G, Schlievert PM, et al. Comparative proteomic profiling of patients with atopic dermatitis based on history of eczema herpeticum infection and Staphylococcus aureus colonization. J Allergy Clin Immunol. 2011;127(1):186-93, 93 e1-11.

36. Simpson EL, Villarreal M, Jepson B, Rafaels N, David G, Hanifin J, et al. Patients with Atopic Dermatitis Colonized with Staphylococcus aureus Have a Distinct Phenotype and Endotype. J Invest Dermatol. 2018;138(10):2224-33.
37. Oh J, Conlan S, Polley EC, Segre JA, Kong HH. Shifts in human skin and nares microbiota of healthy children and adults. Genome Med. 2012;4(10):77.
38. Kong HH, Oh J, Deming C, Conlan S, Grice EA, Beatson MA, et al. Temporal shifts in the skin microbiome associated with disease flares and treatment in children with atopic dermatitis. Genome Res. 2012;22(5):850-9.
39. Grice EA, Segre JA. The skin microbiome. Nat Rev Microbiol. 2011;9(4):244-53.

SUPPLEMENTARY MATERIAL

Table S1. Swab locations for lesional and nonlesional skin

	Wild type (n=46)	*FLG* mutation carriers[a] (n=31)	p-value[b]
Swab location lesional skin			0.2
Antecubital fold	38 (82.6)	26 (83.9)	
Back	1 (2.2)	-	
Neck	6 (13.0)	1 (3.2)	
Popliteal fossae	1 (2.2)	3 (9.7)	
Swab location nonlesional skin			0.6
Coeur	7 (15.2)	4 (12.9)	
Leg	1 (2.2)	2 (6.5)	
Volar arm	38 (82.6)	24 (77.4)	

[a]Missing n=1; [b]Chi-square test.

Table S2. Comparison of the most common West-European *FLG* mutations and additional findings using smMIP-NGS strategy

	four most common mutations in European population						all mutations					
ID	**Mutation 1**	**Protein change**	**rpt**	**Mutation 2**	**Protein change**	**rpt**	**Mutation 1**	**Protein change**	**rpt**	**Mutation 2**	**Protein change**	**rpt**
113	c.2282_2285del	p.Ser761Cysfs*36	1				c.2282_2285del	p.Ser761Cysfs*36	1	c.10225C>T	p.(Arg3409)	10[1]
119	c.2282_2285del	p.Ser761Cysfs*36	1	c.2282_2285del	p.Ser761Cysfs*36	1	c.2282_2285del	p.Ser761Cysfs*36	1	c.2282_2285del	p.Ser761Cysfs*36	1
120	c.1501C>T	p.(Arg501*)	1				c.1501C>T	p.(Arg501*)	1	c.4420C>T	p.(Arg1474*)	4
122							c.5128G>T	p.(Gly1710*)	4			
126	c.1501C>T	p.(Arg501*)	1				c.1501C>T	p.(Arg501*)	1			
169	c.2282_2285del	p.Ser761Cysfs*36	1				c.2282_2285del	p.Ser761Cysfs*36	1			
207	c.2282_2285del	p.Ser761Cysfs*36	1				c.2282_2285del	p.Ser761Cysfs*36	1	c.2929C>T	p.(Gln977*)	2
297	c.7339C>T	p.(R2447*)	7				c.7339C>T	p.(R2447*)	7			
298	c.7339C>T	p.(R2447*)	7				c.7339C>T	p.(R2447*)	7			
315	c.1501C>T	p.(Arg501*)	1				c.1501C>T	p.(Arg501*)	1			
330	c.7339C>T	p.(R2447*)	7				c.7339C>T	p.(R2447*)	7			
338	c.2282_2285del	p.Ser761Cysfs*36	1				c.2282_2285del	p.Ser761Cysfs*36	1			
384	c.7339C>T	p.(R2447*)	7				c.7339C>T	p.(R2447*)	7			
385							c.4420C>T	p.(Arg1474*)	4	c.4420C>T	p.(Arg1474*)	4
386							c.7211C>G	p.(Ser2404*)	6			
407	c.7339C>T	p.(R2447*)	7				c.7339C>T	p.(R2447*)	7			
504	c.2282_2285del	p.Ser761Cysfs*36	1	c.2282_2285del	p.Ser761Cysfs*36	1	c.2282_2285del	p.Ser761Cysfs*36	1	c.2282_2285del	p.Ser761Cysfs*36	1
540	c.2282_2285del	p.Ser761Cysfs*36	1				c.2282_2285del	p.Ser761Cysfs*36	1			
544	c.2282_2285del	p.Ser761Cysfs*36	1				c.2282_2285del	p.Ser761Cysfs*36	1			
549	c.2282_2285del	p.Ser761Cysfs*36	1				c.2282_2285del	p.Ser761Cysfs*36	1			
597							c.7487del	p.(Thr2496Asnf)	7	c.7487del	p.(Thr2496Asnf)	7
612	c.2282_2285del	p.Ser761Cysfs*36	1				c.2282_2285del	p.Ser761Cysfs*36	1			
626							c.4420C>T	p.(Arg1474*)	4			

Table S2. Comparison of the most common West-European *FLG* mutations and additional findings using smMIP-NGS strategy (*continued*)

	four most common mutations in European population			all mutations				
648	c.2282_2285del	p.Ser761Cysfs*36	1	c.2282_2285del	p.Ser761Cysfs*36	1		
655	c.2282_2285del	p.Ser761Cysfs*36	1	c.2282_2285del	p.Ser761Cysfs*36	1		
677	c.2282_2285del	p.Ser761Cysfs*36	1	c.2282_2285del	p.Ser761Cysfs*36	1	c.10051_10054del	9
694	c.7339C>T	p.(R2447*)	7	c.7339C>T	p.(R2447*)	7		
699				c.4420C>T	p.(Arg1474*)	4		
700	c.2282_2285del	p.Ser761Cysfs*36	1	c.2282_2285del	p.Ser761Cysfs*36	1		
705	c.1501C>T	p.(Arg501*)	1	c.1501C>T	p.(Arg501*)	1		
723	c.2282_2285del	p.Ser761Cysfs*36	1	c.2282_2285del	p.Ser761Cysfs*36	1		

Abbreviations: ID, study number; rpt, filaggrin gene repeat region.

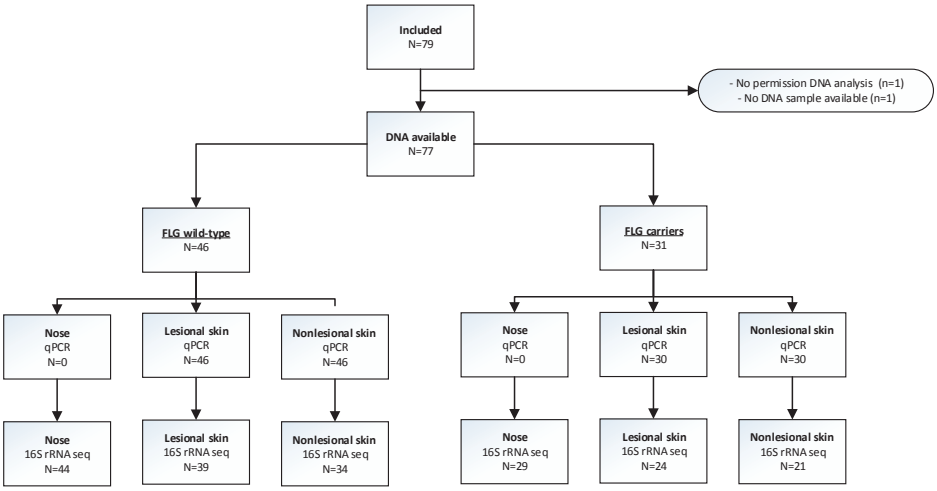

Figure S1. Flowchart of available cultures for qPCR and 16S rRNA-sequencing
Samples containing insufficient amounts of DNA did not result in usable sequence data and were therefore omitted.

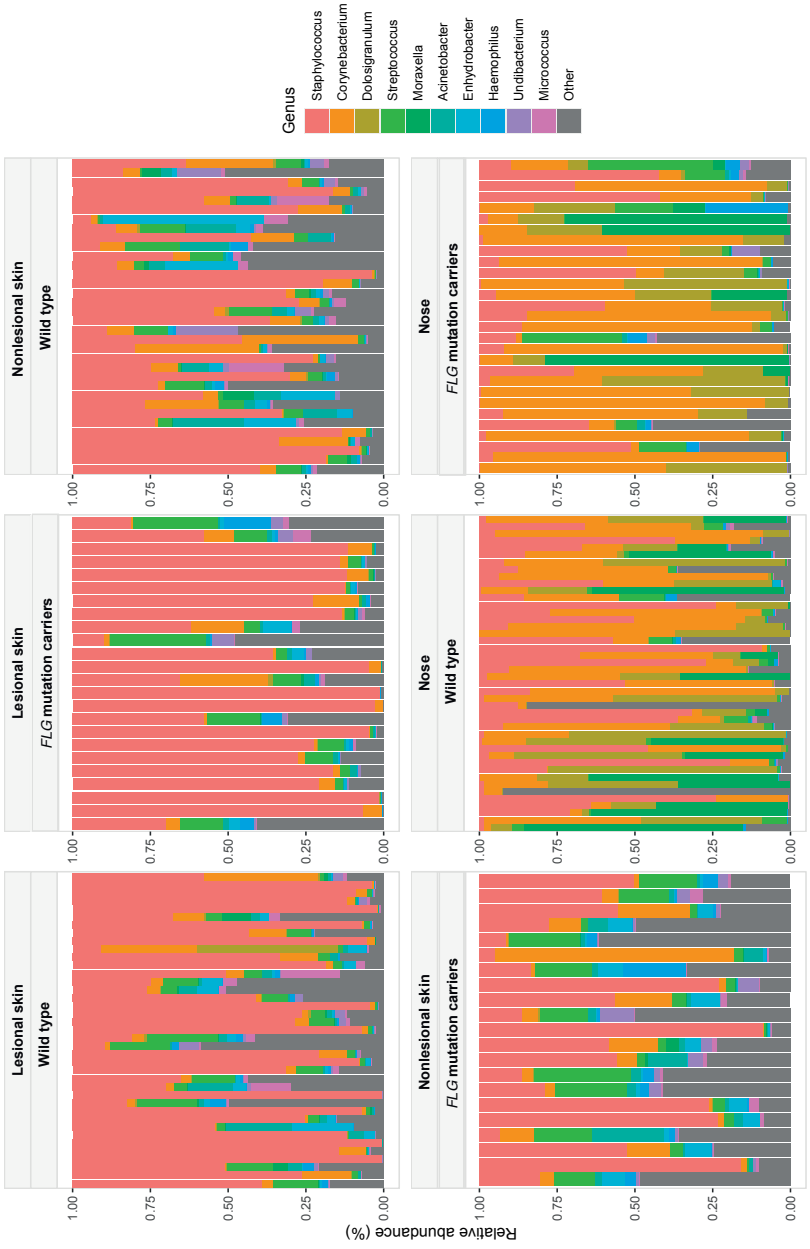

Figure S2. Relative abundance of the most common genera per niche and *FLG* mutation status
Relative abundance (RA) of the most common genera were calculated on the 81 genera after filtering of non-subsampled genus tables.

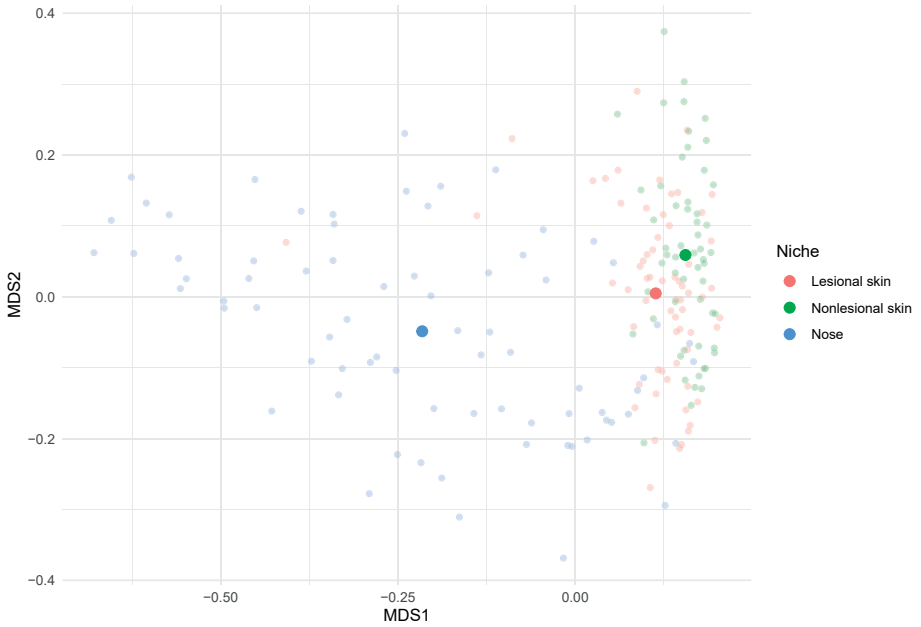

Figure S3. Bray-Curtis-distance based multidimensional scaling plot showing the microbiota for nose, lesional and nonlesional skin.

For ordination analysis, genus tables were standardized using square-root transformation with subsequent application of Wisconsin double standardization. All 3 niches harbored a significantly different microbiome (lesional vs nonlesional skin, nose vs lesional skin, nose vs nonlesional skin: PERMANOVA R^2=0.027, p<0.01; R^2=0.190, p<0.01 and R^2=0.139, p<0.01 respectively).

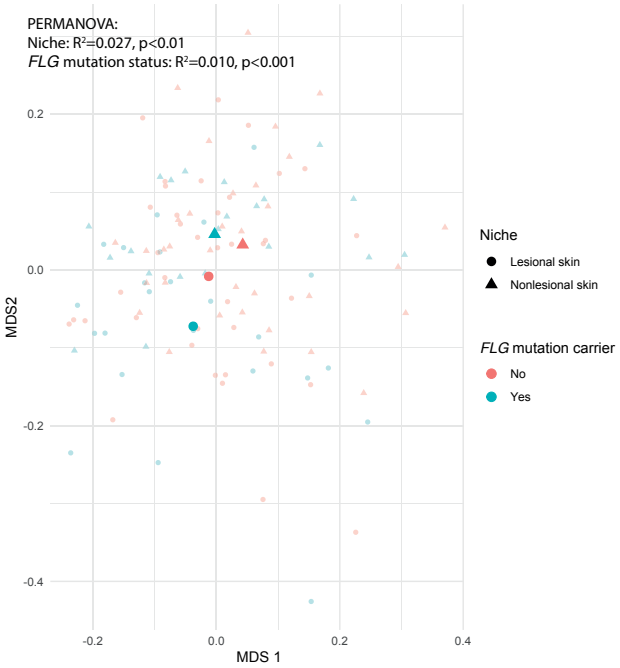

Figure S4. Bray-Curtis-distance based multidimensional scaling plot showing the microbiota for lesional and nonlesional skin in relation to *FLG* mutation status

For ordination analysis, genus tables were standardized using square-root transformation with subsequent application of Wisconsin double standardization. *FLG* mutation status was significantly associated with the overall microbial composition using PERMANOVA analysis (niche: R^2=0.027, p<0.01; *FLG* status: R^2=0.010, p<0.01; interaction 'niche: *FLG* status': R^2=0.005, p=0.56).

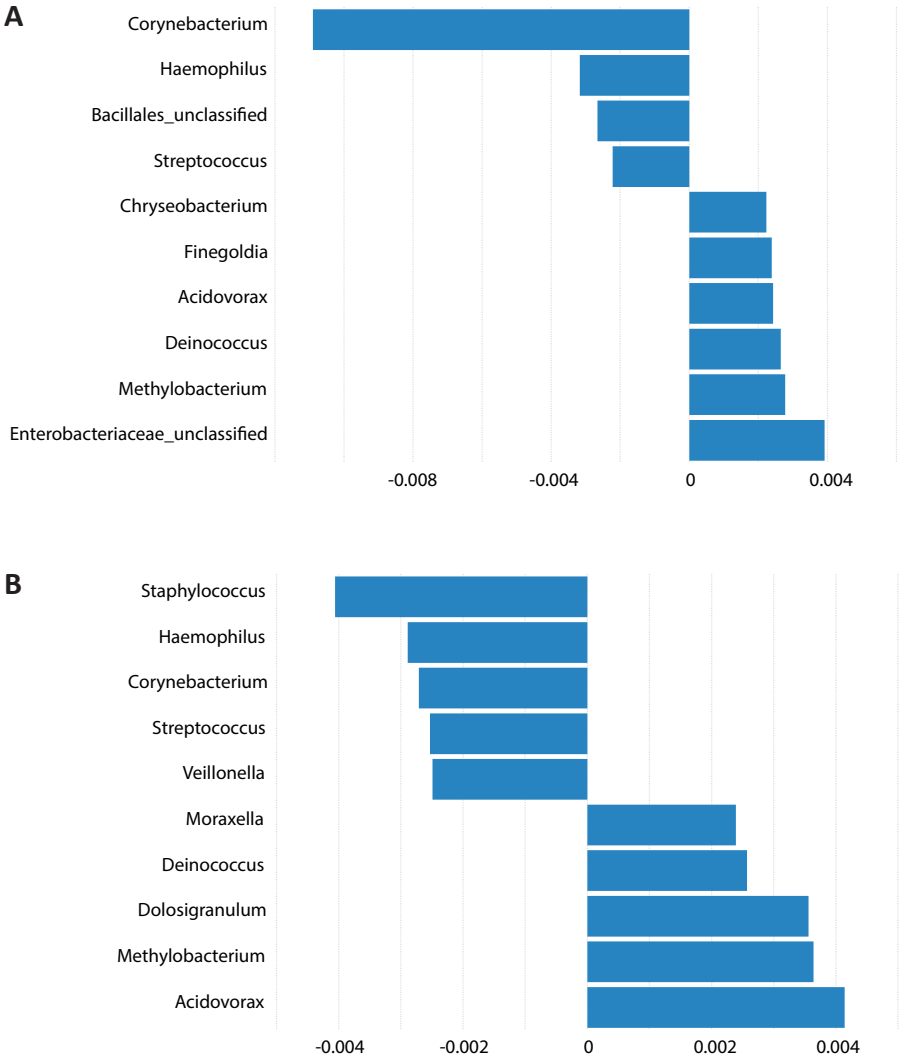

Figure S5. Top 10 genera driving the association between *FLG* mutation status and microbial composition
The x-axis displays coefficients that represent the contribution of a certain genus to the association between *FLG* mutation status and (A) the microbial composition of the nose, lesional skin and nonlesional skin and (B) the microbial composition of the lesional skin and nonlesional skin.

Chapter 3.2

Increased Th22 cell numbers in a general pediatric population with filaggrin haploinsufficiency: The Generation R Study

M.M.F. van Mierlo*
K.I.M. Looman*
M.C. van Zelm
C Hu
L Duijts
J.C. de Jongste
T.E.C. Nijsten
L.M. Pardo
J.C. Kiefte-de Jong
H.A. Moll
S.G.M.A. Pasmans
**These authors contributed equally to this work*

Accepted for publication in Pediatric Allergy and Immunology

ABSTRACT

Background

Mutations in the filaggrin gene (*FLG*) affect epidermal barrier function and increase the risk of atopic dermatitis (AD). We hypothesized that *FLG* mutations affect immune cell composition in a general pediatric population. Therefore, we investigated whether school-aged children with and without *FLG* mutations have differences in in T- and B-cell subsets.

Methods

This study was embedded in a population-based prospective cohort study, the Generation R Study, and included 523 children of European genetic ancestry aged 10 years. The most common *FLG* mutations in the European population (R501X, S1085CfsX36, R2447X and S3247X) were genotyped. Additionally, 11-color flow cytometry was performed on peripheral blood samples to determine helper T (Th), regulatory T (Treg) and $CD27^+$ and $CD27^-$ memory B cells. Subgroup analyses were performed in 358 non-AD and 102 AD cases, assessed by parental questionnaires.

Results

FLG mutations were observed in 8.4% of the total population and in 15.7% of the AD cases. Children with any *FLG* mutation had higher Th22 cell numbers compared to *FLG* wild-type children in the general and non-AD population. Children with and without *FLG* mutations had no difference in Th1, Th2, Th17, Treg or memory B cell numbers. Furthermore, in children with AD, *FLG* mutation carriership was not associated with differences in T- and B-cell subsets.

Conclusions

School-aged children of a general population with *FLG* mutations have higher Th22 cell numbers, which reflects the immunological response to the skin barrier dysfunction. *FLG* mutations did not otherwise affect the composition of the adaptive immunity in this general pediatric population.

INTRODUCTION

Filaggrin is a filament-associated protein that is encoded by the filaggrin gene (*FLG*) and is an important contributor to the preservation of the skin barrier.[1, 2] Approximately 10% of the European population is a heterozygote carrier of a disrupting mutation in *FLG*.[3] Both complete loss-of-function and reduced functional activity of filaggrin lead to destruction of the stratum corneum (SC) and consequently skin barrier dysfunction.[1, 4] This barrier dysfunction due to *FLG* mutations is presumed to be caused by lower numbers of tight junctions, reduced density of the protein corneodesmosin, and impaired maturation and excretion of lamellar bodies in the epidermis which are important in maintaining cell-to-cell integrity.[2]

Failure in barrier function through mutations in *FLG* results in increased skin permeability for percutaneous transfer of exogenous particles including allergens and pollutants.[1, 2, 4] Accordingly, *FLG* mutations are the strongest genetic risk factor for atopic dermatitis (AD).[1, 3, 5] A previous meta-analysis showed that *FLG* haploinsufficiency results in an odds ratio (OR) of 3.12 for the incidence of AD.[6] In addition, *FLG* mutations are associated with a form of AD that starts in early infancy and persists into adulthood, a higher incidence of skin infections and a higher likelihood of having asthma, inhalant or food allergies.[2, 7-9]

The increased permeability of the skin as a result of *FLG* mutations is thought to affect immune responses and maturation of adaptive immune cells. Filaggrin is also expressed in the thymus, the primary lymphoid organ in which T cells are formed.[10] Hence, *FLG* mutations potentially affect the peripheral immune cell compartment through effects in skin and thymus. Previous studies observed higher γδT17 and T helper (Th) 17 in filaggrin-deficient flaky tail (ft/ft) mice.[10, 11] In addition, a case study reported higher numbers of circulating thymus-emigrated regulatory T (Treg) cells and Th2 in 6 AD patients with a heterozygote *FLG* mutation.[12] Another study, including 2 heterozygous, 2 homozygous and 1 compound heterozygous AD patient, showed increased Th17 cells in the *FLG* mutation group.[11] On the other hand, literature on the role of B cell dysregulation in AD is scarce and conflicting.[13-16] It can be hypothesized that mutations in *FLG* can affect B-cell numbers due to skewing of the Th-cell populations.

We hypothesized that *FLG* mutations affect T- and B-cell maturation in children through effects on the skin and thymus. Until now, no studies on this association have been performed in the general pediatric population and only case studies have been performed in AD patients.[11, 12] By examining the effect of *FLG* mutations on the adaptive immune cells, we will gain more insights into the role of *FLG* in the immune maturation and development of AD. Therefore, we here studied the associations between common *FLG* mutations in the European population and immune cell numbers, as determined using with 11-color flow

cytometry, within a population-based birth cohort study including a subgroup non-AD and AD patients.

METHODS

Study design

This study was embedded within the Generation R Study, a prospective birth cohort study conducted in Rotterdam, the Netherlands. The Medical Ethical Committee of the Erasmus MC, University Medical Center Rotterdam approved the study (MEC-2012-165).[17] Written informed consent was obtained from parents or legal representatives of all children. We included all children of European genetic ancestry[18] with information on *FLG* mutation status (homozygous, compound heterozygous, heterozygous or wild type) and information on at least one of the immune cell outcomes. This resulted in a total number of 523 children (Figure 1). AD was defined as physician-diagnosed eczema from parental questionnaires obtained at the child's age of 10 years ('Was your child ever diagnosed by a physician with atopic dermatitis', 'yes; no'). This information was available for 470 children, including 102 subjects with AD.[16]

FLG genotype

DNA samples obtained from umbilical cord blood were genotyped by modified Taqman allelic discrimination assays for common European mutations in *FLG* (R501X (rs61816761), S1085CfsX36 (rs41370446), R2447X (rs138726443), and S3247X (rs150597413)) with the use of primers as described previously.[19, 20] The distribution of the *FLG* mutations was as follows: 4.2% had R501X (rs61816761), 1.1% had S1085CfsX36 (rs41370446), 3.5% had R2447X (rs138726443), and 0.0% had S3247X (rs150597413). Because S3247X (rs150597413) was not present within our study population, this mutation was not included in the analyses. Children were classified as having a *FLG* mutation if they were homozygous, compound heterozygous or heterozygous for any of the 3 mutations that were present in our study population. Children without any of the mutations were classified as wild type.

Immune cell numbers

Peripheral blood samples from children were obtained at the age of 10 years.[16] The analyses were performed on fresh blood cells within 24 hours of sampling. Absolute counts of $CD3^+$ T cells and $CD19^+$ B cells per µLblood were determined with diagnostic lyse-no-wash protocol and detailed immunophenotyping was performed with 11-color flow cytometry (LSR Fortessa, BD Biosciences). We determined naïve T cells ($CD45RO^-CCR7^+$), effector memory RO-positive T cells (TemRO; $CD45RO^+CCR7^-$) and effector memory RA-

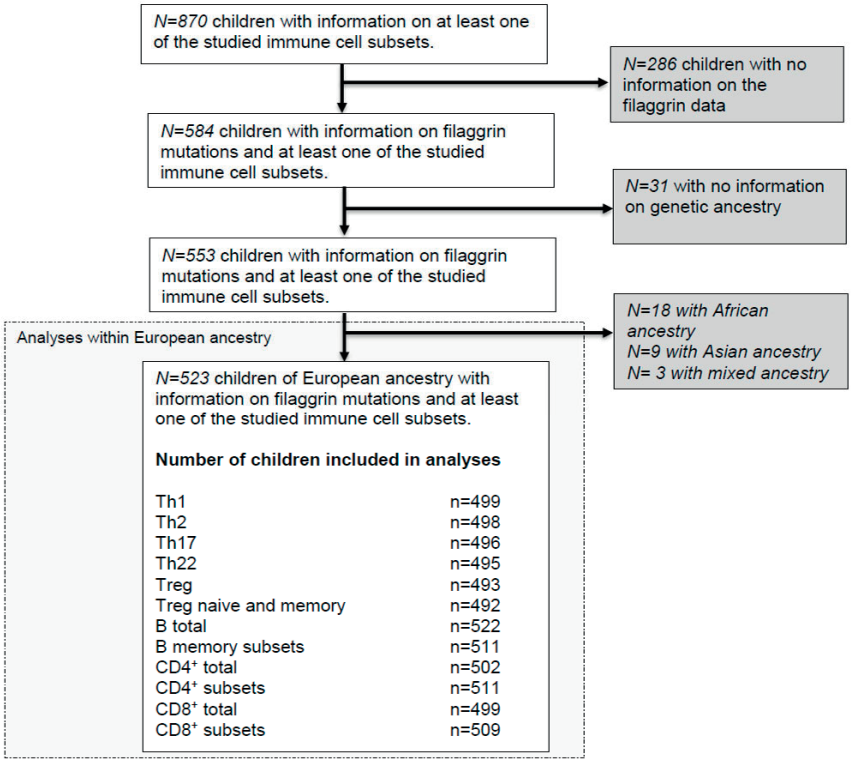

Figure 1. Flow chart of participants included in the study
Abbreviations: Th, helper T cell; Treg, regulatory T cell.; Tcm, central memory T lymphocytes; TemRA, effector memory RA-positive T lymphocytes; TemRO, effector memory RO-positive T lymphocytes

positive T cells (TemRA; CD45RO$^-$CCR7$^-$) within CD4$^+$ and CD8$^+$ lineages.[16, 21, 22] Within Treg cells, the differentiation in naive (CD45RA$^+$) and memory (CD45RA$^-$) was determined.[16] Finally, the following Th cell subsets (CD4$^+$CD45RA$^-$) were determined after exclusion of Treg cells on the basis of chemokine receptor profiles as defined previously:[16, 23-27] Th1 (CCR6$^-$CXCR3$^+$CCR4$^-$), Th2 (CCR6$^-$CXCR3$^-$CCR4$^+$), Th17 (CCR6$^+$CXCR3$^-$CCR4$^+$CCR10$^-$), Th17.1(CCR6$^+$CXCR3$^+$CCR4$^-$) and Th22 (CCR6$^+$CXCR3$^-$CCR4$^+$CCR10$^+$). In addition CD27$^+$ and CD27$^-$ IgG$^+$, IgA$^+$, IgE$^+$ CD19$^+$CD38^{dim}IgD$^-$ memory B cell subsets were defined.[16] Gating strategies for Th cell determination are presented in Figure S3.

Statistical analysis

First, characteristics of the study population were determined, stratified for *FLG* mutation status and AD diagnosis. *P*-values for determining differences between the categorical variables of both groups were calculated with chi-squared tests. Next, median cell numbers with interquartile range (IQR) were determined. Differences in cell numbers between

children with and without *FLG* mutations were assessed with the non-parametric Mann-Whitney U test. Subgroup analyses on the associations of *FLG* genotype with immune cell numbers were performed within non-AD and AD children. The possibility of effect modification by AD diagnosis was tested by performing linear regression analysis between immune cell numbers and *FLG* mutation status with the addition of an interaction term between *FLG* mutation status and AD diagnosis. To assure a normal distribution of the outcome in the linear regression analysis, a natural-log transformation for the immune cell outcomes was used. No adjustment for multiple testing was performed because of strong correlation between the immune cells studied. Statistical analyses were performed with SPSS version 21.0 (IBM Corp.) and R version 3.6.1 (R Foundation for Statistical Computing).

RESULTS

Study population

Characteristics of the study population are presented in Table 1. Within the total group of 523 children with European ancestry, *FLG* mutations were detected in 44 (8.4%) children, including 3 biallelic mutations (2 compound heterozygous and 1 homozygous). The proportion of patients with AD was lower in the wild-type group compared to the group with *FLG* mutations (20% versus 42%; $p<0.01$). Within the non-AD population, 6.1% of the children had a *FLG* mutation, including one compound heterozygous. Within the AD population, 15.7% of the children had a *FLG* mutation, including one homozygous and one compound heterozygous.

Higher Th22 cell counts in children of the general population with *FLG* mutations

Children of the general population with a *FLG* mutation had higher Th22 cell numbers compared to children of the wild-type population (Figure 2A, Table S1). The median cell number within the *FLG* mutation group was 5.60/µL (IQR 4.04;8.94) and 4.5/µL (IQR 2.5;7.4, $p=0.03$) within the wild-type group. To determine if this association between Th22 and *FLG* was different between children with and without AD, we performed a linear regression analysis with the following interaction term: *FLG* mutation status*AD. This interaction term was non-significant ($p=0.13$) and therefore effect modification by AD in this association is not likely. However, when the analyses were stratified, the association between *FLG* and Th22 was slightly stronger in the non-AD group with a median cell number of 6.8/µL (IQR 4.9;11.4) in non-AD children with *FLG* mutations compared to 4.5/µL (IQR 2.4;7.5, $p=0.006$) in non-AD children without *FLG* mutations (Table S2). In contrast, when studying the differences in absolute Th22 cell numbers between children with and without *FLG* mutations in the AD population, no significant differences were observed (median 5.0/µL (IQR 3.6;6.4) and 4.6/µL (IQR 2.9;7.4) respectively ($p=0.64$, Figure S1a)).

Table 1. Details of the study population

	Total population					Subgroup analyses	
Child characteristics	**Total (n=523)**	**Wild-type population (n=479)**	***FLG* mutation population (n=44)**	**P-value**	**Missing, n (%)**	**Non-atopic dermatitis (n=358)**	**Atopic dermatitis (n=102)**
Sex, n (%)							
Female	280 (53.5)	256 (53.4)	24 (54.5)	1.0	0.0	192 (53.6)	48 (47.1)
Male	243 (46.5)	223 (46.6)	20 (45.5)			166 (46.4)	54 (52.9)
***FLG* mutations, n (%)**							
Wild type	479 (91.6)	479 (100.0)	-	-	0.0	336 (93.9)	86 (84.3)
1 or more mutations	44 (8.4)[a]	-	44 (100.0)			22 (6.1)[b]	16 (15.7)[c]
Type *FLG* mutations, n (%)							
S1085CfsX36 (rs41370446)	22 (4.2)	-	22 (4.2)	-	0.0	12 (3.4)	7 (6.9)
R2447X (rs138726443)	6 (1.1)	-	6 (1.1)	-	0.2	4 (1.1)	1 (1.0)
R501X (rs61816761)	18 (3.5)	-	18 (3.5)	-	0.4	7 (2.0)	9 (9.0)[d]
S3247X (rs150597413)	0 (0.0)	-	0 (0.0)	-	0.4	0 (0.0)	0 (0.0)
Ever physician diagnosed AD, n (%)[e]	102 (22.2)	86 (20.4)	16 (42.1)	**0.004**	12.0	0 (0.0)	102 (100.0)

Child characteristics for the study population stratified for *FLG* mutation status. Values are based on the non-imputed dataset and represented as number (%). Chi-squared tests were conducted to examine possible differences in baseline characteristics between the different genotypes. *Abbreviations*: AD, atopic dermatitis; *FLG*, filaggrin gene; N, number; [a]Including 3 biallelic mutations (2 compound heterozygous and 1 homozygous). [b]1 compound heterozygous biallelic mutation. [c]1 compound heterozygous and 1 homozygous mutation.. [d]Including one biallelic mutations (homozygous). [e]Based on parental-reported questionnaires obtained at the child's age of 10 years: 'Was your child ever diagnosed by a physician with atopic dermatitis' (no or yes)

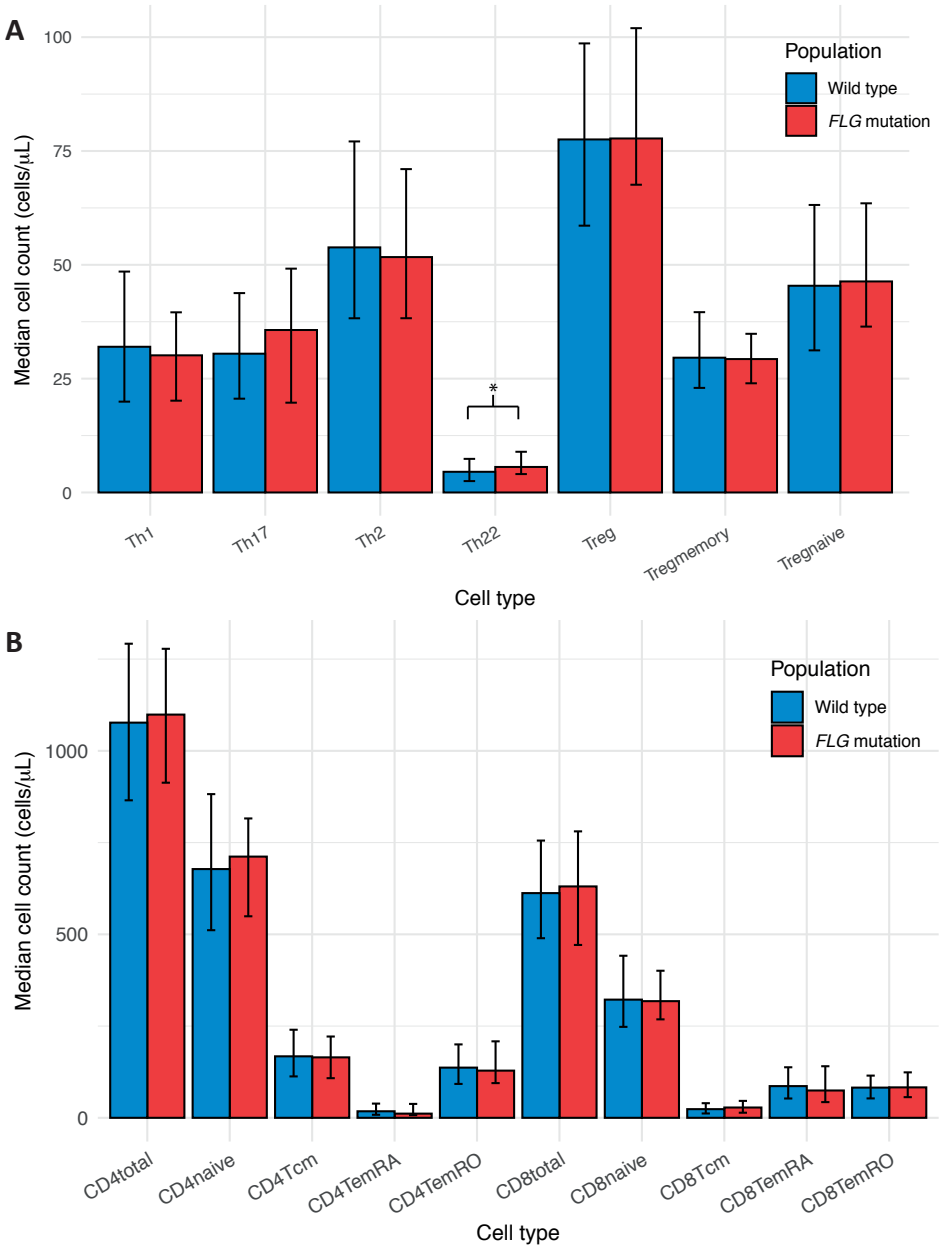

Figure 2. Absolute numbers of blood T cell subsets stratified by *FLG* mutation status
(A)The median (IQR) cell count per µLblood for Th and Treg cell numbers stratified for *FLG* mutation status. (B) The median (IQR) cell count per µL blood for $CD4^+$ and $CD8^+$ effector memory T cell numbers stratified for *FLG* mutation status. *Abbreviations*: IQR, interquartile range; Tcm, central memory T lymphocytes; TemRA, effector memory RA-positive T lymphocytes; TemRO, effector memory RO-positive T lymphocytes; Th, helper T cell; Treg, regulatory T cell. * denotes a two-sided P-value <0.05. Table S1 and Table S3 show absolute numbers and p-values.

No associations between Th1, Th2, Th17 and Treg and *FLG* mutations

No differences in median cell numbers between *FLG* mutation and wild-type group were observed for Th1, Th2, Th17 and Treg (Figure 2a, Table S1). This was similar in the subgroup analyses that were stratified for AD diagnosis (Figure S1a, Table S2). No differences in median cell numbers between the *FLG* mutation and the wild-type group were observed for the effector memory $CD4^+$ and $CD8^+$ T cell subsets: naive, Tcm, TemRA, TemRO (Figure 2b, Figure S1b).

No associations between memory B cells and *FLG* mutations

No differences in median cell numbers between the *FLG* mutation and the wild-type group were observed for total B cells and naive mature B cells (Table S1). In addition, no associations between *FLG* mutations and the following $CD27^+$ and $CD27^-$ memory B cell subsets were observed: IgA^+, IgE^+, IgG^+, IgM^+ (Figure 3). Similarly, no changes were observed in the subgroup analyses that stratified the analyses for AD diagnosis (Figure S2, Table S2).

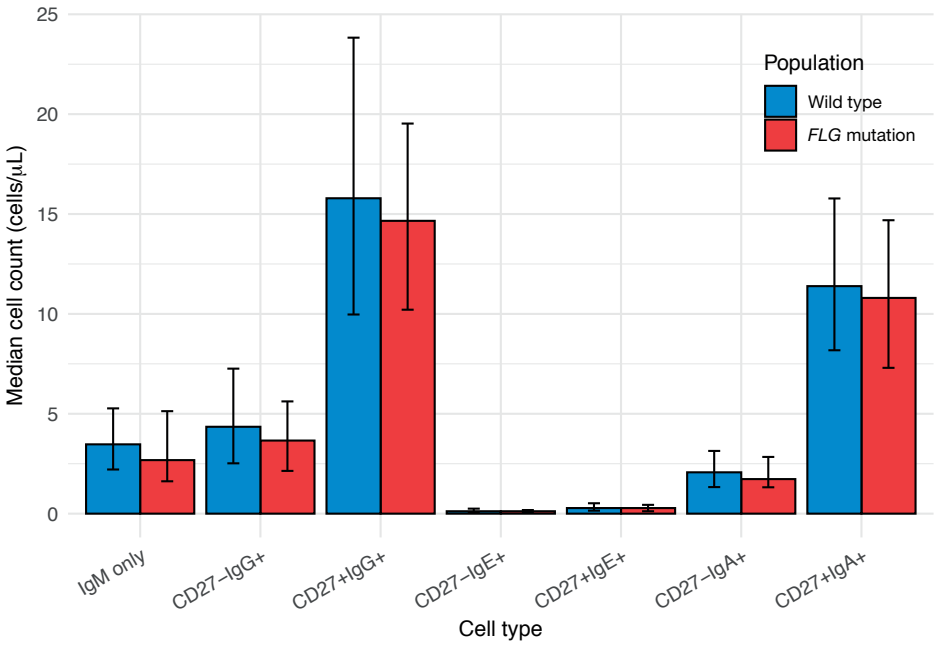

Figure 3. Absolute numbers of blood memory B cell subsets stratified by *FLG* mutation status
The median (IQR) cell count per µL blood for B memory cell numbers stratified for *FLG* mutation status are shown. *Abbreviations*: IQR, interquartile range; Ig, Immunoglobulin. Table S2 shows absolute numbers and p-values.

DISCUSSION

In this population-based study among children of European genetic ancestry, we observed a prevalence of 8.4% for *FLG* mutations. In addition, we demonstrated that children with *FLG* mutations had higher Th22 cell numbers than children without *FLG* mutations. In contrast, the Th1, Th2, Th17, Treg and memory B cell numbers were comparable between children with and without FLG mutations. In addition, among children with AD, those with or without *FLG* mutations had no differences in B- or T-cell subsets.

Comparison with literature and interpretation

All previous studies on *FLG* mutations and immune cell numbers have been performed within mice models or smaller numbers of AD patients.[10-12, 28] This is the first study that provides insight into the role of *FLG* mutations on immune cell numbers in school-aged children of a general population. The setting of this study within a population-based pediatric cohort study is therefore unique to study the association between *FLG* and immune cell numbers.

We observed higher Th22 cell numbers in children with *FLG* mutations in the general population, with a slightly higher median Th22 cell number in non-AD children with a mutation in *FLG*. No previous studies that assess the association between *FLG* mutations and Th22 have been performed within a general population. Interestingly, in contrast to our findings, several studies have observed increased Th22 cell numbers in the skin and circulation of patients with AD.[29, 30] Within AD patients, the role of Th22 is still not fully elucidated. Current literature suggests both protective and pro-inflammatory roles for Th22 by the production of IL-22.[31] IL-22 contributes to skin integrity and is known for its role in the defense against different pathogens in the skin by the production of antimicrobial proteins.[32, 33] However, the combined secretion of IL- 22 and TNF- α is thought to have an pro-inflammatory effect as observed in AD.[31] Possible explanations for the higher number of Th22 cell numbers in children without AD but with *FLG* mutations could be the following. First, the increase in Th22 could represent some level of inflammation due to *FLG* mutations without apparent clinical symptoms. Second, hypothetically, the increase in Th22 could contribute to skin homeostasis in children without AD to prevent further inflammatory processes leading to AD. However, further studies are needed to elaborate on the Th22 cell function in children with and without AD.

In contrast to previous studies investigating the effect of the *FLG* mutation status, we did not observe differences in Th2, Th17 and Treg cell numbers between children with and without *FLG* mutations, both in the total study population and in the subgroup of patients with AD.[10-12, 28] The discrepancies between previous studies and this study could

be explained by differences in investigated populations and species. Previous mice studies and skin equivalents studied the effect of complete absence of filaggrin, compared to the filaggrin haploinsufficiency in our study population which leads to 50% reduction in filaggrin expression.[2, 10, 28] In addition, these studies could represent a different immunological setting than is present in human skin. [28] It is also expected that previous results on immune cell numbers in AD populations are affected by disease severity. Namely, the presence of different immune cells is dependent on disease state, including disease flare and chronic AD.[29, 30] In turn, immune cells in active AD skin can induce downregulation of filaggrin protein expression in the skin independent of *FLG* mutations, subsequently affecting immune cell composition.[20] Although we do not have information on disease severity in our AD population, this study included a population-based, relatively healthy cohort in which we expect most children to have mild AD. Therefore, we suspect that alterations in immune cell numbers is probably not only dependent on *FLG* mutation genotype, but also on AD severity and epigenetic and environmental factors.

In addition, we did not observe differences in memory B cell numbers between children with and without *FLG* mutations. This is in line with our previous study in which we did not observe any association between B cells and AD.[16] No previous studies have investigated B cell subsets in relation to *FLG* mutations.

Finally, within our study, we studied the associations between *FLG* mutations and the adaptive immunity. It can be speculated that *FLG* mutations cause alterations in the innate immunity such as eosinophilic granulocytes and ILC2 cell numbers. Future studies are needed to determine if *FLG* mutations are associated with altered innate immunity cell numbers.

Methodological considerations

A major strength is that this study investigated the association between *FLG* genotype and a large panel of T- and B-cells in the general population for the first time. We had detailed and extensive information on immune cell numbers from 11-color flow cytometry and obtained objective information on genetic ancestry. However, the following four limitations need to be addressed. First, we used chemokine receptor profiles which are surrogate markers of Th cells. Due to the large scale of our study and the need to process fresh blood within 24 hours, in vitro activation and cytokine staining were not feasible. Importantly, multiple studies have shown that the use of surface chemokine receptors is a robust approach to define Th subsets with the corresponding cytokine profiles.[23, 24] Second, the AD population for the subgroup analyses was relatively small which could have limited the power in the statistical analyses. Nevertheless, in comparison to previous studies, only including a maximum of 6 AD patients with *FLG* mutations, this is the largest

study on *FLG* mutations in both the general population and in AD patients. Third, our AD population was defined by ever having physician diagnosed AD before or at the age of 10 years and no information on current disease activity was available. Therefore, it is likely that a part of the children has outgrown AD at the age of 10 and this might have affected their immunophenotype. Fourth, as mentioned previously, our study included the four most common *FLG* mutations in the European population. To prevent misclassification, we selected children with genetic European ancestry for the current study. Although the choice for including the most common *FLG* mutations in European populations is in line with previous studies,[11, 12] other less frequent *FLG* mutations could exist in low numbers since up to 113 *FLG* mutations resulting in premature protein termination have been described.[34] A recent study including patients with AD and Ichthyosis Vulgaris (IV), showed that screening the entire encoding region of *FLG* for mutations led to an improvement of the diagnostic yield.[34] As this is the first study in a general cohort addressing the association between *FLG* mutation and immune cell numbers, future studies are needed to validate our results.

In conclusion, school-aged children of a general population with *FLG* mutations have higher Th22 cell numbers, which might reflect the skin barrier dysfunction that is caused by decreased filaggrin expression in the epidermis. In our study population, *FLG* mutations do not otherwise affect the composition of the adaptive immune cells in a general pediatric population, nor in the children with AD.

REFERENCES

1. Sandilands A, Sutherland C, Irvine AD, McLean WH. Filaggrin in the frontline: role in skin barrier function and disease. J Cell Sci. 2009;122(Pt 9):1285-94.
2. Irvine AD, McLean WH, Leung DY. Filaggrin mutations associated with skin and allergic diseases. N Engl J Med. 2011;365(14):1315-27.
3. Thyssen JP, Elias PM. It Remains Unknown Whether Filaggrin Gene Mutations Evolved to Increase Cutaneous Synthesis of Vitamin D. Genome Biol Evol. 2017;9(4):900-1.
4. Goleva E, Berdyshev E, Leung DY. Epithelial barrier repair and prevention of allergy. J Clin Invest. 2019;129(4):1463-74.
5. McAleer MA, Irvine AD. The multifunctional role of filaggrin in allergic skin disease. J Allergy Clin Immunol. 2013;131(2):280-91.
6. Rodriguez E, Baurecht H, Herberich E, Wagenpfeil S, Brown SJ, Cordell HJ, et al. Meta-analysis of filaggrin polymorphisms in eczema and asthma: robust risk factors in atopic disease. J Allergy Clin Immunol. 2009;123(6):1361-70 e7.
7. Brown SJ, Asai Y, Cordell HJ, Campbell LE, Zhao Y, Liao H, et al. Loss-of-function variants in the filaggrin gene are a significant risk factor for peanut allergy. J Allergy Clin Immunol. 2011;127(3):661-7.
8. Rice NE, Patel BD, Lang IA, Kumari M, Frayling TM, Murray A, et al. Filaggrin gene mutations are associated with asthma and eczema in later life. J Allergy Clin Immunol. 2008;122(4):834-6.
9. Chan A, Terry W, Zhang H, Karmaus W, Ewart S, Holloway JW, et al. Filaggrin mutations increase allergic airway disease in childhood and adolescence through interactions with eczema and aeroallergen sensitization. Clin Exp Allergy. 2018;48(2):147-55.
10. Jee MH, Johansen JD, Buus TB, Petersen TH, Gadsboll AO, Woetmann A, et al. Increased Production of IL-17A-Producing gammadelta T Cells in the Thymus of Filaggrin-Deficient Mice. Front Immunol. 2018;9:988.
11. Bonefeld CM, Petersen TH, Bandier J, Agerbeck C, Linneberg A, Ross-Hansen K, et al. Epidermal filaggrin deficiency mediates increased systemic T-helper 17 immune response. Br J Dermatol. 2016;175(4):706-12.
12. Moosbrugger-Martinz V, Gruber R, Ladstatter K, Bellutti M, Blunder S, Schmuth M, et al. Filaggrin null mutations are associated with altered circulating Tregs in atopic dermatitis. J Cell Mol Med. 2019;23(2):1288-99.
13. 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. 2017;140(1):134-44 e9.
14. Czarnowicki T, Gonzalez J, Bonifacio KM, Shemer A, Xiangyu P, Kunjravia N, et al. Diverse activation and differentiation of multiple B-cell subsets in patients with atopic dermatitis but not in patients with psoriasis. J Allergy Clin Immunol. 2016;137(1):118-29 e5.
15. Heeringa JJ, Hajdarbegovic E, Thio HB, van Zelm MC. Systemic B-cell abnormalities in patients with atopic dermatitis? J Allergy Clin Immunol. 2016;138(1):317-8.
16. Looman KIM, van Meel ER, Grosserichter-Wagener C, Vissers FJM, Klingenberg JH, de Jong NW, et al. Associations of Th2, Th17, Treg cells, and IgA(+) memory B cells with atopic disease in children: The Generation R Study. Allergy. 2020;75(1):178-87.
17. Kooijman MN, Kruithof CJ, van Duijn CM, Duijts L, Franco OH, van IMH, et al. The Generation R Study: design and cohort update 2017. Eur J Epidemiol. 2016;31(12):1243-64.

18. Medina-Gomez C, Felix JF, Estrada K, Peters MJ, Herrera L, Kruithof CJ, et al. Challenges in conducting genome-wide association studies in highly admixed multi-ethnic populations: the Generation R Study. Eur J Epidemiol. 2015;30(4):317-30.
19. Elbert NJ, Duijts L, den Dekker HT, Jaddoe VW, Sonnenschein-van der Voort AM, de Jongste JC, et al. Role of environmental exposures and filaggrin mutations on associations of ethnic origin with risk of childhood eczema. The Generation R Study. Pediatr Allergy Immunol. 2016;27(6):627-35.
20. Kezic S, O'Regan GM, Yau N, Sandilands A, Chen H, Campbell LE, et al. Levels of filaggrin degradation products are influenced by both filaggrin genotype and atopic dermatitis severity. Allergy. 2011;66(7):934-40.
21. Appay V, van Lier RA, Sallusto F, Roederer M. Phenotype and function of human T lymphocyte subsets: consensus and issues. Cytometry A. 2008;73(11):975-83.
22. Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature. 1999;401(6754):708-12.
23. Paulissen SM, van Hamburg JP, Dankers W, Lubberts E. The role and modulation of CCR6+ Th17 cell populations in rheumatoid arthritis. Cytokine. 2015;74(1):43-53.
24. Pandya JM, Lundell AC, Hallström M, Andersson K, Nordström I, Rudin A. Circulating T helper and T regulatory subsets in untreated early rheumatoid arthritis and healthy control subjects. J Leukoc Biol. 2016;100(4):823-33.
25. Heeringa JJ, Karim AF, van Laar JAM, Verdijk RM, Paridaens D, van Hagen PM, et al. Expansion of blood IgG(4)(+) B, T(H)2, and regulatory T cells in patients with IgG(4)-related disease. J Allergy Clin Immunol. 2018;141(5):1831-43 e10.
26. Grosserichter-Wagener C, Radjabzadeh D, van der Weide H, Smit KN, Kraaij R, Hays JP, et al. Differences in Systemic IgA Reactivity and Circulating Th Subsets in Healthy Volunteers With Specific Microbiota Enterotypes. Front Immunol. 2019;10:341.
27. Edwards ESJ, Bosco JJ, Aui PM, Stirling RG, Cameron PU, Chatelier J, et al. Predominantly Antibody-Deficient Patients With Non-infectious Complications Have Reduced Naive B, Treg, Th17, and Tfh17 Cells. Front Immunol. 2019;10:2593.
28. Wallmeyer L, Dietert K, Sochorova M, Gruber AD, Kleuser B, Vavrova K, et al. TSLP is a direct trigger for T cell migration in filaggrin-deficient skin equivalents. Sci Rep. 2017;7(1):774.
29. Czarnowicki T, He H, Krueger JG, Guttman-Yassky E. Atopic dermatitis endotypes and implications for targeted therapeutics. J Allergy Clin Immunol. 2019;143(1):1-11.
30. 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(5):1347-58.
31. Cavani A, Pennino D, Eyerich K. Th17 and Th22 in skin allergy. Chem Immunol Allergy. 2012;96:39-44.
32. Eyerich K, Eyerich S. Th22 cells in allergic disease. Allergo J Int. 2015;24(1):1-7.
33. Fujita H. The role of IL-22 and Th22 cells in human skin diseases. J Dermatol Sci. 2013;72(1):3-8.
34. van Leersum FS, Nagtzaam IF, van Oosterhoud CN, Ghesquiere SAI, Brandts R, Gostynski A, et al. Improving the diagnostic yield for filaggrin; concealed mutations in the Dutch population. J Allergy Clin Immunol. 2020;145(6):1704-6 e2.

SUPPLEMENTARY MATERIAL

Table S1. The median (IQR) cell counts per µL blood for T- and B-cells subsets stratified by *FLG* mutation status

Total study population (n=523)			
	Wild-type population (n=479)	***FLG* mutation carriers (n=44)**	**P-value[a]**
Th1	32 (20.0-48.5)	30.1 (20.2-39.6)	0.31
Th2	53.8 (38.3-77.1)	51.7 (38.3-71.0)	0.99
Th17	30.5 (20.6-43.8)	35.7 (19.7-49.2)	0.13
Th22	4.5 (2.5-7.4)	5.6 (4.04-8.9)	**0.03**
Treg	77.5 (58.6-98.6)	77.8 (67.6-102.0)	0.48
Treg naive	45.4 (31.2-63.2)	46.4 (36.4-63.5)	0.25
Treg memory	29.6 (23.0-39.6)	29.3 (24.0-34.9)	0.74
Total B cells	478.5 (370.0-632.0)	493.0 (389.5-575.8)	0.69
B naive	303.1 (228.9-416.6)	323.2 (245.6-366.9)	0.90
IgM only	3.5 (2.2-5.27)	2.7 (1.6-5.1)	0.06
CD27-IgG+	4.4 (2.5-7.3)	3.7 (2.1-5.6)	0.14
CD27+IgG+	15.8 (10.0-23.8)	14.7 (10.2-19.5)	0.29
CD27-IgE+	0.1 (0.1-0.3)	0.1 (0.1-0.2)	0.69
CD27+IgE+	0.3 (0.2-0.5)	0.3 (0.1-0.4)	0.33
CD27-IgA+	2.1 (1.3-3.1)	1.7 (1.3-2.8)	0.26
CD27+IgA+	11.4 (8.2-15.8)	10.8 (7.3-14.7)	0.30
$CD4^+$ total	1076.8 (865.2-1291.9)	1098.8 (913.2-1278.1)	0.76
$CD4^+$ naive	677.8 (511.3-882.0)	711.8 (549.2-815.8)	0.49
$CD4^+$ Tcm	167.5 (112.8-240.0)	164.8 (108.1-221.5)	0.41
$CD4^+$ TemRA	17.8 (8.3-38.8)	11.4 (7.3-37.9)	0.39
$CD4^+$ TemRO	136.8 (92.4-200.0)	128.6 (94.6-208.4)	0.75
$CD8^+$ total	612.3 (489.4-755.5)	630.5 (471.1-780.6)	0.96
$CD8^+$ naive	322.1 (247.9-441.6)	317.9 (268.3-400.8)	0.94
$CD8^+$ Tcm	23.6 (11.8-39.9)	28.1 (14.0-46.2)	0.37
$CD8^+$ TemRA	86.4 (52.7-137.8)	74.5 (42.8-140.6)	0.45
$CD8^+$ TemRO	82.3 (52.9-115.0)	83.0 (56.3-123.9)	0.45

Abbreviations: Ig, immunoglobulin; IQR, interquartile range; Tcm, central memory T lymphocytes; TemRA, effector memory RA-positive T lymphocytes; TemRO, effector memory RO-positive T lymphocytes; Th, helper T cell; Treg, regulatory T cell. [a] Two-sided P-value determined by Mann-Withney U test.

Table S2. The median (IQR) cell counts per µL blood for T- and B-cells subsets stratified by *FLG* mutation status divided into the non-atopic dermatitis and atopic dermatitis subgroup

	Non atopic dermatitis population (n=358)			Atopic dermatitis (n=102)		
	Wild-type population (n=393)	***FLG* mutation carriers (n=28)**	**P-value[a]**	**Wild-type population (n=86)**	***FLG* mutation carriers (n=16)**	**P-value[a]**
Th1	31.3 (18.3-47.3)	31.6 (24.3-44.5)	0.79	30.9 (20.0-49.2)	30.7 (21.4-40.4)	0.43
Th2	54.0 (37.1-78.9)	44.5 (37.4-69.0)	0.42	54.7 (41.6-65.24)	53.4 (46.8-63.0)	0.76
Th17	30.4 (20.7-42.9)	34.6 (23.3-47.0)	0.24	31.9 (20.3-45.0)	34.8 (19.7-59.5)	0.51
Th22	4.50 (2.4-7.5)	6.8 (4.9-11.4)	**0.006**	4.6 (2.9-7.4)	5.0 (3.6-6.4)	0.64
Treg	76.0 (57.0; 95.8)	75.4 (66.3-107.0)	0.36	86.2 (65.6-104.2)	80.3 (74.3-89.5)	0.68
Treg naive	44.8 (31.2-61.7)	46.1 (36.2-68.0)	0.24	51.2 (32.5-66.8)	48.0 (43.3-57.3)	0.80
Treg memory	28.4 (21.9-37.5)	29.6 (26.1-33.7)	0.67	32.1 (24.5-42.0)	28.5 (23.2-37.5)	0.43
Total B cells	481.0 (375.5-628.5)	505.0 (407.3-590.8)	0.75	463.0 (360.0-604.3)	446.5 (332.5-545.0)	0.51
B naive	300.9 (231.1-416.9)	333.5 (255.3-403.2)	0.55	306.4 (212.4-407.4)	280.9 (198.0-363.3)	0.50
IgM only	3.4 (2.1-5.3)	2.5 (2.0-4.8)	0.24	3.4 (2.3-5.1)	3.0 (1.9-5.2)	0.52
CD27-IgG+	4.4 (2.5-7.4)	3.8(2.3-5.8)	0.49	3.4 (2.3-5.5)	3.3 (2.3-5.5)	0.93
CD27+IgG+	15.7 (9.9-23.6)	15.3 (11.5-21.6)	0.94	14.9 (10.3-20.0)	15.1 (10.5-18.9)	0.78
CD27-IgE+	0.1 (0.1-0.3)	0.1 (0.1-0.2)	0.64	0.1 (0.1-0.2)	0.1 (0.1-0.2)	0.63
CD27+IgE+	0.3 (0.2-0.5)	0.3 (0.1-0.5)	0.63	0.3 (0.1-0.4)	0.3 (0.2-0.4)	0.94
CD27-IgA+	2.1 (1.4-3.1)	1.8 (1.2-2.8)	0.28	1.9 (1.9-2.8)	1.6 (1.4-3.5)	0.95
CD27+IgA+	2.1 (1.4-3.1)	1.8 (1.2-2.8)	0.81	11.3 (8.4-15.1)	11.1 (7.8-14.9)	0.76
$CD4^+$ total	1073.3 (866.1-1288.3)	1098.9 (901.8-1309.2)	0.49	1117.5 (938.1-1271.8)	1096.6 (935.8-1213.3)	0.82
$CD4^+$ naive	667.0 (512.1-864.9)	773.5 (587.8-832.7)	0.25	708.9 (540.8-850.2)	711.8 (583.3-815.8)	0.92
$CD4^+$ Tcm	166.6 (111.0-237.0)	158.1 (107.1-218.5)	0.65	169.4 (109.0-258.0)	164.8 (120.1-223.3)	0.85
$CD4^+$ TemRA	16.8 (8.7-36.5)	11.4 (8.4-37.4)	0.54	22.4 (7.4-44.9)	12.4 (5.3-39.2)	0.54
$CD4^+$ TemRO	136.8 (89.1-199.2)	149.7 (91.2-216.1)	0.47	132.5 (95.7-212.8)	118.1 (94.5-207.7)	0.78
$CD8^+$ total	597.2 (507.3-747.3)	681.9 (587.5; 824.6)	0.19	604.5 (457.5-785.1)	543.8 (463.9-644.4)	0.53
$CD8^+$ naive	321.1 (246.1-431.6)	300.6 (267.9-369.6)	0.93	310.7 (248.4-452.6)	356.9 (277.1-411.8)	0.72
$CD8^+$ Tcm	24.2 (11.9-40.3)	26.3 (14.2-44.6)	0.79	22.5 (10.0-38.2)	26.6 (14.2-47.1)	0.38
$CD8^+$ TemRA	84.4 (50.2-136.1)	79.6 (60.9-135.2)	0.72	87.8 (47.1-139.2)	62.1 (42.4-123.4)	0.30
$CD8^+$ TemRO	81.1 (52.5-112.2)	96.1 (56.3-133.9)	0.25	85.0 (52.6-125.0)	74.9 (60.6-95.0)	0.69

Abbreviations: Ig, immunoglobulin; IQR, interquartile range; Tcm, central memory T lymphocytes; TemRA, effector memory RA-positive T lymphocytes; TemRO, effector memory RO-positive T lymphocytes; Th, helper T cell; Treg, regulatory T cell. [a] Two-sided P-value determined by Mann-Withney U test.

Table S3. Number of children included in the individual analyses

	Total population (n=523)	Non-atopic dermatitis (n=358)	Atopic dermatitis (n=102)
$CD4^+$ T cells	502	339	101
$CD4^+$ T naive	511	347	102
$CD4^+$ T_{CM}	511	347	102
$CD4^+$ T_{EMRO}	511	347	102
$CD4^+$ T_{EMRA}	511	347	102
Th1 cells	499	336	101
Th2 cells	498	335	101
Th17 cells	496	335	100
Th22 cells	495	334	100
Treg cells	493	334	98
Treg naïve	492	334	98
Treg memory	492	334	98
$CD8^+$ T cells	499	337	101
$CD8^+$ T naive	509	345	102
$CD8^+$ T_{CM}	509	345	102
$CD8^+$ T_{EMRO}	509	345	102
$CD8^+$ T_{EMRA}	509	345	102
Total B cells	522	357	102
B naive	512	347	102
IgM only	511	346	102
CD27-IgG+	511	346	102
CD27+IgG+	511	346	102
CD27-IgE+	511	346	102
CD27+IgE+	511	346	102
CD27-IgA+	511	346	102
CD27+IgA+	511	346	102

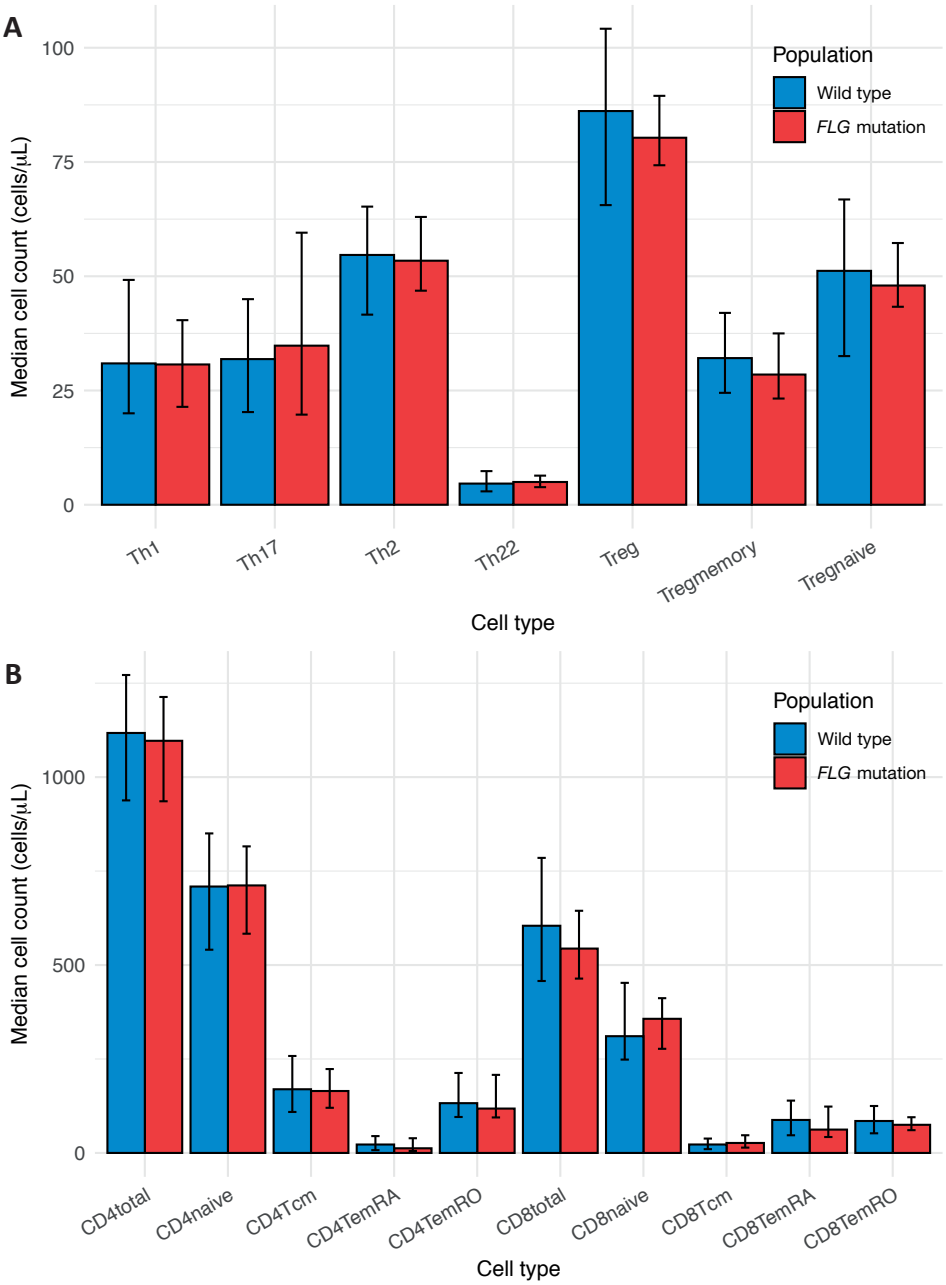

Figure S1. Absolute numbers of blood T cell subsets stratified by *FLG* mutation status in children with atopic dermatitis

(A-B) The median (IQR) Th, Treg, $CD4^+$ and $CD8^+$ Tem cell count per µL blood stratified for *FLG* mutation status. *Abbreviations*: Tcm, central memory T-lymphocytes; Tem, effector memory T-lymphocytes; Th, helper T-cell; Treg, regulatory T-cell.

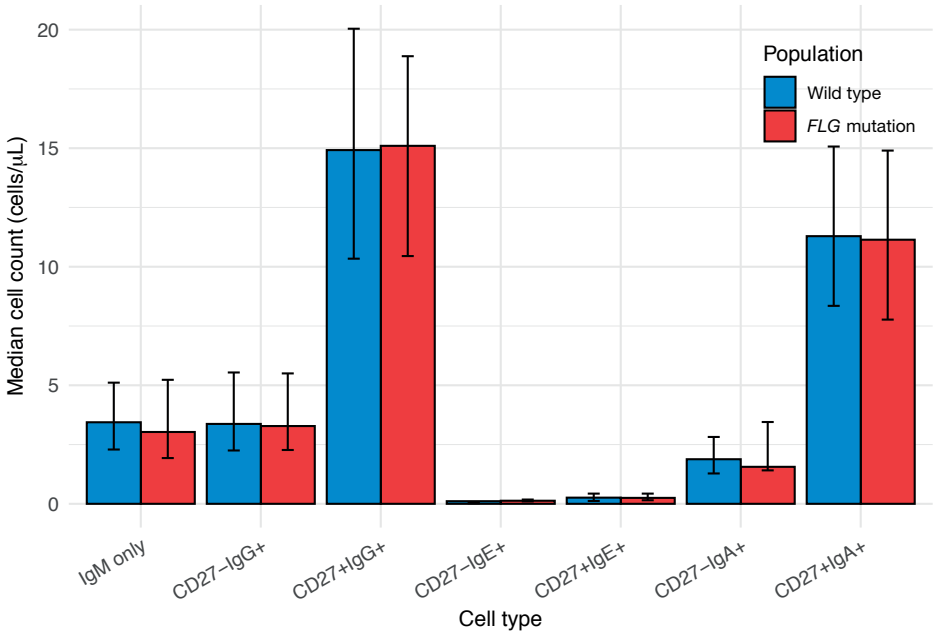

Figure S2. Absolute numbers of blood memory B cell subsets stratified by *FLG* mutation status in children with atopic dermatitis.

The median (IQR) cell count per µL blood for B memory cell numbers stratified for *FLG* mutation status. *Abbreviations*: IQR, interquartile range.

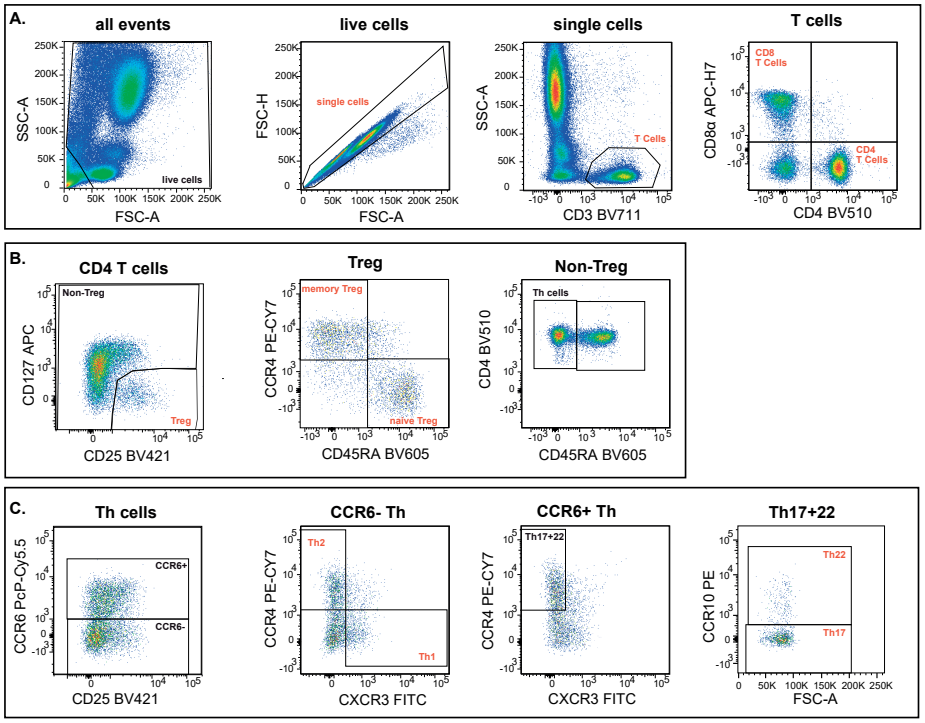

Figure S3. Gating strategy T helper subsets

Chapter 4

Natural moisturizing factor as a biomarker for filaggrin mutation status in a multi-ethnic pediatric atopic dermatitis cohort

M.M.F. van Mierlo
P.J. Caspers
M.S. Jansen
G.J. Puppels
A.E.M. Nouwen
M.B. Bronner
L.M. Pardo
M. van Geel
S.G.M.A. Pasmans

Submitted

ABSTRACT

Background

Mutations in the filaggrin gene (*FLG*) result in reduced levels of filaggrin-derived components of the natural moisturizing factor (NMF) and predispose a distinct atopic dermatitis (AD) phenotype. Our first aim was to validate a non-invasive NMF measurement as a proxy for *FLG* mutation status in an unselected multi-ethnic pediatric AD cohort. Second, we aimed to assess if the NMF content in the nonlesional skin of the thenar eminence is affected by acute disease severity.

Methods

The NMF content was measured in 780 AD children in the nonlesional stratum corneum (SC) of the thenar eminence using confocal Raman spectroscopy. The presence of *FLG* mutations was assessed in 101 children by screening the entire encoding region of *FLG* with single molecule molecular inversion probes (smMIPS) and barcoded next-generation sequencing (NGS). Acute disease severity was determined using the Eczema Area and Severity Index (EASI).

Results

Within the group of 101 children, a total of 12 different *FLG* mutations were identified, corresponding to 30 (30%) patients with ≥ 1 mutation in *FLG.* NMF content in the SC could accurately predict *FLG* mutation status (wild type vs. ≥ 1 mutation) with an area under the curve of 0.93 (95% CI 0.87-0.99). No correlation was found between EASI and NMF, thereby excluding the potential influence of acute disease severity on NMF values measured on the thenar eminence's nonlesional skin.

Conclusion

NMF measurement with Raman spectroscopy can be used as a non-invasive biomarker to stratify patients based on *FLG* mutation status in an unselected clinical AD cohort with diverse ethnical backgrounds.

INTRODUCTION

Atopic dermatitis (AD) is a common inflammatory skin disease among children with increasing prevalence in the past decades.[1] The symptoms of AD are highly heterogeneous and the etiology is complex. The strongest and most widely replicated genetic risk factor for AD is a null mutation in the filaggrin gene (*FLG*) located on chromosome 1q21. *FLG* encodes the protein filaggrin, which is involved in the formation and homeostasis of the skin barrier.[2]

Previous research showed that AD patients with a mutation in *FLG* have a different phenotype, characterized by early onset disease with persistence into adulthood, increased severity and increased risk of asthma and allergic sensitization.[2, 3] Additionally, it has been suggested that patients with a mutation in *FLG* respond differently to immunosuppressive treatment compared to wild-type patients.[4] This suggests that *FLG* mutation profiling could be used to stratify patients in terms of clinical course and to develop personalized treatment strategies. However, genotyping is time consuming, expensive and DNA collection poses ethical considerations, which hampers its use in daily practice.[5, 6] In addition, up to 113 different mutations in *FLG* have been described with prevalence varying widely based on ethnic background.[7, 8] Hence, most studies selectively screen for a small portion of mutations, mainly driven by detection difficulties and cost-effectiveness. This underlines the importance to investigate alternative biomarkers for *FLG* genotype.

Recent literature suggests that decreased concentrations of filaggrin-derived components of the natural moisturizing factor (NMF) are a proxy for the presence of *FLG* null mutations.[9, 10] During the terminal differentiation of keratinocytes, profilaggrin is dephosphorylated and enzymatically degraded into a highly hygroscopic mixture of amino acids and amino acid derivatives, including pyrrolidone carboxylic acid (PCA), histidine and its metabolite urocanic acid (UCA).[9, 11] The free amino acids and their derivatives constitute the majority of NMF in the stratum corneum (SC).[9] NMF has impact on moisturization, skin pH gradient and the cutaneous antimicrobial defense.[12] Raman spectroscopy can measure the NMF concentration in skin rapidly, non-invasively and provides an instant result, which could be used as a biomarker for the presence of *FLG*-null variants.[9, 10, 13] A previous study in a selected Irish pediatric population of AD patients showed that NMF could discriminate between wild-type patients and *FLG* mutation carriers with a sensitivity of 98.73% and a specificity of 86.89% using 1.07 arbitrary units (a.u.) as the cutoff value.[14] It has not been investigated whether this cutoff value could be applied to assess *FLG* mutation status in different clinical cohorts.

The first aim of our study was to screen the entire encoding region of *FLG* for potential mutations and validate if NMF could be used as a biomarker for the *FLG* genotype in an unselected multi-ethnic clinical cohort of children with mild to severe disease. Second, we wanted to ensure that the thenar eminence's nonlesional skin is a suitable location for NMF measurement by excluding a potential influence of the acute disease severity on the NMF content in this area. These results present a step forward in the clinical use of NMF content as an accurate proxy for a *FLG* mutation status to stratify patients for personalized treatment.

METHODS

Study design

This cross-sectional study was conducted at the tertiary referral center for Pediatric Dermatology in KinderHaven, an outpatient clinic and pediatric multidisciplinary allergology center of the Erasmus MC University Medical Center-Sophia Children's Hospital, Rotterdam, the Netherlands. This study is part of the clinical and translational studies in our center to understand the etiology of AD, aimed at improving the management and quality of life of patients with this condition. One of our future aims is to stratify patients based on NMF content to enable personalized treatment. Assessment of the NMF content by Raman spectroscopy is part of the clinical care in KinderHaven since March 2016. From June 2018, DNA specimens were obtained, in addition to the NMF measurement, in all patients that participated in this study. All patients and/or their parents (guardians), signed informed consent. Study procedures were approved by the medical ethical committee of the Erasmus MC University Medical Center (MEC-2017-370).

Participants

Children (0-18 years) diagnosed with AD according to the UK Working Party criteria consulting the dermatologist at KinderHaven were included in this study.[15] Acute AD severity was assessed using the Eczema Area and Severity Index (EASI) score on the same day as the research assistant took the NMF measurements. Ethnic origin was retrieved from questionnaires and was based on the parents' country of birth, in accordance with the Statistics Netherlands.[16] Children were classified as being of non-Dutch ethnic origin if at least one parent was born abroad. If both parents were born abroad, the mother's birth country was decisive.[17, 18]

FLG mutation analysis

FLG mutations were determined on DNA isolated from buccal swabs using Isohelix SK-1S swabs (manufacturer). Single molecule molecular inversion probes (smMIPS) and bar-

coded next-generation sequencing (NGS) was performed to screen the entire encoding region of *FLG* for all mutations resulting in premature protein termination as previously described.[7, 19] Since this publication showed that more than two mutations could be present in *FLG*, we could not exclude that patients with two different mutations, of which at least one is rare, were present on the same (in cis) or different alleles (in trans).[7] All patients with a mutation in *FLG* were referred to as patients with ≥1 mutation(s)(*FLG*$^-$). Wild-type patients were referred to as *FLG*$^+$.

Natural moisturizing factor measurement

The NMF content was measured in the SC of the thenar eminence (nonlesional skin) using confocal Raman spectroscopy (gen2- SCA Skin Composition Analyzer, RiverD International B.V.) as described previously.[20] In short, an average of 8 profiles from different locations on the thenar eminence were measured at a range of 20 µm – 40 µm below the skin surface in 5 µm depth intervals. The result for a patient was included as 'valid measurement' if at least 10 valid NMF values were measured in at least two profiles at different locations. These profiles resulted in an average NMF value (a.u.). Patients were asked to withhold topical therapies, including emollients, for 24 hours preceding the measurement. Furthermore, prior to the measurements, the skin surface was cleaned with a paper towel. The NMF values are determined from the Raman spectra by ordinary least squares fitting using a reference spectrum of NMF constructed from a superposition of the spectra of pyrrolidone-5-carboxylic acid, ornithine, serine, proline, glycine, histidine and alanine.[10]

Statistical analysis

Patient characteristics of *FLG*$^+$ and *FLG*$^-$ were presented as proportions, median (interquartile (IQR)) or mean (standard deviation (SD)) when appropriate. A Receiver Operating Characteristic (ROC) curve was constructed to measure the diagnostic ability of NMF to predict *FLG* mutation status (wild type vs. ≥ 1 mutation) by mapping the sensitivity versus 1-specificity for all possible values of the cutoff point. The optimal cutoff point was determined by maximizing the Youden function. A multivariate linear regression model was used to examine the association between EASI (as independent variable) and NMF content, corrected for *FLG* mutation status, age and sex.

The NMF content that was measured in a total of 780 children was used to give an indication of mutation carriers in a tertiary referral center. In addition, this was used to compare our cutoff value to a previously determined cutoff value in a selected pediatric AD cohort of Irish ancestry.[14]

RESULTS

Study population

A total of 837 children have been included in the KinderHaven cohort. From 780 patients a valid NMF measurement was available, from which 111 patients were screened for the presence of a *FLG* mutation. In 10 children parts of the encoding gene were not covered and were therefore excluded, leaving 101 patients with a valid NMF measurement and a *FLG* determination who were included for analysis. The median NMF value of these 101 patients was 1.20 a.u. (IQR 0.91-1.32). We identified 30 (30%) patients with ≥ 1 mutation in *FLG,* including 25 heterozygous mutation carriers and 5 patients with more than one mutation in *FLG* (including one patient with a homozygous mutation). Demographic data and clinical characteristics for FLG^+ and FLG^- patients are outlined in Table 1. As shown in this table, the ethnic origin from the included patients was highly diverse.

Table 1. Patient characteristics

	FLG^+ (n=71)	FLG^- (n=30)	p-value
Gender, n(%)			0.24[a]
Male	35 (49.3)	11 (36.7)	
Age, mean±SD (years)	8.6±5.3	8.2±4.4	0.55[b]
Ethnic origin[d]			0.17[a]
Cape Verdean	2 (2.8)	1 (3.3)	
Dutch	30 (42.3)	19 (63.3)	
Dutch Antillean	6 (8.4)	-	
Moroccan	6 (8.4)	-	
Surinamese-Creole	7 (9.9)	5 (16.7)	
Turkish	1 (1.4)	1 (3.3)	
Other non-Western	11 (15.5)	3 (10.0)	
Western	4 (5.6)	-	
Number of mutations, n(%)			
1	-	25 (83.3)	
2	-	5 (16.7)[e]	
NMF content a.u., median (IQR)	1.26 (IQR 1.18-1.37)	0.82 (IQR 0.56-0.92)	**<0.01**[c]
EASI score, median (IQR)[f,g]	6.1 (IQR 2.3-14.5)	7.8 (3.3-15.6)	0.22[c]

Abbreviations: a.u., arbitrary unit; EASI, Eczema Area and Severity Index; FLG^+, wild-type patients; FLG^-, patients with ≥1 mutation(s) in filaggrin gene; IQR, interquartile range; NMF, natural moisturizing factor; n, number; SD, standard deviation. [a]Chi-square test; [b]independent t-test; [c]Mann-Withney U test; [d]Information on ethnicity was missing for 4 (5.6%) patients in FLG^+ and 1 (3.3%) patient in FLG^- group; [e]including one homozygous mutation carrier; [f]EASI score was missing for 4 (5.6%) patients in FLG^+ group and 3 (10.0%) patient in FLG^- group; [g]The distribution of EASI score is shown in Table S1.

The association between NMF and *FLG* mutation status

A total of 12 different mutations were detected among *FLG* mutation carriers, of which five have not been reported before (Table 2). The median NMF content in FLG^{+} patients was 1.26 a.u. (IQR 1.18-1.37), compared to a median of 0.82 a.u. (IQR 0.56-0.94) in FLG^{-} patients (Figure 1, p<0.01). Furthermore, the NMF content in patients with a single mutation

Table 2. *FLG* loss of function mutations

ID	Ethnic origin	Mutation 1	Protein change	rpt	Mutation 2	Protein change	rpt
391	Unknown	c.6453del	p.(Ser2152Profs*7)	6			
615	Dutch	c.2282_2285del	p.(Ser761Cysfs*36)	1			
652	Dutch	c.3418C>T	p.(Arg1140*)	3			
662	Dutch	c.1501C>T	p.(Arg501*)	1	c.2282_2285del	p.(Ser761Cysfs*36)	1
664	Dutch	c.1501C>T	p.(Arg501*)	1			
665	Dutch	c.2282_2285del	p.(Ser761Cysfs*36)	1			
705	Dutch	c.1501C>T	p.(Arg501*)	1			
714	Other non-Western	c.2282_2285del	p.(Ser761Cysfs*36)	1	c.2976_2977del	p.(Arg992Serfs*31)	2
719	Surinamese-Creole	c.1501C>T	p.(Arg501*)	1			
723	Dutch	c.1501C>T	p.(Arg501*)	1			
729	Dutch	c.1501C>T	p.(Arg501*)	1			
733	Surinamese-Creole	c.6109C>T	p.(Arg2037*)	5			
736	Dutch	c.2282_2285del	p.(Ser761Cysfs*36)	1			
737	Dutch	c.1501C>T	p.(Arg501*)	1	c.1501C>T	p.(Arg501*)	1
745	Other non-Western	c.1217C>G	p.(Ser406*)	0			
749	Other non-Western	c.2282_2285del	p.(Ser761Cysfs*36)	1			
752	Dutch	c.2282_2285del	p.(Ser761Cysfs*36)	1			
755	Surinamese-Creole	c.6950_6957del	p.(Ser2317*)	6			
756	Turkish	c.1501C>T	p.(Arg501*)	1			
764	Dutch	c.2282_2285del	p.(Ser761Cysfs*36)	1			
766	Dutch	c.6950_6957del	p.(Ser2317*)	6	c.9894del	p.(Gly3299Glufs*92)	9
771	Dutch	c.2282_2285del	p.(Ser761Cysfs*36)	1			
775	Dutch	c.2282_2285del	p.(Ser761Cysfs*36)	1			
777	Surinamese-Creole	c.10354C>T	p.(Gln3452*)	10[1]			
780	Cape Verdean	c.1501C>T	p.(Arg501*)	1	c.2282_2285del	p.(Ser761Cysfs*36)	1
781	Dutch	c.1501C>T	p.(Arg501*)	1			
792	Dutch	c.2282_2285del	p.(Ser761Cysfs*36)	1			
799	Surinamese-Creole	c.8702C>G	p.(Ser2901*)	8[1]			
802	Dutch	c.1501C>T	p.(Arg501*)	1			
805	Dutch	c.2282_2285del	p.(Ser761Cysfs*36)	1			

Abbreviations: ID, study number; rpt, filaggrin gene repeat region. Underlined mutations have not been reported before.

in *FLG* was significantly different compared to both wild-type patients and patients with 2 mutations in *FLG* (p<0.01, Figure S1). ROC curves were constructed to test the diagnostic ability of NMF measured using Raman spectroscopy. The optimal cutoff value for NMF to distinguish between *FLG*$^{+}$ and *FLG*$^{-}$ patients was 1.03 a.u. with an area under the curve (AUC) of 0.93 (95% CI 0.87-0.99) (Figure 2). This resulted in a sensitivity of 97%, specificity of 87%, positive predictive value of 76%, and a negative predictive value of 98% (Table 3).

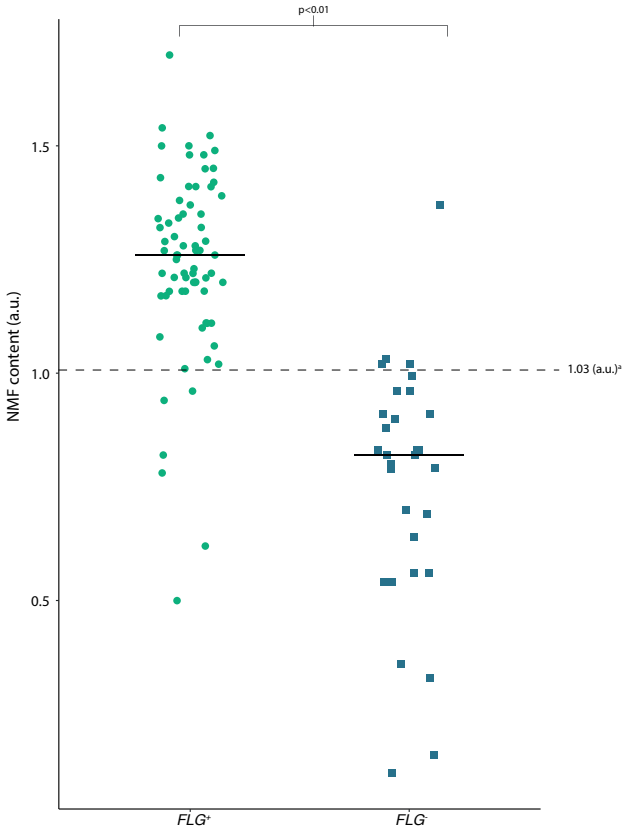

Figure 1. NMF content by *FLG* genotype
Wild-type patients (*FLG*$^{+}$) had median NMF content of 1.26 a.u. (IQR 1.18-1.37), compared to 0.82 a.u. (IQR 0.56-0.94) in *FLG*$^{-}$ (p<0.01, using a Mann-Whitney U test). [a]Cutoff value 1.03 (a.u.)

When this threshold was applied to the NMF measurements of the entire cohort (n=780), 215 (28%) patients would be classified as low NMF and 565 (72%) as normal NMF. Compared to the previous determined cutoff value by O'Reagan of 1.07 a.u., 10 (1.3%) patients would be classified differently, going from low NMF content to normal NMF content (Table S2).[14]

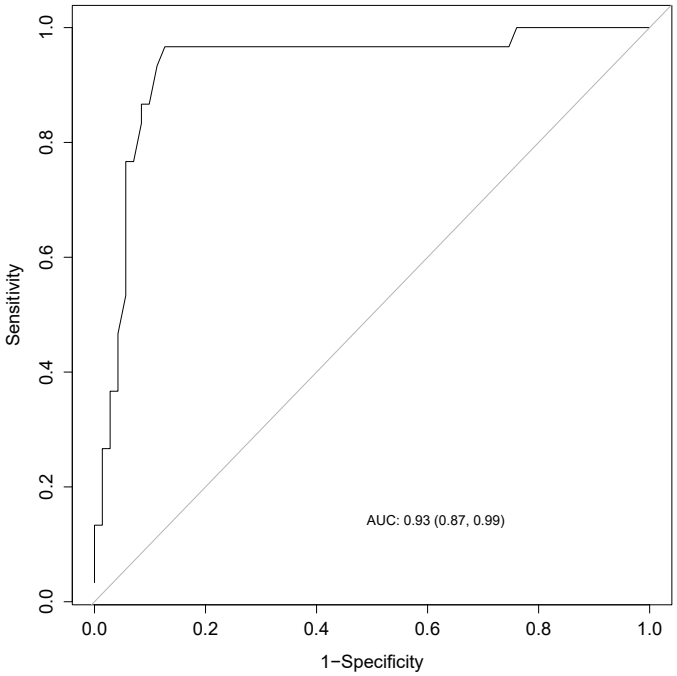

Figure 2. ROC curve determining the discriminatory power for NMF content per *FLG* genotype
The cutoff value of 1.03 a.u. was determined by maximizing the sum of sensitivity and specificity with an AUC of 0.93 (95% CI 0.87-0.99). This resulted in a sensitivity of 96.67, specificity of 87.32, positive predictive value of 76,32 and negative predictive value of 98.41.

Table 3. *FLG* genotype per NMF category based on the cutoff value (1.03 a.u.)

	***FLG*$^+$ (n=71)**	***FLG*$^-$ (n=30)**
Low NMF content a.u.[a] (n=38)	9	29
Normal NMF content a.u.[a] (n=63)	62	1

Abbreviations: a.u, arbitrary unit; *FLG*$^+$, wild-type patients; *FLG*$^-$, patients with ≥1 mutation(s) in filaggrin gene; NMF, natural moisturizing factor. [a]Based on the cutoff value of 1.03 a.u.

Correlation of NMF with acute disease severity

The patient group consisted mainly of patients with mild (39%) and moderate (34%) disease based on the EASI score (Table S1). The EASI score and NMF content are plotted in Figure 3 separated for *FLG*$^+$ and *FLG*$^-$. We did not find a significant association between the EASI score and NMF content measured at the thenar eminence's nonlesional skin (corrected for *FLG* mutation status, age and sex) (beta -0.555 (95% C.I. -0.005 – 0.003), p=0.58, Table S3).

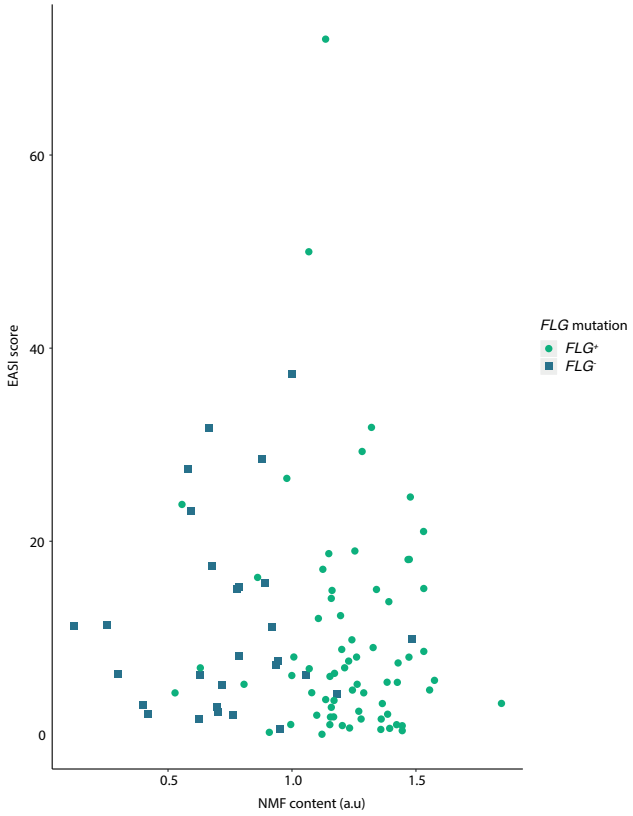

Figure 3. Correlation between NMF content and disease severity
There is no significant association between the EASI score and NMF content measured at the thenar eminence (beta -0.555 (95% C.I. -0.005 – 0.003), p=0.58)) (Table S3).

DISCUSSION

In our multi-ethnic population of children with mild to severe AD, 30% of the patients had a mutation in *FLG.* In this cohort, 12 different mutations were found. We showed that the NMF content in the SC of the thenar eminence could accurately discriminate between *FLG*$^{+}$ and *FLG*$^{-}$ patients with an AUC of 0.93 (95% CI 0.87-0.99). The optimal cutoff value of 1.03 a.u. was very close to the previously determined cutoff value of 1.07 a.u.[14] Applying the two cutoff values to the NMF values of the total KinderHaven cohort results in only 10 out of the 780 (1.3%) patients who were classified differently.

The use of the cutoff value of 1.03 a.u. resulted in high sensitivity and specificity (i.e., 97% and 87%).[14] Interestingly, 13% of the wild-type patients had low NMF content in the SC. Since we used the smMIP-NGS technique, this could not be attributed to a rare or new mutation in *FLG.*[7] These results underline the importance of other factors affecting the

NMF content in the SC that are not a direct result of *FLG* mutations. These factors could also justify interpersonal variation in NMF content. Previous research showed a positive correlation between intragenetic copy number variation (CNV) and the amount of filaggrin breakdown products.[21] In addition, there is an important role for proteases in this multi-stage breakdown process (ie., bleomycin hydrolase (BH) and Caspase-14) which can be less effective due to genetic variants influencing enzyme activity or the effect of external humidity on the proteolysis of filaggrin.[22-25] Last, the presence of other mutations in the epidermal differentiation complex (EDC) could account for low NMF values and should be investigated further. Since a previous study showed that patients with low NMF content in the SC had a more intensive treatment history (more potent topical corticosteroid and systemic treatment) compared to patients with normal NMF value,[26] we suggest that determination of NMF content as an independent biomarker for AD phenotype has added value to the determination of *FLG* mutation status alone.

Our results showed no correlation between EASI score and NMF value, making the non-lesional skin of the thenar eminence a suitable location to predict *FLG* mutation status without direct interference of acute disease severity. Previous studies have shown that filaggrin degradation products in the SC of the nonlesional skin of the forearm are affected by both a mutation in *FLG* and disease severity.[27, 28] The effect of disease severity can be attributed to the activated immune system in both lesional and nonlesional skin. Previous research, using a tape stripping technique, showed an upregulation of markers for AD severity, including T helper 2 (Th2)-skewed markers (interleukin (IL)-13, CCL17, CCL22, IL-5) in the nonlesional skin of the forearm, which was associated with a reduced NMF content.[29] The current results do not show a correlation between acute disease severity and NMF measurement on the thenar eminence. The hypothesis is that the NMF in the SC of the thenar eminence is less affected by an acute upregulation of the immune response because the SC on the thenar is much thicker and has a much slower turnover time as compared to the SC on the forearm. Determining both cytokines and NMF values in the SC of the thenar eminence and correlating this to disease severity could support our hypothesis.

Major strengths of this study are the application of the non-invasive NMF measurement in an unselected multi-ethnic patient cohort and the use of a novel technique to detect all mutations leading to a premature protein termination in *FLG*. Especially in this multi-ethnic population, screening of only the most common mutations is not sufficient and will lead to an underrepresentation of patients with *FLG* mutations. A limitation is that patients were instructed not to apply any topical therapies on the thenar eminence 24 hours before the measurement, but information on the use of these ointments during the rest of the week before the visit was missing. Previous research has suggested that topical corticosteroid

treatment could decrease the level of NMF in the SC of both mice and humans.[30] Because of the relative thickness of the SC on the thenar and since eczematous lesions on the thenar eminence, needing control by topical corticosteroids are uncommon, and in part of the children ointments are applied by their parents/guardians, we think this had a minor impact on our results.

Future research should focus on other factors, next to *FLG*-null variants, leading to a reduction in NMF. This knowledge will lead to a better understanding of the pathogenesis in AD since a large proportion of AD patients does not have a mutation in *FLG*. Furthermore, the use of NMF should be further evaluated as a predictor for treatment outcome to enable personalized treatment.

In conclusion, we validated that the NMF content in the SC of the thenar eminence can be used as a biomarker for *FLG* mutation status in an unselected clinical cohort of children with AD from different ethnical backgrounds. NMF measurement can be used in daily practice to identify patients with a high risk to develop a more severe phenotype and to stratify patients in future clinical studies to evaluate treatment response.

REFERENCES

1. Odhiambo JA, Williams HC, Clayton TO, Robertson CF, Asher MI, Group IPTS. Global variations in prevalence of eczema symptoms in children from ISAAC Phase Three. J Allergy Clin Immunol. 2009;124(6):1251-8 e23.
2. Irvine AD, McLean WH, Leung DY. Filaggrin mutations associated with skin and allergic diseases. N Engl J Med. 2011;365(14):1315-27.
3. Irvine AD. Fleshing out filaggrin phenotypes. J Invest Dermatol. 2007;127(3):504-7.
4. Roekevisch E, Leeflang MMG, Schram ME, Campbell LE, Irwin McLean WH, Kezic S, et al. Patients with atopic dermatitis with filaggrin loss-of-function mutations show good but lower responses to immunosuppressive treatment. Br J Dermatol. 2017;177(6):1745-6.
5. Irvine AD, McLean WH. Breaking the (un)sound barrier: filaggrin is a major gene for atopic dermatitis. J Invest Dermatol. 2006;126(6):1200-2.
6. Botkin JR. Ethical issues in pediatric genetic testing and screening. Curr Opin Pediatr. 2016;28(6):700-4.
7. van Leersum FS, Nagtzaam IF, van Oosterhoud CN, Ghesquiere SAI, Brandts R, Gostynski A, et al. Improving the diagnostic yield for filaggrin; concealed mutations in the Dutch population. J Allergy Clin Immunol. 2020;145(6):1704-6 e2.
8. Chen H, Common JE, Haines RL, Balakrishnan A, Brown SJ, Goh CS, et al. Wide spectrum of filaggrin-null mutations in atopic dermatitis highlights differences between Singaporean Chinese and European populations. Br J Dermatol. 2011;165(1):106-14.
9. Kezic S, Kammeyer A, Calkoen F, Fluhr JW, Bos JD. Natural moisturizing factor components in the stratum corneum as biomarkers of filaggrin genotype: evaluation of minimally invasive methods. Br J Dermatol. 2009;161(5):1098-104.
10. Kezic S, Kemperman PM, Koster ES, de Jongh CM, Thio HB, Campbell LE, et al. Loss-of-function mutations in the filaggrin gene lead to reduced level of natural moisturizing factor in the stratum corneum. J Invest Dermatol. 2008;128(8):2117-9.
11. Mlitz V, Latreille J, Gardinier S, Jdid R, Drouault Y, Hufnagl P, et al. Impact of filaggrin mutations on Raman spectra and biophysical properties of the stratum corneum in mild to moderate atopic dermatitis. J Eur Acad Dermatol Venereol. 2012;26(8):983-90.
12. Miajlovic H, Fallon PG, Irvine AD, Foster TJ. Effect of filaggrin breakdown products on growth of and protein expression by Staphylococcus aureus. J Allergy Clin Immunol. 2010;126(6):1184-90 e3.
13. Ní Chaoimh C, Nico C, Puppels GJ, Caspers PJ, Wong X, Common JE, et al. In vivo Raman spectroscopy discriminates between FLG loss-of-function carriers vs wild-type in day 1-4 neonates. Ann Allergy Asthma Immunol. 2020;124(5):500-4.
14. O'Regan GM, Kemperman PM, Sandilands A, Chen H, Campbell LE, Kroboth K, et al. Raman profiles of the stratum corneum define 3 filaggrin genotype-determined atopic dermatitis endophenotypes. J Allergy Clin Immunol. 2010;126(3):574-80 e1.
15. Williams HC, Burney PG, Hay RJ, Archer CB, Shipley MJ, Hunter JJ, et al. The U.K. Working Party's Diagnostic Criteria for Atopic Dermatitis. I. Derivation of a minimum set of discriminators for atopic dermatitis. Br J Dermatol. 1994;131(3):383-96.
16. Netherlands S. Annual report on integration 2016. The Hague/Heerlen: Statistics Netherlands. 2016.
17. Elbert NJ, Duijts L, den Dekker HT, Jaddoe VW, Sonnenschein-van der Voort AM, de Jongste JC, et al. Role of environmental exposures and filaggrin mutations on associations of ethnic origin with risk of childhood eczema. The Generation R Study. Pediatr Allergy Immunol. 2016;27(6):627-35.

18. Kooijman MN, Kruithof CJ, van Duijn CM, Duijts L, Franco OH, van IMH, et al. The Generation R Study: design and cohort update 2017. Eur J Epidemiol. 2016;31(12):1243-64.
19. Eijkelenboom A, Kamping EJ, Kastner-van Raaij AW, Hendriks-Cornelissen SJ, Neveling K, Kuiper RP, et al. Reliable Next-Generation Sequencing of Formalin-Fixed, Paraffin-Embedded Tissue Using Single Molecule Tags. J Mol Diagn. 2016;18(6):851-63.
20. Caspers PJ, Lucassen GW, Carter EA, Bruining HA, Puppels GJ. In vivo confocal Raman microspectroscopy of the skin: noninvasive determination of molecular concentration profiles. J Invest Dermatol. 2001;116(3):434-42.
21. Brown SJ, Kroboth K, Sandilands A, Campbell LE, Pohler E, Kezic S, et al. Intragenic copy number variation within filaggrin contributes to the risk of atopic dermatitis with a dose-dependent effect. J Invest Dermatol. 2012;132(1):98-104.
22. Riise R, Odqvist L, Mattsson J, Monkley S, Abdillahi SM, Tyrchan C, et al. Bleomycin hydrolase regulates the release of chemokines important for inflammation and wound healing by keratinocytes. Sci Rep. 2019;9(1):20407.
23. Hoste E, Kemperman P, Devos M, Denecker G, Kezic S, Yau N, et al. Caspase-14 is required for filaggrin degradation to natural moisturizing factors in the skin. J Invest Dermatol. 2011;131(11):2233-41.
24. Scott IR, Harding CR. Filaggrin breakdown to water binding compounds during development of the rat stratum corneum is controlled by the water activity of the environment. Dev Biol. 1986;115(1):84-92.
25. Cau L, Pendaries V, Lhuillier E, Thompson PR, Serre G, Takahara H, et al. Lowering relative humidity level increases epidermal protein deimination and drives human filaggrin breakdown. J Dermatol Sci. 2017;86(2):106-13.
26. Nouwen AEM, Karadavut D, Pasmans S, Elbert NJ, Bos LDN, Nijsten TEC, et al. Natural moisturizing factor as a clinical marker in atopic dermatitis. Allergy. 2020;75(1):188-90.
27. Kezic S, O'Regan GM, Yau N, Sandilands A, Chen H, Campbell LE, et al. Levels of filaggrin degradation products are influenced by both filaggrin genotype and atopic dermatitis severity. Allergy. 2011;66(7):934-40.
28. Jurakic Toncic R, Kezic S, Jakasa I, Ljubojevic Hadzavdic S, Balic A, Petkovic M, et al. Filaggrin loss-of-function mutations and levels of filaggrin degradation products in adult patients with atopic dermatitis in Croatia. J Eur Acad Dermatol Venereol. 2020;34(8):1789-94.
29. McAleer MA, Jakasa I, Hurault G, Sarvari P, McLean WHI, Tanaka RJ, et al. Systemic and stratum corneum biomarkers of severity in infant atopic dermatitis include markers of innate and T helper cell-related immunity and angiogenesis. Br J Dermatol. 2019;180(3):586-96.
30. Tanizaki H, Amano W, Rerknimitr P, Miyachi Y, Kabashima K. Effect of topical steroid on the str atum corneum compositions by using confocal Raman microscopy. Journal of Dermatological Science. 2016;84(1):e29-e30.

SUPPLEMENTARY MATERIAL

Table S1. Disease severity per category

	Total study population[a] (n=101)	*FLG*$^{+}$ [b] (n=71)	*FLG*$^{-}$ [c] (n=30)	p-value[d]
EASI score[e], n(%)				0.24
Clear	1 (1.0)	1 (1.4)	-	
Almost clear	10 (9.9)	9 (12.7)	1 (3.3)	
Mild	39 (38.6)	28 (39.4)	11 (36.7)	
Moderate	34 (33.7)	23 (32.4)	11 (36.7)	
Severe	9 (8.9)	5 (7.0)	4 (13.3)	
Very severe	1 (1.0)	1 (1.4)	-	

Abbreviations: EASI, Eczema Area and Severity Index; *FLG*$^{+}$, wild-type patients; *FLG*$^{-}$, patients with ≥1 mutation(s) in filaggrin gene; n,number. [a]Information on disease severity was missing for 7 (6.9%) patients in the total study population. [b]Information on disease severity was missing for 4 (5.6%) patients in the *FLG*$^{+}$ group. [c]Information on disease severity was missing for 3 (10.0%) patients in *FLG*$^{-}$ group. [d]Chi-square test. [e]0 = clear; 0.1– 1.0 = almost clear; 1.1–7.0 = mild; 7.1–21.0 = moderate; 21.1–50.0 = severe; 50.1–72.0 = very severe[2]

Table S2. Comparison of new determined cutoff value with previously published cutoff value

	Number of patients[a] (n=780)
Low NMF, n(%)	
Cutoff 1.03 a.u.	215 (27.6)
Cutoff 1.07 a.u.	225 (28.8)
Normal NMF, n(%)	
Cutoff 1.03 a.u.	565 (72.4)
Cutoff 1.07 a.u.	555 (71.2)

Abbreviations: a.u, arbitrary unit; n, number; NMF, natural moisturizing factor. [a]The percentages shown are derived from the total study population with a valid NMF measurement (n=780). The cutoff value of 1.03 a.u. was determined with the data used in this manuscript. The cutoff value of 1.07 a.u. was previously determined by O'Regan et al.[1]

Table S3. Multivariate linear regression model testing the association between EASI score and NMF value.

	Standardized beta (95% CI)	p-value
***FLG* mutation status[a]**	-0.70 (-0.59, -0.38)	<0.01
Sex	-0.06 (-0.13, 0.01)	0.45
Age	-0.06 (-0.13, 0.01)	0.43
EASI score[b]	-0.04 (-0.01, 0.00)	0.58

Abbreviations: CI, confidence interval; EASI, Eczema Area and Severity Index; *FLG,* filaggrin gene; n,number. [a]*FLG* mutation status (wild type vs. mutation carriers). [b]EASI score (range 0-72).

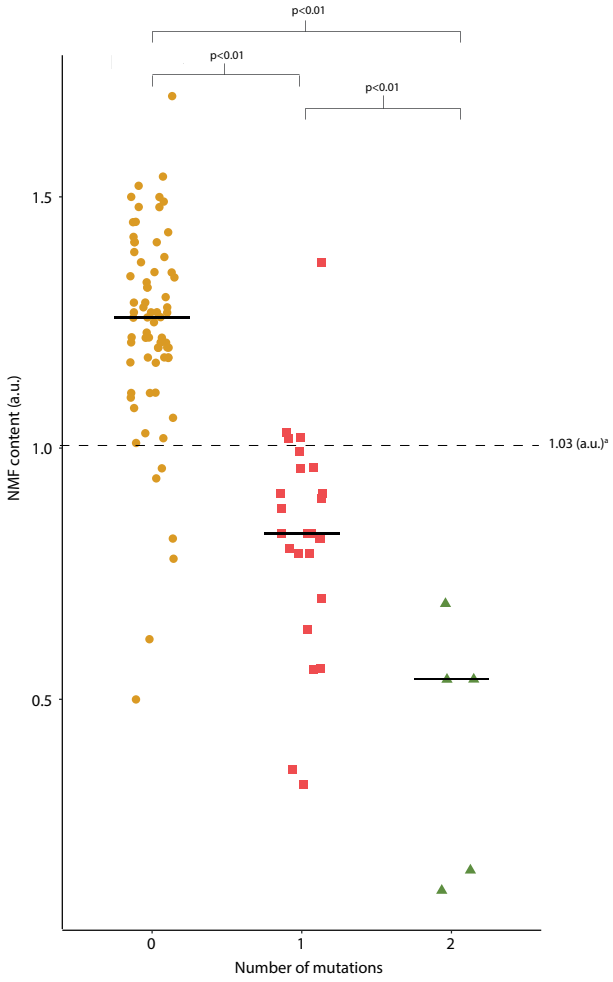

Figure S1. NMF content by *FLG* genotypes

Pairwise comparison showed significant different NMF amount between three *FLG* genotypes (Mann-Whitney U test). Wild-type patients had median NMF content of 1.26 a.u. (IQR 1.18-1.37) and median value of 0.83 (IQR 0.75-0.96) and 0.54 (0.14-0.62), respectively for patients with one and two mutations in *FLG*. [a]Cutoff value 1.03 (a.u.)

REFERENCES SUPPLEMENTARY MATERIAL

1. O'Regan GM, Kemperman PM, Sandilands A, Chen H, Campbell LE, Kroboth K, et al. Raman profiles of the stratum corneum define 3 filaggrin genotype-determined atopic dermatitis endophenotypes. J Allergy Clin Immunol. 2010;126(3):574-80 e1.
2. 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(5):1353-7.

Chapter 5.1

The influence of treatment in alpine and moderate maritime climate on the composition of the skin microbiome in patients with difficult to treat atopic dermatitis

M.M.F. van Mierlo
J.E.E. Totté
K.B. Fieten
T.J. van den Broek
F.H.J. Schuren
L.M. Pardo
S.G.M.A. Pasmans

Clin Exp Allergy. 2019 Nov;49(11):1437-1445

ABSTRACT

Background

The skin microbiome, characterized by an overgrowth of *Staphylococcus (S.) aureus*, plays an important role in the pathogenesis of atopic dermatitis (AD). Multidisciplinary treatment in alpine climate is known for its positive effect on disease severity in children with AD and can result in a different immune response compared with moderate maritime climate. However, the effect on the composition of the skin microbiome in AD is unknown.

Objective

To determine the effect of treatment in alpine climate and moderate maritime climate on the microbiome for lesional and nonlesional skin in children with difficult to treat AD.

Methods

This study is part of the DAVOS trial (ISRCTN88136485), a pragmatic randomized controlled trial in which 84 children with difficult to treat AD were randomized to a six-week personalized integrative multidisciplinary treatment in either alpine climate (intervention) or moderate maritime climate (control). Before and directly after treatment, swabs were collected from the lesional and nonlesional skin and analyzed using 16S rRNA sequencing. Additional quantitative (q)PCR for *S. aureus* and *S. epidermidis* was performed.

Results

Alpine climate treatment led to a significant change in the microbiome on lesional skin, whereas no significant change was found after moderate maritime climate. On both lesional and nonlesional skin, we observed a significant increase in Shannon diversity and a significant decrease in both *Staphylococcus* genus abundance and *S. aureus* load after alpine climate treatment. The decrease in *S. aureus* was significantly larger on lesional skin following alpine climate treatment compared with moderate maritime climate treatment. *S. epidermidis* load was stable over time.

Conclusion

Alpine climate treatment leads to significant changes in the composition of the skin microbiome in children with AD, mainly caused by a reduction in the *Staphylococcus* genus. This study shows new perspectives in the potential mode of action for therapies in AD.

INTRODUCTION

Atopic dermatitis (AD) is a chronic, relapsing skin disease with a prevalence of 10-20% in West-European children.[1] AD is characterized by immunological changes, skin barrier dysfunction and changes in the microbial composition on the skin, influenced by genetics and environmental factors. While the skin barrier and the immune system were the most important factors in AD etiology, the role of the skin microbiome gained more attention due to development of new molecular methods.[2, 3] The skin microbiome is in constant interaction with the skin barrier and immune system, reinforcing the process of inflammation.[4-6] The skin in AD is characterized by an overgrowth of *Staphylococcus (S.) aureus* and analysis of the skin microbiome in different disease states of AD showed a correlation between the abundance of *S. aureus* and disease severity, with a higher load during disease flares.[7] Also, a reduced diversity of other bacteria on the skin was found.[6-8]

AD treatment involves epidermal barrier repair using emollients, anti-inflammatory therapy using corticosteroids and trigger avoidance. In cases of severe (or infected) AD, systemic treatment and antimicrobial therapy is used.[9, 10] Alpine climate therapy has been used in patients with asthma and AD for decades. This climate is characterized by lower exposure to allergens and pollution and an increased ultraviolet radiation (UV-R).[11-13] The rationale of alpine climate therapy is mainly based on trigger avoidance and dampening the immune response. A recent study exploring the underlying immunological effects of alpine climate therapy found a significant reduction in blood eosinophils and an increase in circulating memory B cells, CD8+ T cells and T helper (Th)2 cells which reflected a reduction in disease severity.[14-16]

It is known that climate factors can influence the skin microbiome. A study performed in healthy individuals within different humidity and temperature conditions showed an effect on the quantities of bacteria on the skin.[17] Furthermore, UV-R can modulate the skin microbiome by causing direct microbial DNA damage and by affecting the immune system.[18-21]

Evaluation of alpine climate treatment has mainly focused on the immune system. The effect of alpine climate on the skin microbiome is still unclear. Identification of the skin microbiome and factors influencing the microbial composition might help in developing treatment strategies that improve disease severity by targeting the microbiome. Therefore, the aim of this study was to assess the effect of alpine climate treatment on the skin microbiome in children with difficult to treat AD in a randomized controlled trial (RCT), comparing 6 weeks of alpine climate treatment with treatment in moderate maritime climate.[22]

METHODS

Study design

This study is incorporated in the DAVOS-trial, a pragmatic RCT including children with difficult to treat AD. The trial is registered at Current Controlled Trials (ISRCTN88136485). The detailed study protocol and primary outcomes have been published previously.[22, 23] Briefly, Dutch children and adolescents were randomized to a six-week personalized integrative multidisciplinary (PIM) treatment in either a clinic in the Swiss alps at 1560 meters (intervention, alpine climate group) or an outpatient treatment program in the Netherlands in moderate maritime climate (control, moderate maritime climate group). Patients were assessed before the start of treatment (time point T0) and within 72 hours after the end of the six-week treatment (time point T1). All study assessments were performed in the Netherlands. Study procedures were reviewed and approved by the Medical Ethics Committee of the University Medical Center Utrecht, the Netherlands (reference 09-192/K). This study involves secondary outcomes of this trial.

Participants

Dutch children between 8 and 18 years old with difficult to treat AD were eligible for participation in the study. We defined difficult to treat AD as the need for at least a class 3 topical corticosteroid and not being able to step down, or current use of systemic immunosuppressive treatment, or repeated treatment with potent topical corticosteroids or systemic immunosuppressive treatment, or a history of use of systemic treatment, or a significant impact of AD on the child's or the families quality of life, or seemingly unresponsive to conventional therapy according to current guidelines.[23] All patients and if needed, their parents provided written informed consent. Demographic data were extracted from questionnaires and the electronic patient file. Microbiome samples were obtained prior to and after the end of the six-week treatment in the Wilhelmina Children's Hospital Utrecht, the Netherlands.

Microbial samples

Microbial samples were collected from the lesional and nonlesional skin. Samples taken from the lesional skin were preferably taken from the antecubital fold or the popliteal fold. Nonlesional skin samples were taken from the volar arm if possible. Sterile cotton swabs soaked in NaCl 0.9% were used to collect all samples. Skin samples were collected by rubbing the skin for 30 seconds. All samples were stored at -80°C until further processing.

DNA isolation and qPCR

For DNA isolation phenol extraction and magnetic beads were used (Agowa mag Mini DNA isolation kit; LCG). First, 150 µL from the sample was added to 350 µL lysis buffer, 500 µL

Phenol (Tris pH 8) and 500 µL 0,1 mm zirconium beads. This mixture was mechanically disrupted with a beadbeater (Biospec products, Bartelsville) twice for 2 minutes, followed by centrifuging for 10 minutes at 1690 RCF to separate the aqueous and phenolic phases. The aqueous phase was purified using AGOWA mag Mini DNA isolation kit. The bacterial DNA concentration measured after DNA extraction was performed using universal 16-S qPCR (16S-uni-I-F (5'- CGA AAG CGT GGG GAG CAA A -3'), 16S-uni-I-R (5'- GTT CGT ACT CCC CAG GCG G -3'), 16S-uni-I MGB Taqman® probe (5'- ATT AGA TAC CCT GGT AGT CCA -3') with FAM™ label). *S. aureus* and *S. epidermidis* load were quantified using multiplex qPCR with the following combination of primers and probes: 16S-S.aur-F1 (5'-GCG AAG AAC CTT ACC AAA TCT TG-3'), 16S-S.aur-R1 (5'-TGC ACC ACC TGT CAC TTT GTC-3'), 16S-S.aur MGB Taqman® probe (5'-CAT CCT TTG ACA ACT CT-3') with NED™ label. 16S-S.epi-R1 (CAT GCA CCA CCT GTC ACT CTG T) and the 16S-S.epi MGB Taqman probe (CCT CTG ACC CCT CTA G) with VIC label. 40 cycli of qPCR were performed. The DNA concentration was reported as log10 transformed, femtogram per microliter (fg/ul) in this paper. Detailed information about the DNA concentrations before log transformation are noted in Table S1.

16S rRNA sequencing and taxonomic classification

Microbiome analysis was performed with massively sequencing of the 16S rRNA gene using V4 hypervariable region on the Illumina MiSeq sequencer (Illumina, San Diego, CA). Barcoded DNA fragments spanning the V4 hypervariable region were amplified with a standardizing level of template DNA (1 ng). This was used to prevent over-amplification. Amplicons, generated using adapted primers F515 and R806 (using 30 PCR cycles), were bidirectionally sequenced using the MiSeq system.[24] Samples containing insufficient amounts of DNA did not result in usable sequence data and were therefore omitted. Pre-processing and classification of sequences was performed using the Mothur V.1.31.1 software platform. To assign taxonomic names, the Ribosomal Database Project (RDP) Classifier was used.[25] Technical performance was checked by using standardized mock communities. Negative control samples of the lysis buffer did not show signs of contamination. A genus table with raw read counts was generated for downstream analysis.

Statistical analysis

Our statistical analyses were performed in patients with available data at both time points per outcome. Shannon diversity index was calculated at genus level on non-subsampled unfiltered data. For further analysis of the microbiome we used non-subsampled genus tables and excluded genera with a relative abundance lower than 0.0001. Prior to ordination analysis, the filtered genus tables were square root transformed with subsequent application of Wisconsin double standardization. To visualize bacterial community compositions, Bray-Curtis distance based multidimensional scaling (MDS) was used. Permutational multivariable analysis of variance (PERMANOVA) was used to determine

significant changes in microbiome. To assess if the change in microbiome was significantly different between both treatment groups we used the covariates 'time point' (T0 and T1) and 'treatment group' (alpine climate and moderate maritime climate) as interaction terms in this model. If any statistically significant difference was detected, we obtained PERMANOVA coefficients to determine which genera contributed most to this change.[26] To detect changes in relative abundance within the 10 most abundant genera over time, we performed univariate analysis using a negative binomial generalized linear model.[27]

In depth analyses were performed for *S. aureus* and *S. epidermidis* by comparing log10 transformed concentrations (fg/µL). Undetectable DNA concentrations were noted as equal to zero and referred to as negative. A Linear Mixed-Effect model with post hoc analysis was used to assess the changes in *S. aureus* and *S. epidermidis* abundance. The differences between both treatment groups were assessed by calculating the interaction between covariates 'time point*treatment group'. This statistical model was also applied to Shannon diversity index.

Statistical analyses were performed in SPSS (version 21) and R software (version 3.5.1). Linear Mixed-Effect models were performed using 'lme' and 'lme4' package. Post hoc analysis was performed with 'multcomp' package and corrected for multiple testing.[28, 29] We used the packages 'ape' and 'vegan' for MDS and PERMANOVA respectively.[30] In this model, we accounted for repeated measurements using the 'strata' argument. The changes in relative abundance for the 10 most abundant genera were analyzed using package 'DESeq2'.[27] 'ggplot 2' was used for visualization.[31] A *p*-value of ≤ 0.05 was considered statistically significant.

RESULTS

Study subjects

A total of 84 patients were randomized of whom 79 patients started the intervention. Two patients from the moderate maritime climate group did not complete the intervention and sequencing data and qPCR data was missing for two other patients in this group. This resulted in 75 patients in our study: 38 patients in the alpine climate group and 37 patients in the moderate maritime climate group (Figure 1). Demographic data, comorbidities and disease severity were not significantly different between both groups at the start of intervention (Table 1). The median EASI score at T0 was 39.0 (interquartile range (IQR) 18.7-59.3) for the alpine climate group and 40.8 (IQR 22.2-52.8) for the moderate maritime climate group.

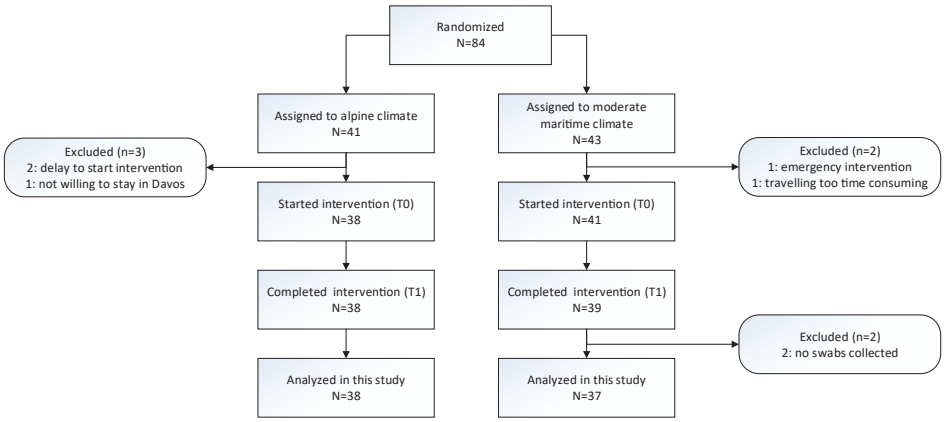

Figure 1. Flowchart of study participants

Table 1. Baseline characteristics

	Alpine climate group (n=38)	Maritime climate group (n=37)
Sex (female), n(%)	17 (44.7)	19 (51.4)
Age, mean ± SD	13.1±2.5	12.8±2.4
Age of AD onset < 6 months, n(%)	6 (15.8)	5 (13.5)
Asthma[a], n(%)	25 (65.8)	29 (78.4)
Rhinitis[b], n(%)	34 (89.5)	32 (86.5)
Food allergy[c], n(%)	26 (68.4)	26 (70.3)
SA-EASI, median (IQR)	39.0 (18.7-59.3)	40.8 (22.2-52.8)
Topical corticosteroids[d], n(%)		
None	1 (2.6)	1 (2.7)
Moderate	1 (2.6)	3 (8.1)
Potent	35 (92.1)	30 (81.1)
Very potent	1 (2.6)	3 (8.1)
Systemic medication, n(%)		
Prednisone	3 (7.9)	-
Cyclosporine	1 (2.6)	4 (10.8)
Oral antibiotics, n(%)	-	1 (2.7)
Positive for *S. aureus*, n(%)[e]		
Lesional skin	29 (76.3)	28 (75.7)
Non-lesional skin	19 (50.0)	18 (48.6)
Positive for *S. epidermidis*, n(%)[e]		
Lesional skin	35 (92.1)	36 (97.3)
Non-lesional skin	36 (94.7)	35 (94.6)

Abbreviations, AD, atopic dermatitis; SA-EASI, Self-Administered Eczema Area and Severity Index; SD, standard deviation. [a]Asthma was diagnosed based on spirometry reversibility testing and Methacholine Challenge Test. [b]Rhinitis was diagnosed based on assessment by a pediatrician. [c]Food allergy was defined as a positive double-blind placebo-controlled food challenge (DBPCFC) or convincing clinical history (a reported Type I allergic reaction with acute symptoms within 2 hours after ingestion of the food) in combination with sensitization to the specific food allergen. [d]UK potency system used. [e]determined using qPCR methods

Sample characteristics

Lesional and nonlesional skin samples were collected from all 75 patients in this study at both time points and were analyzed using qPCR techniques. Sufficient amount of DNA to perform 16S rRNA sequencing was available at both time points in 49 patients for lesional skin and 45 patients for nonlesional skin (Figure S1). Patient characteristics of missing data did not differ from the study group. A total of 1603092 sequences (median 9600; IQR 3516-26420) were obtained from the 98 lesional samples and 1949477 sequences (median 28780; IQR 6007-347885) from the 90 nonlesional samples. All sequences belonged to 603 genera, of which 213 remained after filtering. The 10 most abundant genera on lesional and nonlesional skin, before and after both treatment regimens, are shown in Figure S2. *Staphylococcus* was predominant in all groups followed by the *Corynebacterium* genus and *Streptococcus* genus.

The effect of alpine climate treatment on the microbial composition

We visualized the microbiome separated for lesional (Figure 2) and nonlesional skin (Figure S3). Before the start of treatment, the microbial composition did not differ significantly be-

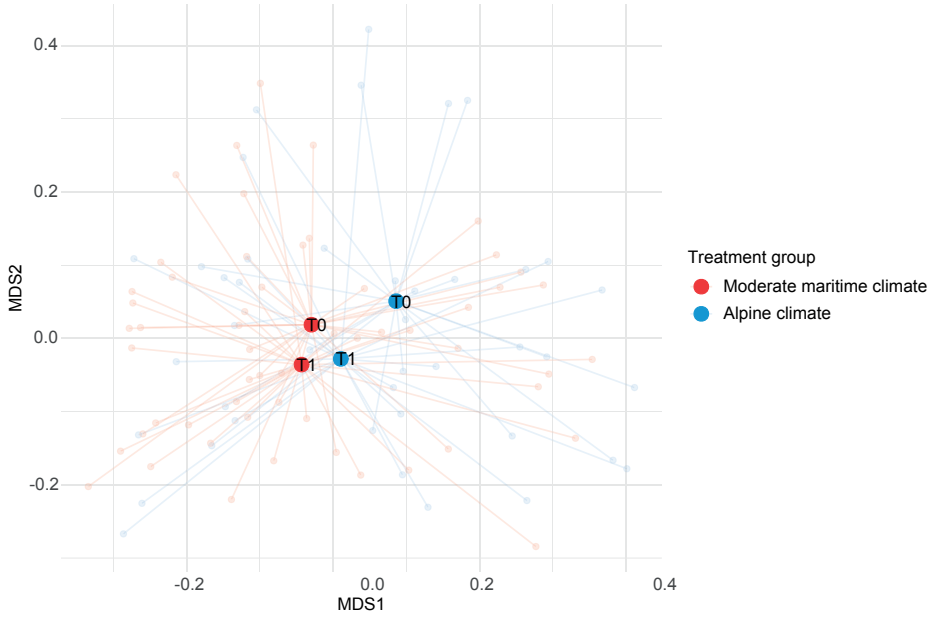

Figure 2. Bray-Curtis-distance based multidimensional scaling plot showing the microbiota of the lesional skin samples before and after the six week intervention period in alpine climate and moderate maritime climate For ordination analysis, genus tables were standardized using square root transformation with subsequent application of Wisconsin double standardization. The change in microbiome was significant for patients treated in alpine climate (PERMANOVA: R2=0.035, p=0.01). No significant change was observed after moderate maritime climate treatment (PERMANOVA: R2=0.011, p=0.81). *Abbreviations*: T0, before the start of the intervention period; T1, after six weeks of treatment.

tween both treatment groups (PERMANOVA: lesional skin: R^2=0.025, p=0.25, nonlesional skin: R^2=0.025, p=0.22). After six weeks of alpine climate treatment, a significant shift in the microbiome was observed on lesional skin (PERMANOVA: R^2=0.035, p=0.01), whereas no significant change occurred after treatment in moderate maritime climate (PERMANOVA: R^2=0.011, p=0.81). The observed change on lesional skin after alpine climate was mainly driven by the *Staphylococcus* genus with a coefficient which was 2.7 times larger than for other genera (Figure S4). The interaction 'time point*treatment group' was not significant, indicating that the change in the microbiome was not significantly different affected by treatment protocol (p=0.19). The microbiome on nonlesional skin did not change significantly following either treatment (Figure S3).

Compared to baseline, Shannon diversity index was significantly increased after alpine climate treatment on both lesional and nonlesional skin (p<0.01 and p=0.02, respectively). This was not observed after moderate maritime climate treatment (p=0.26 and p=0.70, respectively). The change in Shannon diversity index was not significantly different between both treatment groups (lesional skin p=0.26, nonlesional skin p=0.07) (Figure 3).

Effect of alpine climate treatment on the abundance of the 10 most abundant genera

The *Staphylococcus* genus showed a significant reduction in the group treated in alpine climate on both lesional and nonlesional skin (p<0.01). In the moderate maritime climate group, a significant reduction in *Staphylococcus* genus was found on lesional skin (p<0.01). The other 10 most abundant genera, as noted in Figure S2, were not significantly affected by both treatment regimens (Figure S5).

Effect of alpine climate treatment on *S. aureus* and *S. epidermidis*

To gain more insight in the *Staphylococcus* genus, additional qPCR was performed to identify *S. aureus* and *S. epidermidis*. qPCR data was available for all participants and included in our analysis (n=75). In the total study population, 57 (76.0%) patients were positive for *S. aureus* on lesional skin at T0 (alpine climate group: n=29 (76.3%), moderate maritime climate group: n= 28 (75.7%), Table 1). At T1, 40 (53.3%) patients remained positive on lesional skin (alpine climate group: n=16 (42.1%), moderate maritime climate group: n=24 (64.9%)). The *S. aureus* colonization rates were slightly lower on nonlesional skin (Table 1). After six weeks of treatment, the decrease in *S. aureus* load on lesional skin was significantly different in patients treated in alpine climate compared to moderate maritime climate (p=0.02)(Figure 4). After alpine climate treatment *S. aureus* reduced from a median of 2.6 fg/µL log10 (IQR 0.1-3.4) at T0 to 0.0 fg/µL log10 (IQR 0.0-0.9) at T1 (p<0.01), due to the proportion of patients with undetectable *S. aureus* concentration at T1. In the moderate maritime climate group, *S. aureus* decreased from a median of 2.0 fg/µL log10 (IQR 0.3-3.0) to 1.1 fg/µL log10 (IQR 0.0-2.7) (p=0.11). On nonlesional skin, a significant

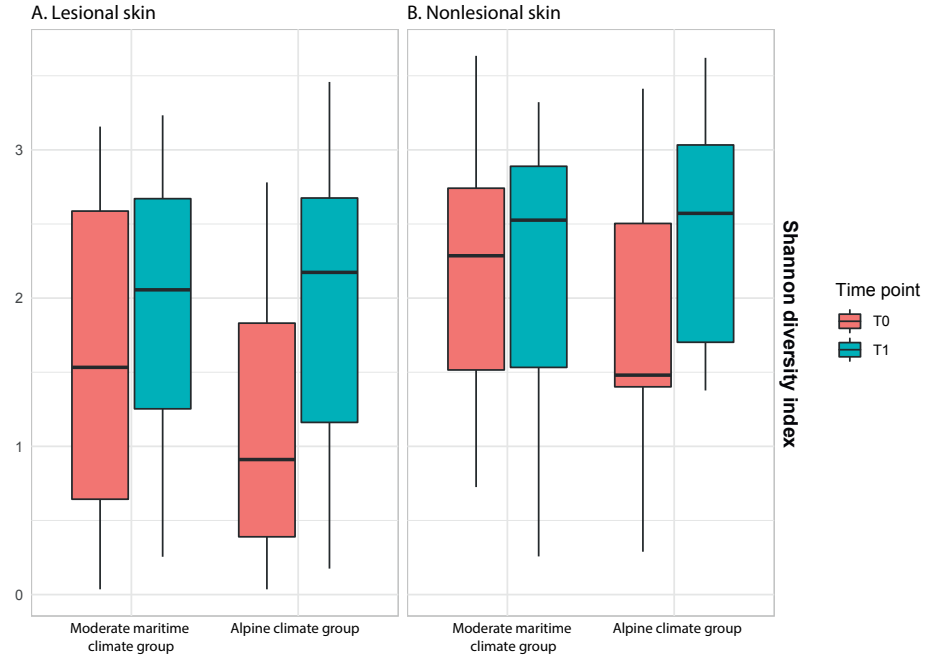

Figure 3. Shannon diversity index of skin samples before and after the six week intervention period in alpine climate and moderate maritime climate

Shannon diversity index represents the number of different genera (richness) and how even they are distributed (evenness). The boxes represent the 25th percentile, median, and 75th percentile. Statistical analysis was performed using a Linear Mixed-Effect model with post-hoc analysis. A: Lesional skin samples. Compared to baseline, significant differences in Shannon diversity index were found after treatment in alpine climate ($p<0.01$). No significant change was observed after moderate maritime climate ($p=0.09$) or between both treatment groups ($p=0.26$). B: Nonlesional skin samples. Compared to baseline, significant differences in Shannon diversity index were found after treatment in alpine climate ($p=0.02$). No significant change was observed after moderate maritime climate ($p=0.70$) or between both treatment groups ($p=0.07$). *Abbreviations*: T0, before the start of the intervention period; T1, after six weeks of treatment.

drop in *S. aureus* load was observed after treatment in alpine climate with a median of 0.4 fg/µL log10 (IQR 0.0-2.1) at T0 and 0.0 fg/µL log10 (IQR 0.0-1.0) at T1 ($p<0.01$) (Figure S6). *S. epidermidis* was positive in 71 (94.7%) patients in the total study group on both lesional and nonlesional skin at T0, compared to 72 (96%) and 74 (98.7%) for lesional and nonlesional skin at T1 (Table 1). *S. epidermidis* load was not affected by either treatment protocol (Figure 4 and S6).

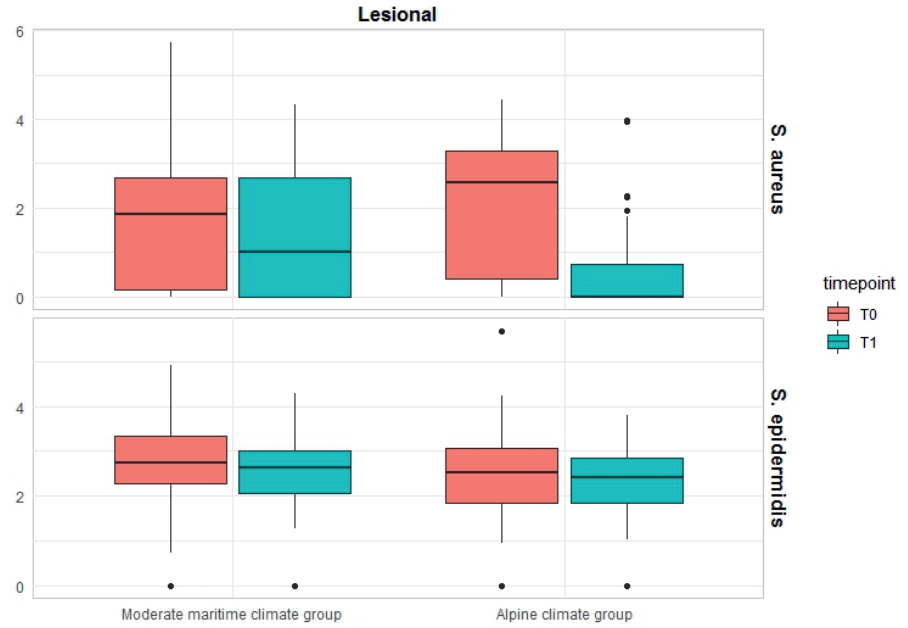

Figure 4. Quantitative PCR results of the lesional skin samples for *S. aureus* and *S. epidermidis* before and after the six week intervention period in alpine climate and moderate maritime climate
The results for *S. aureus* and *S. epidermidis* loads are shown using log10 transformed data. A significant difference in *S. aureus* load was found after alpine climate treatment ($p<0.01$). *S. epidermidis* was stable during the treatment period. The decrease in *S. aureus* load was significantly larger after alpine climate compared to moderate maritime climate ($p=0.02$). The boxes represent the 25th percentile, median, and 75th percentile. Dots represent individual samples. Statistical analysis was performed using a Linear Mixed-Effect model with post-hoc analysis). *Abbreviations*: T0, before the start of the intervention period; T1, after six weeks of treatment.

DISCUSSION

This study showed that alpine climate treatment affects the microbiome on both lesional and nonlesional skin in children with difficult to treat AD. We found a significant change in the overall skin microbiome on lesional skin after six weeks of alpine climate treatment, whereas no significant change was observed after moderate maritime climate treatment. Moreover, a significant change was observed on both lesional and nonlesional skin in Shannon diversity index, *Staphylococcus* abundance and *S. aureus* load in particular. The reduction in *S. aureus* load was significantly larger when compared to moderate maritime climate.

This is the first study describing the effect of alpine climate treatment on the skin microbiome in patients with AD. Alpine climate offers favorable features for patients with AD,

including lower exposure to allergens and pollution and an increased UV-R.[11, 12] Moreover, children treated in alpine climate were separated from their parents and intensively monitored by the multidisciplinary treatment team. This treatment setting in alpine climate has beneficial effects on disease severity and was shown to affect the immune response (blood eosinophils, memory B cells, CD8+ T cells and Th2 cells).[14, 18, 32] Besides effects on the immune system, studies also proposed that geographical variability, with variation in UV-R, can influence the skin barrier and the microbiome.[2, 33] Although this study shows a change in the skin microbiome after alpine climate treatment, we cannot prove that the observed effect is directly caused by the alpine climate or through the effect on the immune system or treatment setting.

After alpine climate treatment, we found a significant increase in Shannon diversity index and a significant reduction in *S. aureus* load on both lesional and nonlesional skin. Moreover, the decrease in *S. aureus* load on lesional skin was significantly different from the maritime climate group (p=0.02). In a previous paper, describing the effectiveness of alpine climate treatment in this study population, a significantly larger decrease in disease severity was observed following alpine climate treatment than maritime climate treatment (p<0.01). The SA-EASI score decreased from a median of 39.0 (IQR 18.7-59.3) to 2.6 (IQR 0.3-6.2) and 40.8 (IQR 22.2-52.8) to 12.0 (IQR 3.6-22.1) after six-week treatment in alpine climate and moderate maritime climate respectively.[23] Literature shows a positive correlation between the abundance of *Staphylococcus*, in particular *S. aureus*, and disease severity in patients with AD.[6-8, 34] A decrease in disease severity also leads to higher bacterial diversity.[7] It is likely that our results are affected by the differences in disease severity. However, it is unknown whether the *S. aureus* abundance is a result or a cause of changes in disease severity. More studies with frequent sampling around flares are needed to answer this question.

Compared to lesional skin, we were not able to detect a change in the microbiome on nonlesional skin after alpine climate treatment. However, the changes in Shannon diversity index, *Staphylococcus* abundance and *S. aureus* after alpine climate treatment were significant for both lesional and nonlesional skin. Nonlesional skin in AD is known to differ from both lesional AD skin and healthy skin.[35, 36] This can be explained by the impaired skin barrier in patients with AD, which also affects nonlesional skin and makes it more susceptible to penetration of allergens and bacteria than healthy skin.[37] A previous study investigating the effect of topical corticosteroids and bleach baths in patients with AD, also showed a significant change in microbial composition after treatment on lesional skin, but not on nonlesional skin.[8] This is in line with our findings. A possible explanation can be the higher diversity and lower *Staphylococcus* abundance, compared to lesional skin, making changes more subtle. Another explanation can be the lesser impact of disease severity

and thus inflammation on this skin. Nonlesional skin might tell us more about the effect of climate than lesional skin which is more subject to the secondary effects of inflammation.

In this study, both lesional and nonlesional skin in AD patients were dominated by the S*taphylococcus* genus. These results support previous literature describing an excess of *Staphylococcus,* and more specific of *S. aureus,* in the skin microbiome of patients with AD.[3, 7, 36] Other abundant genera included *Streptococcus* (known to be more present in children with AD) and *Corynebacterium* (common in healthy skin microbiome).[3, 35, 38] Looking in more depth at the S*taphylococcus* genus, the prevalence of *S. aureus* at the start of intervention was 76.0% and 49.3% for lesional and nonlesional skin respectively. These percentages are slightly higher than described in a recent meta-analysis on this subject and might be explained by our inclusion criteria, selecting patients with moderate to severe AD.[39] Although patients showed a significant reduction in disease severity in this study, a large proportion remained positive for *S. aureus* on the skin (lesional T0: 76,0%, T1: 53,3%. Nonlesional T0: 49,3%, T1: 32,0%). These results suggest that AD symptoms are not only associated with the presence or absence of *S. aureus*, but more importantly with the total *S. aureus* load on the skin.

We did not find a significant change in *S. epidermidis* load in our study. The role of *S. epidermidis* in the pathogenesis of AD is still unclear and literature on this subject is conflicting.[7, 40, 41] Due to the inhibitory effect of *S. epidermidis* on *S. aureus*, by the production of bacteriocins, serine protease Esp and phenol-soluble modulins, some correlation may be expected.[42-44] In a previous study, an increase in *S. epidermidis* was found during a disease flare in patients with AD.[7] In our study the quantities of *S. epidermidis* were stable over time despite a drop in *S. aureus* load and severity following treatment. It is possible that *S. epidermidis* is elevated in the acute stadium (flares) as a compensatory mechanism to control *S. aureus*, but in a chronic stadium these levels normalized.

This study has a pragmatic design, which makes it hard to assess what contributed most to the observed outcomes. A characteristic of the alpine climate group, besides the unique aspects of this climate, was the supervision leading to optimal treatment compliance. It might be that due to supervision, the application of topical corticosteroids was more adequately and frequent in this group and could have affected disease severity and the skin microbiome. Moreover, this study describes secondary outcomes of this trial and medication use was not applied as exclusion criteria. Previous studies showed an effect of medication use on the skin microbiome. However, it was not possible to discontinue medication use in this group of patients with difficult to treat, moderate to severe disease.[8, 45] During this study, the use of medication was carefully monitored and showed no significant differences between both treatment groups. Results for patients using systemic

medication at T0 did not deviate from the rest of the study population (data not shown). Moreover, the proportion of patients using topical and/or systemic medication during the intervention was stable (Table S2).

A limitation of this study was the use of the V4 hypervariable region for sequencing. With this variable region it is not possible to properly detect the *Propionibacterium* or to classify the *Staphylococci* at species level. To overcome this problem for the *Staphylococci*, we determined *S. aureus* and *S. epidermidis* abundance with qPCR methods. Since the body sites which were mainly sampled for this study are usually low or devoid of *Propionibacterium* this should only have had a minor to negligible effect on the data presented here.[2]

This study encourages to perform explanatory studies with a similar treatment setting in both climates to confirm the effect of climate conditions alone on the skin microbiome. Including samples from the skin of healthy subjects in future studies might give us more information about the differences in the skin microbiome between AD and controls and assess if a decrease in disease severity (which was observed at T1) leads to a microbiome more comparable to healthy subjects. Furthermore, it would be interesting to assess if residents of moderate maritime climate and alpine climate have a distinct microbiome.

In conclusion, six weeks of treatment in the alpine climate affects the skin microbiome in children with difficult to treat AD which might reflect successful treatment. This study shows new perspectives in the potential mode of action for therapies in patients with AD and encourages further investigation of skin microbiome modulating therapies.

REFERENCES

1. Odhiambo JA, Williams HC, Clayton TO, Robertson CF, Asher MI, Group IPTS. Global variations in prevalence of eczema symptoms in children from ISAAC Phase Three. J Allergy Clin Immunol. 2009;124(6):1251-8 e23.
2. Grice EA, Segre JA. The skin microbiome. Nat Rev Microbiol. 2011;9(4):244-53.
3. Bjerre RD, Bandier J, Skov L, Engstrand L, Johansen JD. The role of the skin microbiome in atopic dermatitis: a systematic review. Br J Dermatol. 2017;177(5):1272-8.
4. Geoghegan JA, Irvine AD, Foster TJ. Staphylococcus aureus and Atopic Dermatitis: A Complex and Evolving Relationship. Trends Microbiol. 2017.
5. Travers JB. Toxic interaction between Th2 cytokines and Staphylococcus aureus in atopic dermatitis. J Invest Dermatol. 2014;134(8):2069-71.
6. Clausen ML, Agner T, Lilje B, Edslev SM, Johannesen TB, Andersen PS. Association of Disease Severity With Skin Microbiome and Filaggrin Gene Mutations in Adult Atopic Dermatitis. JAMA Dermatol. 2018;154(3):293-300.
7. Kong HH, Oh J, Deming C, Conlan S, Grice EA, Beatson MA, et al. Temporal shifts in the skin microbiome associated with disease flares and treatment in children with atopic dermatitis. Genome Res. 2012;22(5):850-9.
8. Gonzalez ME, Schaffer JV, Orlow SJ, Gao Z, Li H, Alekseyenko AV, et al. Cutaneous microbiome effects of fluticasone propionate cream and adjunctive bleach baths in childhood atopic dermatitis. J Am Acad Dermatol. 2016;75(3):481-93 e8.
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 I. J Eur Acad Dermatol Venereol. 2012;26(8):1045-60.
10. 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(9):1176-93.
11. Vocks E. Climatotherapy in atopic eczema. In: Ring J PB, Ruzicka T, eds. Handbook of Atopic Eczema. Springer-Verlag BerlinHeidelberg: Springer; 2006:507-523.
12. Rijssenbeek-Nouwens LH, Bel EH. High-altitude treatment: a therapeutic option for patients with severe, refractory asthma? Clin Exp Allergy. 2011;41(6):775-82.
13. Fieten KB, Weststrate AC, van Zuuren EJ, Bruijnzeel-Koomen CA, Pasmans SG. Alpine climate treatment of atopic dermatitis: a systematic review. Allergy. 2015;70(1):12-25.
14. Heeringa JJ, Fieten KB, Bruins FM, van Hoffen E, Knol EF, Pasmans S, et al. Treatment for moderate to severe atopic dermatitis in alpine and moderate maritime climates differentially affects helper T cells and memory B cells in children. Clin Exp Allergy. 2018.
15. Simon D BS. The effects of high altitude climate therapy. Phys Med Rehab Kuror. 2001;11:104–9.
16. Triebskorn A GH, Gloor M, Borelli S. Hydration of the horny layer and barrier function in atopic dermatitis before and after therapy in high-altitude climate. Z Hautkr. 1991:66:145–7.
17. McBride ME, Duncan WC, Knox JM. The environment and the microbial ecology of human skin. Appl Environ Microbiol. 1977;33(3):603-8.
18. Faergemann J, Larko O. The effect of UV-light on human skin microorganisms. Acta Derm Venereol. 1987;67(1):69-72.
19. Patra V, Byrne SN, Wolf P. The Skin Microbiome: Is It Affected by UV-induced Immune Suppression? Front Microbiol. 2016;7:1235.
20. Thyssen JP, Zirwas MJ, Elias PM. Potential role of reduced environmental UV exposure as a driver of the current epidemic of atopic dermatitis. J Allergy Clin Immunol. 2015;136(5):1163-9.

21. Wang Y, Zhu W, Shu M, Jiang Y, Gallo RL, Liu YT, et al. The response of human skin commensal bacteria as a reflection of UV radiation: UV-B decreases porphyrin production. PLoS One. 2012;7(10):e47798.
22. Fieten KB, Zijlstra WT, van Os-Medendorp H, Meijer Y, Venema MU, Rijssenbeek-Nouwens L, et al. Comparing high altitude treatment with current best care in Dutch children with moderate to severe atopic dermatitis (and asthma): study protocol for a pragmatic randomized controlled trial (DAVOS trial). Trials. 2014;15:94.
23. Fieten K, Schappin R, Zijlstra W, Figee L, Beutler J, Raymakers F, et al. Effectiveness of alpine climate treatment for children with difficult to treat atopic dermatitis: results of a pragmatic randomized controlled trial (DAVOS trial). Clin Exp Allergy. 2017.
24. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, et al. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. Isme J. 2012;6(8):1621-4.
25. Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol. 2007;73(16):5261-7.
26. Gonzáles I DS, Martin P.G.P et al. CCA: An R Package to extend canonical correlation analysis., 23. JoSS.
27. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 2014;15(12):550.
28. Harrison XA, Donaldson L, Correa-Cano ME, Evans J, Fisher DN, Goodwin CED, et al. A brief introduction to mixed effects modelling and multi-model inference in ecology. PeerJ. 2018;6:e4794.
29. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. Journal of the Royal Statistical Society Series B (Methodological). 1995;57(1):289-300.
30. Oksanen J BF, Friendly M et al. The Vegan Package. Community Ecology Package. [28-02-2018]; Available from: https://cran.r-project.org/web/packages/vegan/vegan.pdf.
31. Wickham H. ggplot2: Elegant Graphics for Data Analysis Springer-Verlag. 2009.
32. Hamann CR, Andersen YMF, Engebretsen KA, Skov L, Silverberg JI, Egeberg A, et al. The effects of season and weather on healthcare utilization among patients with atopic dermatitis. J Eur Acad Dermatol Venereol. 2018;32(10):1745-53.
33. Engebretsen KA, Johansen JD, Kezic S, Linneberg A, Thyssen JP. The effect of environmental humidity and temperature on skin barrier function and dermatitis. J Eur Acad Dermatol Venereol. 2016;30(2):223-49.
34. Gong JQ, Lin L, Lin T, Hao F, Zeng FQ, Bi ZG, et al. Skin colonization by Staphylococcus aureus in patients with eczema and atopic dermatitis and relevant combined topical therapy: a double-blind multicentre randomized controlled trial. Br J Dermatol. 2006;155(4):680-7.
35. Shi B, Bangayan NJ, Curd E, Taylor PA, Gallo RL, Leung DYM, et al. The skin microbiome is different in pediatric versus adult atopic dermatitis. J Allergy Clin Immunol. 2016;138(4):1233-6.
36. Seite S, Flores GE, Henley JB, Martin R, Zelenkova H, Aguilar L, et al. Microbiome of affected and unaffected skin of patients with atopic dermatitis before and after emollient treatment. J Drugs Dermatol. 2014;13(11):1365-72.
37. Irvine AD, McLean WH, Leung DY. Filaggrin mutations associated with skin and allergic diseases. N Engl J Med. 2011;365(14):1315-27.
38. Grice EA, Kong HH, Conlan S, Deming CB, Davis J, Young AC, et al. Topographical and temporal diversity of the human skin microbiome. Science. 2009;324(5931):1190-2.
39. Totte JE, van der Feltz WT, Hennekam M, van Belkum A, van Zuuren EJ, Pasmans SG. Prevalence and odds of Staphylococcus aureus carriage in atopic dermatitis: a systematic review and meta-analysis. Br J Dermatol. 2016;175(4):687-95.

40. Higaki S, Morohashi M, Yamagishi T, Hasegawa Y. Comparative study of staphylococci from the skin of atopic dermatitis patients and from healthy subjects. Int J Dermatol. 1999;38(4):265-9.
41. Hon KL, Tsang YC, Pong NH, Leung TF, Ip M. Exploring Staphylococcus epidermidis in atopic eczema: friend or foe? Clin Exp Dermatol. 2016;41(6):659-63.
42. Sugimoto S, Iwamoto T, Takada K, Okuda K, Tajima A, Iwase T, et al. Staphylococcus epidermidis Esp degrades specific proteins associated with Staphylococcus aureus biofilm formation and host-pathogen interaction. J Bacteriol. 2013;195(8):1645-55.
43. Otto M. Staphylococcus colonization of the skin and antimicrobial peptides. Expert Rev Dermatol. 2010;5(2):183-95.
44. Cogen AL, Yamasaki K, Sanchez KM, Dorschner RA, Lai Y, MacLeod DT, et al. Selective antimicrobial action is provided by phenol-soluble modulins derived from Staphylococcus epidermidis, a normal resident of the skin. J Invest Dermatol. 2010;130(1):192-200.
45. Callewaert C, Nakatsuji T, Knight R, Kosciolek T, Vrbanac A, Kotol P, et al. IL-4Ralpha Blockade by Dupilumab Decreases Staphylococcus aureus Colonization and Increases Microbial Diversity in Atopic Dermatitis. J Invest Dermatol. 2019.

Table S1. qPCR results before log10 transformation

Alpine climate group								
Patient	*S. epidermidis* load on lesional skin (fg/µl)		*S. epidermidis* load on non-lesional skin (fg/µl)		*S. aureus* load on lesional skin (fg/µl)		*S. aureus* load on non-lesional skin (fg/µl)	
	T0	T1	T0	T1	T0	T1	T0	T1
Patient	*S. epidermidis* load on lesional skin (fg/µl)		*S. epidermidis* load on non-lesional skin (fg/µl)		*S. aureus* load on lesional skin (fg/µl)		*S. aureus* load on non-lesional skin (fg/µl)	
	T0	T1	T0	T1	T0	T1	T0	T1
117	1820,00	10,60	808,77	148,99	325,00	undetectable	38,88	undetectable
126	332,00	1330,00	undetectable	125,77	undetectable	undetectable	undetectable	undetectable
128	146,00	70,70	2092,86	223,92	8,91	undetectable	undetectable	undetectable
169	477,00	124,00	256,74	44,30	982,00	undetectable	undetectable	undetectable
207	15,10	142,00	26,87	7,95	21,00	undetectable	undetectable	undetectable
209	373,00	1970,00	649,89	869,95	2650,00	88,30	855,02	10,89
292	1690,00	167,00	180,67	172,25	1520,00	8,21	undetectable	undetectable
295	undetectable	undetectable	149,89	506,45	27000,00	8790,00	768,47	undetectable
297	3940,00	355,00	1316,59	224,34	undetectable	undetectable	undetectable	undetectable
298	17600,00	1900,04	1036,50	279,35	undetectable	undetectable	undetectable	undetectable
328	6325,80	12,25	undetectable	7,23	440,89	undetectable	undetectable	89,07
330	undetectable	28,27	47,12	84,20	1341,63	4,59	undetectable	9,06
338	490389,00	388,47	593,74	223,49	undetectable	188,55	188,70	219,20
384	12098,80	4586,14	192,03	218,41	undetectable	undetectable	undetectable	undetectable
385	589,40	70,21	58,03	35,10	219,18	undetectable	undetectable	undetectable
386	44,75	348,71	74,79	undetectable	undetectable	undetectable	undetectable	undetectable
387	444,10	153,81	6850,94	451,05	undetectable	undetectable	68,86	9,59

Alpine climate group								
Patient	*S. epidermidis* load on lesional skin (fg/µl)		*S. epidermidis* load on non-lesional skin (fg/µl)		*S. aureus* load on lesional skin (fg/µl)		*S. aureus* load on non-lesional skin (fg/µl)	
	T0	T1	T0	T1	T0	T1	T0	T1
394	1506,14	6447,28	1580,27	365,69	20681,30	undetectable	undetectable	undetectable
504	874,40	78,15	4072,35	2474,45	17,56	3,49	undetectable	undetectable
506	132,90	394,22	441,16	211,87	4718,33	undetectable	2979,19	undetectable
507	281,60	5624,08	440,52	1105,88	undetectable	undetectable	undetectable	undetectable
540	23,77	691,39	13,37	202,48	3,37	undetectable	undetectable	undetectable
543	1086,11	170,85	192,00	188,88	3308,54	undetectable	1830,00	undetectable
545	243,87	49,09	373,80	128,11	195,69	3,69	60,98	21,67
586	28,35	236,40	5,53	45,29	undetectable	undetectable	undetectable	undetectable
605	495,42	undetectable	313,57	605,67	1602,22	undetectable	48,68	undetectable
612	315,21	772,14	442,03	399,05	1763,07	undetectable	38,53	undetectable
617	8,48	295,48	3,35	273,96	204,65	9232,65	21,51	288,69
626	undetectable	52,00	50,17	31,39	1072,14	2,56	153,05	4,70
644	612,35	1129,35	808,65	154,77	14602,00	61,73	941,96	undetectable
648	41,87	627,56	36,15	408,13	13,61	8,82	undetectable	42,02
649	216,22	842,25	90,75	152,20	741,10	170,39	324,50	12,49
660	81,08	300,64	90,52	258,25	4831,87	31,86	452,77	undetectable
694	90,04	693,90	357,58	627,80	10595,40	3,22	45,26	undetectable
699	7529,78	undetectable	423,60	1585,04	8049,45	undetectable	105,71	undetectable
705	243,00	109,00	416,34	182,00	57,71	7,87	undetectable	undetectable
717	undetectable	38,68	11,30	34,69	445,00	28,43	9,42	undetectable
731	146,33	166,35	56,50	294,90	859,89	undetectable	53,86	undetectable

Moderate maritime climate group								
Patient	*S. epidermidis* load on lesional skin (fg/µl)		*S. epidermidis* load on non-lesional skin (fg/µl)		*S. aureus* load on lesional skin (fg/µl)		*S. aureus* load on non-lesional skin (fg/µl)	
	T0	T1	T0	T1	T0	T1	T0	T1
110	2640,00	3000,00	1295,18	2778,81	50,50	undetectable	undetectable	undetectable
113	633,00	383,00	432,75	163,19	undetectable	undetectable	undetectable	undetectable
119	81100,00	930,00	1367,46	159,37	undetectable	undetectable	undetectable	undetectable
120	62900,00	882,00	110,11	1387,39	undetectable	undetectable	undetectable	undetectable
122	199,00	77,80	554,64	166,72	4,33	579,00	246,04	undetectable
208	70700,00	19,10	10517,60	4,28	133000,00	undetectable	5787,50	undetectable
213	30700,00	922,00	2643,66	131,25	undetectable	15,70	undetectable	undetectable
214	8,07	94,00	92,31	55,35	10500,00	21400,00	52,77	13,17
215	329,00	20,00	2162,04	62,20	95,10	1140,00	46,27	9,45
273	388,00	120,00	202,07	106,08	478,00	66,70	undetectable	24,72
293	20,00	120,00	402,09	8327,01	51,80	undetectable	undetectable	undetectable
294	2190,00	1590,00	13312,40	4269,05	106,00	105,00	150,21	1126,02
299	743,86	389,01	213,32	361,57	5,35	534,18	6,70	4,33
312	302,24	127,32	120,67	39,56	489,13	undetectable	undetectable	31,14
313	794,49	27,12	982,88	50,64	undetectable	undetectable	undetectable	undetectable
315	182,71	20,87	53,16	5,78	undetectable	undetectable	undetectable	undetectable
329	446,97	20303,30	357,54	6435,84	165,44	76,87	31,67	315,76
358	28,96	97,33	46,06	10,49	4,49	29,17	undetectable	undetectable
383	2302,75	631,82	23,80	21,77	29,59	undetectable	undetectable	12,85
407	38195,20	558,65	84,71	405,16	1224,52	undetectable	undetectable	undetectable
431	201,22	463,60	294,73	9446,68	undetectable	17,97	undetectable	undetectable
530	2148,42	325,61	180,30	30,78	3427,54	459,74	29,46	6,36

Moderate maritime climate group								
Patient	*S. epidermidis* load on lesional skin (fg/µl)		*S. epidermidis* load on non-lesional skin (fg/µl)		*S. aureus* load on lesional skin (fg/µl)		*S. aureus* load on non-lesional skin (fg/µl)	
	T0	T1	T0	T1	T0	T1	T0	T1
544	100,67	4399,20	15,18	415,43	1,56	undetectable	9,26	undetectable
549	1183,48	9303,25	661,70	444,01	325,83	273,76	214,52	112,27
579	5,31	15590,90	89,46	370,25	196,44	undetectable	8,69	undetectable
590	100,77	37,40	30,24	47,29	487,74	1,91	216,19	undetectable
597	1235,33	328,17	222,10	1122,16	1505,40	1433,27	13,57	601,05
613	2402,87	1325,62	795,81	293,08	496,48	704,46	160,99	90,27
623	232,86	4281,11	undetectable	276,55	undetectable	2985,42	undetectable	undetectable
627	81,96	448,91	6,49	1006,78	undetectable	8,46	undetectable	49,12
655	3032,51	197,86	40,31	155,36	30988,00	1,12	14,57	undetectable
661	812,18	978,30	48,41	32,39	42,26	4,11	undetectable	undetectable
677	1136,92	986,20	518,81	233,63	221,18	2183,76	undetectable	undetectable
700	192,95	136,72	398,45	1182,59	25,14	12,04	undetectable	undetectable
703	506,01	3042,22	965,81	803,18	4224,02	1,53	2252,61	undetectable
724	72884,50	675,00	145,70	116,00	71146,90	43,30	314,26	undetectable
728	undetectable	0,76	undetectable	88,10	542792,00	5946,21	80,51	258,17

S. aureus and *S. epidermidis* load were quantified using multiplex quantitative (q)PCR with the following combination of primers and probes: 16S-S.aur-F1 (5'-GCG AAG AAC CTT ACC AAA TCT TG-3'), 16S-S.aur-R1 (5'-TGC ACC ACC TGT CAC TTT GTC-3'), 16S-S.aur MGB Taqman® probe (5'-CAT CCT TTG ACA ACT CT-3') with NED™ label. 16S-S.epi-R1 (CAT GCA CCA CCT GTC ACT CTG T) and the 16S-S.epi MGB Taqman probe (CCT CTG ACC CCT CTA G) with VIC label. 40 cycli of qPCR were performed. *Abbreviations*: fg/µL, femtogram per microliter; T0, before the start of the intervention period; T1, after six weeks of treatment.

Table S2. Use of concomitant medication during the study period

	Alpine climate group N=38	Moderate maritime climate N=37
No topical corticosteroid[a], n(%)		
T0	1 (2.6)	1 (2.7)
T1	3 (8.1)	-
Moderate topical corticosteroid[a], n(%)		
T0	1 (2.6)	3 (8.1)
T1	5 (13.2)	7 (18.9)
Potent topical corticosteroid[a], n(%)		
T0	35 (92.1)	30 (81.1)
T1	30 (78.9)	27 (73.0)
Very potent topical corticosteroid[a] , n(%)		
T0	1 (2.6)	3 (8.1)
T1	-	3 (8.1)
Prednisone, n(%)		
T0	3 (7.9)	-
T1	-	-
Cyclosporine, n(%)		
T0	1 (2.6)	4 (10.8)
T1	-	1 (2.7)
Oral antibiotics , n(%)		
T0	-	1 (2.7)
T1	-	1 (2.7)

Abbreviations: T0, before the start of the intervention period; T1, after six weeks of treatment. [a]UK potency system used.

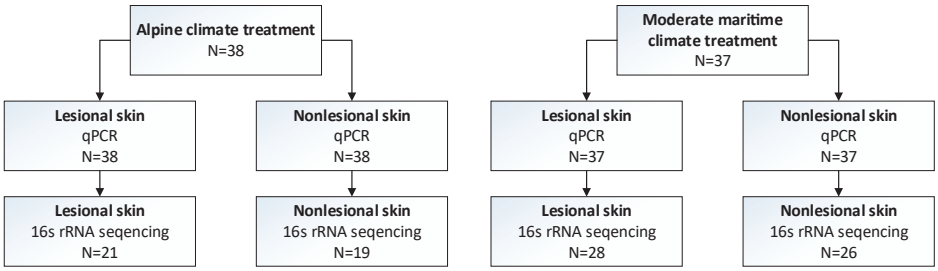

Figure S1. Flowchart showing the number of patients included in our statistical analysis
Samples containing insufficient amounts of DNA did not result in usable sequence data and were therefore omitted. Only the patients with available data at both time points were included in this study.

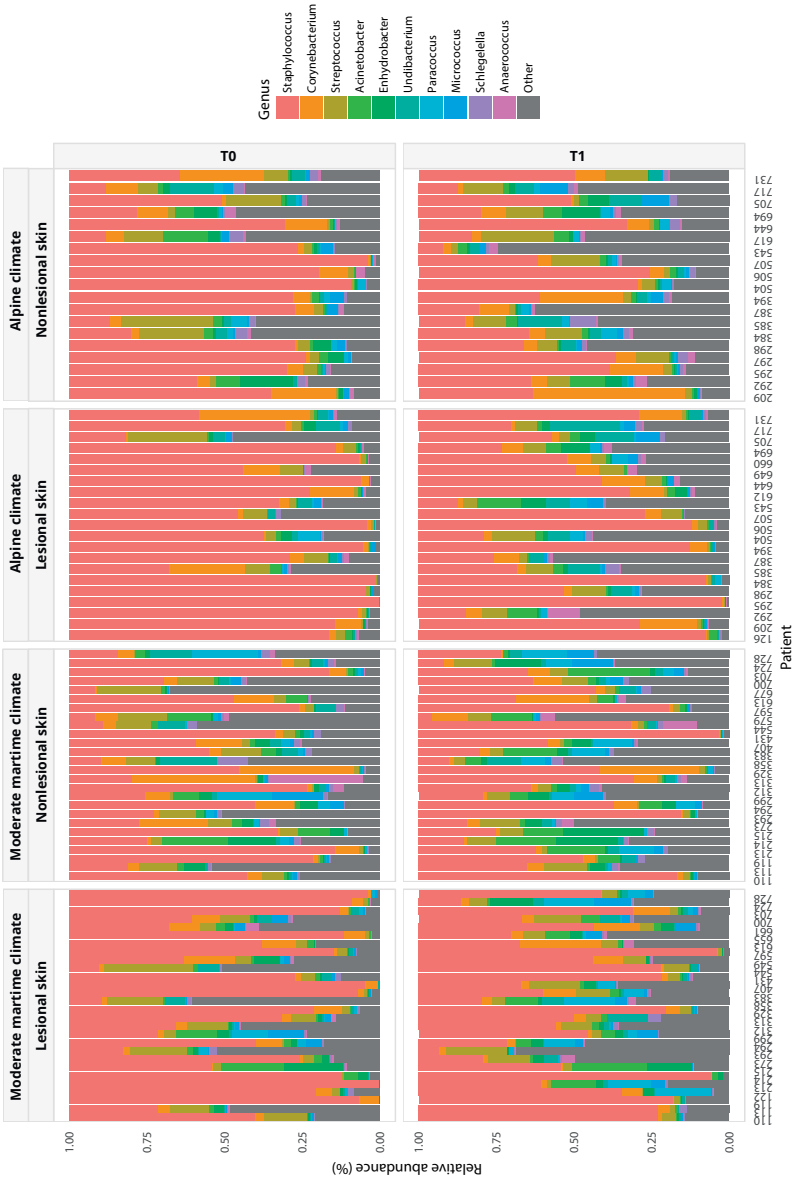

Figure S2. Relative abundance of the most common genera in skin samples before and after the six week intervention period in alpine climate and moderate maritime climate per individual patient

Relative abundance (RA) of the most common genera on lesional and nonlesional skin sites were calculated for the remaining 213 genera after filtering of non-subsamples genus tables. Genera with RA <0.0001 were excluded. *Abbreviations*: T0, before the start of the intervention period; T1, after six weeks of treatment.

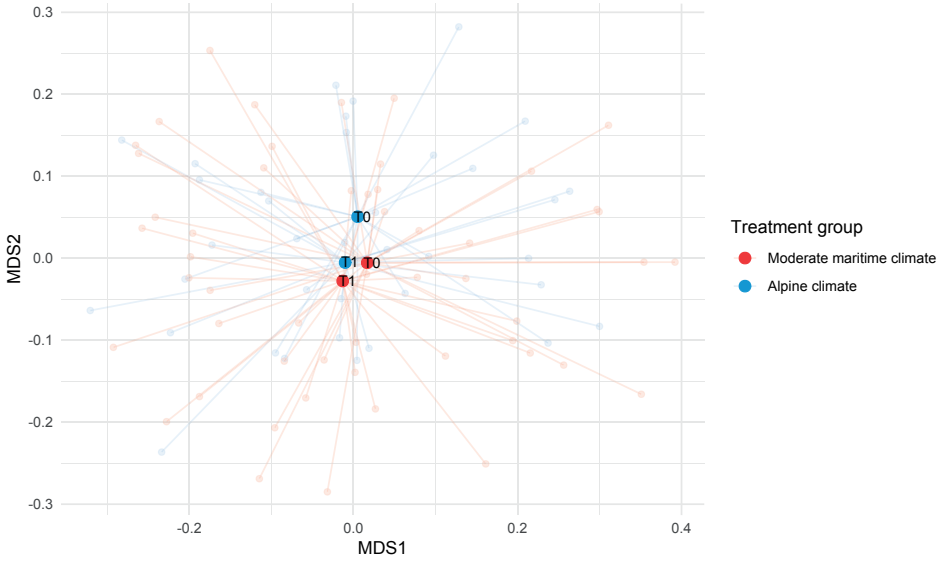

Figure S3. Bray-Curtis-distance based multidimensional scaling plot showing the microbial communities of the nonlesional skin samples before and after the six week intervention period in alpine climate and moderate maritime climate

For ordination analysis, genus tables were standardized using square-root transformation with subsequent application of Wisconsin double standardization. No significant changes were found after both treatment regimens. Alpine climate group (PERMANOVA R^2=0.026, p=0.15), moderate maritime climate group (PERMANOVA R^2=0.018, p=0.23). *Abbreviations*: T0, before the start of the intervention period; T1, after six weeks of treatment.

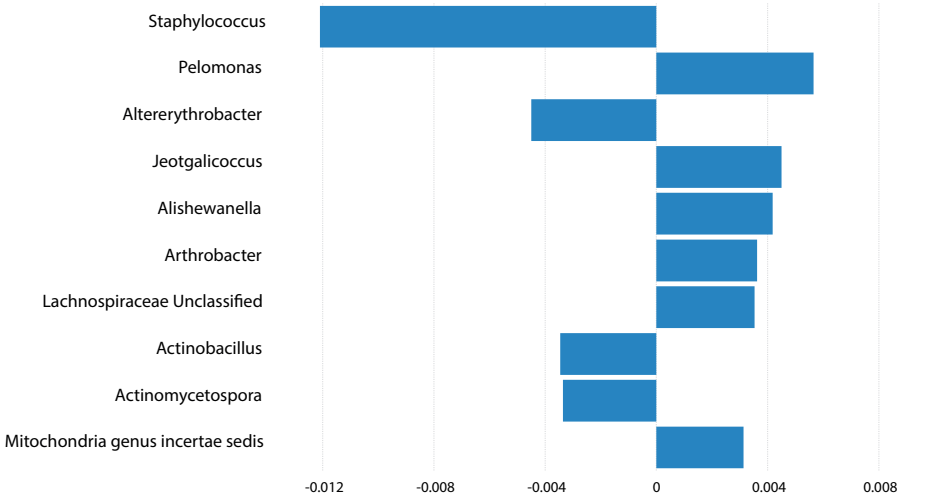

Figure S4. Top 10 genera driving the change in microbial composition on lesional skin after alpine climate treatment

The x-axis displays coefficients that represent the contribution of a certain genus to the change in microbial composition on lesional skin after alpine climate treatment. The contribution of the *Staphylococcus* genus was 2.7 times larger than for other genera.

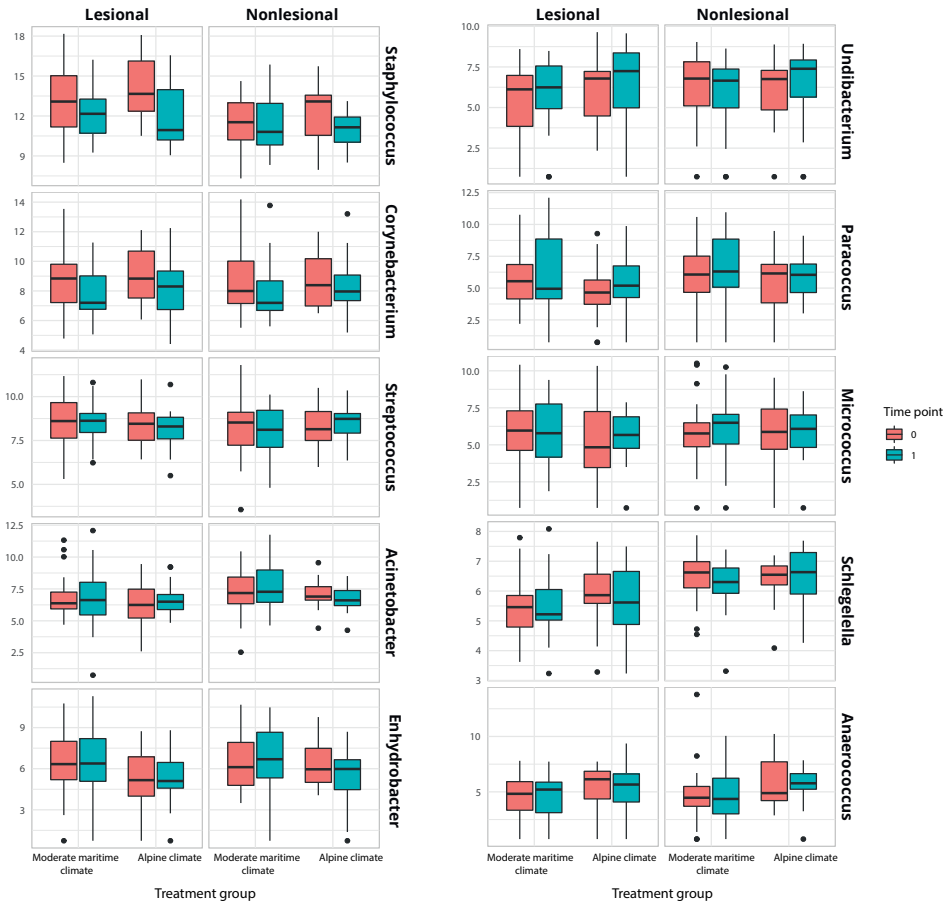

Figure S5. Change in relative abundance for the 10 most abundant genera before and after the six week intervention period in alpine climate and moderate maritime climate

Genus counts were normalized using DESeq regularized-logarithm transformation. Statistical analysis was performed using univariate analysis using a negative binomial generalized linear model. The *Staphylococcus* genus showed a significant reduction in the group treated in alpine climate on both lesional and nonlesional skin (both $p<0.01$). In the moderate maritime climate group, a significant reduction in *Staphylococcus* genus was found on lesional skin ($p<0.01$).

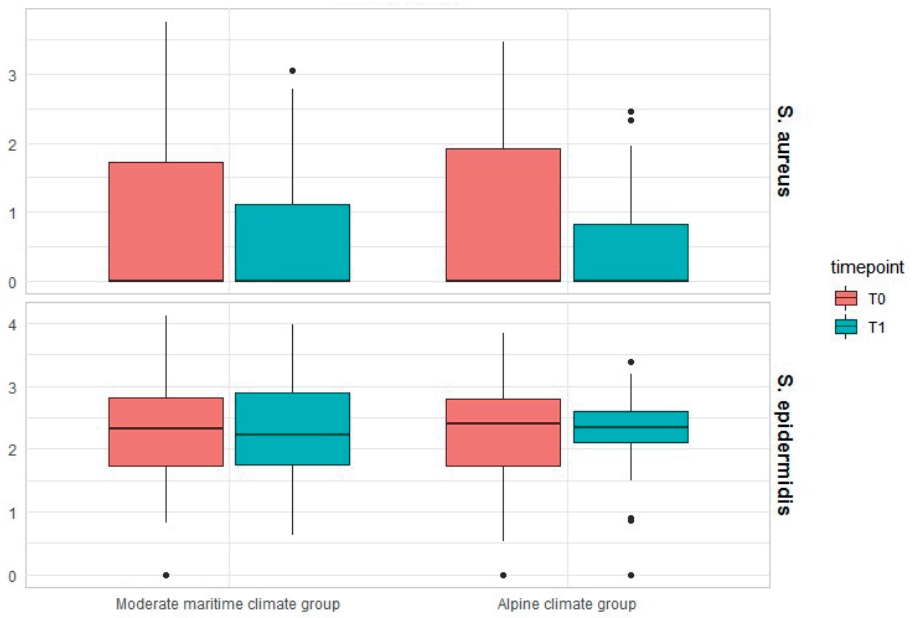

Figure S6. qPCR results of the nonlesional skin samples for *S. aureus* and *S. epidermidis* before and after the six week intervention period in alpine climate and moderate maritime climate
The results for *S. aureus* and *S. epidermidis* load are shown using log10 transformed data. A significant difference in *S. aureus* load was found after alpine climate treatment ($p<0.01$). The boxes represent the 25th percentile, median, and 75th percentile. Dots represent individual samples. Statistical analysis was performed using a Linear Mixed-Effect model with post-hoc analysis. *Abbreviations*: T0, before the start of the intervention period; T1, after six weeks of treatment

Chapter 5.2

Endolysin treatment against *Staphylococcus aureus* in adults with atopic dermatitis: a randomized controlled trial

J. de Wit
M.M.F. van Mierlo*
J.E.E. Totté*
J. van Veldhuizen
M.B.A. van Doorn
F.H.J. Schuren
S.P. Willemsen
L.M. Pardo
S.G.M.A. Pasmans
**These authors contributed equally to this work.*

J Allergy Clin Immunol. 2019 Sep;144(3):860-863

To the Editor:

Staphylococcus (*S.) aureus* density is increased in many patients with atopic dermatitis (AD) and is thought to contribute to the disease pathogenesis, interacting with an altered skin barrier and immunological changes.[1] *S. aureus* might induce or aggravate inflammation via different mechanisms, for example through excretion of virulence factors, even if the *S. aureus* overgrowth is primarily caused by other factors.[2] Current guidelines only recommend antimicrobial therapy directed against *S. aureus* in clinically infected AD based on a Cochrane review where no clinical benefit of short-term antimicrobial treatment in non-infected AD was found.[3]

Arguably, long-term anti-staphylococcal treatment, such as antibiotics, might reduce symptoms in AD.[4] However, this is undesired because antibiotics can affect the commensal microbiota and could induce bacterial resistance.[5] In contrast, long-term treatment of AD with an endolysin that targets only *S. aureus* is feasible. It might improve AD symptoms and reduce the use of corticosteroids consecutively.[2, 6] Therefore, we aimed to determine the topical corticosteroid (TCS)-sparing effect and safety of 12 weeks of endolysin treatment against *S. aureus* in patients with AD.

We performed a double-blind, vehicle-controlled superiority trial (MAAS trial, ClinicalTrials.gov NCT02840955) in 100 adult patients with TCS-treated, not clinically infected, moderate to severe AD, which was defined by an Eczema Area and Severity Index (EASI) of 7.1 to 50.0. After a two-week run-in period to standardize the TCS treatment with triamcinolone acetonide 0.1% cream, patients were randomly assigned 1:1 to a 12-week intervention with either a topical endolysin against *S. aureus* or a vehicle twice daily, followed by an eight-week follow-up period. The vehicle and recombinant chimeric endolysin, Staphefekt™ SA.100, were provided by Micreos Human Health (Bilthoven, The Netherlands) and topically applied in a cetomacrogol cream. Details on patient inclusion, randomization, and study procedures at six assessments are described in a previously published study protocol (Figure S1).[2]

The primary outcome, the TCS-sparing effect of endolysin treatment against *S. aureus*, was evaluated by the patients, who registered daily use of a TCS (yes/no) over 12 weeks. Secondary outcomes included differences in TCS use measured in grams, clinical efficacy, quality of life using the Skindex-29, *S. aureus* load on the skin, and safety and tolerability of endolysin treatment. Clinical efficacy was measured using the EASI, Investigators Global Assessment, Patient-Oriented Eczema Measure, pruritus Numeric Rating Scale, and by registration of the number of flares. The *S. aureus* load on the skin was assessed by a semi-quantitative culture and quantitative polymerase chain reaction (qPCR, Appendix S1).

Generalized linear mixed-effect models for repeated measurements were used to analyze the primary and dichotomous secondary outcomes, and linear mixed-effect models were used for continuous secondary outcomes. Data were analyzed as intention-to-treat and per-protocol. Furthermore, a subgroup analysis was performed in patients with a positive *S. aureus* skin culture at two time points before start of the intervention.

Eighty-eight (88.0%) patients completed the intervention, and 87 (87.0%) completed follow-up (13% dropout rate, Figure S2). Patients' characteristics were comparable between the endolysin and vehicle groups (Table 1). Over the 12-week intervention period (corresponding to 8400 days for 100 patients), patients in the endolysin group used a TCS for 1889 (45.0%) days compared with 1566 (37.3%) days in the vehicle group. There was no statistically significant difference in the probability of TCS use per day between the groups in the intention-to-treat analysis, per-protocol analysis, and in the subgroup of *S. aureus*-positive patients ($p = 0.97$, $p = 0.40$ and $p = 0.08$, respectively, Table 2). Sensitivity analyses showed no differences in the odds ratio of TCS use per assessment day. Except for the number of doctor-reported AD flares during the intervention period (per-protocol, n=2 in endolysin group vs. n=10 in vehicle group, $p = 0.03$), no statistically significant differences were found in the secondary outcomes after both intention-to-treat and per-protocol analyses (Table S1 and S2). At baseline, 62 (64.6%) patients had positive results for *S. aureus* based on skin culture and 24 (24.7%) by qPCR. Both methods showed no significant difference in *S. aureus* reduction (Table S3 and S4). During the study, one serious adverse event occurred in the endolysin group eight weeks after the last application of endolysin cream (pleural effusion with hospitalization), which was considered unlikely to be related to the study intervention (Table S5 and S6).

Our results are in accordance with data from a Cochrane review showing no significant effect of short-term anti-*S. aureus* therapy in patients with non-infected AD.[3] We cannot confirm the positive results of other longer-term studies. However, these studies used broad-spectrum antimicrobials and mainly included patients with signs of bacterial infection.[4] Patients with clinically infected AD were excluded from our study, and a possible effect of anti-*S. aureus* endolysins in this patient group should be determined in future studies.

Several hypotheses could explain our results. First, use of triamcinolone in the run-in phase resulted in a decrease in AD severity (Table 1), which might have masked a possible benefit of endolysin treatment. Second, daily use of an emollient and good compliance with the treatment could have resulted in a reduction of triamcinolone use in both the endolysin and vehicle groups.[7] Because AD is a heterogeneous disease, anti-*S. aureus* treatment might not be suitable for all patients with AD, indicating the need for subphenotyping.

Table 1. Baseline characteristics

	Total (n=100)	Endolysin (n=50)	Vehicle (n=50)
Age			
years; median (IQR)	33.5 (25.5-47.5)	36.5 (25.0-51.0)	32.5 (24.0-44.0)
Sex (male)			
n (%)	55 (55.0)	24 (48.0)	31 (62.0)
Race, n (%)			
American Indian or Alaska Native	5 (5.0)	2 (4.0)	3 (6.0)
Asian	10 (10.0)	2 (4.0)	8 (16.0)
Black or African American	8 (8.0)	5 (10.0)	3 (6.0)
White	77 (77.0)	41 (82.0)	36 (72.0)
Atopic disease, n (%)			
Food allergy	43 (43.0)	18 (36.0)	25 (50.0)
Rhinoconjunctivitis	63 (63.0)	28 (56.0)	35 (70.0)
Asthma	47 (47.0)	25 (50.0)	22 (44.0)
EASI, median (IQR)			
Screening (V1)	12.9 (9.2-19.0)	13.7 (8.9-19.1)	12.5 (9.2-19.0)
Baseline (V2)	8.0 (5.0-13.5)[a]	8.3 (5.0-14.7)[b]	8.0 (4.9-12.9)[c]
IGA, median (IQR)			
Baseline (V2)	2.0 (2.0-3.0)[a]	2.0 (2.0-3.0)[b]	2.0 (2.0-3.0)[c]
POEM, mean (SD)			
Baseline (V2)	12.9 (6.2)[a]	14.5 (8.3-17.0)[b]	13.0 (8.0-15.0)[c]
Pruritus NRS, median (IQR)			
Baseline (V2)	3.0 (2.0-4.0)[a]	3.0 (2.0-4.0)[b]	3.0 (2.0-4.0)[c]
Skindex-29, mean (SD)			
Baseline (V2)	35.1 (17.1)[a]	37.3 (15.3)[b]	32.9 (18.7)[c]
Use of topical corticosteroids at screening, n (%)			
Class 1	3 (3.0)	1 (2.0)	2 (4.0)
Class 2	13 (13.0)	6 (12.0)	7 (14.0)
Class 2-3	11 (11.0)	3 (6.0)	8 (16.0)
Class 3	44 (44.0)	22 (44.0)	22 (44.0)
Class 3-4	3 (3.0)	2 (4.0)	1 (2.0)
Class 4	18 (18.0)	11 (22.0)	7 (14.0
Unknown	8 (8.0)	6 (12.0)	4 (8.0)
***S. aureus* skin culture[d], n (%)**			
Positive	56 (56.0)	32 (64.0)	24 (48.0)
Intermediate	20 (20.0)	7 (14.0)	13 (26.0)
Negative	20 (20.0)	9 (18.0)	11 (22.0)
Missing	4 (4.0)	2 (4.0)	2 (4.0)

Abbreviations: EASI, Eczema Area and Severity Index; IGA, Investigators Global Assessment; IQR, interquartile range; NRS, Numeric Rating Scale; POEM, Patient Oriented Eczema Measure; SD, standard deviation, *S. aureus*, *Staphylococcus aureus*. Missings: [a]n=3 (3.0%), [b]n=2 (4.0%), [c]n=1 (2.0%). [d]Positive is defined as having a positive culture at visit 1 and visit 2a; intermediate is defined as having one positive culture and one negative culture at visit 1 and visit 2a; negative is defined as having two negative cultures at visit 1 and visit 2a.

Table 2. Generalized Linear Mixed-Effect model results for the difference in topical corticosteroid use per day during the 12-week intervention period

Analysis	Time period or time point in days from baseline	Patients included in analysis		Topical corticosteroid use 'yes', n (%)		OR (95% CI)	*P*-value[a]
		Endolysin, n	**Vehicle**, n	**Endolysin**, n (%)	**Vehicle**, n (%)		
Intention-to-treat	Intervention						0.97
	14	40	37	26 (65.00)	20 (54.05)	0.99 (0.95 – 1.03)	0.49
	42	37	37	26 (70.27)	22 (59.46)	0.99 (0.78 – 1.24)	0.91
	84	30	34	18 (60.00)	21 (61.76)	1.04 (0.87 – 1.23)	0.68
Per-protocol	Intervention						0.40
	14	22	21	14 (63.64)	10 (47.62)	1.09 (0.98 – 1.21)	0.10
	42	17	18	12 (70.59)	11 (61.11)	1.36 (0.84 – 2.20)	0.21
	84	13	13	7 (53.85)	5 (38.46)	1.69 (1.14 – 2.51)	**0.01**
***S. aureus* positive**[b]	Intervention						0.08
	14	26	16	17 (65.38)	7 (43.75)	0.87 (0.78 – 0.96)	**0.01**
	42	22	17	16 (72.73)	8 (47.06)	0.85 (0.49 – 1.48)	0.56
	84	18	15	11 (61.11)	11 (73.33)	1.29 (0.84 – 1.96)	0.24

Given the low number of patients using escape medication (n=5), we did not correct for its use. [a]Overall effect of endolysin treatment during intervention period was calculated with a Likelihood-Ratio test, the effect per time point using a Wald test with t-distribution. [b]Defined as having a positive culture both at visit 1 and visit 2a (endolysin n=32, vehicle n=24). Sensitivity analyses, performed by adding/subtracting 0.25 times the standard deviation to/of the odds, showed no differences in the odds ratio of topical corticosteroid use per assessment day. *Abbreviations*: *S. aureus*, *Staphylococcus aureus*

Because only 56% of our study population had two consecutive positive *S. aureus* skin cultures (indicating persistent colonization) before start of the intervention, the target population that would probably benefit the most from endolysin treatment was small.

Our data suggest that endolysin treatment has no effect on *S. aureus in vivo.* However, patients might have been recolonized with *S. aureus* from the nose because 73% of them were nasal carriers (data not shown). Alternatively, cetomacrogol as the basis of the endolysin cream might have created a barrier on the skin that prevented the endolysin to reach and subsequently kill *S. aureus*. However, some reduction in *S. aureus* load would have been expected in both treatment groups because of the use of TCSs and emollients in this study, which both have been shown to reduce the *S. aureus* load on the skin.[8, 9] Nonetheless, it is unclear whether complete eradication of *S. aureus* is required for clinical improvement because a case series showed a clear clinical improvement without *S. aureus* reduction using a qualitative culture in *S. aureus*-related dermatoses.[6] In addition, the discrepant results between culture and qPCR indicate the complexity of the interpretation of *S. aureus* testing. Despite the limitations and outcome, this study provides estimates of

AD symptoms, use of TCSs, and the percentage of persistent *S. aureus* carriers that can be used for future clinical studies.

In conclusion, long-term targeted endolysin treatment against *S. aureus* in this study was well tolerated but had no TCS-sparing effect in patients with AD. However, an effect cannot be excluded because good compliance with the treatment and concurrent application of TCSs, emollients, or both might have masked a clinical benefit.

REFERENCES

1. Totte JE, van der Feltz WT, Hennekam M, van Belkum A, van Zuuren EJ, Pasmans SG. Prevalence and odds of Staphylococcus aureus carriage in atopic dermatitis: a systematic review and meta-analysis. Br J Dermatol. 2016;175(4):687-95.
2. Totte J, de Wit J, Pardo L, Schuren F, van Doorn M, Pasmans S. Targeted anti-staphylococcal therapy with endolysins in atopic dermatitis and the effect on steroid use, disease severity and the microbiome: study protocol for a randomized controlled trial (MAAS trial). Trials. 2017;18(1):404.
3. Bath-Hextall FJ, Birnie AJ, Ravenscroft JC, Williams HC. Interventions to reduce Staphylococcus aureus in the management of atopic eczema: an updated Cochrane review. Br J Dermatol. 2010;163(1):12-26.
4. Wong SM, Ng TG, Baba R. Efficacy and safety of sodium hypochlorite (bleach) baths in patients with moderate to severe atopic dermatitis in Malaysia. J Dermatol. 2013;40(11):874-80.
5. Niebuhr M, Mai U, Kapp A, Werfel T. Antibiotic treatment of cutaneous infections with Staphylococcus aureus in patients with atopic dermatitis: current antimicrobial resistances and susceptibilities. Exp Dermatol. 2008;17(11):953-7.
6. Totté JEE, van Doorn MB, Pasmans S. Successful Treatment of Chronic Staphylococcus aureus-Related Dermatoses with the Topical Endolysin Staphefekt SA.100: A Report of 3 Cases. Case Rep Dermatol. 2017;9(2):19-25.
7. Ng JP, Liew HM, Ang SB. Use of emollients in atopic dermatitis. J Eur Acad Dermatol Venereol. 2015;29(5):854-7.
8. Angelova-Fischer I, Neufang G, Jung K, Fischer TW, Zillikens D. A randomized, investigator-blinded efficacy assessment study of stand-alone emollient use in mild to moderately severe atopic dermatitis flares. J Eur Acad Dermatol Venereol. 2014;28 Suppl 3:9-15.
9. Gonzalez ME, Schaffer JV, Orlow SJ, Gao Z, Li H, Alekseyenko AV, et al. Cutaneous microbiome effects of fluticasone propionate cream and adjunctive bleach baths in childhood atopic dermatitis. J Am Acad Dermatol. 2016;75(3):481-93 e8.

SUPPLEMENTARY MATERIAL

Appendix S1. Microbial methods

a. Microbial sampling methods

Samples of the skin were collected using sterile Copan 490CE.A swabs for culture analysis and skin scrubs for qPCR analysis. Skin swabs were collected from a skin lesion at the first visit, preferably located at the antecubital fold or the popliteal fold. For all consecutive visits the swab was collected from the location chosen at the first visit. The scrubs were collected from the lower arm, adjacent to the antecubital fold according to methods described previously.[23] Scrub samples were stored at -80 °C.

b. Semi-quantitative culture and qPCR for *S. aureus*

Bacterial cultures were performed using routine diagnostic culture procedures, using blood agar plates and specific *S. aureus* culture plates (ChromID *S. aureus* Elite agar (SAIDE), Biomérieux, France) for overnight incubation and subsequent species determination by MALDI-TOF (Bruker Daltonics, Bremen, Germany). For DNA isolation 150 µl from the sample was added to 350 µl lysis buffer, 500 µl Phenol (Tris pH 8) and 500 µl 0.1 mm zirconium beads. This mixture was mechanically disrupted by bead beating twice for 2 minutes, followed by centrifuging for 10 minutes at 1690 RCF to separate the aqueous and phenolic phases. The aqueous phase was purified using AGOWA mag Mini DNA isolation kit. After elution, we used qPCR to determine the total load of *S. aureus* with the following primers and probes: 16S-S.aur-F1 (5'-GCG AAG AAC CTT ACC AAA TCT TG-3'), 16S-S.aur-R1 (5'-TGC ACC ACC TGT CAC TTT GTC-3'), 16S-S.aur MGB Taqman® probe (5'-CAT CCT TTG ACA ACT CT-3') with NED™ label.

Table S1. Results for the difference in secondary clinical outcomes – intention-to-treat analysis

Outcome	Time period or visit	Endolysin	Vehicle	Difference in change (95% CI)[a]	OR (95% CI)[b]	*P*-value[c]
Mean grams/week topical corticosteroid use, median (IQR)	Intervention[d]	4.5 (2.2-8.7)	3.6 (1.9-7.4)			0.66
	Intervention + follow-up[e]	5.1 (1.9-8.9)	4.1 (2.3-10.4)			0.91
Proportion of patients who indicated to have used less corticosteroids compared with baseline, n (%)	Intervention + follow-up					0.19
	3	36 (76.60)	34 (72.34)		0.77 (0.45 – 1.29)	0.32
	4	30 (65.22)	27 (60.00)		0.79 (0.51 – 1.22)	0.29
	5	22 (50.00)	20 (45.45)		0.91 (0.61 – 1.38)	0.67
	6	23 (53.49)	13 (29.55)		0.32 (0.21 – 0.51)	<0.001[†]
Change in EASI from baseline, mean (SD)	Intervention + follow-up					0.57
	3	-0.61 (4.88)	-1.63 (5.09)	1.03 (-1.30 – 3.36)		0.38
	4	0.11 (6.40)	-0.88 (6.22)	1.08 (-1.41 – 3.57)		0.39
	5	0.29 (7.40)	-1.08 (6.16)	1.28 (-1.48 – 4.04)		0.36
	6	0.43 (7.83)	-1.71 (7.26)	2.61 (-0.53 – 5.75)		0.10
IGA corrected for baseline	Intervention + follow-up					0.78
Proportion of patients with a reduction of ≥2 points in IGA from baseline, n (%)	3	0 (0.00)	2 (4.26)			0.46[f]
	4	1 (2.17)	2 (4.44)			0.62[f]
	5	1 (2.27)	3 (6.82)			0.62[f]
	6	1 (2.33)	5 (11.36)			0.20[f]

Table S1. Results for the difference in secondary clinical outcomes – intention-to-treat analysis (*continued*)

Outcome	Time period or visit	Endolysin	Vehicle	Difference in change (95% CI)[a]	OR (95% CI)[b]	*P*-value[c]
Change in POEM from baseline, mean (SD)	Intervention + follow-up					0.98
	3	-3.15 (4.64)	-2.94 (5.52)	-0.23 (-2.55 – 2.09)		0.84
	4	-2.93 (5.80)	-2.09 (5.43)	-0.54 (-2.87 – 1.78)		0.64
	5	-2.43 (6.13)	-2.02 (6.76)	-0.57 (-3.01 – 1.88)		0.65
	6	-1.79 (6.32)	-1.09 (6.68)	-0.79 (-3.57 – 1.99)		0.57
Change in pruritus NRS from baseline, mean (SD)	Intervention + follow-up					0.56
	3	0.26 (1.57)	0.21 (1.64)	0.03 (-0.63 – 0.69)		0.92
	4	0.20 (1.77)	-0.09 (1.43)	0.37 (-0.31 – 1.05)		0.28
	5	0.23 (1.85)	-0.05 (1.70)	0.32 (-0.43 – 1.07)		0.39
	6	-0.05 (2.23)	0.14 (1.98)	-0.09 (-0.97 – 0.79)		0.84
Proportion of patients with a reduction of ≥2 points in pruritus NRS from baseline, n (%)	Intervention + follow-up					0.65
	3	14 (29.79)	10 (21.28)		0.56 (0.24 – 1.28)	0.17
	4	16 (34.78)	11 (24.44)		0.57 (0.26 – 1.23)	0.15
	5	14 (31.82)	17 (38.64)		1.44 (0.71 – 2.94)	0.31
	6	16 (37.21)	15 (34.09)		0.84 (0.42 – 1.68)	0.62
Proportion of patients with a reduction of ≥3 points in pruritus NRS from baseline, n (%)	Intervention + follow-up					0.71
	3	7 (14.89)	3 (6.38)		0.41 (0.11 – 0.53)	0.18
	4	5 (10.87)	4 (8.89)		0.69 (0.17 – 2.77)	0.60
	5	7 (15.91)	6 (13.64)		0.83 (0.32 – 2.18)	0.71
	6	11 (25.58)	8 (18.18)		0.50 (0.22 – 1.13)	0.09

Table S1. Results for the difference in secondary clinical outcomes – intention-to-treat analysis (*continued*)

Outcome	Time period or visit	Endolysin	Vehicle	Difference in change (95% CI)[a]	OR (95% CI)[b]	*P*-value[c]
Change in Skindex-29 from baseline, mean (SD)	Intervention + follow-up					0.71
	3	-7.15 (9.83)	-4.86 (9.32)	-2.63 (-6.81 – 1.54)		0.21
	5	-7.35 (10.54)	-4.82 (12.96)	-2.35 (-7.22 – 2.51)		0.34
	6	-8.38 (12.98)	-4.66 (13.87)	-3.35 (-8.97 – 2.26)		0.24
Number of doctor reported flares from baseline, n (%)	Intervention[g]	14 (10.22)	17 (12.50)			0.55
Number of patient reported flares from baseline, n (%)	Intervention[g]	14 (10.22)	17 (12.50)			0.58
Mean time (days) to doctor reported flare from baseline, median (IQR)	Intervention[h]	44.50 (35.25-84.00)	43.00 (30.50-84.50)			0.95
Mean time (days) to patient reported flare from baseline, median (IQR)	Intervention[h]	40.50 (20.00-72.25)	30.00 (15.00-64.00)			0.71
	Follow-up[i]	30.50 (16.75-50.50)	22.00 (8.50-43.50)			0.52
Number of patients with at least one (serious) AE, n	Intervention + follow-up	40	40			1.00
Number of (serious) AEs, n	Intervention + follow-up	82	74		1.14 (0.83 – 1.56)[j]	0.42

Abbreviations: AE, adverse event; CI, confidence interval; EASI, Eczema Area and Severity Index; IGA, Investigators Global Assessment; IQR, interquartile range; NRS, Numeric Rating Scale; POEM, Patient Oriented Eczema Measure; OR, odds ratio; SD, standard deviation. Patients included in analyses per visit in endolysin and vehicle group: visit 3, n=47 and n=47; visit 4, n=46 and n=45; visit 5, n=44 and n=44; visit 6, n=43 and n=44. NOTE: Given the low number of patients using escape medication (n=5), we did not correct for its use in the analysis of the mean grams/week topical corticosteroid use. [a]Differences in change presented for analyses using a Linear Mixed-Effect model. [b]OR presented for analysis using a Generalized Linear Mixed-Effect model. [c]*P*-values were calculated using a Chi-Square test or Fisher's Exact test for categorical data, where appropriate. A non-parametric Mann-Whitney U Test for independent samples was used for continuous variables. The overall effect of endolysin treatment during the intervention and follow-up period ((Generalized) Linear Mixed-Effect models) was analyzed with a Likelihood-Ratio test and per visit using a Wald test with t-distribution. [d]Endolysin group n=32, vehicle group n=34. [e]Endolysin group n=32, vehicle group n=32. [f]Since the number of patients per cell are ≤5 a Fisher's Exact test per visit was used instead of a Generalized Linear Mixed-Effect model. A *P*-value of 0.0125 will be considered significant after Bonferroni correction. [g]For every visit from baseline through week 12, 137 visits in endolysin group and 136 visits in vehicle group, it was registered if a flare occurred yes/no. [h]Endolysin group n=14, vehicle group n=17. [i]Endolysin group n=8, vehicle group n=5. [j]Rate ratio with 95% CI. [†]Significant result.

Table S2. Results for the difference in secondary clinical outcomes – per-protocol analysis

Outcome	Time period or visit	Endolysin	Vehicle	Difference in change (95% CI)[a]	OR (95% CI)[b]	*P*-value[c]
Mean grams/week topical corticosteroid use, median (IQR)	Intervention[d]	6.0 (3.3-10.9)	4.1 (2.6-7.9)			0.44
	Intervention + follow-up[e]	6.1 (4.2-7.3)	5.0 (2.6-14.7)			1.00
Proportion of patients who indicate to have used less corticosteroids compared with baseline, n (%)	Intervention + follow-up					0.40
	3	19 (76.00)	21 (72.41)		0.91 (0.41 – 2.00)	0.81
	4	8 (42.11)	11 (52.38)		1.52 (0.70 – 3.34)	0.30
	5	7 (43.75)	11 (73.33)		3.17 (1.06 – 9.48)	0.04
	6	8 (50.00)	5 (33.33)		0.50 (0.18 – 1.39)	0.18
Change in EASI from baseline, mean (SD)	Intervention + follow-up					0.81
	3	-1.17 (5.70)	-0.64 (4.99)	-0.45 (-3.64 – 2.73)		0.78
	4	-0.58 (7.19)	1.73 (6.14)	-1.95 (-5.55 – 1.65)		0.28
	5	-0.59 (7.79)	-0.71 (4.16)	-0.77 (-4.83 – 3.30)		0.71
	6	-0.94 (6.44)	-0.20 (5.61)	-1.45 (-5.92 – 3.03)		0.52
Change in IGA from baseline	Intervention + follow-up					0.86
Proportion of patients with a reduction of ≥2 points in IGA from baseline, n (%)	3	0 (0.00)	1 (3.45)			1.00[f]
	4	0 (0.00)	1 (4.76)			1.00[f]
	5	1 (6.25)	1 (6.67)			1.00[f]
	6	1 (6.25)	2 (13.33)			0.60[f]
Change in POEM from baseline, mean (SD)	Intervention + follow-up					0.77
	3	-4.04 (4.72)	-3.14 (5.78)	-0.91 (-4.11 – 2.30)		0.57
	4	-3.63 (5.56)	-2.76 (6.31)	-0.78 (-4.25 – 2.70)		0.66
	5	-3.06 (5.95)	-2.93 (7.44)	-0.14 (-3.83 – 3.54)		0.94
	6	-3.00 (3.93)	-0.87 (6.55)	-2.13 (-5.82 – 1.55)		0.25

Table S2. Results for the difference in secondary clinical outcomes – per-protocol analysis (*continued*)

Outcome	Time period or visit	Endolysin	Vehicle	Difference in change (95% CI)[a]	OR (95% CI)[b]	*P*-value[c]
Change in pruritus NRS from baseline, mean (SD)	Intervention + follow-up					0.50
	3	0.08 (1.53)	0.34 (1.88)	-0.24 (-1.13 – 0.65)		0.59
	4	-0.16 (1.34)	-0.10 (1.38)	-0.29 (-1.29 – 0.71)		0.56
	5	0.19 (1.56)	0.00 (1.69)	0.11 (-1.09 – 1.31)		0.85
	6	-0.06 (2.41)	0.80 (1.86)	-0.89 (-2.38 – 0.60)		0.24
Proportion of patients with a reduction of ≥2 points in pruritus NRS from baseline, n (%)	Intervention + follow-up					0.58
	3	8 (32.00)	7 (24.14)		0.63 (0.22 – 1.78)	0.39
	4	7 (36.84)	3 (14.29)		0.32 (0.07 – 1.38)	0.13
	5	5 (31.25)	5 (33.33)		1.06 (0.23 – 5.01)	0.94
	6	8 (50.00)	5 (33.33)		0.42 (0.10 – 1.81)	0.24
Proportion of patients with a reduction of ≥3 points in pruritus NRS from baseline, n (%)	3	3 (12.00)	3 (10.34)		0.41 (0.11 – 0.53)	1.00[f]
	4	0 (0.00)	2 (9.52)		0.69 (0.17 – 2.77)	0.49[f]
	5	2 (12.50)	3 (20.00)		0.83 (0.32 – 2.18)	0.65[f]
	6	5 (31.25)	4 (26.67)		0.50 (0.22 – 1.13)	1.00[f]
Change in Skindex-29 from baseline, mean (SD)	Intervention + follow-up					0.66
	3	-6.62 (9.39)	-5.41 (10.26)	-1.53 (-7.33 – 4.27)		0.60
	5	-6.14 (9.67)	-5.00 (15.72)	-2.21 (-10.58 – 6.16)		0.60
	6	-8.41 (14.56)	-2.99 (14.05)	-6.60 (-16.48 – 3.27)		0.19
Number of doctor reported flares from baseline, n (%)	Intervention[g]	2 (3.33)	10 (15.38)			0.03[†]
Number of patient reported flares from baseline, n (%)	Intervention[g]	5 (8.33)	6 (9.23)			0.86
Mean time (days) to doctor reported flare from baseline, median (IQR)	Intervention[h]	22.50[11]	42.00 (11.75-53.25)			0.76

Table S2. Results for the difference in secondary clinical outcomes – per-protocol analysis (*continued*)

Outcome	Time period or visit	Endolysin	Vehicle	Difference in change (95% CI)[a]	OR (95% CI)[b]	*P*-value[c]
Mean time (days) to patient reported flare from baseline, median (IQR)	Intervention[i]	39.00 (15.00-53.50)	29.00 (5.00-47.00)			0.66
	Follow-up[j]	14.00[k]	32.00[k]			NA
Number of patients with at least one (serious) AE, n[l]	Intervention + follow-up	33	33			1.00
Number of (serious) AEs, n[l]	Intervention + follow-up	61	53		1.20 (0.84 – 1.72)[m]	0.31

Abbreviations: AE, adverse event; CI, confidence interval; EASI, Eczema Area and Severity Index; IGA, Investigators Global Assessment; IQR, interquartile range; NA, not available; NRS, Numeric Rating Scale; POEM, Patient Oriented Eczema Measure; OR, odds ratio; SD, standard deviation. Patients included in analyses per visit in Staphefekt and placebo group: visit 3, n=25 and n=29; visit 4, n=19 and n=21; visit 5, n=16 and n=15; visit 6, n=16 and n=15. NOTE: Given the low number of patients using escape medication (n=5), we did not correct for its use in the analysis of the mean grams/week topical corticosteroid use. [a]Differences in change presented for analyses using a Linear Mixed-Effect model. [b]OR presented for analysis using a Generalized Linear Mixed-Effect model. [c]*P*-values were calculated using a Chi-Square test or Fisher's Exact test for categorical data, where appropriate. A non-parametric Mann-Whitney U Test for independent samples was used for continuous variables. The overall effect of endolysin treatment during the intervention and follow-up period ((Generalized) Linear Mixed-Effect models) was analyzed with a Likelihood-Ratio test and per visit using a Wald test with t-distribution. [d]Endolysin group n=14, vehicle group n=10. [e]Endolysin group n=15, vehicle group n=10. [f]Since the number of patients per cell are ≤5 a Fisher's Exact test per visit was used instead of a Generalized Linear Mixed-Effect model. A *P*-value of 0.0125 will be considered significant after Bonferroni correction. [g]For every visit from baseline through week 12, 60 in endolysin group and 65 in vehicle group, it was registered if a flare occurred yes/no. [h]Endolysin group n=2, vehicle group n=10. [i]Endolysin group n=5, vehicle group n=6. [j]Endolysin group n=1, vehicle group n=2. [k]No IQR because n≤2. [l]AEs that occurred until 8 weeks (follow-up period in intention-to-treat analysis) after the first protocol deviation, i.e. no use of endolysin or vehicle on total skin surface twice daily, were analyzed. [m]Rate ratio with 95% CI. [†]Significant result.

Table S3. Results for the difference in reduction of *S. aureus* 0.5 hour after first application determined by semi-quantitative culture

Analysis	Decrease in semi-quantitative culture of *S. aureus*[a]	Endolysin, n (%)[b]	Vehicle, n (%)[c]	*P*-value[d]
Intention-to-treat	Yes	6 (12.5)	4 (8.3)	0.74
	No	42 (87.5)	44 (91.7)	

Abbreviations: *S. aureus*, *Staphylococcus aureus*. [a]Decrease is defined as a decrease of at least 1 point on semi-quantitative scale (scale ranges from 0-4). [b]Patients included in analysis in endolysin group: n=49. [c]Patients included in in analysis in vehicle group: n=48. [d]*P*-values were calculated using a Fisher's Exact test for categorical data.

Table S4. Results for the difference in reduction of *S. aureus* from baseline determined by qPCR

Analysis	Time period	Log10 reduction in qPCR for *S. aureus*		*P*-value[a]
		Endolysin, n (%)	**Vehicle**, n (%)	
Intention-to-treat[b]	Visit 2a to visit 3	3 (6.5)[g]	4 (8.5)[g]	1.00
	Visit 2a to visit 5	3 (6.8)[h]	8 (18.2)[h]	0.20
Per-protocol[c]	Visit 2a to visit 3	2 (7.1)	3 (9.4)	1.00
	Visit 2a to visit 5	1 (6.3)	5 (35.6)[i]	0.07
***S. aureus* positive**[d,e,f]	Visit 2a to visit 3	3 (33.3)	4 (30.8)	1.00
	Visit 2a to visit 5	3 (33.3)	9 (66.7)	0.20

Abbreviations: *S. aureus*, *Staphylococcus aureus*; qPCR, quantitative polymerase chain reaction. [a]*P*-values were calculated using a Fisher's Exact test for categorical data. [b]Patients included in endolysin and vehicle group: visit 2a to visit 3, n=47 and n=47; visit 2a to visit 5, n=44 and n=44. [c]Patients included in endolysin and vehicle group: visit 2a to visit 3, n=25 and n=29; visit 2a to visit 5, n=16 and n=15. [d]Patients included in endolysin and vehicle group: visit 2a to visit 3, n=9 and n=13; visit 2a to visit 5, n=9 and n=12. [e]Analysis additionally to analyses described in the study protocol. [f]*S. aureus* positive is defined as having a positive qPCR at visit 2a (endolysin group n=10, vehicle group n=14). Missings: [g]n=1 (2.1%), [h]n=1 (2.3%), [i]n=1 (6.7%).

Table S5. Incidence of (non-) Treatment Emergent Adverse Events – Overall and per study phase

	Total (n=100)		**Endolysin (n=50)**		**Vehicle (n=50)**	
	Patients, n (%)	**Events**, n	**Patients**, n (%)	**Events**, n	**Patients**, n (%)	**Events**, n
Overall (V1-V6)	83 (83.0)	183	43 (86.0)	96	40 (80.0)	87
Run-in (V1-V2)						
At least 1 non-TEAE	21 (21.0)	27	11 (22.0)	14	10 (20.0)	13
At least 1 serious non-TEAE	0 (0.0)	0	0 (0.0)	0	0 (0.0)	0
At least 1 non-TEAE leading to study discontinuation	0 (0.0)	0	0 (0.0)	0	0 (0.0)	0
At least 1 non-TEAE leading to death	0 (0.0)	0	0 (0.0)	0	0 (0.0)	0
Intervention (V2-V5)						
At least 1 TEAE	73 (73.0)	125	36 (72.0)	67	37 (74.0)	58
At least 1 serious TEAE	0 (0.0)	0	0 (0.0)	0	0 (0.0)	0
At least 1 TEAE leading to study discontinuation	3 (3.0)	3	1 (2.0)	1	2 (4.0)	2
At least 1 TEAE leading to death	0 (0.0)	0	0 (0.0)	0	0 (0.0)	0
Follow-up (V5-V6)						
At least 1 TEAE	25 (25.0)	31	15 (30.0)	15	10 (20.0)	16
At least 1 serious TEAE	1 (1.0)	1	1 (2.0)	1	0 (0.0)	0
At least 1 TEAE leading to study discontinuation	1 (1.0)	1	1 (2.0)	1	0 (0.0)	0
At least 1 TEAE leading to death	0 (0.0)	0	0 (0.0)	0	0 (0.0)	0

Abbreviations: TEAE, Treatment Emergent Adverse Event (adverse events after the first endolysin or vehicle administration); V, visit.

Table S6. Overall incidence of Treatment Emergent Adverse Events specified

	Total (n=100)		Endolysin (n=50)		Vehicle (n=50)	
	Patients, n (%)	**Events**, n	**Patients**, n (%)	**Events**, n	**Patients**, n (%)	**Events**, n
Any Treatment Emergent Adverse Event	83 (83.0)	183	43 (86.0)	96	40 (80.0)	87
Infectious Diseases (including bacterial, viral and fungal infections)	**47 (47.0)**	**65**	**26 (52.0)**	**40**	**21 (42.0)**	**25**
Common cold	26 (26.0)	29	18 (36.0)	20	8 (16.0)	9
Dental inflammation	1 (1.0)	1	1 (2.0)	1	0 (0.0)	0
Ear infection	1 (1.0)	1	0 (0.0)	0	1 (2.0)	1
Influenza	15 (15.0)	15	7 (14.0)	7	8 (16.0)	8
Herpes labialis	5 (5.0)	6	3 (6.0)	4	2 (4.0)	2
Impetiginized dermatitis	1 (1.0)	1	1 (2.0)	1	0 (0.0)	0
Joint and muscle inflammation	3 (3.0)	3	2 (4.0)	2	1 (2.0)	1
Ophthalmic infection	7 (7.0)	7	4 (8.0)	4	3 (6.0)	3
Pneumonia	2 (2.0)	2	1 (2.0)	1	1 (2.0)	1
Allergies	**6 (6.0)**	**6**	**0 (0.0)**	**0**	**6 (12.0)**	**6**
Food allergy	1 (1.0)	1	0 (0.0)	0	1 (2.0)	13
Allergic rhinoconjunctivitis	3 (3.0)	3	0 (0.0)	0	3 (6.0)	2
Allergic rash	2 (2.0)	2	0 (0.0)	0	2 (4.0)	2
Metabolic/Endocrine/Nutritional	**0 (0.0)**	**0**	**0 (0.0)**	**0**	**0 (0.0)**	**0**
Musculoskeletal	**2 (2.0)**	**2**	**1 (2.0)**	**1**	**1 (2.0)**	**1**
Joint and muscle pain	1 (1.0)	1	1 (2.0)	1	0 (0.0)	0
Lower back pain	1 (1.0)	1	0 (0.0)	0	1 (2.0)	1
Dermatologic	**24 (24.0)**	**26**	**13 (26.0)**	**14**	**11 (22.0)**	**12**
Culicosis	1 (1.0)	1	1 (2.0)	1	0 (0.0)	0
Dermatomycosis	5 (5.0)	5	4 (8.0)	4	1 (2.0)	1
Hyperkeratotic eczema of the hands	2 (2.0)	2	2 (4.0)	2	0 (0.0)	0
Intertrigo	1 (1.0)	1	0 (0.0)	0	1 (2.0)	1
Local dry skin	1 (1.0)	1	1 (2.0)	1	0 (0.0)	0
Papules	2 (2.0)	2	2 (4.0)	2	0 (0.0)	0
Paronychia	1 (1.0)	1	1 (2.0)	1	0 (0.0)	0
Perioral dermatitis	2 (2.0)	2	0 (0.0)	0	2 (4.0)	2
Pustulosis	6 (6.0)	6	3 (6.0)	3	3 (6.0)	3
Rosacea	2 (2.0)	2	0 (0.0)	0	2 (4.0)	2
Skin lesions of unknown origin	2 (2.0)	2	0 (0.0)	0	2 (4.0)	2
Tinea versicolor	1 (1.0)	1	0 (0.0)	0	1 (2.0)	1
HEENT[a]	**5 (5.0)**	**5**	**2 (4.0)**	**2**	**3 (6.0)**	**3**
Increased eye pressure	1 (1.0)	1	1 (2.0)	1	0 (0.0)	0
Tinnitus	1 (1.0)	1	0 (0.0)	0	1 (2.0)	1

Table S6. Overall incidence of Treatment Emergent Adverse Events specified (*continued*)

	Total (n=100)		**Endolysin (n=50)**		**Vehicle (n=50)**	
	Patients, n (%)	**Events**, n	**Patients**, n (%)	**Events**, n	**Patients**, n (%)	**Events**, n
Tooth extraction	3 (3.0)	3	1 (2.0)	1	2 (4.0)	2
Respiratory	**5 (5.0)**	**5**	**3 (6.0)**	**3**	**2 (4.0)**	**2**
Breathing problems	1 (1.0)	1	0 (0.0)	0	1 (2.0)	1
Dyspnea	1 (1.0)	1	1 (2.0)	1	0 (0.0)	0
Exacerbation asthma	2 (2.0)	2	1 (2.0)	1	1 (2.0)	1
Pleural effusion	1 (1.0)	1	1 (2.0)	1	0 (0.0)	0
Cardiovascular	**0 (0.0)**	**0**	**0 (0.0)**	**0**	**0 (0.0)**	**0**
Gastrointestinal/Hepatic	**6 (6.0)**	**6**	**3 (6.0)**	**3**	**3 (6.0)**	**3**
Gastroenteritis	6 (6.0)	6	3 (6.0)	3	3 (6.0)	3
Genitourinary/Renal	**0 (0.0)**	**0**	**0 (0.0)**	**0**	**0 (0.0)**	**0**
Neurologic	**2 (2.0)**	**2**	**1 (2.0)**	**1**	**1 (2.0)**	**1**
Headache	2 (2.0)	2	1 (2.0)	1	1 (2.0)	1
Psychiatric/Psychosocial	**2 (2.0)**	**2**	**1 (2.0)**	**1**	**1 (2.0)**	**1**
Depression	2 (2.0)	2	1 (2.0)	1	1 (2.0)	1
Gynaecologic/Obstetric	**1 (1.0)**	**1**	**0 (0.0)**	**0**	**1 (2.0)**	**1**
Non-specific menstrual complaints	1 (1.0)	1	0 (0.0)	0	1 (2.0)	1
Skin symptoms after application of study medication	**45 (45.0)**	**48**	**21 (42.0)**	**23**	**24 (48.0)**	**25**
Local reaction after application of study medication (e.g. wounds, itch, irritated skin)	45 (45.0)	48	21 (42.0)	23	24 (48.0)	25
Other	**14 (14.0)**	**15**	**8 (16.0)**	**8**	**6 (12.0)**	**7**
Bruises	1 (1.0)	1	0 (0.0)	0	1 (2.0)	1
Fatigue	2 (2.0)	2	1 (2.0)	1	1 (2.0)	1
Malaise	11 (11.0)	12	7 (14.0)	7	4 (8.0)	5

[a]HEENT: Head, Eye, Ear, Nose and Throat.

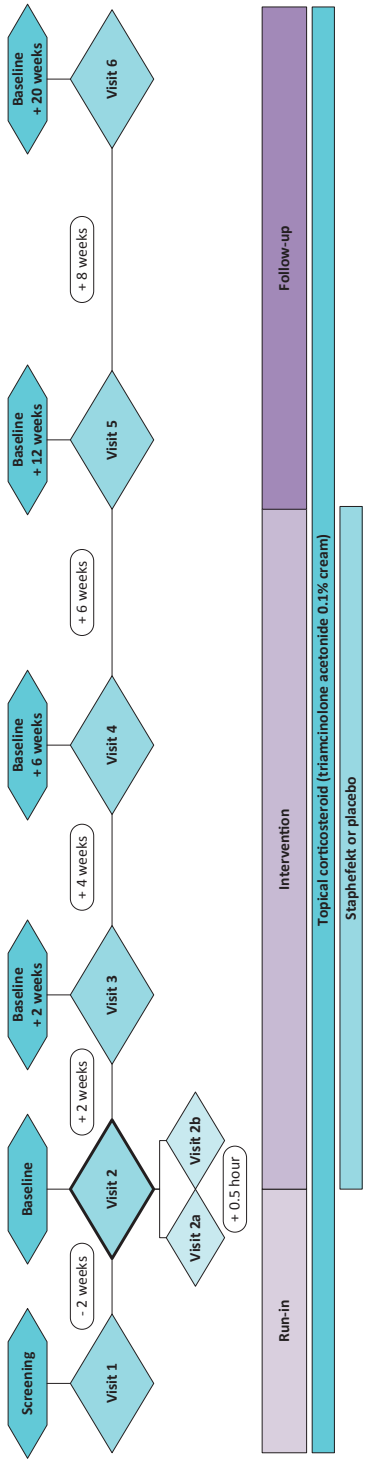

Figure S1. Study timeline

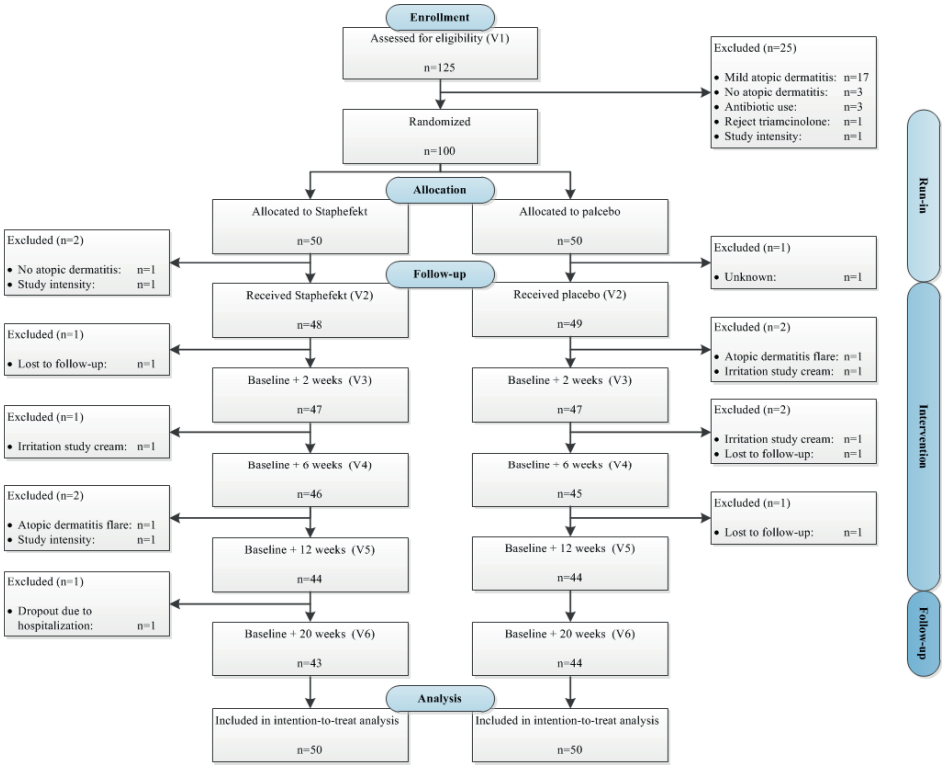

Figure S2. Flowchart of the study design

Chapter 6

General discussion

Atopic dermatitis (AD) is the most common inflammatory skin disease, with a great impact on the quality of life of both patients and their families. Both the pathophysiology and the clinical presentation of AD are highly heterogeneous due to underlying changes in the microbial composition, an impaired skin barrier and an altered immunological response. Although multiple risk factors and key pathogenic aspects have been identified for AD, it remains largely unknown how these mechanisms interact and how they result in the inflammation as seen in AD. Increasing insights into the various mechanisms underlying AD may help to develop preventive and therapeutic interventions. The aim of this thesis was to gain more insight into the role of the main players in the pathophysiology of AD. In particular, we focused on (i) the microbiome, characterized by increased *Staphylococcus (S.) aureus* abundance and (ii) skin barrier defects caused by mutations in the filaggrin gene (*FLG*). This chapter provides a general overview of the main findings and clinical implications, considering methodological considerations. In addition, we suggest directions for future research.

THE ROLE OF *S. AUREUS* IN THE PATHOGENESIS OF AD

Higher *S. aureus* colonization rates in AD patients have been known for a long time. However, it remains a topic of discussion whether *S. aureus* is actively involved in AD development or is merely a consequence of AD-related skin alterations that are caused by both physical barrier and immunological defects. Studies investigating whether *S. aureus* colonization precedes the clinical diagnosis of AD show conflicting results.[1-4] Although some studies showed associations between early *S. aureus* colonization and subsequent development of AD, this does not prove causality.[4] As described in Chapter 1, the skin microbiome, skin barrier and immune system form a well-balanced eco-system that contributes to the mechanical and immunological barrier function of the skin. Any dysfunction in one of the three elements will lead to downstream effects in the other elements, causing inflammation characteristic of AD. These interactions were also demonstrated in recent AD studies that observed a decrease in *S. aureus* abundance after treatment with corticosteroids or coal tar, that do not have any antimicrobial properties.[5, 6] It can be hypothesized that skin barrier defects and immunological changes occur well before diagnosis and lead to increased *S. aureus* colonization.

Although *S. aureus* colonization might not be the primary cause in the pathogenesis of AD, there is evidence that *S. aureus* can aggravate skin barrier damage and inflammation by the secretion of virulence factors, including surface proteins and toxins (Chapter 1). However, this bacterium can be present both as human commensal organism and as a pathogen causing inflammation.[7] Literature on the mechanisms that enable *S. aureus* to

transform from commensal colonizer to a pathogenic organism in AD is lacking. We aimed to further investigate the role of *S. aureus* in the pathogenesis of AD and to assess the effect of treatment interfering with the skin microbiome.

S. aureus strain diversity in AD

One of the discussed topics is whether AD patients are colonized by different *S. aureus* strains as compared to the general population. In addition, it has been suggested that specific *S. aureus* strains are associated with a more severe disease and that temporal variation in colonizing strain can aggravate inflammation.[8, 9] However, literature on this topic is contradicting.[9-12]

In Chapter 2, *S. aureus* strains from both nose and skin were characterized using the *spa*-typing technique in 96 adult AD patients. Temporal variation in colonizing strain was investigated over the course of two weeks, in which treatment was standardized with topical corticosteroids (TSC), and links to disease severity were examined. Our results showed a great strain heterogeneity among the AD patients, comparable to the general population. Interestingly, the intra-individual variation was small, with the majority of the individual patients colonized by the same *spa*-type in both nose and skin, as well as after a two-week treatment period that improved the clinical symptoms. No predominant *spa*-type nor temporal variation was associated with more severe disease.

Our results were in line with previous publications investigating the genetic composition of *S. aureus*, concluding that there are no specific *S. aureus* strains colonizing AD patients compared to the general population.[10, 11] In addition, our results were in agreement with previous studies that did not show correlations between *S. aureus* strains and disease severity.[12, 13] Of important notice is that genetic diversity between strains can be missed with *spa*-typing since it is based on a single gene. Whole-genome sequencing (WGS) allows a more detailed analysis of the relationship between strains.[14] Nonetheless, a recent study in pediatric AD patients, using WGS to discriminate between *S. aureus* strains, showed results that were similar to our findings.[15] During disease flares, an increased growth of the endogenous *S. aureus* strain was observed. This strain was unique per patient, confirming the individuality of strains per patient that is likely affected by host, genetic and environmental factors.[16]

Lessons learned from treatment affecting the microbiome

We can learn a lot about the pathogenesis in AD from evaluating the effect of its treatment. As mentioned above, previous agents without specific anti-microbial effects led to a reduction in *S. aureus* abundance by dampening inflammation and restoring the skin barrier function.[5, 6] This is in line with results from our thesis. In chapter 2 and 5.2 we showed

that treatment with TCS in 100 adult AD patients led to a significant reduction in disease severity and *S. aureus* load. In addition, children with difficult to treat AD that were randomized to treatment in alpine climate (Chapter 5.1), showed that clinical improvement was accompanied by a reduction in *S. aureus* abundance on both lesional and nonlesional skin. We also observed that complete eradication of *S. aureus* was not needed for disease control. These results suggest that *S. aureus* can be present on AD skin without causing overt inflammation and that increased abundance is associated with pathogenicity.[6, 17]

By specifically targeting *S. aureus* on the skin of patients with AD, we might learn how a reduction and/or eradication of *S. aureus* affects the clinical symptoms in AD. In Chapter 5.2, we evaluated the effect of treatment with an endolysin that selectively targets *S. aureus* in adult AD patients with clinically non-infected AD. After a two-week run-in period to standardize TCS treatment, 100 patients were assigned to a 12-week intervention with topical endoloysin or placebo. In this study we did not observe a reduction in *S. aureus* abundance, the use of TCS or clinical disease severity scores. Based on these results we could not further unravel the contribution of *S. aureus* to the pathogenesis of AD. Our results can be explained by several hypotheses that should be considered in future studies. First, the use of TCA and emollients in the run-in phase resulted in a significant decrease in AD severity, which could have masked a possible benefit of endolysin treatment. Second, since only 56% of the patients were persistently colonized by *S. aureus* before the start of intervention, the group of patients that would possibly benefit most from this treatment was small. Third, both the pathogenesis and clinical symptoms in AD are highly heterogeneous. Therefore, it is possible that not all AD patients benefit from treatment that reduces and/or eradicates *S. aureus* on the skin.

THE ROLE OF *FLG* SKIN BARRIER DEFECTS IN THE PATHOGENESIS OF AD

FLG mutations are a significant risk factor for the development of AD (odds ratio (OR) of 3.12).[18] However, *FLG* mutations are neither necessary nor sufficient to develop AD. This can be demonstrated by the prevalence estimates in the study cohorts that were included in this thesis. Chapter 3.1 included 358 non-AD children of the Generation R cohort, of which 6% had a mutation in *FLG*.[19] The prevalence of mutation carriers in AD patients ranged from 16% in the 103 AD children of the Generation R cohort (Chapter 3.2) to 40% in the 77 children of the DAVOS cohort (Chapter 3.1). The difference in the prevalence estimates is also indirect evidence that *FLG* mutations are associated with a distinct phenotype. Namely, the Generation-R children were a sample of the general population in

which we expect most children to have mild AD, whereas the children in the DAVOS study were included based on having difficult to treat AD.

Indeed, numerous studies have associated *FLG* mutations with AD and show that a mutation in this gene predisposes for early onset AD that persists into adulthood, increased AD severity and other allergic conditions (i.e., food allergies, asthma, and allergic rhinitis).[20-22] The exact mechanisms contributing to this disease modification effect remain largely unknown. Therefore, we aimed to investigate whether *FLG* mutations lead to alterations in the microbiome and/or immune cell composition, contributing to the symptoms in AD. In addition, we investigated the use of a non-invasive biomarker to stratify patients based on *FLG* mutation status.

FLG mutations and the microbiome in AD

In Chapter 3.1, we investigated the association between *FLG* genotypes and the microbiome composition of the nose, lesional and nonlesional skin in 79 children with difficult to treat AD. We showed that the overall microbial composition was significantly affected by the sampled niche (nose, lesional skin, nonlesional skin) and *FLG* mutation status (wild type vs. mutation carriers). We also performed subgroup analyses to investigate the effect of *FLG* mutation status on the microbiome in each separate niche. Within the nose, lesional and nonlesional skin separately, no significant association with *FLG* mutation status was found. Shannon diversity and *S. aureus* abundance were significantly affected by the niche, but not by the presence of a *FLG* mutation.

The absence of associations between *FLG* mutation status and *S. aureus* load was somewhat surprising in comparison to previous *in vitro* studies.[23-25] *FLG* is mainly thought to exert its effect through the production of natural moisturizing factor (NMF) in the stratum corneum (SC).[26] *In vitro* studies have shown that decreased NMF led to increased skin pH and altered corneocyte adhesion, that facilitated colonization by *S. aureus*.[23-25] Clinical studies investigating whether *S. aureus* colonization is more common in *FLG* mutation carriers compared to wild-type AD patients, showed conflicting results.[25, 27-30] Until now, one previous study investigated the association between *FLG* mutation status and the entire bacterial microbiome on the skin and in the nasal cavity.[31] Within this population of 56 adult AD patients, the microbiome on the nonlesional skin was significantly associated with *FLG* genotype.[31]

The results from our study indicated that there was only a small effect for *FLG* mutation status and/or that there was a lack of statistical power to identify differences in the subgroup analyses. Another possible explanation for the results in our study and the varying results among clinical AD studies, is the presence of other factors that affect filaggrin

expression and contribute to the barrier defect in AD. Especially disease severity has been shown to downregulate the levels of NMF in both lesional and nonlesional skin through the overexpression of T helper (Th)22 cytokines.[32] As the vast majority of the children in the DAVOS trial had severe or very severe disease at the moment of microbiome sampling, filaggrin expression was likely downregulated in both *FLG* mutant carriers and wild-type patients.

FLG mutations and T- and B-cell subsets in the general pediatric population and in children with AD

Chapter 3.2 has been the first study that investigated the impact of *FLG* mutation status on circulating immune cell numbers in a general pediatric population. Common *FLG* mutations (2282del4, R2447*, R501* and S3247*) and T- and B-cell subsets were determined in a population-based birth cohort study (Generation R), including 523 children of European genetic ancestry and a subgroup of 102 AD patients, selected by parental questionnaires.

In this general pediatric population, we observed higher Th22 cell numbers in children with a mutation in *FLG*. This finding might represent the immunological response to an altered skin barrier caused by *FLG* mutations. Interestingly, other than the findings in Chapter 3.2, increased numbers of Th22 cells have also been observed in the skin and circulation of patients with AD.[33, 34] As IL-22 contributes to the skin integrity and the defense against pathogens, we hypothesized that the increased Th22 cell numbers might contribute to skin homeostasis to prevent further inflammatory processes leading to AD.[35, 36] Another theory is that the higher Th22 could represent some level of inflammation without apparent clinical AD symptoms as is seen in nonlesional skin. However, more studies are needed to further elucidate the effect of skin barrier defects on immune cells in the skin and circulation, and the role of Th22 in both AD and non-AD children.

In the subgroup of children with AD, no associations between *FLG* mutation status and T- and B-cell subsets were found. This was an interesting finding since *FLG* mutations allow increased penetration of irritants, antigens and allergens into the epidermis where they can interact with antigen-presenting cells and provoke an immunological response.[37] In addition, previous case series with a maximum of six adult AD patients, showed increased thymus-emigrated regulatory T (Treg) cells, Th2 and Th17 in the circulation of *FLG* mutation carriers compared to wild-type patients.[38, 39] It should be taken into account that our subgroup of AD patients was relatively small. Since the AD definition in our study population was based on ever having physician diagnosed AD before or at the age of 10 year, some children might have outgrown their disease, which could have affected their immunophenotype. In addition, as we only measured immune cell numbers in the circulation, we could therefore not exclude a migration of immune cells to the skin.[40] Also in light

of these findings, we highlight that the pathogenesis of AD is highly complex with potential other genetic and environmental factors leading to skin barrier defects that might affect immune cell numbers in AD. Identifying differences in immune cell numbers in the skin and circulation in larger groups of individuals might help identify immune profiles based on *FLG* mutation status that could help to develop targeted therapies to prevent and/or treat the symptoms of AD in these subgroups.

NMF as biomarker for *FLG* mutation status in AD

The exact mechanisms that lead to the transition from inherited barrier defect, caused by *FLG* mutations, to the clinical manifestations of inflammatory eczematous lesions remains to be fully elucidated. However, *FLG* mutations are a widely replicated major risk factor for AD and might be used both as a screening marker and as a prognostic biomarker for early onset, severe AD.[41, 42] Considering that AD commonly affects children, the development of non-invasive biomarkers are of added value. The NMF content in the SC of the thenar eminence has been proposed as biomarker to stratify patients based on *FLG* genotype.[26, 43, 44] The SC of the thenar eminence is approximately 100µm, compared to a thickness of 10-15µm on the volar aspect of the arm. Therefore it regenerates less quickly and is thought to be less affected by acute changes in disease severity and to have more stable levels of NMF.[45]

In Chapter 5, we investigated the use of measuring NMF in the thenar eminence's non-lesional SC with Raman Spectroscopy as potential biomarker for *FLG* mutation status in a multi-ethnic population of 101 children with mild to severe AD. Raman spectroscopy can measure the NMF content in skin rapidly, non-invasively and provides an instant result. We concluded that the NMF content in the SC of the thenar eminence could accurately predict *FLG* mutation status (wild type vs ≥ 1 mutation) with an area under the curve of 0.93 (95% CI 0.87-0.99). No correlation was found between acute disease severity and NMF content, thereby excluding a potential influence of acute disease severity on NMF values measured on the thenar eminence's nonlesional skin. The cutoff value for NMF (1.03 a.u.), that was determined by maximizing of the sum of sensitivity and specificity (97% and of 87%, respectively), was highly comparable to a previous determined cutoff value in a selected AD cohort including children from Irish ancestry.[43]

Among the wild-type patients, 13% had low NMF content in the SC. These low NMF values in the SC of the thenar eminence were not associated with disease severity. Other factors that can influence the levels of NMF in the SC are *FLG* intragenetic copy number variation (CNV), protease activity (e.g. bleomycin hydrolase (BH) and Caspase 14) and humidity.[46-49] Therefore we can argue that NMF measurement is potentially more useful to screen

for skin barrier defects than genetic analysis, because it measures the functional consequences of all genetic and environmental factors that affect NMF production.

FUTURE PERSPECTIVES

Investigating *S. aureus* gene expression and antibody responses in the pathogenesis of AD

Based on our results and previous literature, we assume that no specific strains of *S. aureus* colonize AD patients in comparison to healthy individuals. In addition, the majority of the AD patients is persistently colonized by the same *S. aureus* strain over the course of disease, varying from mild/stable disease to disease flare. Therefore it remains to be elucidated further how this bacterium can be present both as commensal organism and as pathogen leading to the symptoms of AD.[7] We suggest two possible mechanisms that are of interest for future research.

First, it is possible that AD patients have an altered immunological response against *S. aureus* that causes the symptoms in AD. A previous publication found that AD patients more often had IgE antibody response against *S. aureus* superantigens compared to healthy controls.[50] In addition, IgG mediated immune response against immune modulating *S. aureus* antigens was associated with disease severity in children with AD.[51] As the antibody response was only directed towards a subset of *S. aureus* antigens rather than the entire range of antigens, these results suggest an altered immune response against specific antigens instead of a reflection of increased *S. aureus* load in AD.

A second possible mechanism is that *S. aureus* expresses different genes during different disease states. This varying gene expression provokes an altered immunological response leading to the symptoms of AD, varying from mild disease to disease flares and to the chronic phase characterized by lichenification. Cutaneous AD colonization models, in which mice were topically colonized with human *S. aureus* strains, have investigated the immunological response against 10 phylogenetically distinct *S. aureus* isolates from AD patients and controls.[15] These results showed that cutaneous T-cell infiltration and increased epidermal thickening was a common response to *S. aureus* strains from patients with a more severe AD flares.[15] In addition, sepsis models have investigated *S. aureus* gene expression during different disease states, ranging from asymptomatic colonization to bacteremia.[52] Interestingly, during nasal colonization, adhesin genes were most commonly expressed, whereas upregulation of immune evasion and exotoxin genes were observed during bacteremia.[52] This shows the ability of *S. aureus* to transfer from commensal to pathogen.

Until now, the differential expression of genes has not been proven for AD and studies investigating the immunological response against *S. aureus* are scarce. Therefore, it would be of interest to determine *S. aureus* gene expression and immunological responses over the course of disease and correlate this to the clinical symptoms in AD. This could lead to further insights into the working mechanisms and the pathogenic role of this bacterium in the heterogeneous phenotype of AD.

Identify AD patients who might benefit from targeted anti-*S. aureus* therapy

Based on our results from Chapter 3.2, it can be doubted whether topical endolysin treatment is the most convenient strategy to target *S. aureus* on the skin. However, major drawbacks of currently available antimicrobial therapies (i.e., antibiotics and bleach baths) are the impact on the commensal microbiota and/or the development of bacterial resistance.[53-55] Therefore, it is desirable to develop and investigate pathogen-specific anti-staphylococcal agents for long-term use that do not cause resistance or harm the beneficial commensal microbiome. Novel targeting strategies are under investigation. Mouse models and pre-clinical trials showed reduced *S. aureus* colonization and disease severity after the application of topical coagulase-negative *Staphylococcus* (coNS) that produce highly potent antimicrobial peptides against *S. aureus* molecules.[56, 57] Also other skin commensal species, vaccines and monoclonal antibodies (moAB) that neutralize one or more *S. aureus* toxins are being further investigated, but not yet available for clinical use.[58, 59]

It is of interest to identify patients that might benefit from these anti-staphylococcal agents. Current guidelines do not recommend anti-microbial treatment in clinically non-infected AD.[60, 61] However, based on the results of this thesis we are of opinion that this heterogeneous group of clinically non-infected AD cannot be considered as a single entity. The symptoms can vary from mild symptoms to disease flares and *S. aureus* is also thought to contribute to the development of the chronic AD lesions that are characterized by lichenification.[62] Therefore novel agents, including endolysins, that specifically target *S. aureus* should be investigated in subgroups of AD patients that are clearly defined based on phenotypic characteristics.

Investigating the benefit of intranasal treatment in AD

In addition to the skin, we suggest to further investigate the added value of pathogen-specific anti-staphylococcal agents in different niches of the body. In Chapter 2 we showed high *S. aureus* colonization rates in the nose of patients with AD. In addition, up to 79% of the patients positive for *S. aureus* had identical *spa*-types in nose and skin, indicating a role for endogenous *S. aureus* transmission from nose to skin and/or vice versa. Intranasal treatment with mupirocin is common in hospitalized patients and has shown

to be successful in decolonizing other body sites and preventing infection in surgical and dialysis patients.[63-65] A randomized placebo-controlled study observed that bleach baths in combination with intranasal mupirocin over the course of 3 months improved AD symptoms, compared to intranasal petrolatum ointment treatment and plain water baths.[66] Since other studies investigating the added value of intranasal anti-staphylococcal agents in AD are lacking, more research is needed.

The use of NMF in future association studies with *FLG* mutations in AD

FLG genotype is the major determinant of NMF levels in the SC.[67] However, reduced levels of NMF have been observed in both wild-type patients and *FLG* mutation carriers, making skin barrier defects a common trait in AD.[67, 68] Especially disease severity is thought to impact the levels of NMF in the SC through the increased presence of Th2 cytokines (IL-4 and IL-13).[32] Also other mechanisms have impact on the levels of NMF, including *FLG* CNV, protease function, humidity and possible unknown mutations in other genes of the epidermal differentiation complex (EDC).[46, 47] Therefore, it is of interest to investigate the relative impact of *FLG* mutations in future association studies. In line with this, we suggest to measure the functional consequences of the skin barrier defects (i.e., NMF) and correct for *FLG* genotype. The exact location for this measurement might depend on the research question as regional variations in NMF content have been described.[69] For measuring local processes, including the microbiome on the skin surface, it would be relevant to measure NMF at the same location as the microbial swabs are collected. For more acute systemic processes, including circulating immune cell numbers, nonlesional skin is thought to have higher correlations with disease severity than lesional skin.[70, 71] However, as it was recently shown that the amount of NMF varies in nonlesional skin, depending on distance from the eczematous skin lesion, there is need for standardization of the sampling location.[72]

Further determining the clinical utility of determining *FLG* mutation status in AD

We have highlighted that *FLG* carriers are at increased risk to develop AD that is associated with early onset disease, increased AD severity and other allergic conditions (i.e., food allergies, asthma, and allergic rhinitis).[20-22] The clinical utility of identifying children with a mutation in this gene needs to be further determined. We are only at the beginning of investigating whether AD patients with *FLG* mutations respond differently to available treatments.[73, 74] Interventions to prevent the development of AD in these high risk children are lacking. Two large randomized controlled trials (RCTs) did not found evidence for daily emollient treatment in the first year of life to prevent AD in high-risk children (based on family history and *FLG* mutation status of the child).[75, 76] In addition, AD children with *FLG* mutations are at increased risk to develop other allergic conditions. Early pro-active interventions in this patient group might prevent the development of allergen sensitization and associated food allergy, asthma and allergic rhinitis.[77] As we have argued that NMF

measurement is potentially more useful to screen for skin barrier defects than genetic analysis, clinical trials including both *FLG* mutation status and NMF measurement should further determine the translational value of these measurements.

Integrating biomarkers related to the skin barrier, immune system and skin microbiome

Besides a non-invasive biomarker to determine *FLG* mutation status, there is need for the development of other biomarkers. Especially in a disease such as AD with a complex and heterogeneous pathophysiology and clinical presentation, it is unlikely that currently available and newly developed highly specific treatments are effective in all AD patients. This thesis highlighted that a defect in one of the three main pathological aspects, including the skin microbiome, skin barrier and immune system, is not likely to cause disease on its own. Alterations in all three aspects and their interactions contribute to the pathogenesis of AD and are likely to differ per patient and might also change over the lifetime of an individual. For instance, not all patients are carrier of a *FLG* mutation and not all patients are colonized by *S. aureus*. By defining subgroups of patients, based on the underlying disease pathways, we can stratify this highly heterogeneous disease into endotypes for more personalized care. Previous research has identified clusters with distinct serum biomarker profiles and overlapping clinical phenotypes that represented distinct disease endotypes.[78] They could identify clusters that shared a Th2 cytokine–low and pauci-inflammatory mediator state presenting with a greater frequency of the lichenified skin phenotype.[78] These patients would hypothetically benefit less from Th2-targeting agents. Adding *S. aureus* abundance, *S. aureus* gene expression profiles, skin barrier parameters and immunological parameters in these models and associate clusters with clinical characteristics could identify the underlying pathophysiologic processes and identify targets for personalized treatment. The next step would be to perform clinical intervention trials to evaluate treatment targeting patient specific disease pathways.

CONCLUDING REMARKS

This thesis shows that the microbiome, characterized by an increased *S. aureus* abundance, is an important aggravating factor in AD. The majority of the patients with AD remains colonized with the same *S. aureus* strain over time in both nose and skin. Since the colonizing strains are not unique for AD patients, we hypothesize that this bacterium exerts its effect through increased abundance, gene expression and the immunological response towards it. Another common trait in AD patients, is the impairment of the skin barrier that can be associated with *FLG* mutations in up to 40% of the patients. Mutations in this gene are associated with a more severe phenotype and appear to affect the

overall microbiome in these patients. Although not confirmed in this thesis, the increased penetration of external stimuli is likely to provoke a different immunological response compared to wild-type patients. Multiple other genetic and environmental factors affect the filaggrin expression in the SC, resulting in lower levels of NMF. By measuring both NMF levels and *FLG* mutations we might determine the relative impact of *FLG* mutation status on the microbiome and immune cell numbers.

Our findings highlight that the pathogenesis of AD cannot be explained by only one mechanism but involves interactions between the skin microbiome, skin barrier and immune system. It is of interest to further investigate these interactions to establish a more comprehensive disease model for AD. Large patient groups with patients of different ages and ethical backgrounds are necessary. Last, we advise to further define subgroups of patients based on the underlying disease pathways to enable personalized treatment. More than individual biomarkers, a combination of different biomarkers related to the skin microbiome, skin barrier and immune system can be used for the stratification of this heterogeneous disease for personalized care in the future.

REFERENCES

1. Skov L, Halkjaer LB, Agner T, Frimodt-Moller N, Jarlov JO, Bisgaard H. Neonatal colonization with Staphylococcus aureus is not associated with development of atopic dermatitis. Br J Dermatol. 2009;160(6):1286-91.
2. Lebon A, Labout JA, Verbrugh HA, Jaddoe VW, Hofman A, van Wamel WJ, et al. Role of Staphylococcus aureus nasal colonization in atopic dermatitis in infants: the Generation R Study. Arch Pediatr Adolesc Med. 2009;163(8):745-9.
3. Kennedy EA, Connolly J, Hourihane JO, Fallon PG, McLean WH, Murray D, et al. Skin microbiome before development of atopic dermatitis: Early colonization with commensal staphylococci at 2 months is associated with a lower risk of atopic dermatitis at 1 year. J Allergy Clin Immunol. 2017;139(1):166-72.
4. Meylan P, Lang C, Mermoud S, Johannsen A, Norrenberg S, Hohl D, et al. Skin Colonization by Staphylococcus aureus Precedes the Clinical Diagnosis of Atopic Dermatitis in Infancy. J Invest Dermatol. 2017;137(12):2497-504.
5. Smits JPH, Ederveen THA, Rikken G, van den Brink NJM, van Vlijmen-Willems IMJJ, Boekhorst J, et al. Targeting the Cutaneous Microbiota in Atopic Dermatitis by Coal Tar via AHR-Dependent Induction of Antimicrobial Peptides. Journal of Investigative Dermatology. 2020;140(2):415-24.e10.
6. Kong HH, Oh J, Deming C, Conlan S, Grice EA, Beatson MA, et al. Temporal shifts in the skin microbiome associated with disease flares and treatment in children with atopic dermatitis. Genome Res. 2012;22(5):850-9.
7. Wertheim HF, Melles DC, Vos MC, van Leeuwen W, van Belkum A, Verbrugh HA, et al. The role of nasal carriage in Staphylococcus aureus infections. Lancet Infect Dis. 2005;5(12):751-62.
8. Lomholt H, Andersen KE, Kilian M. Staphylococcus aureus clonal dynamics and virulence factors in children with atopic dermatitis. J Invest Dermatol. 2005;125(5):977-82.
9. Clausen ML, Edslev SM, Norreslet LB, Sorensen JA, Andersen PS, Agner T. Temporal variation of Staphylococcus aureus clonal complexes in atopic dermatitis: a follow-up study. Br J Dermatol. 2019;180(1):181-6.
10. Yeung M, Balma-Mena A, Shear N, Simor A, Pope E, Walsh S, et al. Identification of major clonal complexes and toxin producing strains among Staphylococcus aureus associated with atopic dermatitis. Microbes Infect. 2011;13(2):189-97.
11. Kim DW, Park JY, Park KD, Kim TH, Lee WJ, Lee SJ, et al. Are there predominant strains and toxins of Staphylococcus aureus in atopic dermatitis patients? Genotypic characterization and toxin determination of S. aureus isolated in adolescent and adult patients with atopic dermatitis. J Dermatol. 2009;36(2):75-81.
12. Alsterholm M, Strombeck L, Ljung A, Karami N, Widjestam J, Gillstedt M, et al. Variation in Staphylococcus aureus Colonization in Relation to Disease Severity in Adults with Atopic Dermatitis during a Five-month Follow-up. Acta Derm Venereol. 2017;97(7):802-7.
13. Rojo A, Aguinaga A, Monecke S, Yuste JR, Gastaminza G, Espana A. Staphylococcus aureus genomic pattern and atopic dermatitis: may factors other than superantigens be involved? Eur J Clin Microbiol Infect Dis. 2014;33(4):651-8.
14. Harris SR, Cartwright EJ, Török ME, Holden MT, Brown NM, Ogilvy-Stuart AL, et al. Whole-genome sequencing for analysis of an outbreak of meticillin-resistant Staphylococcus aureus: a descriptive study. Lancet Infect Dis. 2013;13(2):130-6.

15. Byrd AL, Deming C, Cassidy SKB, Harrison OJ, Ng WI, Conlan S, et al. Staphylococcus aureus and Staphylococcus epidermidis strain diversity underlying pediatric atopic dermatitis. Sci Transl Med. 2017;9(397).
16. Sollid JU, Furberg AS, Hanssen AM, Johannessen M. Staphylococcus aureus: determinants of human carriage. Infect Genet Evol. 2014;21:531-41.
17. Gong JQ, Lin L, Lin T, Hao F, Zeng FQ, Bi ZG, et al. Skin colonization by Staphylococcus aureus in patients with eczema and atopic dermatitis and relevant combined topical therapy: a double-blind multicentre randomized controlled trial. Br J Dermatol. 2006;155(4):680-7.
18. Rodriguez E, Baurecht H, Herberich E, Wagenpfeil S, Brown SJ, Cordell HJ, et al. Meta-analysis of filaggrin polymorphisms in eczema and asthma: robust risk factors in atopic disease. J Allergy Clin Immunol. 2009;123(6):1361-70 e7.
19. Kooijman MN, Kruithof CJ, van Duijn CM, Duijts L, Franco OH, van Ijzendoorn MH, et al. The Generation R Study: design and cohort update 2017. European journal of epidemiology. 2016;31(12):1243-64.
20. Drislane C, Irvine AD. The role of filaggrin in atopic dermatitis and allergic disease. Ann Allergy Asthma Immunol. 2020;124(1):36-43.
21. Irvine AD. Fleshing out filaggrin phenotypes. J Invest Dermatol. 2007;127(3):504-7.
22. Brown SJ, Relton CL, Liao H, Zhao Y, Sandilands A, McLean WH, et al. Filaggrin haploinsufficiency is highly penetrant and is associated with increased severity of eczema: further delineation of the skin phenotype in a prospective epidemiological study of 792 school children. Br J Dermatol. 2009;161(4):884-9.
23. Williams MR, Gallo RL. The role of the skin microbiome in atopic dermatitis. Curr Allergy Asthma Rep. 2015;15(11):65.
24. Feuillie C, Vitry P, McAleer MA, Kezic S, Irvine AD, Geoghegan JA, et al. Adhesion of Staphylococcus aureus to Corneocytes from Atopic Dermatitis Patients Is Controlled by Natural Moisturizing Factor Levels. mBio. 2018;9(4).
25. Miajlovic H, Fallon PG, Irvine AD, Foster TJ. Effect of filaggrin breakdown products on growth of and protein expression by Staphylococcus aureus. J Allergy Clin Immunol. 2010;126(6):1184-90 e3.
26. Kezic S, Kemperman PM, Koster ES, de Jongh CM, Thio HB, Campbell LE, et al. Loss-of-function mutations in the filaggrin gene lead to reduced level of natural moisturizing factor in the stratum corneum. J Invest Dermatol. 2008;128(8):2117-9.
27. 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(11):2192-200.
28. ML C. Staphylococcus aureus colonization in atopic eczema and its association with filaggrin gene mutations. Br J Dermatol. 2017.
29. Simpson EL, Villarreal M, Jepson B, Rafaels N, David G, Hanifin J, et al. Atopic dermatitis Subjects Colonized with Staphylococcus aureus have a Distinct Phenotype and Endotype. J Invest Dermatol. 2018.
30. Berents TL, Carlsen KC, Mowinckel P, Skjerven HO, Kvenshagen B, Rolfsjord LB, et al. Skin Barrier Function and Staphylococcus aureus Colonization in Vestibulum Nasi and Fauces in Healthy Infants and Infants with Eczema: A Population-Based Cohort Study. PLoS One. 2015;10(6):e0130145.
31. Clausen ML, Agner T, Lilje B, Edslev SM, Johannesen TB, Andersen PS. Association of Disease Severity With Skin Microbiome and Filaggrin Gene Mutations in Adult Atopic Dermatitis. JAMA Dermatol. 2018;154(3):293-300.

32. 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(3 Suppl 2):R7-R12.
33. Czarnowicki T, He H, Krueger JG, Guttman-Yassky E. Atopic dermatitis endotypes and implications for targeted therapeutics. J Allergy Clin Immunol. 2019;143(1):1-11.
34. 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(5):1347-58.
35. Eyerich K, Eyerich S. Th22 cells in allergic disease. Allergo J Int. 2015;24(1):1-7.
36. Fujita H. The role of IL-22 and Th22 cells in human skin diseases. J Dermatol Sci. 2013;72(1):3-8.
37. Irvine AD, McLean WH, Leung DY. Filaggrin mutations associated with skin and allergic diseases. N Engl J Med. 2011;365(14):1315-27.
38. Moosbrugger-Martinz V, Gruber R, Ladstatter K, Bellutti M, Blunder S, Schmuth M, et al. Filaggrin null mutations are associated with altered circulating Tregs in atopic dermatitis. J Cell Mol Med. 2019;23(2):1288-99.
39. Bonefeld CM, Petersen TH, Bandier J, Agerbeck C, Linneberg A, Ross-Hansen K, et al. Epidermal filaggrin deficiency mediates increased systemic T-helper 17 immune response. Br J Dermatol. 2016;175(4):706-12.
40. Wallmeyer L, Dietert K, Sochorova M, Gruber AD, Kleuser B, Vavrova K, et al. TSLP is a direct trigger for T cell migration in filaggrin-deficient skin equivalents. Sci Rep. 2017;7(1):774.
41. Barker JN, Palmer CN, Zhao Y, Liao H, Hull PR, Lee SP, et al. Null mutations in the filaggrin gene (FLG) determine major susceptibility to early-onset atopic dermatitis that persists into adulthood. J Invest Dermatol. 2007;127(3):564-7.
42. McAleer MA, Irvine AD. The multifunctional role of filaggrin in allergic skin disease. J Allergy Clin Immunol. 2013;131(2):280-91.
43. O'Regan GM, Kemperman PM, Sandilands A, Chen H, Campbell LE, Kroboth K, et al. Raman profiles of the stratum corneum define 3 filaggrin genotype-determined atopic dermatitis endophenotypes. J Allergy Clin Immunol. 2010;126(3):574-80 e1.
44. Ní Chaoimh C, Nico C, Puppels GJ, Caspers PJ, Wong X, Common JE, et al. In vivo Raman spectroscopy discriminates between FLG loss-of-function carriers vs wild-type in day 1-4 neonates. Ann Allergy Asthma Immunol. 2020;124(5):500-4.
45. Caspers PJ, Lucassen GW, Carter EA, Bruining HA, Puppels GJ. In vivo confocal Raman microspectroscopy of the skin: noninvasive determination of molecular concentration profiles. J Invest Dermatol. 2001;116(3):434-42.
46. Riise R, Odqvist L, Mattsson J, Monkley S, Abdillahi SM, Tyrchan C, et al. Bleomycin hydrolase regulates the release of chemokines important for inflammation and wound healing by keratinocytes. Sci Rep. 2019;9(1):20407.
47. Hoste E, Kemperman P, Devos M, Denecker G, Kezic S, Yau N, et al. Caspase-14 is required for filaggrin degradation to natural moisturizing factors in the skin. J Invest Dermatol. 2011;131(11):2233-41.
48. Scott IR, Harding CR. Filaggrin breakdown to water binding compounds during development of the rat stratum corneum is controlled by the water activity of the environment. Dev Biol. 1986;115(1):84-92.
49. Cau L, Pendaries V, Lhuillier E, Thompson PR, Serre G, Takahara H, et al. Lowering relative humidity level increases epidermal protein deimination and drives human filaggrin breakdown. J Dermatol Sci. 2017;86(2):106-13.

50. de Wit J, Totté JEE, van Buchem FJM, Pasmans S. The prevalence of antibody responses against Staphylococcus aureus antigens in patients with atopic dermatitis: a systematic review and meta-analysis. Br J Dermatol. 2018;178(6):1263-71.
51. Totte JEE, Pardo LM, Fieten KB, de Wit J, de Boer DV, van Wamel WJ, et al. The IgG response against Staphylococcus aureus is associated with severe atopic dermatitis in children. Br J Dermatol. 2017.
52. Jenkins A, Diep BA, Mai TT, Vo NH, Warrener P, Suzich J, et al. Differential Expression and Roles of *Staphylococcus aureus*; Virulence Determinants during Colonization and Disease. mBio. 2015;6(1):e02272-14.
53. Shah M, Mohanraj M. High levels of fusidic acid-resistant Staphylococcus aureus in dermatology patients. Br J Dermatol. 2003;148(5):1018-20.
54. Niebuhr M, Mai U, Kapp A, Werfel T. Antibiotic treatment of cutaneous infections with Staphylococcus aureus in patients with atopic dermatitis: current antimicrobial resistances and susceptibilities. Exp Dermatol. 2008;17(11):953-7.
55. Chaptini C, Quinn S, Marshman G. Methicillin-resistant Staphylococcus aureus in children with atopic dermatitis from 1999 to 2014: A longitudinal study. Australas J Dermatol. 2016;57(2):122-7.
56. Nakatsuji T, Chen TH, Narala S, Chun KA, Two AM, Yun T, et al. Antimicrobials from human skin commensal bacteria protect against Staphylococcus aureus and are deficient in atopic dermatitis. Sci Transl Med. 2017;9(378).
57. Nakatsuji T, Yun T, Butcher A, Hayashi A, Chun K, Shafiq F, et al. 426 Clinical improvement in atopic dermatitis following autologous application of microbiome therapy targeting Staphylococcus aureus. Journal of Investigative Dermatology. 2018;138(5, Supplement):S72.
58. Myles IA, Earland NJ, Anderson ED, Moore IN, Kieh MD, Williams KW, et al. First-in-human topical microbiome transplantation with Roseomonas mucosa for atopic dermatitis. JCI Insight [Internet]. 2018 2018/05//; 3.
59. Dayan GH, Mohamed N, Scully IL, Cooper D, Begier E, Eiden J, et al. Staphylococcus aureus: the current state of disease, pathophysiology and strategies for prevention. Expert Rev Vaccines. 2016;15(11):1373-92.
60. 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(8):1045-60.
61. 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(2):327-49.
62. Biedermann T, Skabytska Y, Kaesler S, Volz T. Regulation of T Cell Immunity in Atopic Dermatitis by Microbes: The Yin and Yang of Cutaneous Inflammation. Front Immunol. 2015;6:353.
63. Laupland KB, Conly JM. Treatment of Staphylococcus aureus colonization and prophylaxis for infection with topical intranasal mupirocin: an evidence-based review. Clin Infect Dis. 2003;37(7):933-8.
64. Kluytmans JA, Wertheim HF. Nasal carriage of Staphylococcus aureus and prevention of nosocomial infections. Infection. 2005;33(1):3-8.
65. Peng HM, Wang LC, Zhai JL, Weng XS, Feng B, Wang W. Effectiveness of preoperative decolonization with nasal povidone iodine in Chinese patients undergoing elective orthopedic surgery: a prospective cross-sectional study. Braz J Med Biol Res. 2017;51(2):e6736.
66. Huang JT, Abrams M, Tlougan B, Rademaker A, Paller AS. Treatment of Staphylococcus aureus colonization in atopic dermatitis decreases disease severity. Pediatrics. 2009;123(5):e808-14.
67. Kezic S, O'Regan GM, Yau N, Sandilands A, Chen H, Campbell LE, et al. Levels of filaggrin degradation products are influenced by both filaggrin genotype and atopic dermatitis severity. Allergy. 2011;66(7):934-40.

68. Janssens M, van Smeden J, Gooris GS, Bras W, Portale G, Caspers PJ, et al. Increase in short-chain ceramides correlates with an altered lipid organization and decreased barrier function in atopic eczema patients. J Lipid Res. 2012;53(12):2755-66.
69. McAleer MA, Jakasa I, Raj N, O'Donnell CPF, Lane ME, Rawlings AV, et al. Early-life regional and temporal variation in filaggrin-derived natural moisturizing factor, filaggrin-processing enzyme activity, corneocyte phenotypes and plasmin activity: implications for atopic dermatitis. Br J Dermatol. 2018.
70. Ungar B, Garcet S, Gonzalez J, Dhingra N, Correa da Rosa J, Shemer A, et al. An Integrated Model of Atopic Dermatitis Biomarkers Highlights the Systemic Nature of the Disease. J Invest Dermatol. 2017;137(3):603-13.
71. Esaki H, Brunner PM, Renert-Yuval Y, Czarnowicki T, Huynh T, Tran G, et al. Early-onset pediatric atopic dermatitis is T(H)2 but also T(H)17 polarized in skin. J Allergy Clin Immunol. 2016;138(6):1639-51.
72. Jurakic Toncic R, Kezic S, Jakasa I, Ljubojevic Hadzavdic S, Balic A, Petkovic M, et al. Filaggrin loss-of-function mutations and levels of filaggrin degradation products in adult patients with atopic dermatitis in Croatia. J Eur Acad Dermatol Venereol. 2020;34(8):1789-94.
73. Hotze M, Baurecht H, Rodríguez E, Chapman-Rothe N, Ollert M, Fölster-Holst R, et al. Increased efficacy of omalizumab in atopic dermatitis patients with wild-type filaggrin status and higher serum levels of phosphatidylcholines. Allergy. 2014;69(1):132-5.
74. Roekevisch E, Leeflang MMG, Schram ME, Campbell LE, Irwin McLean WH, Kezic S, et al. Patients with atopic dermatitis with filaggrin loss-of-function mutations show good but lower responses to immunosuppressive treatment. Br J Dermatol. 2017;177(6):1745-6.
75. Chalmers JR, Haines RH, Bradshaw LE, Montgomery AA, Thomas KS, Brown SJ, et al. Daily emollient during infancy for prevention of eczema: the BEEP randomised controlled trial. Lancet. 2020;395(10228):962-72.
76. Skjerven HO, Rehbinder EM, Vettukattil R, LeBlanc M, Granum B, Haugen G, et al. Skin emollient and early complementary feeding to prevent infant atopic dermatitis (PreventADALL): a factorial, multicentre, cluster-randomised trial. Lancet. 2020;395(10228):951-61.
77. Yamamoto-Hanada K, Kobayashi T, Williams HC, Mikami M, Saito-Abe M, Morita K, et al. Early aggressive intervention for infantile atopic dermatitis to prevent development of food allergy: a multicenter, investigator-blinded, randomized, parallel group controlled trial (PACI Study)-protocol for a randomized controlled trial. Clin Transl Allergy. 2018;8:47.
78. Thijs JL, Strickland I, Bruijnzeel-Koomen C, Nierkens S, Giovannone B, Csomor E, et al. Moving toward endotypes in atopic dermatitis: Identification of patient clusters based on serum biomarker analysis. J Allergy Clin Immunol. 2017;140(3):730-7.

Chapter 7

Summary / Samenvatting

SUMMARY

Chapter 1 is a general introduction describing the main objectives of this thesis. Atopic dermatitis (AD), also known as eczema, is a chronic inflammatory skin disease which affects up to 20% of children and 10% of adults in developed countries. This disorder is characterized by recurrent eczematous lesions and intense itch, which have a great impact on the quality of life. The cause of AD is a complex interplay between microbiome abnormalities, an impaired skin barrier and immune dysregulation. Currently, there is no cure for AD and therapy is focused on disease control. For a better understanding of this disease, we aimed to further investigate these three factors that contribute to the pathogenesis of AD and to assess how they interact with each other. In particular, we focused on the role of the microbiome (with the predominance of *Staphylococcus (S.) aureus)* and the skin barrier defect (caused by mutations in the gene encoding filaggrin (*FLG*)). These insights will help us develop new treatment strategies to reduce the symptoms of AD.

Especially the skin (lesional and nonlesional) and the nasal cavity of AD patients are frequently colonized by *S. aureus,* which adversely affects disease severity. Although, with lower prevalence, healthy controls can also be colonized with this bacterium. It remains largely unknown how *S. aureus* can be present on the skin both as a commensal organism and as a pathogen causing inflammation characteristic of AD. It has been suggested that AD patients are colonized by genetically different *S. aureus* strains, as compared to non-AD individuals. Therefore, in **Chapter 2**, we aimed to investigate whether AD patients are colonized by specific *S. aureus* strains and if certain strains are associated with more severe disease. For this purpose, we performed a longitudinal study that included 96 adult AD patients with moderate to severe disease. *Spa*-typing was performed on *S. aureus* isolates from the nose and skin that were collected over the course of two weeks. The *spa*-typing technique discriminates between different *S. aureus* strains by determining the DNA sequence of the *S. aureus*-specific Staphylococcal protein A (*spa*) gene. Our results showed that there is large inter-individual variation in *spa*-types, that is similar to non-AD individuals. In addition, we concluded that the intra-individual variation is minor, with the majority of the individual patients being colonized by the same *spa*-type in both nose and skin and over a period of time. No predominant *spa*-type nor temporal variation in *spa*-types was associated with more severe disease. The presence of a certain strain is likely influenced by host genetic and environmental factors.

Mutations in *FLG* lead to reduced expression of the protein filaggrin that is essential in the development and maintenance of the skin barrier. Individuals with a mutation in this gene are at increased risk to develop a form of AD that starts in early infancy, persists into adulthood and is associated with increased disease severity and the development of other

allergic conditions (i.e., food allergies, asthma and allergic rhinitis). The exact mechanisms contributing to this disease modification effect are largely unknown. It is thought that *FLG* mutations lead to alterations in the skin microbiome and circulating immune cell numbers. In **Chapter 3.1** we aimed to investigate the effect of *FLG* mutation status (wild type versus ≥ 1 mutation in *FLG*) on the microbial composition in a cross-sectional study including 77 children with difficult to treat AD. All patients were screened for mutations in *FLG* and the microbiome of the nose, lesional and nonlesional skin was characterized using 16S rRNA sequencing. Instead of only screening for the most common mutations in Western-European populations, as done in previous research, the entire encoding region of *FLG* was screened to detect loss of function mutations. This new approach led to an improvement of the diagnostic yield, since it enables to detect novel, family- and population specific mutations. We showed that the nose, lesional skin and nonlesional skin harbored different microbial communities. In addition, we concluded that the overall microbiome was affected by the presence of a mutation in *FLG*. These results propose interactions between host genetics and the microbiome. Since literature on this subject is scarce, more studies including larger patient groups are of interest. In **Chapter 3.2**, we explored the effect of mutations in *FLG* on the circulating immune cells (T- and B-cell subsets at the age of 10 years) in 523 children of the general population and a subgroup of 102 children with AD. This research was part of a large prospective birth cohort study of Generation R, Rotterdam. We established that the presence of a mutation in *FLG* was associated with higher T helper (Th)22 cells in children of the general population, but not with other T- and B-cell subsets. In the subgroup of children with AD, *FLG* mutations did not affect the circulating immune cell numbers. Since Th22 is involved in the skin barrier integrity and in the defense against pathogens, we hypothesized that the upregulation of Th22 is an immunological defense mechanism against an altered skin barrier function in children of the general population.

Filaggrin is degraded into free amino acids in the stratum corneum (SC) of the skin. The amino acids and their derivatives form the largest part of the natural moisturizing factor (NMF). NMF plays an important role in skin hydration, maintenance of the skin pH and ultra-violet (UV) protection. It has been suggested that the concentrations of NMF in the SC could predict the presence of *FLG* mutations in patients with AD. In **Chapter 4**, we assessed if the NMF content in the SC of the thenar eminence (nonlesional) could be used as a valid biomarker to stratify AD patients according to *FLG* mutation status. This research was conducted in a multi-ethnic pediatric AD cohort. The entire encoding region of *FLG* was screened for mutations in 101 AD children. NMF was measured using Raman Spectroscopy, a non-invasive optical technique based on light scattering from molecules in the skin. We showed that the NMF content in the SC could accurately stratify patients based on *FLG* mutation status with high sensitivity and specificity, without direct interference

of present disease severity. NMF measurement can be used in daily practice to identify patients with high risk for a more severe AD phenotype caused by a mutation in *FLG*.

Chapter 5 focused on treatment interacting with the skin microbiome to improve AD related symptoms. In **Chapter 5.1,** we studied the effects of alpine climate treatment on the microbiome of 79 children with difficult to treat AD. Alpine climate treatment has been used to treat patients with AD and/or asthma. This climate is characterized by low exposure to allergens, pollutants and increased UV radiation. We described the secondary outcomes of a pragmatic randomized controlled trial in which patients were randomized to a six-week treatment period either in an alpine clinic in Switzerland or in an outpatient setting in the Netherlands. It was previously shown that children in the alpine climate group had a significantly greater improvement in disease severity, compared to children in the moderate maritime climate group. In contrast to treatment in the Netherlands, alpine climate treatment led to a significant change in the microbiome on lesional skin. In addition, the *S. aureus* load on both lesional and nonlesional skin decreased after treatment in alpine climate. This study showed that alpine climate treatment leads to changes in the skin microbiome, reflecting the successful treatment. We encourage further investigation of skin microbiome modulating therapies to improve the symptoms of AD. In **Chapter 5.2** we presented the outcomes of a randomized vehicle-controlled trial that studied the effect of topical treatment with an endolysin targeting *S. aureus* in 100 adult patients with non-infected AD. The advantage of specifically targeting *S. aureus* with an endolysin, as compared to current available anti-microbial agents (i.e., antibiotics), is that it does not lead to bacterial resistance and does not affect other commensal micro-organisms. The results showed that endolysin treatment was safe and well tolerated. No significant differences were found in the use of topical corticosteroid (TCS) between the endolysin and vehicle treated groups, nor in the reduction of clinical disease severity scores. In line with these clinical outcomes, no significant reduction was observed in the *S. aureus* load on the skin. This lack of difference might be explained by the significant decrease in disease severity that was achieved in the run-in-phase of this trial, in which treatment was standardized with topical corticosteroids and emollients. The methodological lessons learned can be used for future clinical studies that evaluate treatment in AD.

Finally, in **Chapter 6** we presented a general overview of the main findings considering the limitations of our research and propose directions for future research. We concluded that the pathogenesis of AD cannot be explained by only one mechanism, but involves multiple interactions between the skin microbiome, skin barrier and immune system. As the role of the three elements is likely to differ per patient and over the lifetime of an individual, we emphasized to further identify subgroups of patients based on the underlying disease pathways. Using this method, we can stratify this heterogeneous disease for more personalized care.

SAMENVATTING

Hoofdstuk 1 is een algemene introductie met een beschrijving van de belangrijkste doelstellingen van dit proefschrift. Constitutioneel eczeem (CE) is een chronische inflammatoire huidziekte die tot 20% van de kinderen en tot 10% van de volwassenen treft in Westerse landen. Deze aandoening wordt gekenmerkt door recidiverende eczemateuze laesies en hevige jeuk, welke een grote impact hebben op de kwaliteit van leven. CE wordt veroorzaakt door een complex samenspel tussen afwijkingen in het microbioom, een verminderde huidbarrière en immuundysregulatie. Tot op heden is er geen remedie voor CE en is de behandeling gericht op het bestrijden van symptomen. Wij stelden ons ten doel om meer inzicht te krijgen in de drie bovengenoemde factoren die betrokken zijn in de pathogenese van CE en te onderzoeken hoe ze met elkaar interacteren. In het bijzonder hebben wij ons geconcentreerd op de rol van het microbioom (gedomineerd door *Staphylococcus (S.) aureus*) en het huidbarrière defect (veroorzaakt door mutaties in het filaggrine gen (*FLG*)). Deze inzichten zullen ons helpen bij de ontwikkeling van nieuwe behandelingsstrategieën om de symptomen van CE te verlichten.

Met name de huid (aangedane en niet aangedane) en de neusholte van CE-patiënten zijn vaak gekoloniseerd met *S. aureus*, wat de ernst van de ziekte nadelig beïnvloedt. Ook gezonde individuen kunnen gekoloniseerd zijn met deze bacterie. Het blijft grotendeels onbekend hoe *S. aureus* zowel een commensaal organisme kan zijn als een ziekteverwekker die bijdraagt aan de symptomen van CE. In **Hoofdstuk 2** stelden we ons ten doel te onderzoeken of patiënten met CE gekoloniseerd zijn door specifieke *S. aureus* stammen en of bepaalde stammen geassocieerd zijn met ernstigere klachten van CE. Met dit doel hebben wij een longitudinale studie uitgevoerd waaraan 96 CE patiënten deelnamen met matig tot ernstige ziekte. *Spa*-typering werd uitgevoerd op *S. aureus*-kweken van de neus en huid, die in de loop van twee weken werden verzameld. De *spa*-typeringstechniek maakt onderscheid tussen verschillende *S. aureus* stammen door de DNA-sequentie van het *S. aureus*-specifieke Staphylococcal proteïne A (*spa*) gen te bepalen. Onze resultaten toonden dat er in de bestudeerde CE-populatie een grote inter-individuele variatie is in *spa*-typen, welke vergelijkbaar is met individuen zonder CE. Daarnaast vonden we dat de intra-individuele variatie gering is, waarbij de meerderheid van de individuele CE patiënten gekoloniseerd is met hetzelfde *spa*-type zowel in de neus als op de huid en over een periode van tijd. Er was geen verband tussen de ernst van de ziekte en de aanwezigheid van een bepaald *spa*-type. We veronderstellen dat de aanwezigheid van een specifieke stam vooral wordt beïnvloed door genetische -en omgevingsfactoren van het individu.

Mutaties in *FLG* leiden tot verminderde expressie van het eiwit filaggrine dat essentieel is voor de ontwikkeling en het behoud van de huidbarrière. Individuen met een muta-

tie in dit gen lopen een verhoogd risico op het ontwikkelen van een vorm van CE die al op jonge leeftijd begint, aanhoudt tot op volwassen leeftijd en gepaard gaat met een ernstiger ziektebeeld en de ontwikkeling van andere allergische aandoeningen (voedselallergieën, astma en allergische rhinitis). De exacte mechanismen die hieraan bijdragen zijn grotendeels onbekend. Er wordt gedacht dat *FLG* mutaties leiden tot veranderingen in het huid-microbioom en het aantal circulerende immuuncellen. In **Hoofdstuk 3.1** hebben we onderzocht of patiënten met ≥1 mutatie in *FLG* een andere samenstelling van het microbioom hebben in vergelijking met patiënten zonder een mutatie in dit gen. Met dit doel werd een cross-sectionele studie uitgevoerd waarin 77 kinderen met moeilijk behandelbaar CE werden geïncludeerd. *FLG* werd gescreend op mutaties en het microbioom van de neus, de aangedane en niet aangedane huid werd in kaart gebracht met behulp van 16S rRNA sequencing. In plaats van te screenen op de meest voorkomende mutaties in West-Europese populaties, wat veelal is gedaan in eerder onderzoek, werd de gehele coderende regio van *FLG* gescreend op mutaties. Deze techniek verbetert de diagnostische opbrengst in vergelijking met het alleen bepalen van populatie-specifieke mutaties. Dit is te verklaren doordat hierbij ook zeldzame, familie specifieke, en niet eerder beschreven mutaties worden gedetecteerd. Daarnaast toonden onze resultaten dat het microbioom verschillend is per afnamelocatie (neus, de aangedane en niet aangedane huid) en dat de samenstelling wordt beïnvloed door de aanwezigheid van een *FLG* mutatie. Deze resultaten suggereren een wisselwerking tussen genetische factoren en het microbioom. Er zijn studies met grotere patiëntengroepen nodig om deze resultaten te bevestigen. In **Hoofdstuk 3.2** onderzochten we het effect van mutaties in *FLG* op de circulerende immuuncellen (T -en B-cellen op de leeftijd van 10 jaar) in een algemene pediatrische populatie (n=523) en een subgroep van 102 kinderen met CE. Het onderzoek maakt deel uit van een grootschalig geboorte cohortstudie in Rotterdam, genaamd Generation R. We toonden aan dat de aanwezigheid van een mutatie in *FLG* is geassocieerd met hogere aantallen T helper (Th)22 cellen in kinderen van de algemene populatie, maar niet met andere T -en B-cellen. De aanwezigheid van een *FLG* mutatie had geen invloed op hoeveelheid immuuncellen in een subgroep van kinderen met CE. Th22 speelt een rol in de integriteit van de huidbarrière en de verdediging tegen ziekteverwekkers. Dit veronderstelt dat de toename van Th22 een verdedigingsmechanisme is van het afweersysteem tegen een verminderde huidbarrièrefunctie in een algemene pediatrische populatie.

Filaggrine wordt in de hoornlaag van de huid afgebroken tot vrije aminozuren. De aminozuren en hun derivaten vormen samen het grootste deel van de 'natural moisterizing factor' (NMF). NMF speelt een belangrijke rol bij de hydratatie van de huid, de instandhouding van de pH-waarde en de bescherming tegen ultraviolet licht (UV). Eerder onderzoek suggereerde dat de hoeveelheid NMF in de hoornlaag de aanwezigheid van *FLG* mutaties bij patiënten met CE zou kunnen voorspellen. In **Hoofdstuk 4** beoordeelden we of de hoe-

veelheid NMF in de hoornlaag van de huid op de duimmuis gebruikt kan worden als biomarker in CE om patiënten te stratificeren op basis van *FLG* mutatie status. Dit onderzoek werd uitgevoerd in een multi-etnisch cohort van kinderen met CE. De gehele coderende regio van *FLG* werd gescreend op mutaties in 101 CE kinderen. De hoeveelheid NMF werd gemeten met Raman Spectroscopy. Dit is een niet-invasieve techniek gebaseerd op de weerkaatsing van invallend licht door moleculen in de huid. We concludeerden dat de hoeveelheid NMF kan worden gebruikt als biomarker om patiënten te stratificeren op basis van *FLG* mutatie status met hoge sensitiviteit en specificiteit, zonder directe inmenging van de actuele ziekte-ernst. De NMF-meting kan in de dagelijkse praktijk worden gebruikt om patiënten te identificeren met een hoog risico op een ernstiger CE fenotype veroorzaakt door een mutatie in *FLG*.

Hoofdstuk 5 richt zich op behandelingen die interacteren met het microbioom van de huid met als doel de klachten van CE te verlichten. In **Hoofdstuk 5.1** bestudeerden we de effecten van hooggebergtebehandeling op het microbioom van 79 kinderen met moeilijk behandelbaar CE. Hooggebergtebehandeling kan gebruikt worden voor de behandeling van patiënten met CE en/of astma. Dit klimaat wordt gekenmerkt door een lage blootstelling aan allergenen, verontreinigende stoffen en verhoogde UV-straling. We beschrijven de secundaire uitkomsten van een pragmatische gerandomiseerde gecontroleerde studie waarin patiënten werden gerandomiseerd voor een 6 weekse behandelperiode in een kliniek in het hooggebergte in Zwitserland of poliklinisch behandeling in gematigd zeeklimaat in Nederland. In deze studiepopulatie werd eerder aangetoond dat er significant meer verbetering was in ziekteactiviteit in de hooggebergte groep. In tegenstelling tot de behandeling in Nederland leidde hooggebergtebehandeling tot een significante verandering in het microbioom op de aangedane huid. Bovendien nam de hoeveelheid *S. aureus* op de aangedane en niet aangedane huid af na behandeling in hooggebergte. Deze studie toont aan dat hooggebergtebehandeling leidt tot veranderingen in het microbioom van de huid, wat de succesvolle behandeling weerspiegelt. We moedigen verder onderzoek aan naar therapieën die het microbioom van de huid kunnen moduleren om de symptomen van CE te verlichten. In **Hoofdstuk 5.2** evalueerden we de werkzaamheid en veiligheid van een op endolysine gebaseerde therapie gericht op de *S. aureus* bacterie in een gerandomiseerde gecontroleerde studie met 100 volwassen patiënten met niet-geïnfecteerd CE. Het voordeel van het specifiek aanpakken van *S. aureus* met een endolysine, in vergelijking met de huidige beschikbare antimicrobiële middelen (zoals antibiotica), is dat het niet leidt tot bacteriële resistentie en geen invloed heeft op andere commensale micro-organismen. De resultaten toonden aan dat de behandeling met endolysine veilig was en goed werd verdragen. Er werd geen significant verschil gevonden in het gebruik van topicale corticosteroïden (TCS) tussen de met endolysine en placebo behandelde groepen. Daarnaast was er geen verschil in de scores voor de ernst van de ziekte. In overeenstemming met deze

klinische resultaten werd geen significante vermindering waargenomen in de hoeveelheid *S. aureus* op de huid. Het uitgebleven voordeel van endolysine behandeling is mogelijk te verklaren door de significante afname in ziekte-ernst in de aanloopfase van dit onderzoek, waarin de behandeling werd gestandaardiseerd met TCS en vette zalf. De geleerde methodologische lessen kunnen gebruikt worden voor toekomstige klinische studies die het effect van behandeling evalueren in CE.

Tot slot presenteerden we in **Hoofdstuk 6** een algemeen overzicht van de belangrijkste bevindingen van dit proefschrift waarbij we de methodologische beperkingen in acht nemen. Daarnaast doen we suggesties voor toekomstig onderzoek. Wij concludeerden dat de pathogenese van CE niet kan worden verklaard door slechts één mechanisme, maar dat er meerdere interacties zijn tussen het huid-microbioom, de huidbarrière en het immuunsysteem. Wij veronderstellen op basis van onze resultaten dat de rol van deze drie elementen verschilt per patiënt en ook kan veranderen over de levensloop van een patiënt. Door het bepalen van microbiële, epidermale en immunologische biomarkers kunnen we subgroepen van patiënten met CE identificeren op basis van de onderliggende ziektemechanismen. Met behulp van deze methode kunnen we deze heterogene ziekte beter stratificeren en daarmee de zorg meer op het individu afstemmen.

Appendices

Abbreviations
List of contributing authors
List of publications
PhD portfolio
Curriculum vitae
Dankwoord

ABBREVIATIONS

AD	Atopic dermatitis
AMP	Anti-microbial peptide
APC	Antigen-presenting cells
AUC	Area under the curve
BH	Bleomycin hydrolase
CC	Clonal complexes
CCA	Canonical correspondence analysis
CI	Confidence interval
CNV	Copy number variation
coNS	Coagulase-negative *Staphylococcus*
DC	Dendritic cell
EASI	Eczema Area and Severity Index
EDC	Epidermal differentiation complex
FLG	Filaggrin gene
FLG-LoF	Filaggrin gene loss of function
GWAS	Genome-wide association studies
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IQR	Interquartile range
IV	Ichthyosis Vulgaris
MDS	Multidimensional scaling
MLST	Multilocus sequence typing
moAB	Monoclonal antibodies
mTEC	Medullary thymic epithelial cells
NGS	Next generation sequencing
NMF	Natural moisturizing factor
NRS	Numeric Rating Scale
OR	Odds ratio
PAMP	Pathogen-associated molecular pattern
PCA	Pyrrolidone carboxylic acid
PERMANOVA	Permutational multivariable analysis of variance
PFGE	Pulsed field gel electrophoresis
PIM	Personalized integrative multidisciplinary
POEM	Patient Oriented Eczema Measure
PRR	Pattern recognition receptor
qPCR	Quantitative polymerase chain reaction

RA	Relative abundance
RDP	Ribosomal Database Project
ROC	Receiver Operating Characteristic
RCT	Randomized controlled trial
SA-EASI	Self-Administered Eczema Area and Severity Index
Sags	Superantigens
SC	Stratum corneum
SD	Standard deviation
smMIPs	Single molecule Molecular Inversion Probes
SNP	Single nucleotide polymorphisms
Spa	Staphylococcal protein A
Staphefekt	Staphefekt SA.100
TCI	Topical calcineurin inhibitors
TCS	Topical corticosteroids
Tem	Effector memory T cell
TEWL	Trans epidermal water loss
Th	T helper lymphocyte
TJ	Tight junctions
TNF	Tumor necrosis factor
Treg	Regulatory T cell
TSLP	Thymic stromal lymphopoietin
UCA	Urocanic acid
UV	Ultra-violet
UV-R	Ultraviolet radiation
WGS	Whole-genome sequencing

LIST OF CONTRIBUTING AUTHORS

Affiliations at the time at which the research was conducted

Broek, T.J. van den
TNO, Microbiology and Systems Biology Group, Zeist, The Netherlands.

Bronner, M.B.
Department of Dermatology-Center of Pediatric Dermatology, Erasmus MC University Medical Center– Sophia Children's Hospital, Rotterdam, the Netherlands

Caspers, P.J.
Center for Optical Diagnostics and Therapy, Department of Dermatology, Erasmus MC, University Medical Center, Rotterdam, The Netherlands
RiverD International B.V., Rotterdam, The Netherlands

Doorn, M.B.A. van
Department of Dermatology, Erasmus MC University Medical Center, Rotterdam, the Netherlands

Duijts, L
Department of Pediatrics, Division of Respiratory Medicine and Allergology, Erasmus MC, University Medical Center Rotterdam, Rotterdam, the Netherlands.

Fieten, K.B.
Department of Dermatology and Allergology, University Medical Center, Utrecht, The Netherlands
Swiss Institute of Allergy and Asthma Research, University of Zürich, Davos, Switzerland
Dutch Asthma Center Davos, Davos, Switzerland

Geel, M. van
Department of Dermatology, Maastricht University Medical Centre, Maastricht, The Netherlands.
Department of Clinical Genetics, Maastricht University Medical Centre, Maastricht, The Netherlands.

Herpers, B.L.
Regional Public Health Laboratory Kennemerland, Haarlem, The Netherlands

Hu, C.
Department of Dermatology, Erasmus MC University Medical Center, Rotterdam, the Netherlands
The Generation R Study Group, Erasmus MC, University Medical Center Rotterdam, Rotterdam, the Netherlands.

Jansen, M.S.
Department of Dermatology-Center of Pediatric Dermatology, Erasmus MC University Medical Center– Sophia Children's Hospital, Rotterdam, the Netherlands

Jongste, J.C. de
Department of Pediatrics, Division of Respiratory Medicine and Allergology, Erasmus MC, University Medical Center Rotterdam, Rotterdam, the Netherlands.
The Generation R Study Group, Erasmus MC, University Medical Center Rotterdam, Rotterdam, the Netherlands.

Kiefte-de Jong, J.C.
Department of Public Health and Primary Care, Leiden University Medical Center/LUMC Campus, The Hague, the Netherlands.

Klaassen, C.H.W.
Department of Medical Microbiology and Infectious Diseases, Erasmus MC University Medical Center Rotterdam, the Netherlands

Looman, K.I.M.
Department of General Pediatrics, , Erasmus MC, University Medical Center Rotterdam-Sophia Children's Hospital, Rotterdam, the Netherlands.
The Generation R Study Group, Erasmus MC, University Medical Center Rotterdam, Rotterdam, the Netherlands.

Moll, H.A.
Department of General Pediatrics, , Erasmus MC, University Medical Center Rotterdam-Sophia Children's Hospital, Rotterdam, the Netherlands.

Mierlo, M.M.F. van
Department of Dermatology, Erasmus MC University Medical Center, Rotterdam, the Netherlands

Nijsten, T.E.C.
Department of Dermatology, Erasmus MC University Medical Center, Rotterdam, the Netherlands

Nouwen, A.E.M.
Department of Dermatology-Center of Pediatric Dermatology, Erasmus MC University Medical Center– Sophia Children's Hospital, Rotterdam, the Netherlands

Pardo. L.M.
Department of Dermatology, Erasmus MC University Medical Center, Rotterdam, the Netherlands

Pasmans, S.G.M.A.
Department of Dermatology-Center of Pediatric Dermatology, Erasmus MC University Medical Center– Sophia Children's Hospital, Rotterdam, the Netherlands

Puppels, G.J.
Center for Optical Diagnostics and Therapy, Department of Dermatology, Erasmus MC, University Medical Center, Rotterdam, The Netherlands
RiverD International B.V., Rotterdam, The Netherlands

Schuren, F.H.J.
TNO, Microbiology and Systems Biology Group, Zeist, the Netherlands.

Totté, J.E.E.
Department of Dermatology, Erasmus MC University Medical Center, Rotterdam, the Netherlands
Department of Medical Microbiology, University Medical Center Utrecht, the Netherlands

Veldhuizen, J. van
Department of Dermatology, Erasmus MC University Medical Center, Rotterdam, the Netherlands

Vos, M.C.
Department of Medical Microbiology and Infectious Diseases, Erasmus MC University Medical Center Rotterdam, the Netherlands

Willemsen, S.P.
Department of Biostatistics, Erasmus University Medical Center, Rotterdam, the Netherlands.

Wit, J. de
Department of Dermatology, Erasmus MC University Medical Center, Rotterdam, the Netherlands

Zelm, M.C. van
Department Immunology and Pathology, Central Clinical School, Monash University and The Alfred Hospital, Melbourne, VIC, Australia.

LIST OF PUBLICATIONS

van Mierlo M.M.F., Pasmans S.G.M.A., Totté J.E.E., de Wit J., Herpers B.L., Vos M.C., Klaassen C.H.W., Pardo L.M. Temporal variation in *Staphylococcus aureus spa*-genotypes from nose and skin in atopic dermatitis patients. *Accepted for publication in Dermatology*

van Mierlo M.M.F., Pardo L.M., Fieten K.B., van den Broek T.J., Schuren F.H.J., van Geel M., Pasmans S.G.M.A. The skin and nose microbiome and its association with filaggrin mutations in children with atopic dermatitis. *Submitted*

van Mierlo M.M.F., Looman K.I.M., van Zelm M.C., Hu C., Duijts L., de Jongste J.C., Nijsten T.E.C., Pardo L.M., Kiefte-de Jong J., Moll H.A., Pasmans S.G.M.A. Increased Th22 cell numbers in a general pediatric population with filaggrin haploinsufficiency: The Generation R Study. *Accepted for publication in Pediatric Allergy and Immunology*

van Mierlo M.M.F., Caspers P.J., Jansen M.S., Puppels G.J., Nouwen A.E.M., Bronner M.B., Pardo L.M., van Geel M., Pasmans S.G.M.A. Validation of natural moisturizing factor as a biomarker for filaggrin mutation status in a multi-ethnic pediatric atopic dermatitis cohort. *Under review*

van Mierlo M.M.F., Totté J.E.E., Fieten K.B., van den Broek T.J., Schuren F.H.J., Pardo L.M., Pasmans S.G.M.A. The influence of treatment in alpine and moderate maritime climate on the composition of the skin microbiome in patients with difficult to treat atopic dermatitis. *Clin Exp Allergy. 2019 Nov; 49(11): 1437-1445*

de Wit J., **van Mierlo M.M.F.**, Totté J.E.E., van Veldhuizen J., van Doorn M.B.A., Schuren F.H.J., Willemsen S.P., Pardo L.M., Pasmans S.G.M.A. Endolysin treatment against Staphylococcus aureus in adults with atopic dermatitis: a randomized controlled trial. *J Allergy Clin Immunol. 2019 Sep;144(3):860-863*

PHD PORTFOLIO

Name PhD student	Minke M.F. van Mierlo
Erasmus MC department	Dermatology
Research school	Postgraduate school Molecular Medicine (MolMed), Netherlands Institute of Health Sciences (NIHES)
PhD period	2017-2021
Promotor	Prof. S.G.M.A. Pasmans, MD PhD
Copromotor	L.M. Pardo, MD PhD

	Year	Workload (ECTS)
Courses		
• Erasmus MC - Microbiomics I	2018	0.6
• Research management	2018	1.0
• Erasmus MC - Scientific Integrity	2018	0.3
• Erasmus MC - Basic Course on 'R'	2017	2.0
• Erasmus MC - Basic Introduction Course on SPSS	2017	1.0
• Erasmus MC - BROK® (Basic course Rules and Organisation for Clinical researchers)	2017	1.5
Workshops		
• Systematic literature retrieval in Pubmed part 1 & 2	2017	0.3
• Erasmus MC - EndNote workshop	2017	0.2
Oral presentations		
• 21th Annual meeting NVED, Lunteren, The Netherlands	2020	1.0
• 20th Annual meeting NVED, Lunteren, The Netherlands	2019	1.0
Conferences attending		
• 10th Georg Rajka International Symposium on Atopic Dermatitis, Utrecht, the Netherlands (attending)	2017	1.0
• Radboud New Frontiers in the Microbiome symposium, Nijmegen, The Netherlands (attending)	2017	1.0
Committees		
• Organizing 6th PhD weekend, Breda	2018	1.0

Teaching

• Supervising master theses of Amber Drost	2019	2.0
• Supervising master theses of Melissa Rodenhuis	2019	2.0
• Supervising master theses of Yasmine Boelhrif	2018	2.0
• Supervising master theses of Marieke Jansen	2018	2.0

Research proposal

• 5th funding call in the framework of the 'Enabling Technologies Hotels' (ETH) program (40k eur)	2019	1.0
• Anti-Bacterial Clothing (ABC) study: writing study protocol and submission METC	2018	2.0

Other

• Annual PhD weekend Dermatology (attending)	2017-2021	1.3
• Skintermezzo, Rotterdam, the Netherlands (quarterly, attending)	2017-2021	2.0
• Weekly 'Research meeting Dermatology and Journal Club', Erasmus MC, Rotterdam	2017-2021	2.0
• Implementation of anakinra treatment in patients with Netherton syndrome	2019-2020	2.0
• Coördinator scientific research group Pediatric Dermatology, Erasmus MC	2018-2020	2.0
• Implementation of Natural Moisterizing factor (NMF) in atopic dermatitis, Pediatric dermatology, Erasmus MC	2018-2019	2.0

CURRICULUM VITAE

Minke van Mierlo was born on the 28th of November 1989 in Nijmegen, the Netherlands. She graduated from secondary school at Canisius College Nijmegen and was admitted to study medicine at the Leiden University Medical Center. After obtaining her bachelor's degree, she took a full-time minor program named managing strategy and marketing at Amsterdam Business School of the University of Amsterdam. She finished her MSc in Medicine by the end of 2016. After traveling through Vietnam, Laos and Cambodia she worked as a medical doctor at the department of internal medicine at the Reiner de Graaf Gasthuis, Delft. Because of her growing interest in dermatology and in research, she started her PhD trajectory in 2017, which resulted in this thesis at the Department of Dermatology at the Erasmus MC University Medical Center under supervision of prof. S.G.M.A Pasmans and co-supervision of L.M. Pardo. In 2021 she started her dermatology residency at the Leiden University Medical Center.

DANKWOORD

Eindelijk is het dan zover: mijn proefschrift is af! Met deze laatste pagina's sluit ik een bijzondere periode af. Een periode waar ik op wetenschappelijk gebied enorm ben gegroeid, maar ik me bovenal op persoonlijk vlak heb ontwikkeld. Ik heb mezelf beter leren kennen, nieuwe vriendschappen gesloten en leven gegeven aan mijn grootste trots: 'Olivier'. Ik ben dankbaar dat ik tijdens mijn promotietraject heb mogen samenwerken met vele anderen die mij geïnspireerd hebben. Zonder jullie vertrouwen, hulp en kennis was dit proefschrift er niet geweest. In het bijzonder wil ik een aantal bedanken:

Allereerst mijn promotor prof. dr. Pasmans, beste Suzanne. Wat hebben we veel meegemaakt in de afgelopen jaren, zowel diepte- als hoogtepunten. Jouw luisterend oor, geduld, enthousiasme en doorzettingsvermogen hebben ervoor gezorgd dat dit proefschrift er ligt. Ondanks jouw duizelingwekkende agenda had je altijd oog en aandacht voor belangrijke life events en maakte je altijd tijd om met elkaar van gedachten te wisselen met de nodige hoeveelheid koffie. Ik bewonder je om jouw tomeloze energie, waarbij je naast werk en gezin ook letterlijk ontelbare kilometers hebt afgelegd om meer aandacht te krijgen voor patiënten met een huidaandoening.

Mijn co-promotor, dr. Pardo, beste Luba. Wat ben ik trots en soms verbaasd dat we samen de eindstreep hebben gehaald. Ik denk dat we veel van elkaar hebben geleerd en zijn gegroeid in het proces. Bedankt voor de begeleiding.

Dr. Kezic, Prof. dr. E.P. Prens en Prof. dr Vos, hartelijk dank voor het beoordelen van mijn proefschrift en jullie deelname in de kleine promotiecommissie. Beste leden van de grote promotiecommissie, bedankt voor de bereidheid om tijdens de verdediging van mijn proefschrift met mij van gedachten te wisselen.

De studies die beschreven zijn in dit proefschrift waren niet mogelijk geweest zonder de betrokkenheid van tal van specialisten, verpleegkundigen en ondersteuning uit zowel het Erasmus MC, UMC Utrecht en Maastricht UMC. Daarnaast wil ik alle deelnemers en ouders/verzorgers van deelnemers bedanken voor hun interesse, deelname en het vertrouwen in ons team. In het bijzonder wil ik nog een aantal mensen bedanken: Michel van Geel, bedankt voor alle moeite rondom het analyseren van de filaggrine samples, alle waardevolle feedback en de leuke dag die ik heb gehad bij jou op de werkvloer. Daarnaast dank ook aan Frank Schuren voor de fijne samenwerking en de deur die altijd open stond voor vragen. Tim van de Broek, door jouw hulp, kennis en geweldige R skills zijn mijn artikelen naar een hoger niveau getild. De complexe microbioom analyses waren me nooit zonder jou gelukt. Daarnaast bedankt voor al je uitleg en geduld. Karin Fieten, het contact

dat we hebben gehad over de manuscripten en in de afrondende fase waren voor mij een enorme steun in de rug. Terwijl je op grote afstand zat was je altijd benaderbaar, betrokken en enthousiast. Peter Caspers, ik vond het leuk hoe jij vanuit de invalshoek als natuurkundige de passie deelt om de zorg voor patiënten met eczeem beter te maken. Dank voor al je betrokkenheid en de wegwijs binnen de Raman Spectroscopie. Corné Klaassen, dank voor de inspanningen voor de *spa*-typering en het laagdrempelige contact. Menno van Zelm, leuk om jou te leren kennen tijdens het schrijven van ons manuscript. Vanaf de andere kant van de wereld was je altijd bereikbaar voor vragen. Kirsten, wat was het fijn om met jou samen te werken. We waren echt een goed team.

Ik wil Micreos Human Health bedanken voor het voor het bieden van de financiële middelen om mijn wetenschappelijk onderzoek uit te voeren in het Erasmus MC. Daarnaast wil ik jullie, en met name Johan Friehling en Mark Offerhaus, bedanken voor de betrokkenheid, interessante discussies en de inzichten die we elkaar hebben gegeven vanuit ons eigen perspectief.

Dank ook aan alle betrokken medewerkers van de samenwerkende laboratoria: genetisch laboratorium interne geneeskunde Erasmus MC, Medische Microbiologie en Infectieziekten Erasmus MC, Streeklaboratorium Kennemerland en TNO Zeist.

Lieve collega's van KinderHaven, bedankt voor alle gezelligheid tijdens het doen van spreekuur. De hechtheid van jullie team en de manier waarop jullie zorg dragen voor alle patiënten zijn inspirerend.

Het eczeemteam van de kinderdermatologie, bedankt voor het prettige vangnet. Joan, mijn voorgangster, wat had jij de lat hoog voor mij gelegd. Bedankt voor de begeleiding in mijn eerste maanden, de betrokkenheid bij de manuscripten en de afronding van mijn promotie. Jouw kritische blik is voor mij heel waardevol geweest en heeft tot nieuwe inzichten geleid. Jill, het eerste jaar hebben wij veel samengewerkt, maar ook hierna hebben we altijd veel contact gehouden. Hieraan hecht ik veel waarde. Ik bewonder jouw nuchterheid en enorme gedrevenheid. Aviel, ook al doe je een ander onderzoek, het voelt soms alsof jij mijn opvolger bent omdat we in de laatste fase nog zoveel hebben samengewerkt. Ik vond het fijn dat we altijd bij elkaar terecht konden.

Mijn geweldige kamergenoten Sven en Selma van Gk-026a en natuurlijk Sterre die ook praktisch op onze kamer woonde. Wat hebben we veel lol gehad op deze 10m2. Vanaf dag één voelde ik me welkom en hebben we altijd alles met elkaar kunnen delen. Later werden we verhuisd naar Na-501, maar ook hier bleef onze band bestaan. Selma en Sterre, jullie zijn een begrip geworden in mijn promotietraject en ik ben blij met onze hechte vriend-

schap. Inmiddels hebben we alle drie ons plekje elders gevonden en ben ik super trots op wat we bereikt hebben door dicht bij onszelf te blijven. Selma, ontzettend fijn dat je tijdens deze dag als paranimf achter me wil staan. Sterre, vanwege de voorbereidingen van jouw huwelijk in Italië kun je er helaas niet bij zijn. Hopelijk kun je van een afstand toch een beetje achter me staan.

Lieve kamergenootjes van Na-501, jullie hebben mijn promotietijd onvergetelijk gemaakt. Bedankt voor alle gezelligheid en betrokkenheid. Linde, ondanks dat we niet op dezelfde kamer zaten, waren we altijd bij elkaar betrokken en heb ik genoten van onze koffie en lunchafspraken. Er zullen er in de toekomst nog veel volgen. Natuurlijk ook alle andere PhD-collega's van Nd, AIOSen, ANIOSen en stafleden, bedankt voor de leuke tijd.

Lieve collega's van de afdeling dermatologie in het LUMC. Allereerst bedankt voor jullie vertrouwen in mij en de kans die jullie mij geven de opleiding op jullie afdeling te volgen. Dank ook voor de tijd en ruimte die jullie bieden om mijn promotie tot een goed einde te brengen. Ik voel me nu al enorm thuis bij jullie en kijk uit naar de komende jaren.

Al mijn lieve vrienden en vriendinnen, wat maken jullie mijn leven leuk. Bedankt voor al jullie betrokkenheid en gezelligheid. Op naar nog meer mooie herinneringen samen.

Lieve Simone, jij bent een categorie apart. Je kent me als geen ander en weet me altijd van goede adviezen te voorzien. Ons dagelijkse contact over alles en over niets zijn voor mij onmisbaar. Wat heerlijk dat we nu bij elkaar om de hoek wonen en we elkaar nog meer kunnen zien.

Schoonfamilie, wat fijn om jullie altijd in de buurt te hebben en bedankt voor alle steun. Lieve (schoon)papa John, jij was meteen erg enthousiast over het lezen van mijn volledige proefschrift. Dit lezende betekent dat je bij de laatste bladzijde bent aangekomen. Dank voor jouw interesse en betrokkenheid.

Lieve familie, wat heb ik een geluk om met jullie te zijn opgegroeid en nog steeds door jullie te zijn omgeven. Jullie oneindige liefde, vertrouwen en support geven mij de kracht om mijn leven op mijn eigen manier vorm te geven. Daarnaast maakt onze humor het leven luchtig. Mama en papa, wat ben ik dankbaar voor ons heerlijke thuis. Het gezin staat bij jullie altijd op de eerste plaats en heeft ervoor gezorgd dat er een warm nestje is waarin iedereen zich welkom voelt. Mama, de sterkste en meest bijzondere vrouw die ik ken. Bedankt voor jouw grenzeloze betrokkenheid, positiviteit en steun. Ik kan alleen maar dromen op te groeien lijkend op jou. Papa, mijn grote fan. Zowel in goede en slechte tijden kan ik letterlijk dag en nacht bij jou terecht. Jij voorziet mij altijd van de beste adviezen en

ik krijg altijd goede zin als ik jou zie. Lieve Masha, jouw uitzonderlijke kracht en doorzettingsvermogen zijn voor mij een grote inspiratie. Wat voel ik me sterk wetende dat jij altijd achter me staat. Nu ook als paranimf tijdens mijn promotie. Je hebt me geleerd dat alles in het leven gevierd moet worden en dat hebben we dan ook goed samen gedaan. Bas, lief broertje, jouw nuchterheid houdt mij altijd bij de les. Jouw arm om mij heen met de woorden 'komt goed' zijn momenten waar ik op vooruit kan.

Lieve kleine Olivier, zonder jou was dit proefschrift er niet geweest. Jouw komst plaatste mijn leven in perspectief en gaf me de motivatie om mijn promotie tot een goed einde te brengen. Wat ben ik gelukkig elke dag met jou te mogen doorbrengen. Jouw knuffels, vrolijkheid en altijd aanwezige lach geven mij enorm veel energie. Je bent een geweldig kind en ik kan niet wachten om te zien hoe jij samen met ons opgroeit.

Dan als laatst, lieve lieve Nathan. Woorden schieten te kort om te beschrijven hoe belangrijk je voor me bent geweest in de jaren die we samen zijn en in het bijzonder tijdens de periode van het schrijven van mijn proefschrift. Jouw vertrouwen in mijn kunnen, het op de goede momenten remmen en stimuleren van mijn ambitie hebben mij door dit traject heen geholpen. Niets is jou ooit te veel en je staat altijd onvoorwaardelijk voor me klaar. Jouw grenzeloze liefde geeft mij vleugels. Ik hou van je!