The microbiome and the human immune response in atopic dermatitis

Exploring microbial targets for personalized treatment

Joan Totté

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Exploring Microbial Targets for Personalized Treatment

Het microbioom en de humane immuunrespons bij constitutioneel eczeem
op zoek naar aangrijpingspunten voor geïndividualiseerde behandelstrategieën

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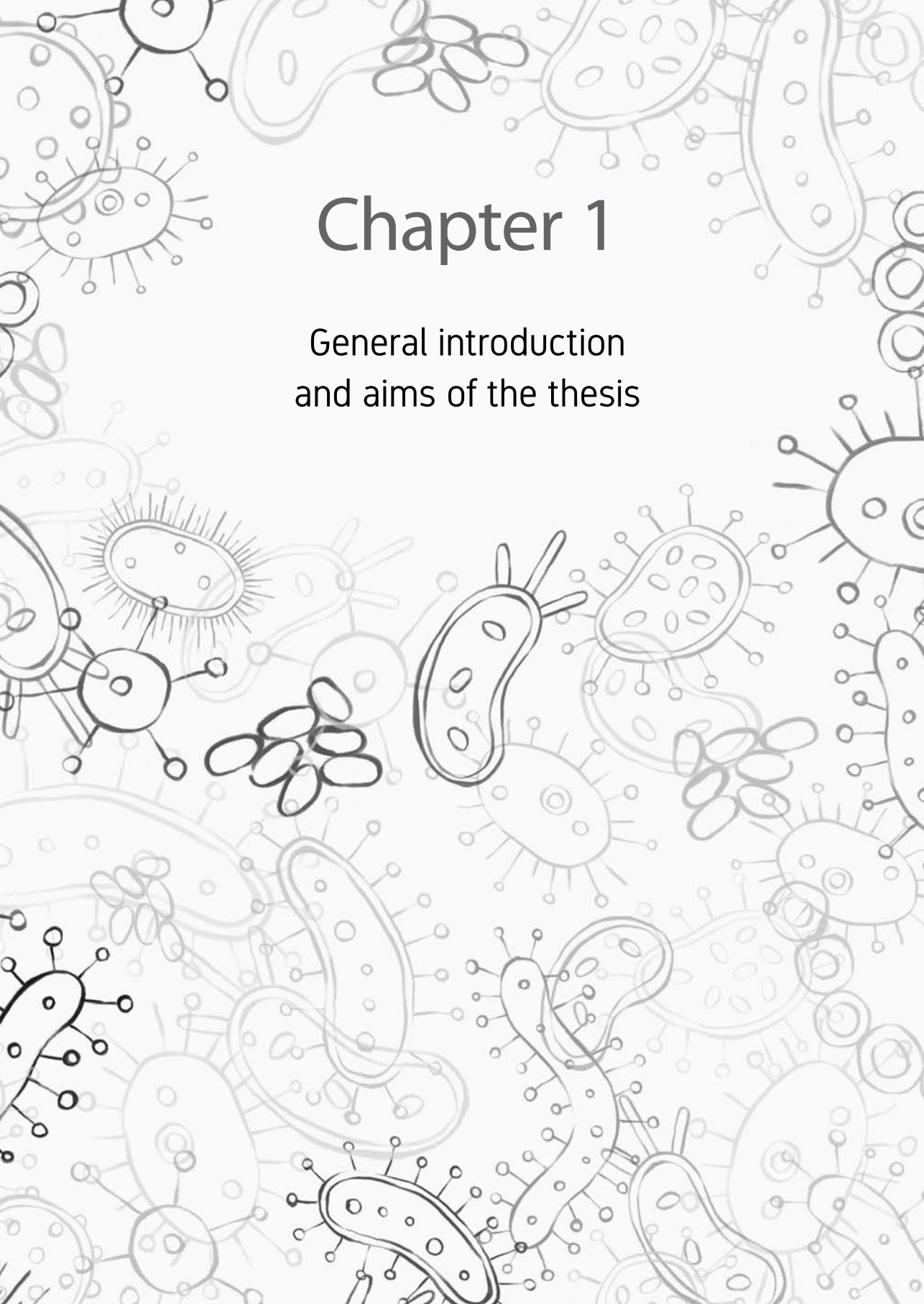
Chapter 1

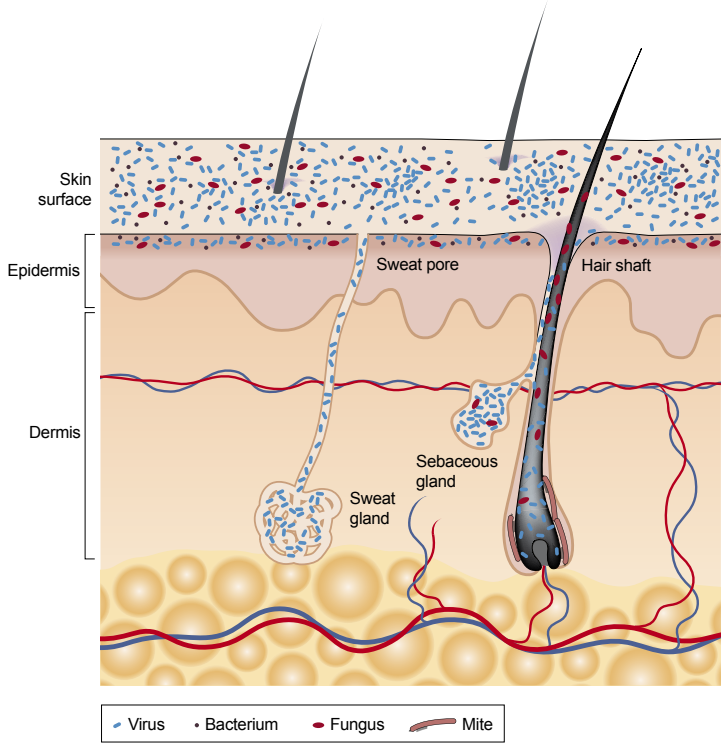
General introduction and aims of the thesis



THE HUMAN MICROBIOME

Our human body is colonized by a diversity of microorganisms. These microorganisms reside in different niches of the body, including the gut, upper respiratory tract and skin (figure 1). Most of them are harmless colonizers, commensals, and it is becoming increasingly clear that these microorganisms participate in important physiological processes, such as metabolism (particularly gut microbes), immunity and barrier integrity.[1-3] The postnatal microbiome is relatively homogeneous and mainly shaped by maternal transmission at birth.[4-7] During childhood the microbiome becomes more diverse and develops into a microbiome which is unique and relatively stable per individual.[4,8,9] Multiple factors influence the microbial composition. A recent population-based metagenomics study found 126 factors of influence on the gut microbiome, together explaining around 20% of the variability between individuals. Major contributing factors were dietary patterns and exposure to medication, in particular proton pump inhibitors and antibiotics.[6,7] The skin microbiome is also known to be influenced by the use of antimicrobial agents. Additionally, UV-light and skin characteristics (such as lipid composition) are of influence.[10,11] Washing and use of soap might also have an effect on the skin microbiome, but these effects are still poorly explored.[12,13] In this thesis we will study the role of the microbiome in the pathogenesis of atopic dermatitis (AD), with a focus on bacteria. Bacteria can be classified according to taxonomic ranks into phyla and further down to genera and species. The most common generum in the gut is *Bacteroides,* followed by *Faecalibacterium* and *Bifidobacterium*, while on the skin *Corynebacterium*, *Propionibacterium* and *Staphylococcus* are most abundant.[14,15] In the last years, a rapid development of high throughput sequencing techniques led to more comprehensive determination of microbial populations, compared to the older culture techniques that focus on single bacteria.[16] For identification of bacteria, sequencing of the well conserved 16S ribosomal RNA gene is often used. More recently, whole genome shotgun metagenomics (WGS) sequencing has been developed, which explores the full genomic complement of bacteria, fungi and viruses, reflecting both the composition and functional profile of the microbiome.[17] These developments led to rapid discoveries of changes in the microbiome (microbial dysbiosis) in relation to different diseases, including inflammatory bowel disease, diabetes type 1 and AD[18,19] However, a lot of basic aspects are still to be explored in microbiome research. For example, it is still unclear what exactly constitutes a 'normal' microbiome (if it exists) and which microbial functions impact human physiology.[6,20] Especially in skin microbiome research, a young field of microbiome research, there are obstacles to overcome. The unique characteristics of the skin, including a site specific microbiota, a distinct immune system and the low microbial biomass, require standardization of methods, including techniques for sample collection and sample processing.[21,22]

Figure 1. The skin microbiome *(Published with permission. Grice et al. Nature Reviews Microbiology 2011).*[23]



NOTE: Microorganisms also reside in the deeper layers of the skin, where the microbial composition differs from that of the skin surface and interaction can take place with living dermal cells.[24]

ATOPIC DERMATITIS BACKGROUND

Prevalence

AD is one of the most common inflammatory diseases affecting up to 25% of children and 1-7% of adults.[25,26] Its incidence has been increasing during the past decades and is still on the rise, especially in developing countries.[27] Although the disease can start at any age, symptoms start in infancy in most patients, followed by long continuous periods of disease or a relapsing-remitting course with symptom-free intervals.[28,29] AD has been found to negatively impact the quality of life of both patients and their families.[30,31]

Clinical features and comorbidities

The characteristic clinical features in AD are intense itch and recurrent eczematous lesions. Infants usually have lesions that show acute inflammation and oozing, while older children have more polymorphous lesions. In adolescents and adults chronic lesions

with lichenification are part of the clinical presentation. Typically, also the location of the lesions changes with the age of the patient. Infants show lesions in the face, at the extensor site of the limbs and sometimes the trunk. In older children the lesions are particularly located in the flexural folds and in adults the flexures, hands, eyelids, head and neck, upper trunk and scalp are sites of predilection.[32,33] Particularly more severe AD is frequently associated with other atopic diseases, including asthma, allergic rhinitis and food allergy.[29] Together with epidemiological and genetic data that associate AD with other diseases, such as rheumatoid arthritis, ulcerative colitis and diabetes type 1, this suggests that AD should be considered as a systemic disease rather than an inflammation limited to the skin.[34,35]

Risk factors

Both genetic and environmental factors underlie the development of AD and the course of the disease. Different environmental risk- and protective factors for AD have been identified until now. The main environmental risk factors for AD are a 'Western' diet (fast-food, low fruit) and broad-spectrum antibiotic exposure in early life.[36] Some studies have shown that air pollution and maternal psychiatric symptoms during pregnancy are associated with an increased risk of eczema.[36,37] The main protective factors that have been identified are UV light and factors related to microbial exposure, such as dog ownership and rural residence.[36]

A positive family history for atopic diseases is a strong risk factor for AD and multiple genetic defects have been identified that explain genetic susceptibility to AD.[38] The best known genetic defect is a null mutation in the gene encoding filaggrin, a protein that helps maintain skin barrier homeostasis.[39] Although a substantial part of the patients with AD do not have a mutation in this gene, it is known that patients who do carry the mutation have more persistent disease and a higher risk of atopic comorbidities, including asthma and allergic rhinitis.[40,41] In recent studies it has been shown that other genes may also play a role in susceptibility for AD. In a review of genome-wide association studies (GWAS) where thousands of AD cases were tested for associations with single nucleotide variants against controls it was shown that a total of 34 gene loci were associated with AD, including genes involved in skin barrier function and innate and adaptive immune defense.[42] One of the included GWAS found that the identified loci explain around 15% of variation of AD in populations due to genetic variation (heritability) in a subset of European studies.[43] Interaction between genes and environmental factors also seems a major modifier of the disease, although large scale studies investigating potential interactions between gene- and environmental effects are lacking.[42]

ATOPIC DERMATITIS PATHOGENESIS – THE ROLE OF THE MICROBIOME

Three major pathophysiologic changes characterize AD, namely: an impaired skin barrier, an altered immune response and changes in microbial composition. The skin epithelial function and immune responses have been extensively studied in AD. They are considered the two major biologic pathways responsible for AD etiology, based on genetic studies.[33,42] The primary event however is continued to be topic of debate.[44] There has been an increased interest in understanding the relation between the microbiome and AD as alterations in the microbiome are associated with AD and its severity. A summary of the known evidence on the main pathological pathways is presented below.

Skin barrier impairment

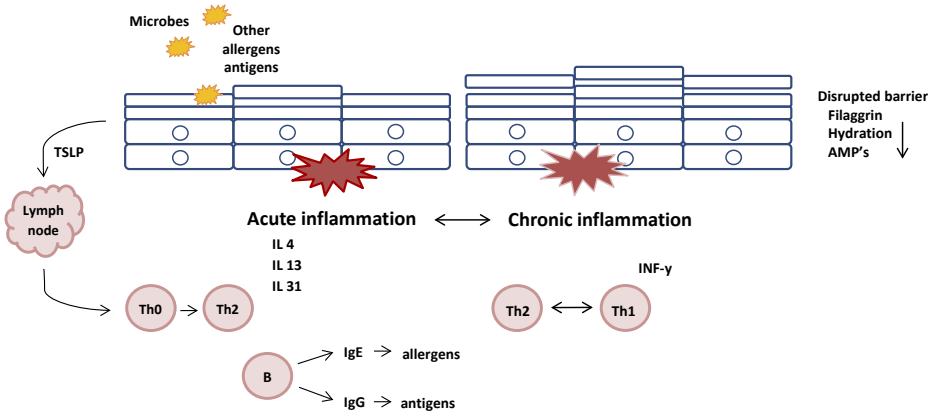
The healthy skin forms a strong barrier against harmful stimuli from the environment, including irritants, allergens, antigens and microorganisms. The outermost layer of the epidermis, the stratum corneum, consists of densely packed corneocytes (terminally differentiated keratinocytes) and proteins that together comprise the mechanical barrier. Thereby, the protective function of the skin is dependent on a balanced activity of lipids, acids, enzymes and the production of pro-inflammatory and antimicrobial molecules by immune cells and keratinocytes lower in the epidermis.[33] Multiple skin barrier abnormalities have been associated with AD. An increased water loss, also in the non-lesional skin, indicates an overall impairment of the barrier function in patients with AD.[45] Furthermore, changes in skin pH, reduced expression of antimicrobial peptides and changes in the composition of lipids that control skin hydration are associated with the disease.[46-48] A deficiency in filaggrin, described above as an important genetic risk factor for AD, affects multiple aspects that are important for a healthy skin barrier, such as water retention and lipid composition.[46] The impaired barrier function in AD causes environmental irritants, antigens and allergens to penetrate into skin, where they can provoke an immune reaction.

Immunological characteristics

One of the immune abnormalities in AD is infiltration of inflammatory cells into the skin. Non-lesional skin and newly developing lesions already show signs of low-level inflammation with increased numbers of Th2, Th22, Th17 cells and their pro-inflammatory cytokines.[34] The pro-inflammatory state in non-lesional skin combined with the existing impaired skin barrier in AD allows irritants, antigens and allergens to penetrate into the skin. This triggers keratinocytes to produce TSLP (Thymic stromal lymphopoietin) and cytokines that stimulate Th2 cell production in the lymph nodes.[49,50] A downstream effector molecule of TSLP, TARC (thymus and activation-regulated chemokine), stimulates migration of these Th2 cells to the skin, resulting in a positive feedback mechanism and

acute inflammation.[51] Acute AD lesions are predominated by infiltration of Th2 cells that produce multiple pro-inflammatory cytokines, including interleukin (IL)-4, 13 and 31, whereas a shift towards Th1 cells promotes chronic inflammation. This shift is thought to occur under the influence of IL-12 produced by dendritic cells, possibly stimulated by *Staphylococcus (S.) aureus*.[49] The Th1 cells in chronic lesions produce interferon-γ. This inhibits keratinocyte differentiation, causing the hyperplastic epidermis seen in these lesions (figure 2).[49,52]

Figure 2. Inflammatory cells in the skin during acute and chronic inflammation *(Adapted from Geoghean et al. Trends Microbiol. 2017).*[53]



The humoral immune response has also been shown to contribute to the pathogenesis of AD. Mainly abnormalities in immunoglobulin E (IgE) production are attributed to the disease. The impaired skin barrier in AD that becomes susceptible to the penetration of allergens causes production of IgE by B cells which are stimulated by Th2 cytokines. Once formed and released into the circulation, IgE binds mast cells, and subsequent re-exposure to the allergen can cause degranulation. Many patients with AD show high IgE concentrations against specific allergens. Up to two-thirds of the infants with moderate to severe AD show sensitization against food allergens, but actual symptoms of a food allergy occur in a smaller subset.[54,55] In older children, additional IgE sensitization towards inhalant allergens is seen.[56] In some patients with AD, increased IgE has also been found against microbial antigens, indicating that microbes might act as allergens and stimulate mast cells in AD.[57-62] Although less in forefront, IgG antibodies have also been studied in AD. IgG subclasses IgG1, IgG2, IgG3 and possibly also IgG4 are able to activate complement.[63] The IgG response in AD has mainly been studied in the context of food antigens that interact with the intestinal mucosa. Contact between these antigens and immune cells in the mucosa leads to production of specific IgG. A next encounter with

the food antigen provokes a pro-inflammatory response leading to phagocytosis of the antigen, which involves activation of the complement cascade.[64] This IgG based immune response probably also occurs in reaction to antigens that penetrate the impaired skin in AD. Studies in this field are still scarce but Sohn *et al.* reported significantly higher levels of IgG against microbial antigens in patients with AD compared to controls.[65] The presence of IgG represents a physiological response to repeated contact with a certain antigen. It is unclear whether IgG is just an 'innocent bystander' and a marker for interaction between antigens and the immune system or if it also contributes to inflammation and barrier dysfunction.[64] Measuring IgG against microbial antigens might help us to understand how microbes interact with the immune system and possibly induce inflammation in the skin.

Microbiome alterations

The microbiome of the skin, but also that of the nose and gut, have gained major interest in AD because of a possible role in inflammation and close interaction with the immune system.[1] The skin is the most well studied niche in AD. Already since the 1970s studies describe an overgrowth of *S.aureus* bacteria on the lesional skin, accompanied by reduced diversity of commensal bacteria on the skin.[66,67] Until now, microbial research has mainly focused on *S. aureus*. Some mechanisms by which the bacterium interacts with the skin barrier and immune system have been unraveled, such as the production of α-toxin by the bacterium that induces keratinocyte damage.[68] *S. aureus* strain-specific differences in eliciting skin inflammation were demonstrated in a cutaneous colonization model.[69] However, the exact role of the skin microbiome in the pathogenesis of AD remains poorly understood. Recently, the first longitudinal studies were published. Meylan *et al.* found in 149 infants that the presence of *S. aureus* on the skin at the age of three months was associated with the development of AD later in life (20% vs. 5.7%; p=0.035).[70] Another small study (n=20) found that staphylococci were less abundant on the skin in infants at the age of two that developed AD at one year of age. Further classification of these staphylococci revealed that *S. epidermidis* and *S. cohnii* were most abundant, while notably no *S. aureus* was present.[71] These findings relate to a mice study that found that early colonization with commensal *Staphylococcus* species might have a role in shaping the adaptive immune response and tolerance against these species.[72] A recent systematic review found that dysbiosis in AD does not only involve increased *S. aureus*. Also other staphylococci and other species such as *Propionibacterium* and *Malassezia* were found to have an altered abundance.[19] At the same time, they state that current data are not sufficiently robust for good characterization which emphasizes further determination of the role of skin microbes in AD.

Studies on the nasal microbiome have mainly focused on carriage of *S. aureus*. Approximately 20% of general population is a persistent carrier of *S. aureus* and in

another 30% the bacterium is intermittently found.[73] Persistent carriers have higher *S. aureus* loads, higher risk of *S. aureus* infection and higher titers of anti-staphylococcal antibodies when compared to intermittent and non-carriers.[74] It is still unclear why humans are not equally susceptible to colonization, as we are all exposed to the bacterium from birth. Multiple factors probably determine carriage, including the genotype of the bacterium, the host immune response and underlying host genetic factors.[75] The nose is also an important niche for microbes in AD as the anterior nares are considered an important reservoir for self-contamination and bacterial spread to the skin. A prospective study showed an association between AD and colonization of the nares with *S. aureus* at the age of 6 months and frequent colonization during the first year of life.[76] However, current literature is conflicting as another study did not find an association between nasal *S. aureus* colonization at 1 month of age and AD development.[77] The role of the nasal microbiome in AD and its interaction with the skin microbiome or vice versa are still unclear.[78,79]

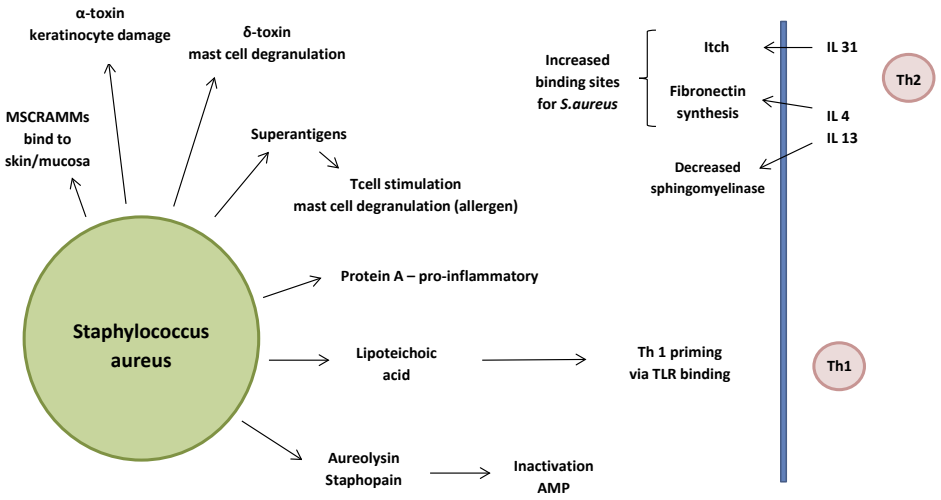
The gut microbiome is thought to play a role in shaping the immune system and it is speculated to influence the development of allergic diseases.[80] Gut microbes can modulate the direction of T-cell differentiation into T regulatory cells or effector T cells (Th1, Th2, Th17), which is important for immune tolerance.[81] Studies investigating the gut microbiome in children showed associations between changes in the gut microbial composition and the development atopic diseases, including asthma and allergic rhinitis.[82,83] The relation between gut microbiome and development of food allergy is less well studied. Literature investigating the actual gut microbiome in relation to AD confirmed associations but is still inconclusive.[84] Some studies found an increase in *Escherichia coli* and *Clostridium difficile* and a decrease in *Bifidobacteria*.[85,86] Intervention studies have also been inconsistent. However, a large study found a deviating microbiota along with reduction in AD after a probiotic intervention, which supports an association between the gut microbiota and AD.[87]

Cross-talk between skin barrier, immune system and microbiome

The skin barrier and immune system are known to interact in a bidirectional way, reinforcing the process of inflammation. For example, pro-inflammatory cytokines (IL-4, IL-13 and IL-22) are strong suppressors of filaggrin causing skin barrier dysfunction.[88] Another example is illustrated by the Th2 cytokine IL-31 that evokes itch, resulting in scratching and further skin barrier dysfunction.[89] Also the microbiome is in constant interaction with the skin barrier and immune system. For example *S. aureus* facilitates colonization and induces inflammation via interactions with the immune system and barrier.[53,90] Using MSCRAMMs (microbial surface component recognizing adhesive matrix molecules), such as clumping factor, *S. aureus* binds to the extracellular matrix.[91] After establishing contact, *S. aureus* can secrete molecules that damage the cell membrane, such as alpha

toxin.[68] Via other antigens, including Protein A and staphylococcal enterotoxins, the bacterium modulates the immune system. The enterotoxins can act as superantigens and allergens which means that they can directly stimulate T cells, causing proliferation and the release of pro-inflammatory cytokines.[67,92,93] They are also thought to stimulate mast cells, both direct and indirect via IgE binding. Thereby, *S. aureus* enterotoxin B can stimulate IL-22 and alfa-toxin can stimulate IL-31.[94,95] At last, binding of *S. aureus* lipoteichoic acid to TLR2 on dendritic cells seems to enhance Th1/Th17 cell priming, suggesting a role for *S. aureus* in the transition towards chronic AD which is more Th1 cell driven.[96] Another study found that, next to lipoteichoic acid, also alpha-toxin might facilitate chronic AD via induction of a Th1 cytokine response.[97] On the other hand, innate immune system abnormalities in AD as well as epidermal barrier abnormalities contribute to *S. aureus* colonization. For example, the inflammatory Th2 milieu induces fibrinectin synthesis and thereby adherence of *S. aureus*.[98] And Th2 cytokines IL-4 and IL-13 can decrease sphingomyelinase, which normally protects against alfa-toxin induced keratinocyte damage.[68,99] Figure 3 illustrates known interactions between microbiome, skin barrier and immune-system, supporting that all three components are important to study the process of inflammation in the skin.

Figure 3. *S. aureus* and its interaction with the skin barrier and immune system



MANAGEMENT OF ATOPIC DERMATITIS

General management

Current AD treatment is based on a 'one size fits all' principle according to the clinical severity of the disease. Basic therapy consists of a daily emollient (and bath oils) and avoidance of triggers. Mild AD requires reactive therapy with anti-inflammatory topical immunosuppressive agents, including corticosteroids. Moderate disease severity needs a more proactive treatment with higher potency topical corticosteroids or calcineurin inhibitors. In case of severe AD systemic immune suppression might be indicated. UVB therapy might be considered in moderate AD and PUVA therapy (only in adults) in severe AD before starting systemic medication.[100] New targeted therapies are under investigation for the treatment of AD, including small molecules and biologics.[101] Two phase 3 trials showed promising results of a biologic drug in AD for the first time (Dupilumab, a human monoclonal IgG4 anti IL-4 and IL-13 antibody).[102] The downside of the current treatment options, especially in moderate to severe AD, is the risk of side effects. Long term use of more potent topical and systemic corticosteroids might result in local and systemic side effects including adrenal suppression.[103] Also systemic therapy can cause serious side effects, such as liver dysfunction, hematological and gastro-intestinal side effects (azathioprine and methotrexate) or kidney failure and high blood pressure (cyclosporine A).[104]

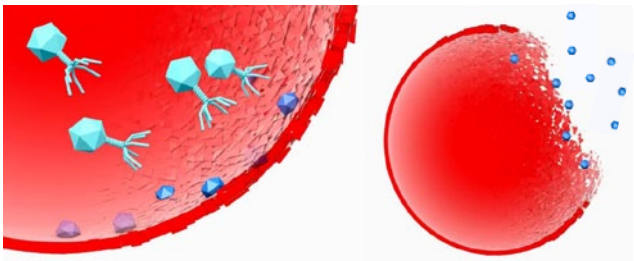
The microbiome as a therapeutic target

Dutch guidelines recommend antimicrobial (anti-staphylococcal) treatment only in cases of fever, high staphylococcal load or clinically infected AD.[100] In these cases treatment with antibiotics might be beneficial, but only short term use is allowed (maximum to 14 days). In case of recurrent infected AD Povidon-iodine (Betadine) scrubs or chlorhexidine can be used. American Academy of Dermatology guidelines from 2014 also mention that in patients with moderate to severe AD and signs of bacterial infection, bleach baths and intranasal mupirocin may be recommended.[105] Guidelines published in 2012 mention that the use of silver-coated antimicrobial textiles can reduce AD severity, but that their use is under investigation and safety concerns exist for the use in infants and toddlers.[106]

According to the Dutch guidelines there is no place for antibiotics in non-clinically infected AD.[100] Although international guidelines support this, European guidelines shortly mention that in severe exacerbations systemic antibiotic treatment may be helpful.[100,105,106] Current guidelines are based on clinical studies that mainly used short antimicrobial interventions and did not clearly show the added value of anti-staphylococcal therapy in non-infected AD.[107] However, recent studies show effectiveness of chloride bathing on AD symptoms after two and three months.[108,109] Modulation of the skin microbiome can still be promising for the treatment of AD, either by finding out how

to apply existing antimicrobials in a way that they result in clinical improvement or via newly developed treatment strategies. However, the role of the microbiome in AD needs to be further identified to guide treatment approaches that target the microbiome. Different new (non-antibiotic) treatment strategies for modulation of the microbiome are under development or clinically tested. Different strategies include either targeting single pathogenic species or providing control over these species using beneficial bacteria. Currently, vaccines and mABs (monoclonal antibodies) are under development that neutralize one or more *S. aureus* toxins, such as alfa toxin.[67] Also, the interest in the use of bacteriophages and bacteriophage lysins has been renewed. Bacteriophages are bacteria-specific viruses that use the host's cellular machinery to reproduce inside the host. They produce endolysins that weaken the bacterial cell wall from inside and make the bacterial cell burst, forced by internal osmotic pressure. Currently, the use of endolysins instead of the whole phage gained particular interest. Lysins typically consist of a domain that allows binding to bacteria-specific structures of the cell wall. Thereby, one or two other domains cleave specific bonds in the peptidoglycan.[110,111] Their advantage over antibiotic and also whole phage therapy is their targeted mode of action, minimal influence on commensal flora and the low risk of bacterial resistance induction (figure 4). Staphefekt SA.100 is an engineered endolysin that specifically lyses the cell membrane of *S.aureus*.[112]

Figure 4. The left figure shows lysis of the bacterial host by endolysins, a critical step in replication cycle of bacteriophages. The right figure shows lyses the cell from the outside by the endolysin. (Courtesy from Micreos)



On the other hand, artificial modification of the skin microbiota using microbes that provide control over dominant species (probiotica) might be a promising strategy, but until now very few studies report on topical probiotic approaches for skin disorders.[113] Few research groups are investigating the effect of adding beneficial bacteria to moisturizers. Seite *et al.* describe a reduction of AD flares after treatment with an emollient containing non-living extract of a Gram-negative proteobacterium, *Vitreoscila filiformis*.[114] An ongoing study of Gallo *et al.* uses beneficial *S. aureus* species.[115] These new strategies mentioned above seem promising. However, we should keep in mind

that modification of the microbiome might have unexpected effects. For example by eliminating a (single) species from the microbiome, one might create a niche for other pathogenic organisms to grow.

AIMS AND SCOPE OF THE THESIS

The impaired skin barrier and altered immune mechanisms in AD are widely studied and considered as the two major players in AD inflammation. A third player, the altered microbiome, is an established finding in AD but its role in the pathogenesis is still poorly understood. Studies have focused mainly on a single species, namely *S. aureus,* and on the cutaneous microbiome. However, other species and other niches might also be involved. In this thesis, we first aimed to characterize the microbial composition of the skin, nose and gut in pediatric patients with mild to severe AD. Our second aim was to estimate the prevalence of *S. aureus* in patients with AD and to study the humoral immune response against *S. aureus*. Third, we aimed to design a clinical study to test the effect of a new endolysin-based therapy that specifically targets *S. aureus* in AD. The results of this research are presented in this thesis. This knowledge will help to better understand the role of the microbiome in the pathogenesis of AD. Furthermore, it will help to determine if there is a role for therapy that targets the microbiome in the treatment of AD, and to identify possible microbial therapeutic targets of interest.

OUTLINE OF THE THESIS

In the first part of the thesis we examined the microbiome composition of different niches of the body in patients with AD. In **Chapter 2** we characterized the bacterial microbiota of the skin and nose using 16S rRNA sequencing in a cohort of children with AD. We tested associations between the microbial composition and AD severity phenotypes and explored the relations between the skin and nasal microbiome. In **Chapter 3** we characterized the gut microbiome in AD and evaluated whether the microbiome can discriminate between children with and without a food allergy.

In the second part we focused on *S. aureus* and the humoral immune response against this bacterium. In **Chapter 4** we estimated the prevalence of *S. aureus* colonization in lesional and non-lesional skin as well as in the nose via a systematic literature search and meta-analysis. We additionally studied the colonization for different disease severity phenotypes. **Chapter 5** outlines a systematic review and meta-analysis that summarizes the available literature on the human antibody responses towards the different *S. aureus*

virulence factors. In **Chapter 6** we studied the serum IgG response against 55 *S. aureus* virulence factors in two cohorts of pediatric patients with AD.

In the third part we work towards studying the effect of new therapeutics that target the skin microbiome in AD. **Chapter 7** of this thesis outlines a comparison between skin swabs and scrubs to identify the most sensitive method for studying microbial outcomes in intervention and cross-sectional studies. Using the results of the chapters described above, we finally designed a protocol for a randomized controlled trial that studies the effect of a new targeted anti-*S. aureus* therapy on the symptoms in AD, described in **Chapter 8**. Finally, the main findings and recommendations for clinical implication and future research were discussed in **Chapter 9**.

STUDY DESIGN

The research described in this thesis was based on two pediatric patient cohorts, the SMA and the DAVOS cohort. SMA included patients with mild to severe AD from 0 to 18, between November 2009 and December 2011. DAVOS included children with difficult to treat eczema from 8 to 18 years, between January 2011 and June 2015. Microbial samples of the skin, nose and gut of the GMA cohort were included to characterize microbial composition in these niches in relation to AD severity and food allergy. Serum samples of both studies were used to study the IgG immune response towards *S. aureus* in relation to AD severity. In both studies, AD severity was assessed using the Self Administrated-Eczema Area and Severity Index (SA-EASI) and levels of thymus and activation-regulated chemokine (TARC), a serum biomarker for AD severity. A third adult patient cohort was included to study different collection methods for skin microbiome sampling, as part of designing a trial to study the effects of long-term microbial modulation in adult patients with AD.

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PART I

The microbiome in patients with atopic dermatitis - *associations with disease phenotypes*

Chapter 2

The nasal and skin microbiome are associated with disease severity in pediatric atopic dermatitis

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ABSTRACT

Background

Changes in the skin microbiome have been associated with atopic dermatitis (AD) and its severity. The role of the nasal microbiome in relation to the severity of AD and its relation with the skin microbiome, are less well studied.

Objective

We aimed to characterize the nasal and skin microbiome in children with AD in relation to disease severity. Additionally, we explored differences and correlations between the nasal and skin communities.

Methods

We characterized the microbial composition of nasal and lesional skin samples from 90 and 108 patients with AD, respectively, using 16S-rRNA sequencing. Additional quantitative (q)PCR for *Staphylococcus (S.) aureus* and *S. epidermidis* was performed on the skin samples. Disease severity was estimated using the self-administered eczema area and severity index.

Results

We found an association between the microbial composition and AD severity in both the nose and skin samples (R^2=2.6%; p=0.017 and R^2=7.0%; p=0.004). Staphylococci were strong drivers for the associations with severity. However, other species also contributed, such as *Moraxella* in the nose. Skin lesions were positive for *S. aureus* in 50% of the children and the presence and load of *S. aureus* was not associated with disease severity. Although the nose and skin harbor distinct microbial communities based on Bray–Curtis dissimilarity (n=48 paired samples; p<0.001), we found that correlations exist between species in the nose and (other) species on the skin.

Conclusion

The results show that both the nasal and skin microbiome are associated with disease severity in children with AD. Next to staphylococci, other species contribute to this association.

INTRODUCTION

Changes in the microbial composition of the skin and nose have been suggested to contribute to the complex pathogenesis of atopic dermatitis (AD).[1] The microbiome of the skin in AD is characterized by an increased abundance of *Staphylococcus (S.) aureus*.[2-4] A recent systematic review on the skin microbiome in AD also reports changes in other bacterial species.[5] For example, *S. epidermidis* has been found increased on the lesional skin, and reductions in *Propionibacterium* and *Streptococcus* were reported during AD flares.[3,5,6] These microbial alterations were reported in small single studies and need further validation.[5] Prospective studies observed increased skin colonization with *S. aureus* at the age of 3 months in infants who later develop AD.[7] Another small study also found an association between skin colonization in the antecubital fold with commensal staphylococci (*S. epidermidis and S. cohnii)* at the age of 2 months, and AD later in life.[8,9] Although data are still limited, these prospective studies suggest that the skin microbiome might contribute to both (severity of the) inflammation in AD and the development of the disease.

There is also evidence that the nasal microbiome is involved in the pathogenesis of AD. For example, it has been shown that patients with AD are almost five times more likely to carry *S. aureus* in the nose compared to healthy controls.[2] In a large birth cohort study, colonization of the nares with *S. aureus* at the age of 6 months and frequent colonization during the first year of life were associated with AD and its severity.[10] However, this was not confirmed in other studies.[3,11] Studies into the nasal microbiome in AD are often limited to *S. aureus* and there is very little known about the nasal microbiome in children in relation to AD severity.

The identification of species that contribute to the pathogenesis of AD is important for the development of new specific treatment strategies that aim to modulate the microbiome. Also, knowledge about which microbial niches are involved in AD and how they interact is needed to guide these developments. Several studies described the importance of relations between the nasal and skin microbiome with regard to *S. aureus*.[4,12,13] The anterior nares could be an important reservoir for self-contamination and bacterial spread from the nose to the skin or vice versa. However, the relation between the skin and nasal microbiome in AD has barely been studied before.

In this study we aimed to characterize the nasal and skin microbiome composition in children with AD and determined its association with AD severity. Additionally, we explored differences and correlations between the nasal and skin microbiome.

METHODS

Study design and patients

This cross sectional study was embedded in a randomized controlled trial that compared group consultation with individual consultations in children with AD, treated in the outpatient clinic of the Wilhelmina Children's Hospital of the University Medical Center Utrecht (ISRCTN08506572).[14] Inclusion criteria were: diagnosis of AD according to UK Working Party criteria, age between 0 and 18 years and parental ability to answer Dutch questionnaires.[15] Microbial swabs of the nose and skin, eczema severity scores and patient characteristics were all obtained at baseline and analyzed in the current study. The medical ethical committee of the University Medical Center Utrecht approved the study (08-368/K) and written informed consent was obtained from all participants. Severity of the AD was estimated using the self-administered eczema area and severity index (SA-EASI) by a research nurse.[16] Patients with a SA-EASI from 0 to 17.35 were classified as mild eczema, from 17.35 to 46.27 as moderate eczema and higher than 46.27 as severe eczema, calculated based on cut-offs of the SCORAD score (Supplementary methods).[17]

Microbial samples

A total of 90 microbial samples were taken from the mucosal surfaces of the anterior nares. Skin microbial samples were taken from the lesional skin (n=108), preferably the antecubital fold or popliteal fossa. Samples were collected by a trained research nurse, according to a standardized procedure and using a sterile swab (Sterile Dryswab™) moistened with sterile NaCl 0.9%. After collection, the samples were aliquoted and frozen at -20^{0}C until further processing.

DNA isolation and qPCR

For DNA isolation approximately 150 ul of cutaneous or nasal material (retained by rinsing the swabs in lysis buffer) was directly transferred to the DNA isolation plate. Then 0.5 mL phenol pH8.0 (Phenol solution, catalogue P4557, Sigma-Aldrich, St Louis, MO) was added and the samples were mechanically disrupted by bead beating 2 times 3 minutes with a 96-well plate Beadbeater (Biospec Products, Bartlesville). Samples were centrifuged at 1880 rcf (4000rpm) for 10 minutes to separate the aqueous and phenolic phases. The aqueous phase was transferred to a new 96-well plate and DNA was purified with the AGOWA mag Mini DNA Isolation Kit (AGOWA, LGC genomics, Berlin, Germany) in accordance with the manufacturer's recommendations. After elution, total load of *S. aureus* and *S. epidermidis* was assessed by quantitative (q)PCR using the following primers and probe: 16S-S.aur-F1 (5'-GCG AAG AAC CTT ACC AAA TCT TG-3') and 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 a FAM label, 16S-S.epi-F1(GCG AAG AAC CTT ACC AAA TCT TG) and

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.

16S rRNA sequencing

The microbial composition of each sample was characterized by mass sequencing of the V4 hypervariable region of the 16S rRNA gene on the Illumina MiSeq sequencer (Illumina, San Diego, CA). To prevent over-amplification, barcoded DNA fragments spanning the Archaeal and Bacterial V4 hypervariable region were amplified with a standardizing level of template DNA (1 ng). These amplicons, generated using adapted primers F515 and R806 (using 30 PCR cycli), were bidirectionally sequenced using the MiSeq system.[18,19] After removing samples with less than 1000 sequences, 89 nasal and 60 skin samples remained (figure S1). Pre-processing and classification of sequences was performed using modules implemented in the Mothur V.1.31.1 software platform.[20] Sequences were trimmed between 243-263nt and chimeric sequences were identified per sample using UCHIME in de novo mode and removed.[21] Sequences with 97% sequence similarity or higher were grouped into operational taxonomic units (OTU) using MOTHUR. Taxonomic names were assigned to all sequences using the Ribosomal Database Project (RDP) naïve Bayesian classifier with confidence threshold of 60% and 1000 iterations and the mothur-formatted version of the RDP training set v.9 (trainset9_032012).[22] For each OTU, the most common sequence was selected as the most representative sequence. Read counts for each OTU were tabulated for downstream analysis. Standardized mock communities were included to check for technical performance of all experimental steps. Negative control samples of the lysis buffer did not show signs of contamination. Based on preliminary cluster analyses we identified three outlier samples in the skin database (data not shown). Two of these samples were dominated by contaminant species (*Bifidobacterium, Enterobacter*) likely transferred via the feces as the samples were collected from the legs of young children. The third skin sample was dominated by *Enhydrobacter*. The three samples were excluded and a total of 89 nasal and 57 skin samples remained for further analysis (figure S1).

Statistical analysis

Differences in baseline characteristics and metadata were statistically tested using the Chi-Square or Fisher's exact test and non-parametric Mann-Whitney U Test for independent samples where appropriate (SPSS version 24). Alpha diversity of the nasal and skin samples was calculated based on unfiltered OTU tables that were subsampled to the sample with the lowest total read count (1160). We calculated richness (number of different OTUs) and Shannon index (number of different OTUs and how evenly they are distributed) and compared these between nose and skin samples using non-parametric independent sample Mann-Whitney-U test. Non-subsampled OTU tables were filtered

for further analysis. OTUs present in less than two samples and with less than 10 counts in total were excluded for downstream analysis. Species and phylum relative abundances were visualized using stacked bar charts. The most dominant species in the nose and skin were estimated based on median relative abundances.

The filtered OTU tables were standardized using Hellinger transformation for further ordination analysis.[23] To test whether AD severity (SA-EASI) significantly drives differences in overall microbial composition, we used a permutational multivariable analysis of variance (PERMANOVA) based on Bray-Curtis dissimilarity.[24] The Bray-Curtis dissimilarity scale measures similarity between communities based on the taxa present and their relative abundances. The PERMANOVA tests were adjusted for age, use of antibiotics and location of sample collection (only skin), and the number of permutations was set on 10000. To identify which species drive the association between the overall microbial composition and AD severity, we obtained PERMANOVA coefficients.[24]

To visualize overall differences in microbial composition between nose and skin samples, we used nonmetric multidimensional scaling (NMDS) plots based on Bray-Curtis dissimilarity. Statistical significance of this difference was assessed using PERMANOVA (10000 permutations). To identify correlations between the microbial communities of the nose and skin, we carried out regularized canonical correlation analysis (RCCA), including the 'ridge' method.[25] For this RCCA we included OTUs that were present in at least 20% of the samples.

The statistical analysis were performed using the R statistical software (RStudio version 1.0.153). We used the packages 'vegan' (version 2.4-6) for NMDS and PERMANOVA [24], 'phyloseq' (version 1.21.0) for alpha diversity [26], 'CCA' (version 1.2) for RCCA [27] and 'ggplot2' (version 2.2.1) for visualization.[28] The set.seed function was used (with seed = 32) to obtain reproducible results.

RESULTS

Characteristics of the study population

A total of 90 nose and 108 skin samples were collected and all 108 skin samples were analysed using qPCR. A total of 89 nose and 57 skin samples were available for analysis after 16S rRNA sequencing (figure S1). The 57 skin samples were collected from the antecubital fold (n=36), popliteal fold (n=9), head/neck (n=5), arm (n=4) and an unknown location (n=3). For 48 children, samples of both the nose and skin were available. Baseline characteristics of the children are described in table 1.

Table 1. Patient characteristics

	Nose cohort (n=89)	Skin cohort (n=57)	p- value
Female sex			
n (%)	42 (47.2)	25 (43.9)	0.694
Age			
months; median (IQR)	30 (11.1-70.6)	22.9 (9.5-62.5)	0.475
SA-EASI			
median (IQR)	30.8 (16.2-52.5) [&]	36.9 (19.2-58.5) ^	0.346
Use of medication, n (%)			
Topical corticosteroids	67 (75.3)	45 (78.9)	0.609
Systemic corticosteroids	0 (0)[$]	0 (0)^	-
Topical antibiotics	7 (7.9)	3 (5.3)	0.741
Systemic antibiotics	2 (2.2)	1 (1.8)	0.663

^ n=55; 2 missing, [&] n=88; 1 missing, [$] n=87; 2 missing

P-values were calculated using Chi-Square test and Fisher's Exact Test for categorical data. A non-parametric Mann-Whitney U Test for independent samples was used for continuous variables

Microbial diversity of nose and skin in children with AD

A total of 2107454 high quality sequences were obtained from the 89 nasal samples (median per sample 22196; range 697-56436). The sequences were assigned to 390 OTUs, 10 different bacterial phyla and one archaeal phylum. Nasal samples contained mainly Proteobacteria (52%), Firmicutes (28%) and Actinobacteria (11%), illustrated in figure 1a. There seems to be a negative correlation between Proteobacteria and Firmicutes, since samples with a high abundance of Firmicutes have lower abundance of Proteobacteria and vice versa. From the 57 skin samples, 1110689 high quality sequences were obtained (median per sample 12449; range 1124-106087), belonging to 358 OTUs of 9 different bacterial phyla and one archaeal phylum. Firmicutes were very predominant on the skin samples with a median relative abundance of 92% of all the bacterial phyla. Proteobacteria, Actinobacteria and Bacteroidetes followed (figure 1b). A comparison of the 10 most abundant OTUs between nose and skin is presented in figure 2. As shown in this figure, *Moraxella, Dolosigranulum* and *Corynebacterium* were prevalent in the nasal microbiome with median relative abundances of 25%, 12% and 7%, respectively. In the skin samples staphylococcal species comprised a large part of the microbial community with a median relative abundance of 87% (figure 2 and figures S2a-b). Alpha diversity, the diversity within a single sample as measured using richness and the Shannon index, shows lower Shannon diversity but not richness in the skin samples compared to the nose samples (figure 3; p-value <0.001 and 0.366).

Figure 1a. Distribution of phyla in the nose samples (n=88, one missing sample)

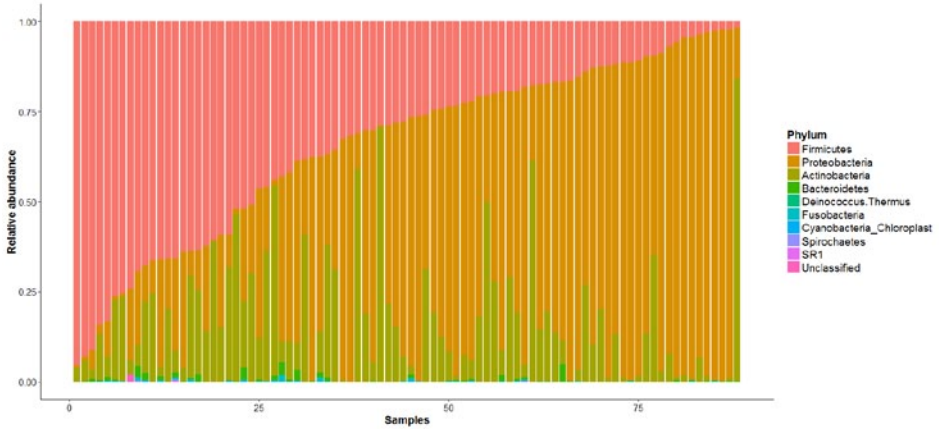
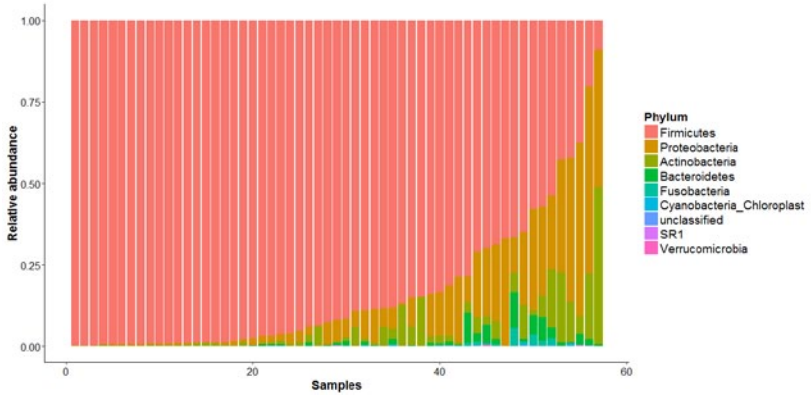


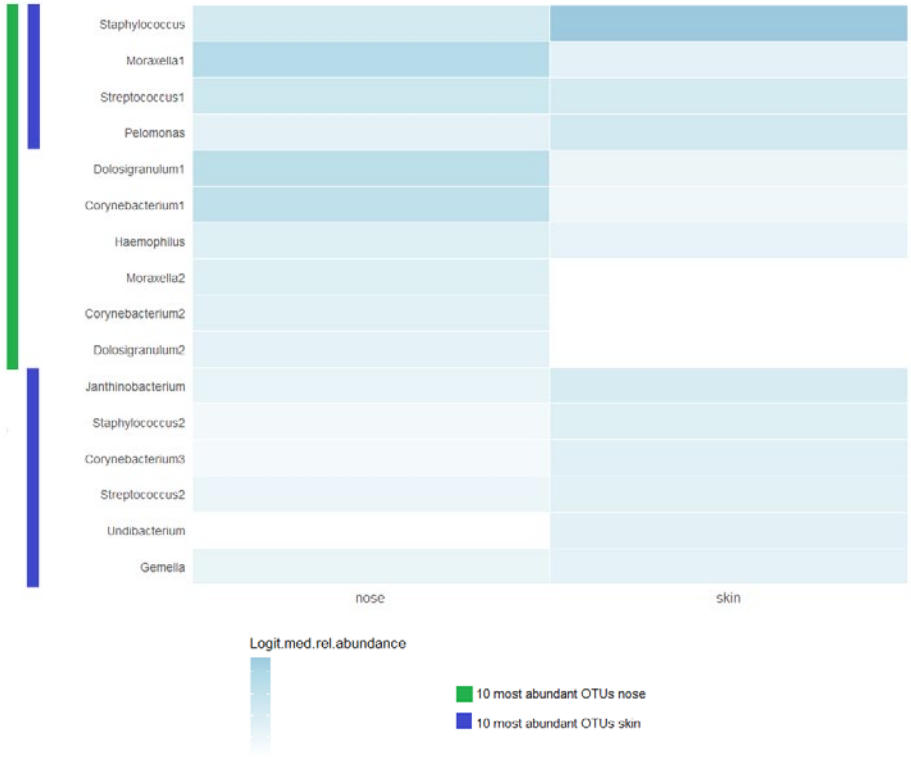
Figure 1b. Distribution of phyla in the skin samples (n=57)



Associations between the microbiome and severity of AD

We found a significant association between the overall microbial composition in the nose and the severity of AD measured by SA-EASI (PERMANOVA, R^2=2.6%; p=0.017; table 2). This association was driven by *Staphylococcus* (increased in more severe AD) and *Dolosigranulum* (decreased in more severe AD) (figure 4a). Also the variance in microbial composition of skin samples was significantly influenced by severity of the AD (R^2=7.0%, p=0.005). Staphylococci were the strongest drivers of the association between skin microbial composition and AD severity. However, other species illustrated in figure 4b might also be implicated. Interestingly, the contribution of the skin microbiome to AD severity is larger than of the nasal microbiome. Next to disease severity, age of the patient was also significantly associated with nasal microbial composition while use of antibiotics and location of sampling influenced the skin microbiome (table 2). To get more insight in the role of the staphylococci on the skin in AD severity, an additional qPCR was performed on the 108 collected skin samples to identify *S. aureus*

Figure 2. 10 most abundant OTU's in the nose and on the skin (n=48)



NOTE: heatmap is based on the logit transformed median relative abundance. Top of blue color gradient bar corresponds with a median relative abundance of 90%. The lowest value (white) lays below 0.01%. On the OTU level, different members of the same species were identified that could not be further classified. The OTUs were named by the species followed by a number (e.g. *Moraxella* 1 and *Moraxella* 2).

and *S. epidermidis* (as a representative for coagulase-negative staphylococci). Of these samples, 50.0% and 79.6% were positive for *S. aureus* and *S. epidermidis,* respectively. Children with moderate and severe AD were colonized with *S. aureus* on their skin more often than children with mild AD (58% and 51% versus 39%), however this was not significantly different (p=0.299; Chi-Square test) and the load of *S. aureus* did not increase with AD severity (p=0.291; figures S3a-b). The prevalence of skin colonization with *S. epidermidis* did not differ between mild, moderate and severe AD (86%, 74% and 80% respectively; p=0.513). However, the load of *S. epidermidis* was significantly higher in severe AD compared to mild AD (p=0.016; figures S3a-b).

Differences and correlations between nasal and skin microbial communities

To visually inspect differences in the microbiome composition between skin and nose we grouped all samples from patients of which both a nose and skin sample were avail-

Figure 3. Richness (Chao1) and Shannon diversity in nose and skin

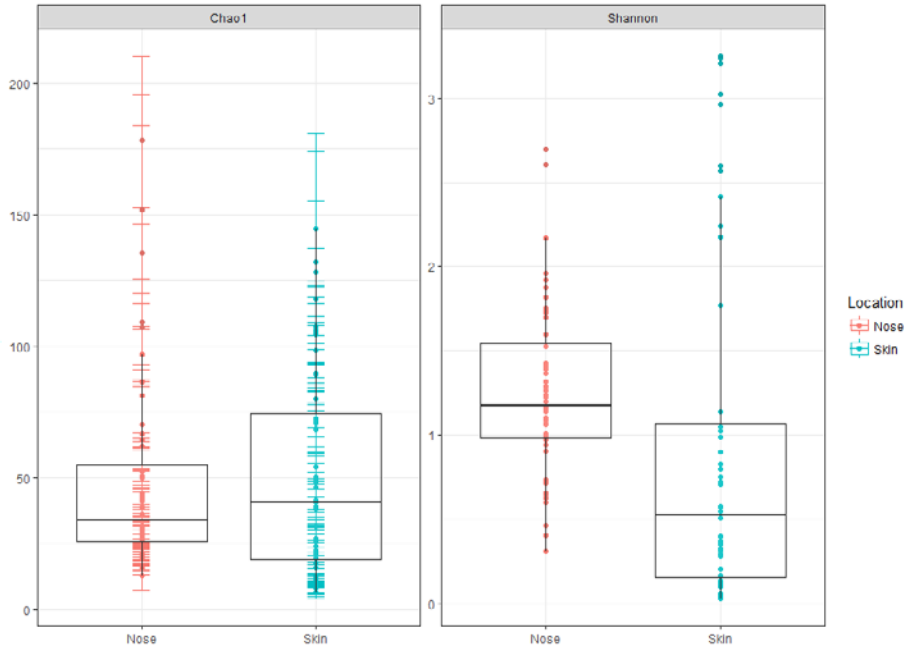


Table 2. Multivariate analysis showing the influence of different covariates on the variance in the nasal and skin microbiome

	Nose (n=88)		Skin (n=53)	
	R^2	P- value	R^2	P- value
SA-EASI #	0.026	0.017*	0.070	0.005*
Age	0.057	<0.001*	0.021	0.212
AB use (topical and oral)	0.006	0.839	0.062	0.007*
Location of sampling ^	-	-	0.052	0.017*

* significant p-value

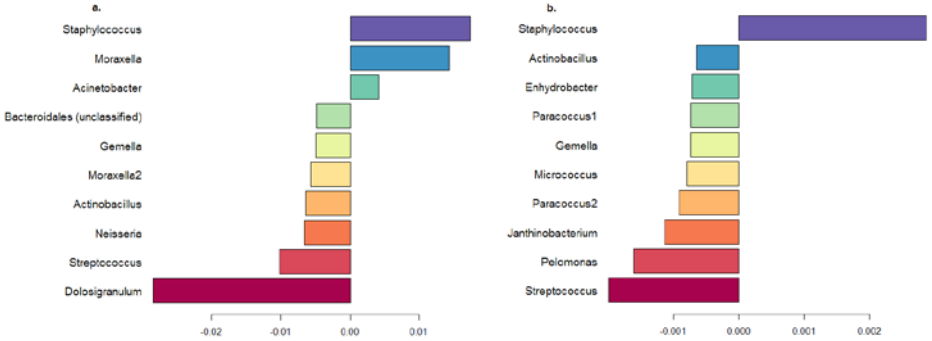
Predetermined order of variables included in the adonis model: SA-EASI, age and antibiotic use for the nose; SA-EASI, sample location, antibiotic use and age for the skin.

^ NB: the homogeneity of dispersion (assumption for adonis) was tested among locations of sampling. P-value of 0.714 indicates that our test results are not an artifact of differences in microbial composition between different locations (beta diversity). As only 4 patients used antibiotics, dispersion could not be reliably tested for these groups.

NOTE: 53 of 57 skin samples were included for analysis due to missing sample location (n=3) and missing SA-EASI score (n=2), one overlapping. 88 nose samples were included, due to one missing SA-EASI score.

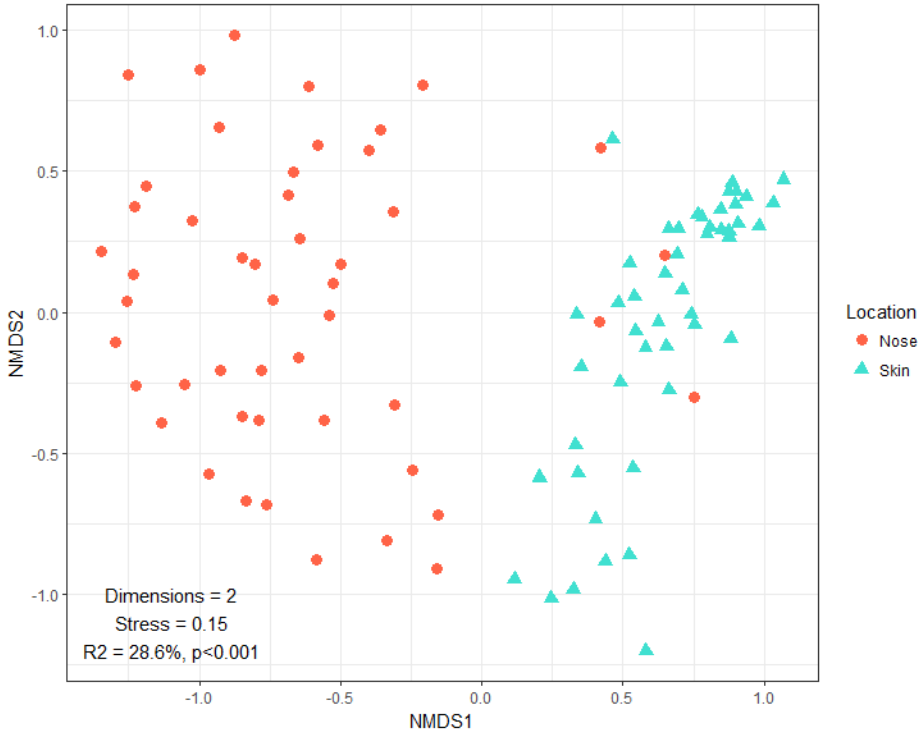
able (n=48). As shown in figure 5 microbial communities of the nose and skin clustered separately (PERMANOVA: R^2=30.9, p<0.001), indicating distinct microbial communities at each location. In all 48 patients, staphylococci were found both in the nose and on the

Figure 4. Top 10 OTUs driving the association between microbial composition and disease severity in nose (a) and skin (b)



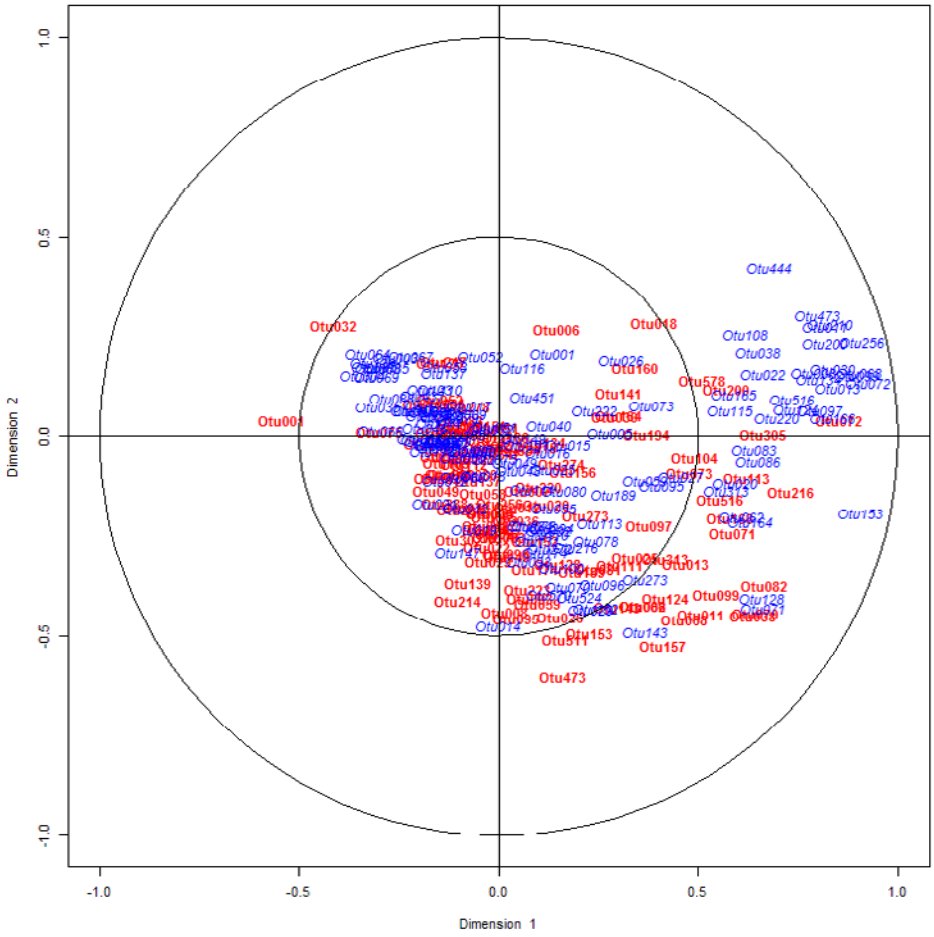
NOTE: The x-axis displays coefficients that represent the contribution of a certain OTU to the association between microbiome composition and severity of the AD.

Figure 5. NMDS plot of skin and nasal samples (n=48). Data are standardized using Hellinger transformation.



NOTE: The microbial composition of a small group of nasal samples resembled the skin samples. A closer view at these four samples showed that these were the samples with highest relative abundance of staphylococci in the nose.

Figure 6. Results from RCCA analysis showing the correlations between the microbiome composition of the nose and skin at OTU level.



NOTE: Canonical correlation dimension 1 and 2: 0.99 and 0.98. Lambda's were 0.00005 and 0.00003. Red OTUs represent the skin and blue represent the nose. Pairs of variables with relatively large weights in the same direction represent positive correlations and variables whose weights have opposite directions exhibit inverse correlations.

skin, but the predominance of the bacterium on the skin was larger (figure S2a-b). Other OTUs that were often found in both niches included *Moraxella* (n=45), *Streptococcus* (n=41), *Pelomonas* (n=39) and *Dolosigranulum* (n=35). To identify correlations between nose and skin microbiome composition, RCCA analysis was performed. Canonical correlations of the first and second dimension were 0.99 and 0.98, indicating that a significant correlation exists between the nasal and the skin databases (figure 6). Most OTUs are placed around the center of the biplot, indicating that these OTUs have a very small

contribution to the correlations between the nose and skin samples (in the first two components). Many skin and nasal OTUs are placed in the same quadrant of the biplot, meaning that they show similar patterns of abundance over the samples. Exceptions are OTU1 and OTU32 in the left upper quadrant, both representing *Staphylococcus* on the skin, that seem to be negatively correlated with most nasal and other skin OTUs.

DISCUSSION

In this study we found distinct microbial communities in the nose and on skin of children with AD. Additionally, we found significant associations between the microbial composition of both the nose and the skin and AD severity in children. Interestingly, this analysis was adjusted for important confounders including age, use of antibiotics and location of sampling.

Our finding that the nose and skin harbor distinct microbial communities is in accordance with earlier studies in healthy subjects.[29] The nares of the children in our cohort were predominated by the phyla Actinobacteria (*Corynebacterium spp.),* Proteobacteria (mainly *Moraxella* but also other species such as *Haemophilus*) and Firmicutes (*Staphylococcus*, *Streptococcus* and *Dolosigranulum* spp.) This is consistent with literature in healthy subjects that describes that the microbiome of the anterior nares is typically enriched for members of these three phyla.[30,31]

Skin lesions were clearly dominated by staphylococci, consistent with literature about skin microbiome in AD.[3,32] While the nasal samples also showed staphylococci, only a few patients showed staphylococcal dominance in the nose. Other OTUs that followed *Staphylococcus* in abundance on the skin of our patients were *Pelomonas*, *Streptococcus* (described especially in young children with AD before) and *Janthinobacterium* (described as an important member in healthy skin microbiome by Grice *et al.*).[32-34] Although the nose and skin harbor distinct microbial communities based on Bray–Curtis dissimilarity, we found that correlations exist between species in the nose and (other) species on the skin. The mechanisms that underlie these findings are not clear yet. Possible mechanisms, such as cross-transmission of bacteria between the niches, need further exploration.

Additional qPCR of the skin samples, showed *S. aureus* and *S. epidermidis* in 50% and 80% of our population, respectively. The prevalence rate of 50% for *S. aureus* on lesional skin is lower compared to a meta-analysis reporting 70%. This might be due to the young age of our cohort (median age of 2.5 years compared to 14 years in the meta-analysis). Kennedy *et al.* also found low *S. aureus* in 2 month old patients with AD.[2] Another study included infants with AD in the first year of life and found positive lesional skin swabs in 21% of the infants, with culture-based analyses.[11] In our cohort, the mean age of the

children that were positive for *S. aureus* did not significantly differ from the group that was negative (Students *T*-test, p=0.705).

We found that both the nasal and the skin microbial composition were associated with AD severity. Cross-sectional studies described associations between an altered microbiome and AD severity before for the skin.[3] For the nose, a single prospective study supports our hypothesis that the nasal microbiome plays a role in (severity of) inflammation in AD. The authors found a relation between nasal colonization with *S. aureus* at 6 months and the development of AD.[10] The timeframe of (nasal) colonization might be important as colonization earlier in life (1 month) was not found correlated with AD development.[11] The strength of the associations between AD severity and microbial composition of the nose and skin was 2.6% and 7.0%, which can be regarded of significant relevance as a large part of the variance in the microbiome can be be explained by host variation.[35] Although an association does not mean causality, our results suggest that both microbial niches (in terms of OTU composition), might play a role in aggravating or worsening the inflammation in pediatric AD. Longitudinal studies that include sampling around AD flares would be needed to confirm if changes in the microbiome precede an increase in AD severity.

Determination of the species that drove the associations between OTU composition and AD severity, showed that in both niches increased staphylococci were of influence. We further classified staphylococci to the species level and found that children with severe AD had *S. aureus* present on the skin more often than patients with mild AD (58% vs 39%) as described earlier.[2] However, this difference was not statistically significant. The presence of *S. epidermidis* on the skin did not significantly differ between mild and severe AD, but the load of *S. epidermidis* was significantly higher in severe AD compared to mild AD. This relates to an earlier study that observed increased bacterial counts of *S. epidermidis* preceding disease flares.[5] Besides staphylococci, we identified other species that drove the association between microbial composition and AD severity. For example, *Moraxella* was found to positively contribute to the association in the nose. An increased abundance of *Moraxella* in the nares of children was not associated with severity of AD before. However, Depner *et al*. found *Moraxella* associated with asthma.[36] In our cohort, 28% of the children had symptoms of asthma or bronchial hyper reactivity (<6 years) reported in their medical file. The association between *Moraxella* and AD severity that we found was not influenced by these diagnosis (p=0.645, data not shown). Thereby, certain species were identified of which a decreased abundance contributed to the association with AD severity in our study. For example *Dolosigranulum* in the nose and *Streptococcus* on the skin (figure 4a-b). High abundance of *Dolosigranulum* was suggested to be beneficial for respiratory health before.[37] *Streptococcus* was observed in lower relative abundance in skin lesions (compared to non-lesional skin) and during flares in children before.[3,32] Future studies should further evaluate the influence of these

species on a lower taxonomic level, as for example different streptococci can vary widely in pathogenic potential.[38] The identification of specific species that contribute to AD and its severity (or protect) is of importance for the development of new treatment strategies for AD.[39,40] Our results can guide future studies in determining their focus for further species specific research.

This study has several limitations. The cross-sectional design of this study precludes statements on cause–effect relationships between microbiota and disease severity. We amplified the V4 variable region using the 806R primer, an often used method at the time of analysis. However, this method does not allow classification of staphylococci at the species level, and recent literature describes that it can be improved by the use of the V1-V3 region or the use of a modified V4 primer.[41, 44] To investigate associations between staphylococci and disease severity to the species level, we performed additional qPCR on *S. aureus* and *S. epidermidis*.[41] Because we were not able to further classify staphylococci into other species, we might have missed correlations between specific staphylococcal species and AD severity.[9] This also holds for strain specific associations with disease severity, as suggested by Byrd et al.[42] In our study *Propionibacterium* was poorly detected in both the skin and nasal samples, while they are described as part of the healthy microbial communities.[43] With regard to the skin samples, the low *Propionibacteria* can still be a true reflection of the eczema lesions as staphylococci are known to overgrow other species. Thereby, we sampled mainly the antecubital and popliteal folds which are known for a low abundance of *Propionibacteria* that prefer more sebaceous environments, such as upper chest.[45] In our study we did not characterize the fungal microbiome, which was found altered in patients with AD before.[5] Despite the limitations, this study shows the relevance of the nasal microbiome in AD and is as far as we know the first to characterize relations between the nose and skin microbiome in AD.

CONCLUSION

This study shows that the severity of AD in children is influenced by the nasal microbial composition. Besides *Staphylococcus*, also other species seem to contribute to the association between skin and nasal microbiome and AD severity. It is important that future studies further explore the role of microbial species in AD, their interaction with the host and other species, and the interaction between different microbial communities of the body, using controlled longitudinal cohorts and adequate sequencing and culture based techniques.

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SUPPLEMENTARY MATERIAL

Figure S1. Flowchart of the sample collection

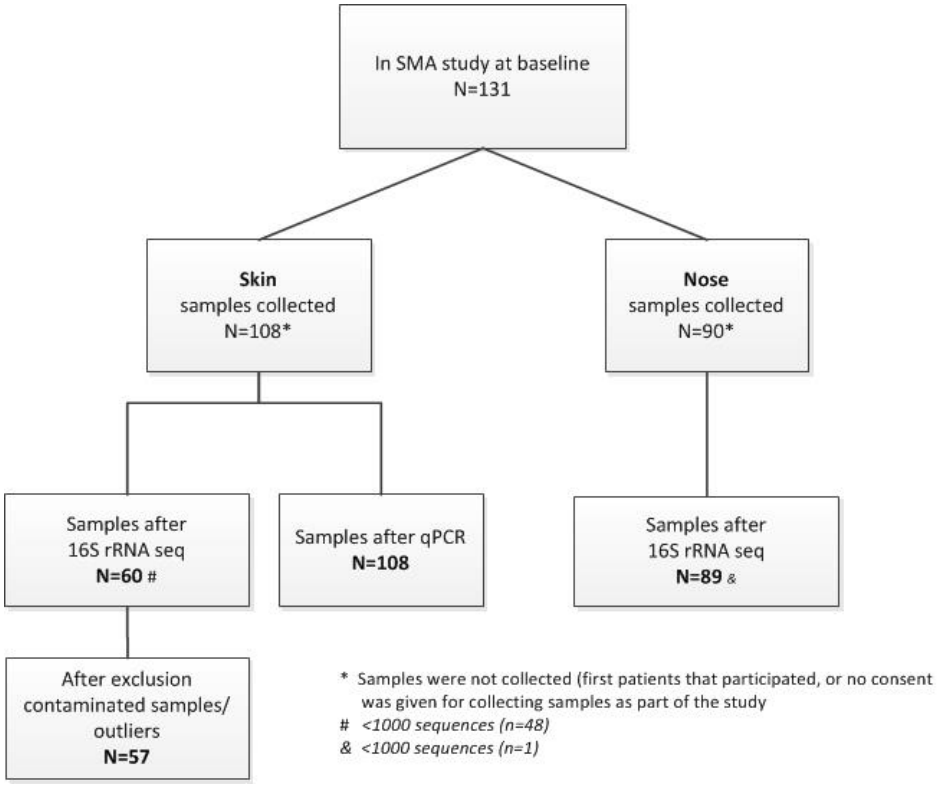


Figure S2a. Distribution of 20 most abundant OTUs in the nose samples (n=89)

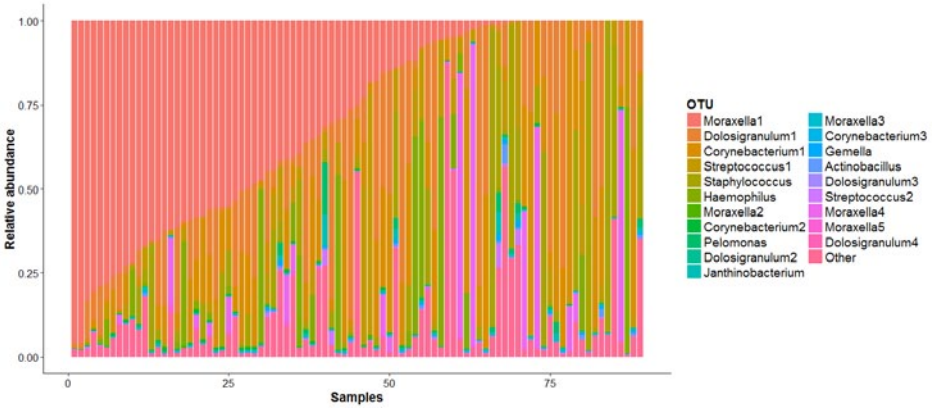


Figure S2b. Distribution of 20 most abundant OTUs in the skin samples (n=57)

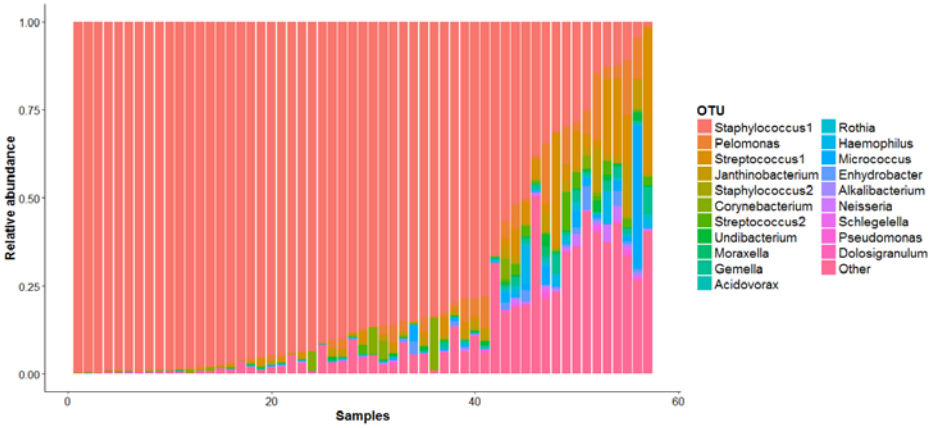
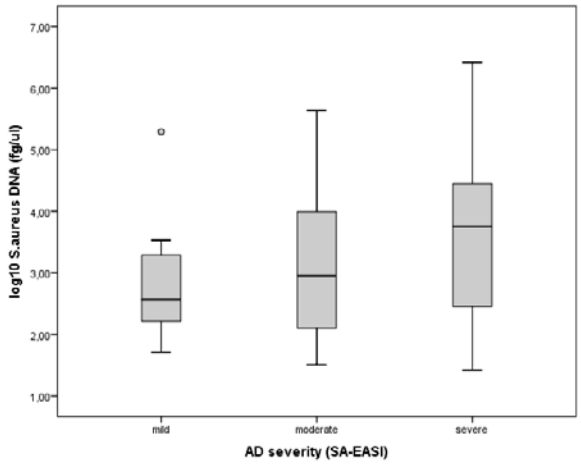
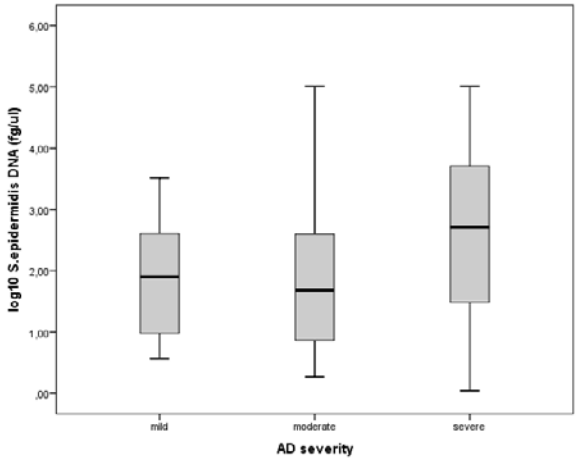


Figure S3a. *S. aureus* load in mild to severe AD



NOTE: no significant differences (p=0.291; ANOVA)

Figure S3b. *S. epidermidis* load in mild to severe AD



NOTE: significant differences (p=0.029; ANOVA), mild versus severe AD (p=0.016; Students t-test)

Supplementary methods

Cut-off values were calculated for the SA-EASI based on the objective SCORing Atopic Dermatitis (SCORAD), a clinical tool used to assess the severity of eczema.[17] The formula to calculate the cut-off points for the SA-EASI included the cut-off points of SCORAD divided by maximum score of SCORAD multiplied by maximum score of the SA-EASI (SA-EASI = cut-off point SCORAD / 83 * 96). Patients with a SA-EASI from 0 to17.35 were classified as mild eczema, from 17.35 to 46.27 as moderate eczema and with a SA-EASI higher than 46.27 as severe eczema.

Chapter 3

Fecal microbiome and food allergy in pediatric atopic dermatitis: a cross-sectional pilot study

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ABSTRACT

Background

Microbial exposure might be important in the development of atopic disease. Atopic diseases have been associated with specific characteristics of the intestinal microbiome. The link between intestinal microbiota and food allergy has rarely been studied and the gold standard for diagnosing food allergy (double blind placebo controlled food challenge (DBPCFC)) has seldom been used. We aimed to distinguish fecal microbial signatures for food allergy in children with AD.

Methods

Pediatric patients with AD, with and without food allergy were included in this cross-sectional observational pilot study. AD was diagnosed according to the UK Working Party criteria. Food allergy was defined as a positive DBPCFC or convincing clinical history in combination with sensitization to the relevant food allergen. Fecal samples were analyzed using 16S rRNA microbial analysis. Microbial signature species discriminating between presence and absence of food allergy were selected with elastic net regression.

Results

82 children with AD (39 girls, median age 2.5 years old) of which 20 were diagnosed with food allergy provided fecal samples. Food allergy to peanut and cow's milk was the most common. Within children with AD, six bacterial species from the fecal microbiome were identified that when combined discriminate between children with and without food allergy: *Bifidobacterium breve, Bifidobacterium pseudocatenulatum, Bifidobacterium adolescentis, Escherichia coli, Faecalibacterium prausnitzii* and *Akkermansia muciniphila (*AUC 0.83, sensitivity 0.77, specificity 0.80).

Conclusions

In this pilot study, we identified a microbial signature in children with AD that discriminates between absence and presence of food allergy. Future studies are needed to confirm our findings.

INTRODUCTION

The worldwide prevalence of atopic disease has been increasing in recent decades.[1] There is no clear reason for this observed increase in prevalence, but reduced early-life exposure to different microbes is thought to be a contributing factor.[2-4] Microbial colonization of the human intestine during infancy is important for the maturation of the immune system.[5, 6] Intestinal microbiota can regulate metabolic and inflammatory responses and also modulate changes in the intestinal barrier. Several studies have shown associations between intestinal microbiota and subsequent development of atopic disease, including atopic dermatitis, asthma or rhinitis. However, few studies have investigated the link between specific patterns of intestinal microbiota and food allergy. Furthermore, the gold standard for diagnosing food allergy (double blind placebo controlled food challenge (DBPCFC)) has rarely been used.[7]

The microbiome can be considered a complex ecosystem where various species interact and group-based correlations have been identified.[5] Therefore the symbiosis of the different bacterial species and their patterns should be taken into account in data analysis. To be able to identify individual species and take the existing structures within the microbiome into account, advanced statistical modelling techniques are needed. Furthermore, assessment of microbial diversity with molecular sequencing techniques, as opposed to culture-based techniques, reveals greater diversity and has shown the importance of uncultured species.[8]

We hypothesize that children have distinct microbial patterns in their fecal microbiome that are associated with a clinical diagnosis of food allergy. In this cross-sectional pilot study, we aimed to identify microbial species in children with AD, using 16S rRNA microbial analysis followed by statistical elastic net regression approaches.

METHODS

Study design and study participants

Children with AD who were treated in the outpatient clinic of the Wilhelmina Children's Hospital of the University Medical Center Utrecht participated in this cross-sectional pilot study. Inclusion criteria were: diagnosis of AD, age between 0 and 18 years, parental ability to answer Dutch questionnaires and the availability of a fecal sample for microbiome analysis. All study participants participated in a randomized controlled trial that compares shared medical appointments with individual consultations (ISRCTN08506572). The medical ethical committee of the University Medical Center Utrecht approved the study and written informed consent was obtained from all participants. Clinical history

and serum samples were taken on the same day, fecal samples were provided within the next days and a DBPCFC was planned within months.

Assessment of AD and food allergy

AD was diagnosed according to the criteria of Williams et al.[9] AD severity was estimated using the self-administered eczema area and severity index (SA-EASI) by the research nurse.[10] Sensitization was determined by measurement of specific IgE against common food allergens (hen's egg, cow's milk, peanut, hazelnut, fish, wheat, soy). Both total and specific IgE were measured according to manufacturer's protocol (Phadia, Uppsala, Sweden). Diagnosis of asthma and allergic rhinitis was based on clinical history.

Food allergy was defined as a positive double blind placebo-controlled food challenge (DBPCFC) or convincing clinical history in combination with sensitization to that specific food or in case of peanut allergy, a sensitization to Ara h 2 above the defined cut off level in our clinic (5.17 kU/L).[11] A convincing clinical history was defined as a reported Type I allergic reaction with acute symptoms within 2 hours after ingestion of the food. DBPCFC was considered positive and terminated when persistent objective symptoms occurred (e.g. vomiting, generalized urticaria, wheezing or a significant drop in blood pressure) or after subjective symptoms (oral allergy symptoms, nausea, abdominal discomfort) on three subsequent doses or a severe subjective symptom (abdominal pain/nausea with discomfort) lasting for more than 45 minutes, according to the international protocol.[12] Late reactions were assessed using follow-up by telephone the next day.

Fecal samples

Children collected fecal samples at home and sent the samples to the laboratory using the regular postal service. The samples were aliquoted and frozen at -20^{o}C until further processing.

Fecal DNA isolation

Approximately 150 mg of fecal material was directly transferred to the DNA isolation plate. Then 0.5 mL phenol pH8.0 (Phenol solution, catalogue P4557, Sigma-Aldrich, St Louis, MO) was added and the samples were mechanically disrupted by bead beating 2 times 3 minutes with a 96-well plate Beadbeater (Biospec Products, Bartlesville). Samples were centrifuged at 1880 rcf (4000rpm) for 10 minutes to separate the aqueous and phenolic phases. The aqueous phase was transferred to a 96-well plate and DNA was purified with the AGOWA mag Mini DNA Isolation Kit (AGOWA, LGC genomics, Berlin, Germany) in accordance with the manufacturer's recommendations. After elution, the total bacterial load in each sample was assessed by quantitative PCR using a universal bacterial primer-probe set.[13]

16S rRNA Illumina sequencing

Analysis of the fecal microbiome composition was performed by mass sequencing of the V4 hypervariable region of the 16S rRNA gene on the Illumina MiSeq sequencer (Illumina, San Diego, CA). Barcoded DNA fragments spanning the Archaeal and Bacterial V4 hypervariable region were amplified with a standardizing level of template DNA (100pg) to prevent over-amplification. These amplicons, generated using adapted primers 533F and 806R, were bi-directionally sequenced using the MiSeq system.[14] Pre-processing and classification of sequences was performed using modules implemented in the Mothur V.1.20.0 software platform.[15] The relative abundance of unique sequences was calculated for every fecal sample. The dataset was transformed using zero mean unit variance transformation for subsequent statistical analyses. The V4 amplicon of the 16S rRNA encoding gene allows for discrimination of several Bifidobacterial species, but not all.[16] Therefore, relevant sequences were blasted in the Ribosomal Database Platform (RDP) to determine a more accurate species level. Shannon diversity indices were calculated to describe the microbial diversity.

Statistical analysis

Descriptive statistics were used to describe patient characteristics. Non-parametric tests were used to compare the groups without and with a confirmed food allergy.

Elastic net regression

Bacterial signature species discriminating between absence and presence of food allergy were selected using elastic net regression. This is a statistical machine learning approach, applicable to large scale, structured and higher dimensional data. The method is regularization-based and combines the advantages of LASSO regression (sparsity, retaining the feature selection property of reducing coefficients to exact zero values provided by LASSO) and ridge regression (smoothness, tendency of shrinking coefficients to small values for correlated trending towards each other).[17, 18] All present species and the correlations between them are taken into account, which allows for the identification of patterns of species rather than individual species.[19] Using elastic net regression, it is not possible to correct for other confounding factors which is common in other types of regression analyses used in medical statistics.[19]

Randomization test and ROC/AUC

A randomization test was conducted to test the statistical validity of the results obtained with elastic net regression. Receiver-Operating-Characteristics/Area-Under-Curve (ROC/AUC) scores were generated multiple times after randomly reshuffling the food allergy diagnoses, while keeping the corresponding microbial profiles intact.[20] The dataset was cross-validated by randomly hiding 30% of the children from the model and evaluating

the prediction quality on that group. The predictive accuracy of the classification model was measured with the ROC/AUC score, using a critical value of 0.05.

SPSS (version 22; IBM, Armonk, NY) was used for descriptive data analysis. GraphPad Prism (version 6.01; GraphPad Software, La Jolla, CA) was used for providing graphs and figures. All other statistical analyses were performed using numerical Python (version 2.7, Python Software Foundation, https://www.python.org).

RESULTS

Atopic dermatitis and food allergy

We included 82 children in this cross-sectional pilot study. There were no significant differences regarding sex or age between the children who were included and those who were not (data not shown). All 82 children were diagnosed with AD, 62 children had no food allergy (AD+FA-) and 20 children had a confirmed food allergy (AD+FA+) (table 1).

Of the 62 children without food allergy, almost half were sensitized to common food allergens without having symptoms of food allergy after ingestions. Among the 20 children with a food allergy, peanut allergy and cow's milk allergy were the most common (table 2). Multiple food allergies were found in two children. On average, a DBPCFC was performed within 10 months of providing the fecal samples (minimum 1 month, maximum 27 months).

Table 1. Patient characteristics

	No food allergy (AD+FA-) (n=62)	**Confirmed food allergy (n=20) (AD+FA-)**	**p-value**
Age in years [median (IQR)]	3.0 (5)	2.2 (5.5)	0.606
Female [n (%)]	32 (52%)	7 (35%)	0.196
SAEASI [median (IQR)]	29 (32)	46 (42)	0.283
TARC (pg/ml) [median (IQR)]	1243 (2247)	2251 (5613)	0.013
Total IgE (kU/L) [median (IQR)]	66 (281)	564 (2912)	<0.001
Sensitization to any food allergen [n (%)]	27 (43%)	20 (100%)	<0.001
Elimination diet for any food [n (%)]	19 (31%)	20 (100%)	<0.001
Diagnosed with asthma [n (%)]	18 (29%)	5 (25%)	0.727
Diagnosed with rhinoconjunctivitis [n (%)]	15 (24%)	5 (25%)	0.942

Data is shown with median and interquartile range or number and percentage. AD = atopic dermatitis, FA = food allergy, SAEASI = self-administered eczema area and severity index scored by research nurse, TARC = thymus and activation regulated chemokine.

Table 2. Children with confirmed food allergies

Food allergy	Confirmed	With DBPCFC*	Obvious clinical history only	Predicted based on elevated sIgE to arah2 > 5.17 KU/L
	n(%)	n(%)	n(%)	n(%) [11]
Peanut	8 (40%)	3 (15%)	1 (5%)	4 (20%)
Hazelnut	1 (5%)	1 (5%)		
Cow's milk	8 (40%)	6 (30%)	2 (10%)	
Hen's egg	4 (20%)	3 (15%)	1 (5%)	
Other nuts	2 (10%)		1 (5%) cashew nut 1 (5%) pistachio	
Soy	0			
Fish / shrimp	0			
Total	20 (100%)			

Multiple food allergies result in multiple entries. One patient has a confirmed food allergy for cow's milk, peanut and hen's egg, another patient has a confirmed allergy for hazelnut and hen's egg.
*including no late reactions

Sequence and microbiota characteristics

A total of 2.609.478 high quality sequences were obtained (mean=27.182, range=5.825 to 105.404 of sequences per sample) that could be assigned to 12 different phyla and 1.000 unique sequences. The most predominant phyla based on mean relative abundance were Firmicutes (47%), Actinobacteria (32%), Bacteroidetes (9%), Proteobacteria (8%) and Verrucomicrobia (2%), which is characteristic for the gut microbiome of children.[21] Predominant families were Bifidobacteriaceae (28%), Lachnospiraceae (27%), Ruminococcaceae (10%), Enterobacteriaceae (5%), Streptococcaceae (4%) and Coriobacteriaceae (3.5%). Median Shannon diversity indices calculated for the group of children with and without a food allergy were 3.61 (IQR 1.16) and 3.93 (IQR 1.09), respectively (p=0.430).

Identification of microbial biomarkers related to food allergy

We identified six microbial species from four families that together discriminate between the absence and presence of food allergy in children with AD: *Bifidobacterium breve, Bifidobacterium pseudocatenulatum, Bifidobacterium adolescentis* (Bifidobacteriaceae), *Escherichia coli* (Enterobacteriaceae), *Faecalibacterium prausnitzii* (Ruminococcaceae) and *Akkermansia muciniphila* (Verrucomicrobiaceae). On the species level, *Bifidobacterium breve/longum* and *Bifidobacterium pseudocatenulatum/ catenulatum/ gallicum/ kashiwanohense* could not be distinguished after additional blasting in the RDP database, and are referred to as *Bifidobacterium breve* and *Bifidobacterium pseudocatenulatum* throughout the manuscript.

Figure 1 shows the relative abundance of the six identified signature species. The fecal microbiome of children with AD and food allergy harbored relatively more *E.coli* and *B.pseudocatenulatum*, and less *B.breve, B.adolescentis, F.prausnitzii,* and *A.muciniphila*, compared to children with AD without food allergy. The randomization test indicates that the combination of these six species is significantly different between the two groups (p = 0.001), even though the relative abundance of some single species may seem similar on a group level (figure 1). Different relative contributions from the single species towards the total distinctive properties are distinguished, with a larger influence of *B.breve*, *B.adolescentis* and *F.prausnitzii* compared to *B.pseudocatenulatum*, *E.coli* and *A.muciniphila*, expressed as importance indices based on the elastic net regression (figure 2). The overall predictive accuracy of the classification model (area under the curve) is 0.83 (figure 3), with a sensitivity of 0.77 and a specificity of 0.80. Supplementary figures S1 to S3 show the relative abundance of the signature species, the distribution of the 30 most abundant species and the individual distribution of the signature species.

Figure 1. Relative abundance of the microbial signature species in children with AD, without and with a confirmed food allergy

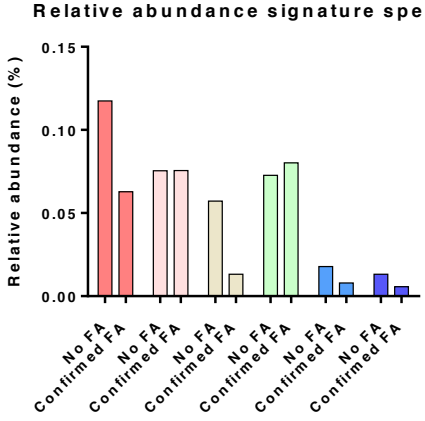
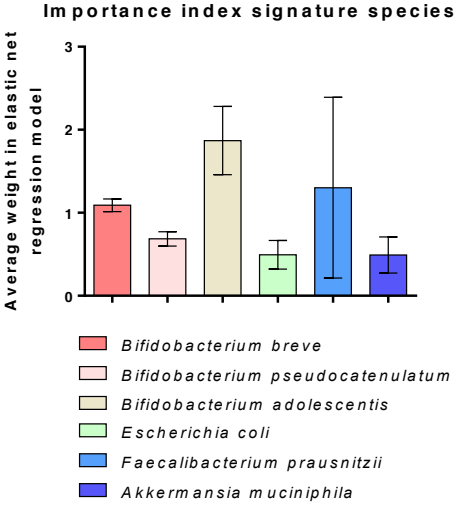


Figure 2. Importance index for signature species in the elastic net regression model



DISCUSSION

We analyzed the fecal microbiome of children with AD with or without a concomitant food allergy and found that a combination of six microbial species, including *E.coli*, *F.prausnitzii, A.muciniphila* and three types of *Bifidobacteria*, discriminates between the presence and absence of food allergy in children with AD (p = 0.001). The fecal

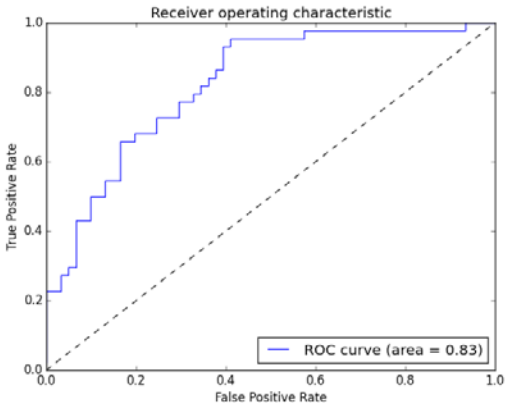


Figure 3. Receiver operating characteristic (ROC) curve of the elastic net regression model

microbiome of children with AD and food allergy harbored relatively more *E.coli* and *B.pseudocatenulatum* and less *B.breve*, *B.adolescentis*, *F.prausnitzii* and *A.muciniphila*, compared to children with AD without food allergy. We found no differences in microbial diversity (according to Shannon index) between the children with AD, with and without food allergy.

This is the first pilot study that identifies microbial signatures specific for food allergy in a group of children with AD using 16S rRNA sequencing techniques generating unique sequences, followed by statistical machine learning approaches. Previous studies mainly used culture-based techniques to analyze the intestinal microbiome or used 16S rRNA sequencing techniques, but subsequently simplified the data in the analysis stage by focusing on key groups of species or analyzing the data on family or genus level. However, this approach leads to less detailed information. For example, the identification of *B.pseudocatenulatum*, *B.breve* and *B.adolescentis* would not have been possible when analyzing the data on a family level. Furthermore, the elastic net regression model takes group-based species interactions into account. Since interactions between species in the gut microbiome occur, this approach may lead to biologically more reliable results compared to other statistical regression approaches.[22]

Our study demonstrates that children with AD and a food allergy had significantly less *Faecalibacterium prausnitzii* and less *Akkermansia muciniphila* compared to children with AD without a food allergy. *F. prausnitzii* and *A. muciniphila* have been gaining interest more recently because of their immune-modulatory properties and possible role in mucosal tolerance. *F. prausnitzii* is the most abundant species in the human intestinal microbiome. Its decreased abundance has been associated with several diseases, including allergic disease and AD. [23-25] *F.prausnitzii* is the main producer of butyrate in the colon, an energy source for colonocytes with important anti-inflammatory effects. It also secretes anti-inflammatory molecules that directly modulate the host immune system, stimulates IL10 producing regulatory T cells and is involved in the balance between

effector and regulatory T cells. [26, 27] *A. muciniphila* is also involved in immunological homeostasis of the gut mucosa and gut barrier function, via an outer membrane protein that stimulates IL10 production.[28]

Bifidobacteria and *E.coli* have been associated with food allergy and AD in other studies.[29] Less *Bifidobacteria* in the feces of children with a confirmed cow's milk allergy has been reported.[30] Cow's milk allergy was a common food allergy in our study population, so it is possible that our results regarding *Bifidobacterium breve* and *Bifidobacterium adolescentis* are mainly contributed by the cow's milk allergic children. Furthermore, we found increased relative abundance of *Escherichia coli* in the food allergic group. *E. coli* has previously been associated with diagnosis of AD, with increasing numbers of *E. coli* further increasing this risk.[31] The children in our study were all diagnosed with AD with varying severity. However, the higher TARC levels in the food allergic group suggest increased AD severity compared to the non-allergic group. This raises the possibility that the selected biomarkers also correlate with AD severity, which fits with the observation that prevalence of food allergy is higher in children with more severe disease.[32]

All microbial species resulting from our analysis have previously been correlated with atopic disease in other studies. This might raise the question whether we are looking at a food allergy specific microbial profile or a profile that is related to atopic diseases in general, as most of these children have or will develop other comorbidities within the atopic syndrome. Atopic disease has been defined differently in previous studies. In our study, all children were clinically diagnosed with AD and in addition asthma and allergic rhinitis was confirmed or ruled out based on clinical history. Food allergy was diagnosed based on DCPCFC in the majority of patients. Post-hoc analyses showed no significant differences in the presence of other atopic diagnoses between the groups with and without food allergy, suggesting that the identified species are indicating food allergy rather than general atopy.

Our study supports the hypothesis that within children with AD the intestinal microbiome differs between children with and without food allergy. Intestinal microbiota regulate the development of a diverse range of T-cell functions, such as Th17, Th1, Th2 and regulatory T cells and modulate innate lymphoid cells.[33, 34] By modifying the response of the gut-associated lymphoid tissues, intestinal microbiota may influence the development of oral tolerance.[35] A recent study in humans showed that delayed colonization with Bacteroidetes is associated with a poorly developed Th1 response, which is important in immune tolerance.[36] It is also possible that disruption of the gut microbiome alters the gut epithelial integrity, thereby increasing the risk of allergic sensitization through direct uptake of allergens.[7] However, the exact mechanisms through which the intestinal microbiome influences food allergy are not elucidated yet. Furthermore, it is not clear whether a change in microbiome precedes or follows the development of food allergy.

Long-term dietary intake affects gut microbiome composition, together with host genetics, age, medication, and general lifestyle.[37] Our study population consumed a Western diet. In addition, established food allergies lead to an elimination diet, where the specific food allergen is excluded from the general diet. We cannot exclude that an elimination diet where one food is excluded from the diet also leads to detectable changes in fecal microbial composition, as has been demonstrated with increased consumption of specific foods. [38] However, in our study one third of children in the non-food allergic group also reported an elimination diet for a specific food because of various reasons. Furthermore, dietary intake varies according to personal preference. Therefore it is unlikely that the observed microbial differences are solely attributed to differences in the consumed diet. Besides a self-reported elimination diet, dietary intake was not further assessed in this study because it is very difficult to assess this accurately.

A limitation of our cross-sectional study is the heterogeneity of the study population. All the children in our study were diagnosed with AD, with varying severity, age and different food allergies, and no healthy controls were included. As expected in children, cow's milk allergy and peanut allergy were the most common food allergies in our study population, so it is possible that our results are influenced by the contribution of these food allergies. It is also plausible that distinct microbes are associated with different food allergies.[29] Due to a lack of statistical power, we were unable to select signature species for specific food allergies. Variables that are known to influence the gut microbiome, such as use of antibiotics, delivery via caesarian-section, or breast feeding, were not assessed in this study.[7] Furthermore, because of the time between the acquisition of the fecal sample and the DBPCFC, transient food allergies could have resulted in the misclassification of some children with cow's milk and hen's egg allergy.

Our findings are based on a study population of children with AD from an academic center. Identifying the microbes that are related to food allergy may help in the development of future interventions. However, future studies are needed to confirm our findings in the community population, preferably with prospective study designs using well-defined patient populations to further explore the potential of fecal microbial colonization patterns associated with specific food allergies in children with AD. Furthermore, future studies should be of sufficient size to allow for stratification of different food allergies and include additional control groups: children with food allergy without AD, children with severe 'extrinsic' AD without food allergy.

CONCLUSION

In this pilot study, we identified a microbial signature in children with AD that discriminates between absence and presence of food allergy. Future studies are needed to confirm our findings.

ACKNOWLEDGEMENT

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SUPPLEMENTARY MATERIAL

Figure S1. Relative abundance of the six signature species in children with AD, without and with food allergy

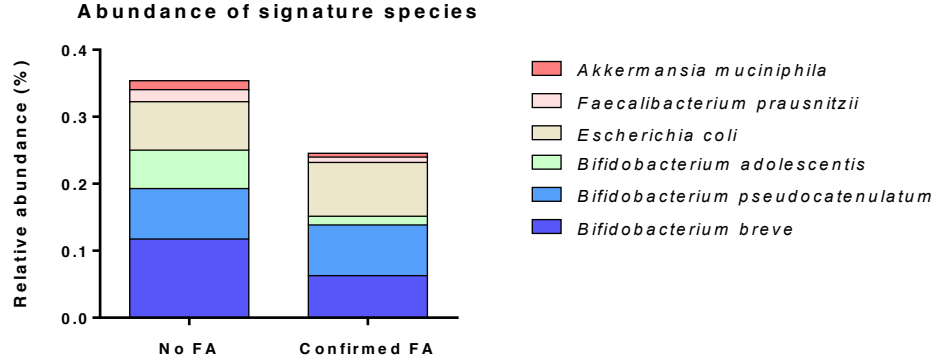


Figure S2. Distribution of 30 most abundant microbial species in children with AD, without and with food allergy

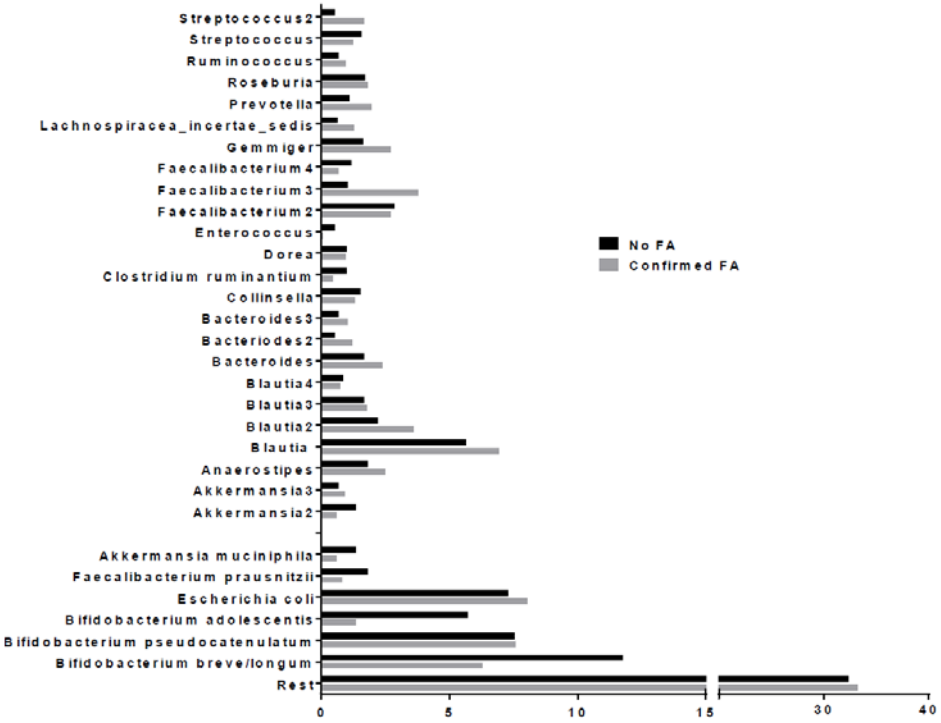
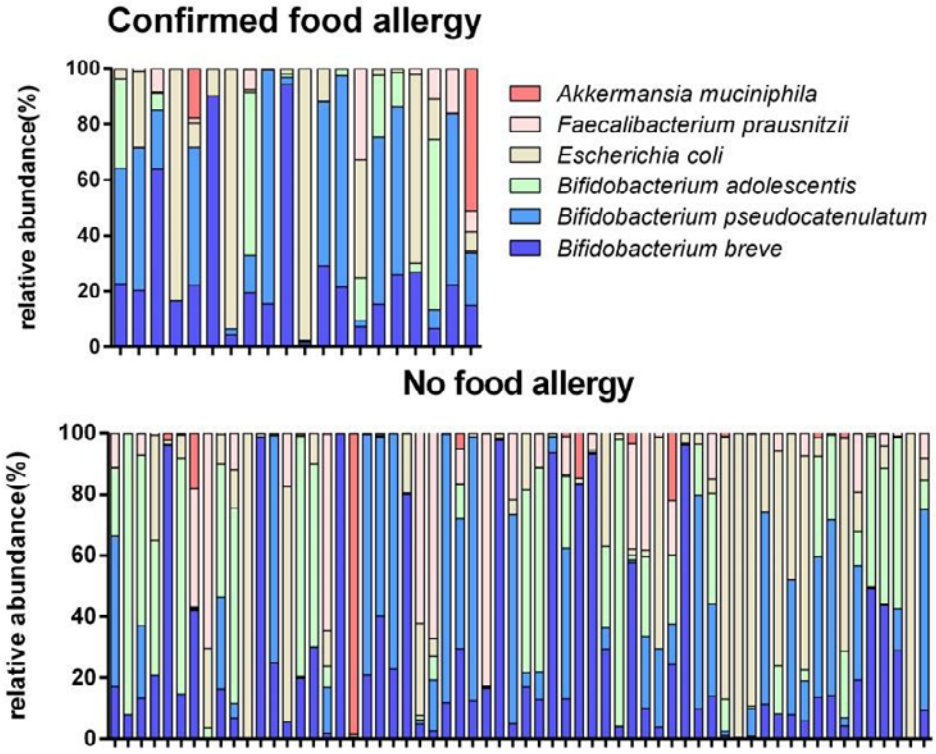


Figure S3. Distribution of six signature species in individual children with AD, without and with food allergy



PART II

The role of *Staphylococcus aureus* in atopic dermatitis and the humoral immune response towards it

Chapter 4

Prevalence and odds of *Staphylococcus aureus* carriage in atopic dermatitis: a systematic review and meta-analysis.

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ABSTRACT

Background

Staphylococcus (S.) aureus is increasingly implicated as a possible causal factor in the pathogenesis of atopic dermatitis (AD). However, the reported prevalence rates of skin and nasal colonization in the literature vary widely.

Objective

This study evaluates the prevalence and odds of skin and nasal colonization with *S. aureus* in patients with AD.

Methods

A systematic literature search was conducted. Odds ratios (ORs) for colonization in patients vs. controls and the prevalence of colonization in patients were pooled using the random-effects model.

Results

Overall, 95 observational studies were included, of which 30 had a control group. The Newcastle-Ottawa Scale was used to assess study quality, with the majority of studies being of fair to poor quality. Patients with AD were more likely to be colonized with *S. aureus* than healthy controls [OR 19.74, 95% confidence interval (CI) 10.88-35.81]. Differences were smaller in nonlesional skin (OR 7.77, 95% CI 3.82-15.82) and in the nose (OR 4.50, 95% CI 3.00-6.75). The pooled prevalence of *S. aureus* colonization among patients was 70% for lesional skin, 39% for nonlesional skin and 62% for the nose. In lesional skin, meta-regression showed that the prevalence of colonization increased with disease severity. Study heterogeneity should be taken into consideration when interpreting the results.

Conclusions

The results demonstrate the importance of colonization with *S. aureus* in AD. Further evaluation of the mechanisms by which *S. aureus* influences inflammation is required in addition to the development of targeted strategies to decrease skin and nasal *S. aureus* load.

INTRODUCTION

Increased colonization with *S. aureus* in the skin of patients with atopic dermatitis (AD) was first described in the 1970s. Multiple studies confirmed this finding, reporting a prevalence of skin colonization with *S. aureus* ranging from around 30% to nearly 100%.[1-4] The underlying pathogenic mechanisms of *S. aureus* in relation to AD have still not been fully elucidated. However, recent studies suggest a causal role in the complex pathogenesis of AD by showing that *S. aureus* colonization precedes (flares of) the disease.[5-9] *S. aureus* can facilitate skin barrier defects and inflammation in AD using different mechanisms.[4,10] Examples of this include the stimulation of mast-cell degranulation by staphylococcal delta toxin, the induction of keratinocyte apoptosis by alpha toxin, the stimulation of T cells by enterotoxins that act as superantigens and the modulation of inflammation by staphylococcal surface proteins, protein A and lipoteichoic acid.[10-14]

As *S. aureus* contributes to both skin barrier defects and to inflammation, a more proactive control of *S. aureus* in certain patients may help to reduce disease severity. However, use of antibiotics can result in resistance of *S. aureus* and perturbation of healthy microbiota, which has been shown to have potentially deleterious health effects.[15-18] At present, new targeted anti-microbial therapies (such as lysins) are being developed, which are directed against single bacteria (e.g. Staphefekt SA.100 against *S. aureus)*.[19-22] Therefore it is important to identify patients with AD who can potentially benefit from antistaphylococcal treatment.

Defining the prevalence of *S. aureus* skin and mucosal colonization in (subgroups of) patients with AD might provide more insight into the importance of *S. aureus* as a contributor to the disease and its severity.

Current prevalence rates of *S. aureus* colonization reported in AD vary widely, mainly depending on the type of patients included, the sample size and the methods used to collect and detect *S. aureus* or its products. The swab and the scrub method are frequently used to collect microorganisms from the skin.[23] Swabs collect bacteria from the superficial layer of the skin, whereas a scrub technique allows collection of superficial skin cells and associated microbes.[24] The detection of *S. aureus* was predominantly based on culture-based methods. In recent years DNA sequencing methods have allowed determination of the complete microbial composition at species level and recently upcoming metagenomics techniques can be used for identification at strain level.[25]

In this systematic review we aim to provide an overview and a pooled estimate of the prevalence and odds of colonization with *S. aureus* in patients with AD.

MATERIALS AND METHODS

Type of study

Both experimental and observational (original, human) studies were included, however, case reports were excluded. No restrictions were made relating to publication date and language.

Type of participants

Patients of all ages with a diagnosis of AD confirmed by a physician were included.

Type of outcome measures

The primary outcome was the proportion of patients with presence of *S. aureus* on the skin (lesional and nonlesional) or in the nose. Secondary outcomes were (i) the presence of *S. aureus* virulence factors on the skin and (ii) the relation between AD severity and colonization with *S. aureus.* In case of intervention studies, only the baseline measurement was included in this review. When studies reported multiple measurements over time taken from the same skin site (without treatment regimen), or when multiple locations were sampled at the same time point, the mean was included in the meta-analysis. Studies that reported solely on methicillin-resistant *S. aureus* were excluded.

Search strategy

The search was conducted in Embase (from 1974), Medline (from 1946), OvidSP (from 1946), Pubmed (from 1947), Web of Science (from 1945) and The Cochrane Central Register of Controlled Trials (CENTRAL) up to 16 September 2014 (table S1). A cross-reference check was performed to identify further relevant studies.

Study selection and data extraction

The titles and abstracts were screened for relevance. Articles or abstracts were selected based on predefined inclusion and exclusion criteria (appendix S1; see Supporting information). Non-English articles were translated by an official translation service when considered relevant. The methodological quality of the articles was rated using an adapted version of the Newcastle-Ottawa Scale (NOS).[26,27] Uncontrolled studies could reach a maximum score of 7 points and studies including a control group could reach a maximum score of 8 points. Using a scoring algorithm (appendix S2; see Supporting information), the controlled studies were classified as being of poor, fair or good methodological quality, based on their NOS scores for patient selection, comparability and outcome.[28] Study selection and quality assessment was conducted independently by two researchers (J.E.E.T. and W.T. v.d.F., J.E.E.T. and M.H. or W.T.v.d.F. and M.H.). Disagreements were resolved and consensus was reached. If identical populations were

described in different publications within an overlapping time period, the study with the most extensive reporting of results was included.

Statistical analysis

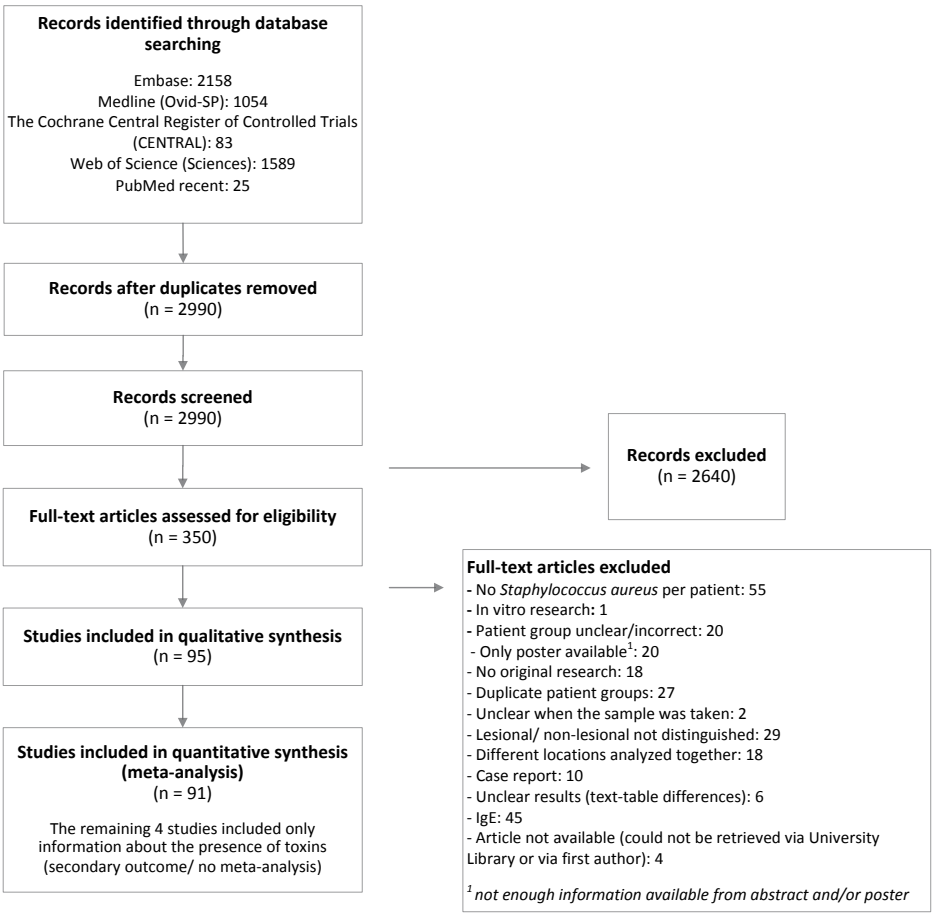
A meta-analysis was performed using a random-effects model. A weighted prevalence of colonization with *S. aureus* on the skin and in the nose was calculated. In controlled studies the prevalence of colonization in patients and controls was compared, expressed as an odds ratio (OR) with a 95% confidence interval (CI). Heterogeneity was assessed using I^2. In cases of substantial heterogeneity between studies ($I^2 > 50\%$) the reasons for heterogeneity were explored using meta-regression (using the unrestricted maximum likelihood method and in cases where there were more than 10 available studies) for the variables NOS score, age and AD severity. For the meta-regression on severity, studies that used the Eczema Area Severity Index (EASI) score or the SCORing Atopic Dermatitis (SCORAD) score were selected. Cut-off values for mild, moderate and severe AD were used as previously described.[29,30] Subgroup analysis was performed for variables that were significant in the meta-regression. Additional subgroup analysis was carried out for studies in which patients were not receiving antibiotic treatment. All statistical analyses were performed using Comprehensive Meta-Analysis Version 2.2 (Biostat, Englewood, NJ, U.S.A.). Publication bias was evaluated using funnel plots, Egger's regression and the trim-and-fill method.[31] The present systematic review was conducted and reported in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines.[32]

RESULTS

Study characteristics

The search yielded a total of 4909 articles, of which 2990 articles remained after deduplications. We used article title and abstract to identify 350 studies (figure 1). After reading the full article texts, 95 studies met our inclusion criteria. All studies had an observational design and 30 studies compared patients with AD with healthy controls. In 77% of the studies AD was diagnosed by clinical assessment (dermatologist or another specialized physician). The other studies did not clearly report who diagnosed the patients. The overall percentage of male patients was 52% and the mean age was 14 years (range 0.8-68.9) based on 58 studies. A total of 11 studies measured disease severity using the EASI, with nine studies reporting a mean EASI [17.7 (range 4.5-51.6)]. Twenty-two of the 40 studies that used SCORAD reported a mean score [48.2 (range 13.5-73.5)]. The remaining studies did not measure the disease severity, used other measuring methods or did not report mean EASI or SCORAD values. Overall, 54% of the studies were conducted

Figure 1. Flow chart of the search strategy and study selection.



in Europe, 27% in Asia and 13% in the U.S. Study characteristics including the methods used to collect and identify *S. aureus* are described in table S2.

Quality of the studies

We rated the quality of the 30 articles that included a control group as good (n=4), fair (n=4) and poor (n=22). The quality of the 65 uncontrolled studies varied from 1 to 6 points out of 7 points on the NOS. (table S2). The main reason for downgrading the quality of controlled studies was incomparability of the patient and control groups. Uncontrolled studies were mainly downgraded owing to a limited description of the methods used for collection and identification of *S. aureus.* The low NOS scores were also partly due to the inclusion of abstracts in this review, which provided limited information on methods.

Prevalence of nasal- and skin colonization with *S. aureus*

Overall, 81 studies (5231 patients) reported on colonization of the lesional skin and 30 studies (1496 patients) reported on colonization of the nonlesional skin. Pooled analysis showed that 70% of the patients with AD carried *S. aureus* on the lesional skin (95% CI 66-74; I^2= 88.31) and 39% on the nonlesional skin (95% CI 31-47; I^2 = 87.39). Pooled results of the 43 studies (2476 patients) that address nasal colonization estimated that 62% of the patients with AD carry *S. aureus* in the nose (95% CI 57-68; I^2 = 85.20) (table 1 and figure S1). The prevalence varied substantially among studies (28% to 99% in lesional skin, 3% to 79% in nonlesional skin and 4% to 95% in the nose). This variation probably resulted in the considerable heterogeneity among studies and might be partly explained by the variation in disease severity and the age of patients included in these studies.

Odds of colonization with *S. aureus*

A total of 26 studies compared colonization of the lesional skin in patients with AD with healthy controls. From 10 studies the OR could not be obtained as the reported percentage of patients colonized with *S. aureus* or controls was either 100% or 0%. A pooled OR based on the remaining 16 studies (including 823 patients and 688 controls) showed that patients were significantly more likely than controls to be colonized with *S. aureus* on the lesional skin (OR 19.74; 95% CI 10.88-35.81; p<0.001; I^2 = 66.04). Overall, 12 out of 20 studies were eligible for inclusion in the pooled analysis for the nonlesional skin (550 patients and 446 controls) (OR 7.77, 95% CI 3.82-15.82; p<0.001; I^2 = 63.08). Pooled analysis of the 19 of 21 studies that evaluated nasal colonization (1051 patients and 1263 controls) showed that 57% of the patients was positive for *S. aureus* in the nose vs. 23% of the controls (OR 4.50; 95% CI 3.00-6.75; p<0.001; I^2 = 70.31) (table 2).

Meta-regression and subgroup analysis

Heterogeneity between the studies was considerable, mainly in the pooled analysis of prevalence (>85%). A meta-regression for the variables AD severity, NOS score and age was performed to identify possible sources of heterogeneity. The prevalence of lesional skin colonization was independent of the NOS score but increased with AD severity (1.02; 95% CI 0.21-1.82) and age (0.64; 95% CI 0.15-1.14). A subgroup analysis of the studies that included patients with mild AD showed colonization of the skin in 43% of the patients (95% CI 31-57; I^2 =79.15) whereas the pooled prevalence for severe AD was 83% (95% CI 74-89; I^2 = 65.78). For the nonlesional skin, colonization decreased with a higher NOS score (-0.27; 95% CI -0.50-(-0.04)). Subgroup analysis of the studies with a higher quality (NOS > 4) showed a pooled prevalence of 31% (95% 23-40; I^2 = 64.62), which is lower than the overall prevalence of 39%. Colonization of the nose was independent of the three variables (table 1).

Table 1. Colonization with *S. aureus* in patients with atopic dermatitis

	Number of studies	Pooled proportion of patients positive for colonization (95% CI)	Heterogeneity (I^2)	Pooled proportion of patients positive for colonization adjusted for publication bias	Meta-regression NOS, B (95% CI)	Meta-regression Severe AD, B (95% CI)	Meta-regression age B (95% CI)
Lesional skin							
All studies	81	0.70 (0.66-0.74)	88.31	0.57 (0.52-0.62)	0.07 (-0.10-0.24)	1.02 (0.21-1.82)[1]*	0.64 (0.15-1.14)[3]*
Studies including mild AD	4	0.43 (0.31-0.57)	79.15				
Studies including severe AD	9	0.83 (0.74-0.89)	65.78				
Studies excluding AB/steroid use	17	0.67 (0.58-0.75)	86.44				
Studies including age<18	29	0.78 (0.71-0.84)	84.19				
Studies including age>18	40	0.65 (0.59-0.71)	89-64				
Nonlesional skin							
All studies	30	0.39 (0.31-0.47)	87.39	0.38 (0.30-0.46)	-0.27 (-0.50-(-0.04))*	-	0.76 (-0.01-1.52)[4]
Studies with a NOS score >4	9	0.31 (0.23-0.40)	64.62				
Studies excluding AB/steroid use	11	0.24 (0.16-0.36)	85.22				
Nose							
All studies	43	0.62 (0.56-0.68)	85.20	0.53 (0.48-0.60)	-0.05 (-0.25-0.15)	0.62 (-0.15-1.39)[2]	0.12 (-0.47-0.72)[5]
Studies excluding AB/steroid use	8	0.58 (0.47-0.69)	78-23				

All estimates were calculated using the random effects model.
See supplementary figures 1 for individual forest plots
- = meta-analysis not performed because <10 studies
[1] 28 studies included
[2] 15 studies included
[3] 69 studies included
[4] 27 studies included
[5] 35 studies included
* = significant result

Table 2. Colonization with *S. aureus* in patients with atopic dermatitis versus healthy controls

	Number of studies	Pooled OR colonization in patients vs controls (95% CI)	Hetero-geneity (I^2)	Pooled OR in patients vs controls adjusted for publication bias	Meta-regression NOS, B (95% CI)	Meta-regression Severity, B (95% CI)	Meta-regression age B (95% CI)
Lesional skin						-	
All studies	16	19.74 (10.88-35.81)*	66.04	10.21 (5.44-19.16)	-0.05 (-0.47-0.37)		-0.55 (-1.84-0.74)[1]
Studies excluding AB/steroid use	6	27.43 (11.20-67.16)*	47.46				
Non- lesional skin						**-**	
All studies	12	7.77 (3.82-15.82)	63.08	3.82 (2.18-6.72)	**-**		0.36 (-1.23-1.95)[2]
Studies excluding AB/steroid use	5	9.70 (3.60-26.13)*	51.06				
Nose						-	
All studies	19	4.50 (3.00-6.75)	70.31	#	0.13 (-0.12-0.39)		0.68 (-0.48-1.84)[3]
Studies excluding AB/steroid use	7	5.54 (3.55-8.65)*	23.70				

All estimates were calculated using the random effects model. Studies that reported event rates of 0 or 1 were excluded as ORs cannot be calculated with these event rates. See supplementary figures 1 for individual forest plots

- = meta-analysis not performed because <10 studies

= no studies were trimmed according to the Trim and Fill method

* = significant result

[1] = 14 studies included

[2] = 10 studies included

[3]= 16 studies included

The ORs for colonization in patients with AD vs. controls were independent of the NOS and age. Severity was not tested as fewer than 10 studies that measured this variable were available (table 2). Additional subgroup analysis, performed with studies that excluded patients who used antibiotics and corticosteroids at the time of inclusion, showed pooled ORs that were higher than the original pooled estimate of all studies (tables 1 and 2).

Enterotoxins prevalence

The prevalence of at least one toxin-producing *S. aureus* strain on the lesional skin in patients varied between 31.5% and 80%. Staphylococcal enterotoxin B was the toxin found most often, with a prevalence of up to 70%. One study reported a prevalence of toxin-producing *S. aureus* of 11.5% on nonlesional skin. Three studies reported the presence of at least one toxin-producing *S. aureus* in the nose, with prevalence rates varying between 32% and 80%. Other studies reported combined results of skin and nose samples and were not taken into consideration in this study (table S3).

Publication bias

The funnel plots for the prevalence of skin and nasal *S. aureus* in patients with AD showed asymmetry (figure S2). The Eggers test confirmed the presence of publication bias with intercepts of 3.68 (95% CI 2.71-4.65, $p<0.001$) for lesional skin, 0.76 (95% CI -3.06-4.85, $p=0.69$) for the nonlesional skin and 2.63 (95% CI 0.84-4.42, $p=0.005$) for the nose. Also the pooled analysis of the odds for colonization showed publication bias with an Eggers regression intercept of 2.47 (95% CI 1.66-3.28, $p<0.001$) for lesional skin, 1.71 (95% CI 0.45-2.97, $p=0.010$) for nonlesional skin and 2.08 (95% CI 0.64-3.52, $p=0.023$) for the nose. Adjusted prevalence rates and ORs according to the trim-and-fill method were all lower than the original estimates (table 1 and 2).

DISCUSSION

In this systematic review we demonstrate that patients with AD are significantly more likely to be colonized with *S. aureus* than healthy controls on both the lesional and nonlesional skin and in the nose. Pooled prevalence of *S. aureus* carriage among patients is 70% for lesional skin, 39% for nonlesional skin and 62% for the nose. For lesional skin the prevalence appeared to be dependent on disease severity and age, however, this could not be confirmed for nonlesional skin or nasal colonization. Substantial to considerable heterogeneity, incomparability of patient and control groups, variation in methods used for sampling and poor description of exposures (such as treatment) downgraded the

quality of the included articles, which should be taken into consideration when interpreting the results.[33]

The typical features of AD skin, such as a comprised barrier integrity, altered sphingolipid metabolism and antimicrobial peptide expression probably facilitate colonization with *S. aureus*.[34,35] The meta-regression analysis finds a higher prevalence of colonization among patients with more severe AD. However, the causal relationship between colonization with *S. aureus* and AD still has to be further clarified. Recent studies often suggest colonization with *S. aureus* as a primary cause rather than only a secondary effect of skin damage or an insufficient antistaphylococcal immune status. According to Kong *et al.* flares in AD accompany temporal microbial dysbiosis, dominated by *S. aureus*.[5] Microbiome analysis of lesions in mice with an eczematous phenotype revealed that dysbiosis was a driving factor for dermatitis formation and bacterial inoculation experiments showed that *S. aureus* could accelerate eczematous inflammation.[36] Despite these studies that suggest a causal relationship, a systematic review by Bath-Hextall *et al.* did not demonstrate a beneficial clinical effect of untargeted anti-*S. aureus* therapy combined with steroids over steroids alone.[37,38] However, other studies including treatment with mupirocin and bleach baths did show a reduction in clinical severity together with a reduction of *S. aureus* skin load.[39,40] In our review we did not investigate the relation between antistaphylococcal interventions and AD severity. We did conduct a subgroup analysis; including patients who were not receiving any antibiotic or corticosteroid treatment. This showed a lower prevalence of *S. aureus* on the skin and nose, which is not in line with the antibacterial effect of both antibiotics and corticosteroids.[41,42] One explanation might be that the inclusion of patients who did not require treatment resulted in a selection of patients with mild AD who were less likely to be colonized with *S. aureus.*

Several natural and technical factors that are known to cause variation in microbiome outcomes might have influenced our results. There is variation between methods used to collect and detect *S. aureus* and its virulence factors on the human skin.[43,44] Also, *S. aureus* might be present not only on the surface of the skin but also in deeper layers.[45] These differences highlight the importance of interpreting the results carefully, taking the methods used into consideration. Subgroup analysis for culture- vs. DNA-based detection methods were not performed owing to a small number of studies using DNA-based methods. Although DNA-based methods only include nonviable bacteria, they might provide more accurate results for quantifying *S. aureus* in the microbiome.

Furthermore, the impact of exposures such as treatment regimen and duration of the disease at the moment of collection were often poorly reported, which might have resulted in performance bias. A subgroup analysis excluding patients using antibiotics or steroids was performed to take the influence of treatment on the results into consideration. However, the use of other (aseptic) products might have also influenced the microbial composition. The duration of the disease might influence the activity of the

host's immune response, which, in turn, could influence the presence of *S. aureus* via an antimicrobial effect.[46] The presence and quantity of microorganisms on the skin is influenced by many factors that naturally give rise to changes in diversity of the microbiota over time and skin site (e.g. ethnicity and climate).[24,47-50 51,52] It should be noted that our review reports on the proportion of *S. aureus* on the skin and mucosa determined at one specific time point.

As a result of underlying factors such as the (genetic) barrier defect and immune pathways enhancing a defective skin barrier, dysbiosis dominated by *S. aureus* is a chronic and recurring factor in AD. [8,53-55] It is important to evaluate further the pathways by which *S. aureus* leads to inflammation and how current therapies already influence these pathways. Antibiotics and antiseptics are used in infected or severe AD.[56,57,58] Functional textiles that are used as complementary treatment in AD might also decrease *S. aureus* colonization.[59] Glucocorticosteroids might also have an antibacterial effect besides their anti-inflammatory effect, probably via an effect on antimicrobial peptides, and even emollient monotherapy was shown to reduce bacterial colonization.[9,60] The current use of antistaphylococcal therapies, together with literature that points to *S. aureus* as a driver in AD pathogenesis, underlines the importance of antistaphylococcal treatment in AD. However, long-term (preventive) use of antibiotics and glucocorticosteroids is undesirable as they can cause side-effects and antibiotic resistance.[16]

To date this is the most comprehensive review that systematically summarizes data regarding *S. aureus* colonization in patients with AD. A large number of studies were included. These studies were mainly observational and often consisted of small numbers of patients. By not restricting the language of the search, selection bias was kept to a minimum. However, selection might have occurred owing to the exclusion of studies that did not report whether samples were taken from lesional or nonlesional skin. The covariate 'severity' in the meta-analysis was based on the level of the study, which may have led to an aggregation bias. As determining the prevalence of *S. aureus* colonization was not the primary objective in a substantial number of the studies, indirectness of evidence with regard to the study population might have occurred. Publication bias changed the outcomes considerably according to the trim-and-fill method. The quality of a large portion of the individual studies was considered to be low. Future studies into the prevalence of *S. aureus* in patients vs. controls should take these quality criteria into consideration to raise the confidence in pooled estimates.

CONCLUSIONS

Despite the low quality of the included studies and the presence of publication bias, this systematic review and meta-analysis demonstrates that patients with AD are more

frequently colonized with *S. aureus* than healthy controls and that colonization is increased in more severe AD. These results provide an indication of the importance of colonization as a factor in the pathogenesis of AD and encourage evaluation of targeted antistaphylococcal therapy for the skin (and nose), for example based on the use of anti-*S. aureus* lysins. Prospective or experimental studies should further investigate causality and the mechanisms by which *S. aureus* colonization leads to inflammation. Host factors such as age and ethnicity, in addition to host-pathogen interaction, should be taken into consideration when investigating these mechanisms. The possible relevance of other microbes in the pathogenesis of AD should also be explored using metagenomic approaches. Additional examination of colonization in patients with different phenotypes (sensitized and non-sensitized, early onset vs. late onset) might provide insight in the type of patients who are likely to benefit most from targeted therapy against *S. aureus*.

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SUPPORTING INFORMATION

Appendix S1. Criteria for selecting studies

Types of studies

- All original human studies that assess the incidence of (virulence factors of) *S. aureus* on the skin and/or nares in patients with atopic dermatitis
- All study designs, except for case reports (<10 studies)

Participants

- Patients with atopic dermatitis of all ages, diagnosed by a medical doctor, included in hospital setting or general population. All disease severity states were included.

Controls

- Subjects with no known skin disease

Outcome measures

- *Primary: S. aureus* (both MRSA and MSSA) on skin (either lesional or non-lesional) and/or nares, isolated through culture or DNA based methods. Samples taken at any time during the disease or treatment were included. Studies that only report on MSSA were excluded.
- *Secondary:* Incidence rate of *S. aureus* and virulence factors on the skin and/or in the nares, measured by PCR.

Appendix S2. Quality assessment score

Modified Newcastle – Ottawa quality assessment scale for cohort or cross sectional studies

Stars indicate the points allocated if the item criterion is met. A maximum score of 8 can be allocated to each article. Uncontrolled studies can reach a maximum score of 7.

Selection

1. Representativeness of the exposed cohort

a) Truly representative of the general atopic dermatitis population *
b) Somewhat representative of the general atopic dermatitis population *
c) Selected group of atopic dermatitis patients (hospital based, tertiary center, inpatients, outpatients)
d) No description of the derivation of the cohort

2. Selection of the non-exposed cohort

a) Representative of the average community (healthy control, community control)*
b) Selected group of controls (hospital controls, other dermatological condition)
c) No control group or no description of control group

3. Ascertainment of atopic dermatitis

a) Diagnosed by dermatologist *
b) Diagnosed by physician other than dermatologist*
c) Diagnosed by clinical assessment*
d) Based on self-report
e) No description of atopic dermatitis case definition

4. Assessment of disease severity

a) Disease severity was assessed with validated score (doctor assessed) EASI, SCORAD, EASI, TIS, POEM*
b) Disease severity was assessed with a validated score (patient assessed). PO-SCORAD, SA-EASI*
c) Disease severity was assessed using another score
d) No disease severity reported

Comparability

1. Comparability of atopic dermatitis and healthy controls on the basis of design or analysis

a) Study controls for confounding using a multivariate model*

b) Atopic dermatitis patients and healthy controls are matched (for age/gender)*
c) No controlling for confounding or matching

Outcome

1. Assessment of outcome: colonization or presence of virulence factors (measurement)

a) Determined by culture, PCR, ELISA or sequencing*
b) Not mentioned

2. Assessment of outcome: method of sample taking

a) Method or sample taking was well described*
b) Not well described or not mentioned

3. Was there treatment during sampling

a) No treatment *
b) Systemic treatment
c) Topical treatment
d) Not mentioned

4. Adequacy of follow up (only in case of cohort studies (prospective))

a) complete follow-up*
b) subjects loss to follow-up unlikely to introduce bias (<10% lost)*
c) follow-up > 10%
d) no statement

Modified Scoring algorithm controlled studies [28]

Quality rating	Points in Selection Domain	Points in Comparability Domain	Points in Outcome domain
Good	≥ 3	≥ 1	≥ 2
Fair	2	0	≥ 2
Poor	0-1	0	0-1

SUPPLEMENTARY FIGURES

Figure S1a. Forest plot: Odds of lesional skin colonization (all studies)

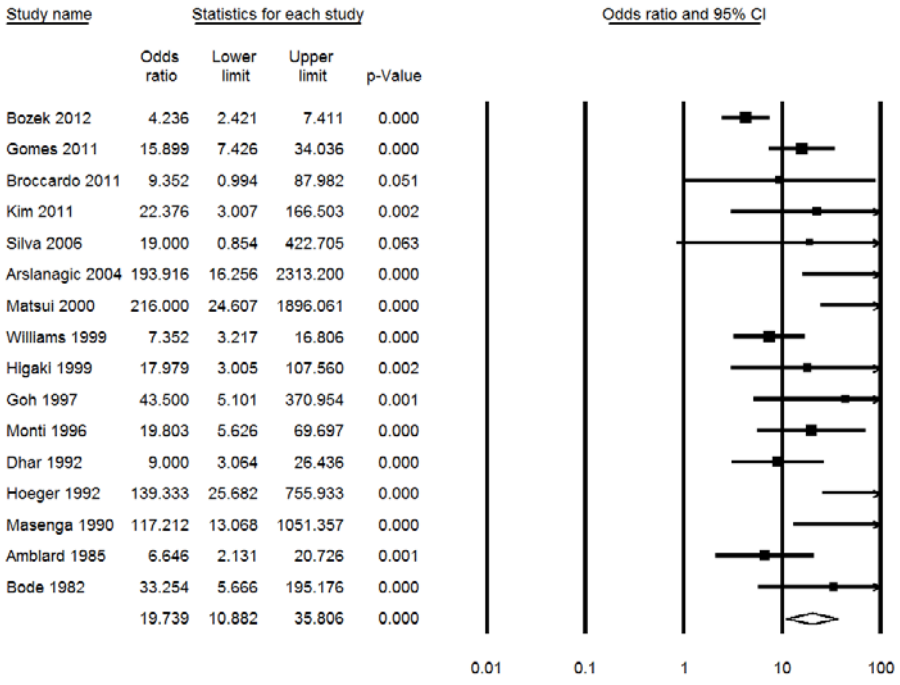


Figure S1b. Forest plot: Odds of non-lesional skin colonization (all studies)

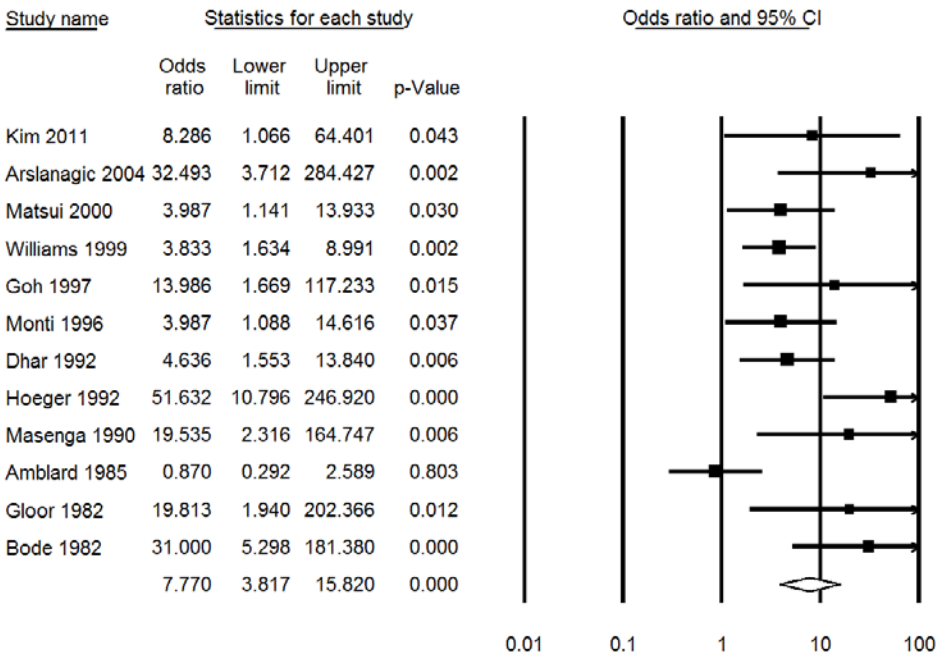


Figure S1c. Forest plot: Odds of nasal colonization (all studies)

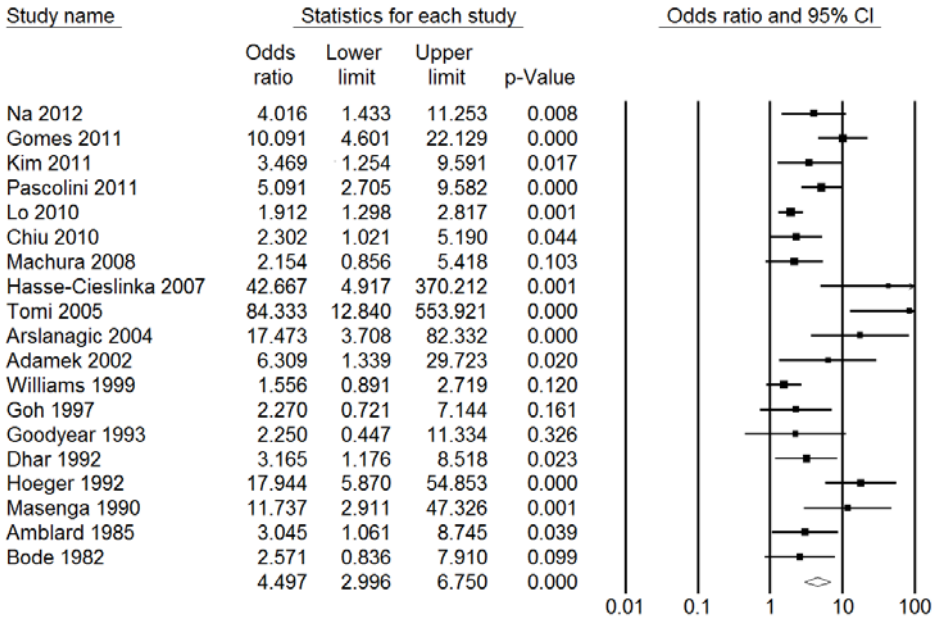


Figure S2a. Funnel plot of studies reporting prevalence of lesional skin colonization with *S. aureus* in patients

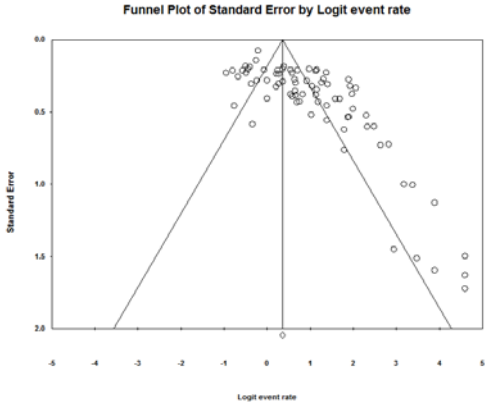


Figure S2b. Funnel plot of studies reporting prevalence of non-lesional skin colonization with *S. aureus* in patients with AD

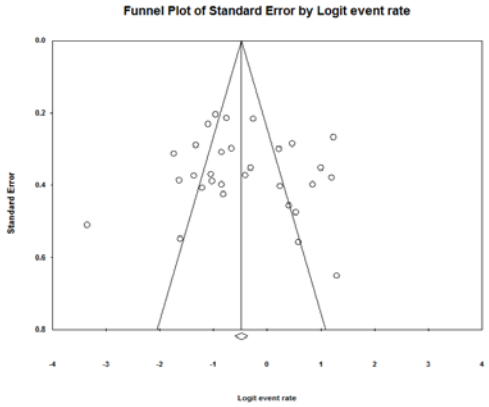


Figure S2c. Funnel plot of studies reporting prevalence of nasal skin colonization with *S. aureus* in patients with AD

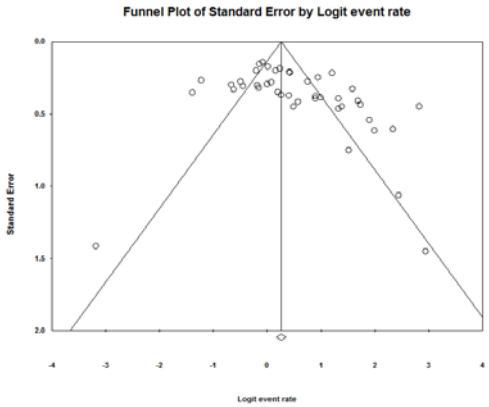


Figure S2d. Funnel plot of studies reporting odds of lesional skin colonization with *S. aureus* in patients

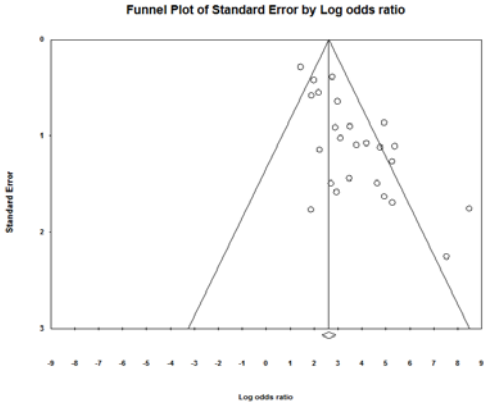


Figure S2e. Funnel plot of studies odds prevalence of non-lesional skin colonization with *S. aureus* in patients with AD

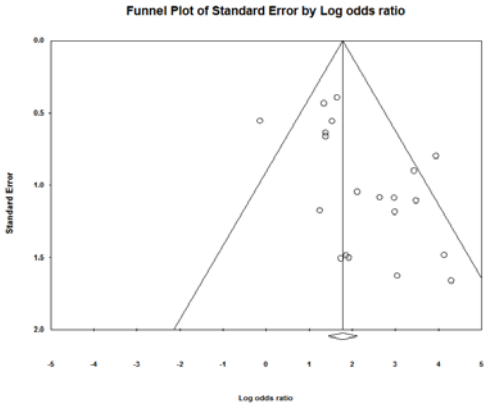
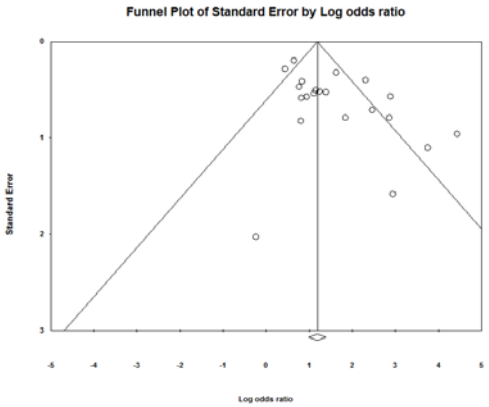


Figure S2f. Funnel plot of studies reporting odds of nasal skin colonization with *S. aureus* in patients with AD



SUPPLEMENTARY TABLES

Table S1. Digital search strategy (last updated 16th of September 2014)

Database	Search string
Embase	('Staphylococcus aureus'/exp OR 'Staphylococcal skin infection'/de ***OR*** 'Microbiome'/de OR 'Skin flora'/de OR ***'Staphylococcus alpha toxin'/de OR 'Staphylococcus toxin'/de OR 'Staphylococcus enterotoxin'/de OR 'Staphylococcus enterotoxin A'/de OR 'Staphylococcus enterotoxin B'/de OR 'Staphylococcus enterotoxin C'/ de OR 'Staphylococcus protein A'/de OR 'Panton Valentine leukocidin'/de OR 'Superantigen'/de OR*** (((cutan* OR skin* OR derma* ***OR nasal OR nose OR nare* OR mucos****) NEAR/3 (flora* OR microflora* OR microbio* OR bacteri* OR staph*)) OR ((staph* OR S OR St) NEAR/3 (aureus* OR pyogenes))):ab,ti OR (***(staph*:ab,ti OR 'Staphylococcus infection'/exp OR 'Staphylococcus'/exp) AND ('Alpha toxin'/de OR 'Bacterial toxin'/ de OR 'Exfoliatin'/de OR 'Leukocidin'/de OR 'Leukotoxin'/de OR 'Bacterial antigen'/de OR 'Cytotoxin'/de OR 'Enterotoxin'/de OR 'Hemolysin'/de OR 'Exotoxin'/de OR (superantigen* OR toxin* OR cytotoxin* OR hemoly* OR haemoly* OR enterotoxin* OR exotoxin* OR exfoliatin* OR leucotoxin* OR leukotoxin* OR leukocidin* OR leucocidin* OR epidermoly* OR dermoly****):ab,ti)))) AND ('Atopic Dermatitis'/de OR ***'Hand eczema'/de*** OR ***'Eczema'/de OR 'Neurodermatitis'/de*** OR (((atopic* OR infant* OR flexur* OR constitution*) NEAR/3 dermatit*) OR neurodermatit* OR eczema*):ab,ti) ***NOT ([animals]/lim NOT [humans]/lim) NOT nare*:ab,ti***
Medline via OvidSP	(exp "Staphylococcus aureus"/ OR "Staphylococcal skin infections"/ ***OR exp*** "Microbiota"/ OR (***staphylococcal alpha toxin OR enterotoxin A, staphylococcal OR enterotoxin B, staphylococcal OR enterotoxin C, staphylococcal OR enterotoxin D, staphylococcal OR enterotoxin E, staphylococcal OR enterotoxin G, staphylococcal OR enterotoxin I, staphylococcal OR staphylococcal enterotoxin J OR staphylococcal enterotoxin H OR SEIO enterotoxin, Staphylococcus aureus OR SEIM enterotoxin, Staphylococcus aureus OR Hlb protein, Staphylococcus aureus OR Gamma-hemolysin, Staphylococcus aureus OR Panton-Valentine leukocidin).mp. OR "Staphylococcal protein A"/ OR "Superantigens"/ OR*** (((cutan* OR skin* OR derma* ***OR nasal OR nose OR nare* OR mucos****) ADJ3 (flora* OR microflora* OR microbio* OR bacteri* OR staph*)) OR ((staph* OR S OR St) ADJ3 (aureus* OR pyogenes))).ab,ti. OR (***(staph*.ab,ti. OR "Staphylococcal infections"/ OR "Staphylococcus"/) AND ("Bacterial toxins"/ OR "Leukocidins"/ OR "Leucocidins"/ OR leukotoxin.mp. OR "Antigens, Bacterial"/ OR exp "Cytotoxins"/ OR "Enterotoxins"/ OR "Hemolysin Proteins"/ OR exp "Exotoxins"/ OR (superantigen* OR toxin* OR cytotoxin* OR hemoly* OR haemoly* OR enterotoxin* OR exotoxin* OR exfoliatin* OR leucotoxin* OR leukotoxin* OR leukocidin* OR leucocidin* OR epidermoly* OR dermoly****).ab,ti.))) AND ("Dermatitis, Atopic"/ OR exp ***"Eczema"/ OR "Neurodermatitis"/*** OR (((atopic* OR infant* OR flexur* OR constitution*) ADJ3 dermatit*) OR neurodermatit* OR eczema*).ab,ti.) ***NOT (animals NOT humans).sh.***
Web of Science	TS=((((cutan* OR skin* OR derma* ***OR nasal OR nose OR nare* OR mucos****) NEAR/3 (flora* OR microflora* OR microbio* OR bacteri* OR staph*)) OR ((staph* OR S OR St) NEAR/3 (aureus* OR pyogenes)) OR (***staph* AND (superantigen* OR toxin* OR cytotoxin* OR hemoly* OR haemoly* OR enterotoxin* OR exotoxin* OR exfoliatin* OR leucotoxin* OR leukotoxin* OR leukocidin* OR leucocidin* OR epidermoly* OR dermoly****))) AND (((atopic* OR infant* OR flexur* OR constitution*) NEAR/3 dermatit*) OR neurodermatit* OR eczema*) NOT ((animal* OR pig* OR sheep* OR horse*) NOT (human* OR patient*)))
Cochrane Central	((((cutan* OR skin* OR derma* ***OR nasal OR nose OR nare* OR mucos****) NEAR/3 (flora* OR microflora* OR microbio* OR bacteri* OR staph*)) OR ((staph* OR S OR St) NEAR/3 (aureus* OR pyogenes))):ab,ti OR (***staph*:ab,ti AND ((superantigen* OR toxin* OR cytotoxin* OR hemoly* OR haemoly* OR enterotoxin* OR exotoxin* OR exfoliatin* OR leucotoxin* OR leukotoxin* OR leukocidin* OR leucocidin* OR epidermoly* OR dermoly****):ab,ti)))) AND ((((atopic* OR infant* OR flexur* OR constitution*) NEAR/3 dermatit*) OR neurodermatit* OR eczema*):ab,ti)
Pubmed	(((cutan*[tiab] OR skin*[tiab] OR dermal[tiab] OR dermatol*[tiab] ***OR nasal[tiab] OR nose[tiab] OR nare*[tiab] OR mucos*[tiab]***) AND (flora*[tiab] OR microflora*[tiab] OR microbio*[tiab] OR bacterial[tiab] OR bacterio*[tiab] OR staph*[tiab])) OR ((staph*[tiab] OR S[tiab] OR St[tiab]) AND (aureus*[tiab] OR pyogenes[tiab])) OR (***staph*[tiab] AND (superantigen*[tiab] OR toxin*[tiab] OR cytotoxin*[tiab] OR hemoly*[tiab] OR haemoly*[tiab] OR enterotoxin*[tiab] OR exotoxin*[tiab] OR exfoliatin*[tiab] OR leucotoxin*[tiab] OR leukotoxin*[tiab] OR leukocidin*[tiab] OR leucocidin*[tiab] OR epidermoly*[tiab] OR dermoly*[tiab]***))) AND (((atopic*[tiab] OR infant*[tiab] OR flexur*[tiab] OR constitution*[tiab]) AND dermatit*[tiab]) OR neurodermatit*[tiab] OR eczema*[tiab]) AND publisher[sb]

Table S2 can be found in the published article online: https://onlinelibrary-wiley-com.eur.idm.oclc.org/doi/abs/10.1111/bjd.14566

Table S3. Presence of enterotoxins in lesional skin of patients with AD and healthy controls

Author	Patients, n/total (%)								Healthy controls, n/total (%)							
	SEA	SEB	SEC	SED	SEG	TSST1	Alpha toxin	At least positive for 1 toxin	SEA	SEB	SEC	SED	SEG	TSST1	Alpha toxin	At least positive for 1 toxin
Lesional skin																
Bozek 2012* [69]	3/121 (2.5)	56/121 (46.3)	8/121 (6.6)	29/121 (24.0)				67/121 (55.4)								5/106 (4.7)
Casas 2011* [74]				7/18 (38.8)	9/18 (50.0)											
Matsui 2000* [2]	4/26 (15.4)	1/26 (3.8)	5/26 (19.2)		0/26 (0.0)	3/26 (11.5)		10/26 (38.5)	2/49 (4.1)	0/49 (0.0)	0/49 (0.0)	0/49 (0.0)	0/49 (0.0)	1/49 (2.0)		2/49 (4.1)
Nada 2012* [71]	1/30 (3.3)	8/30 (26.7)	4/30 (13.3)	1/30 (3.3)		4/30 (13.3)		14/30 (46.7)								
Kozman 2010^ [84]	8/89 (9.0)	20/89 (22.5)		2/89 (2.2)		3/89 (3.4)		28/89 (31.5)								
Wichmann 2009[&] [92]							30/127 (23.6)									
Silva 2006[$] [105]	1/10 (10.0)	7/10 (70.0)	2/10 (20.0)					8/10 (80.0)	0/10 (0.0)	1/10 (10.0)	0/10 (0.0)			0/10 (0.0)		1/10 (10.0)
Leung 1993^ [133]	7/42 (16.7)	8/42 (19.0)	0/42 (0.0)	1/42 (2.4)		7/42 (16.7)		24/42 (57.1)								

* Toxins detected with PCR

^ Toxins detected with ELISA

[&] Toxins detected with synergistic haemolysis test

[$] Toxins detected with dialysis membrane over-agar-method

Chapter 5

The prevalence of antibody responses against *Staphylococcus aureus* antigens in patients with atopic dermatitis: a systematic review and meta-analysis.

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S.G.M.A. Pasmans

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ABSTRACT

Background

Staphylococcus (*S.*) *aureus* plays a role in the pathogenesis of atopic dermatitis (AD), possibly via the expression of various virulence antigens. An altered antibody response towards these antigens might contribute to inflammation.

Objective

To provide an overview of the varying prevalences and odds of antibody responses against *S. aureus* antigens in patients with AD.

Methods

Data were systematically obtained from Embase, MEDLINE, Web of Science, Scopus, Cochrane, PubMed and Google Scholar up to 12 February 2016. We selected all original observational and experimental studies assessing antistaphylococcal antibodies in serum of patients with AD. Prevalences and odds ratios (ORs) of IgE, IgG, IgM, IgA against *S. aureus* in patients with AD vs. healthy controls were pooled using the random-effects model. We calculated I^2 statistics to assess heterogeneity and rated study quality using the Newcastle-Ottawa Scale.

Results

Twenty-six articles (2369 patients) were included, of which 10 were controlled studies. Study quality was fair to poor. Patients with AD had higher prevalences of IgE against staphylococcal enterotoxin (SE)A (OR 8.37, 95% confidence interval 2.93-23.92) and SEB (OR 9.34, 95% confidence interval 3.54-24.93) compared with controls. Prevalences of antistaphylococcal IgE were 33% for SEA, 35% for SEB and 16% for toxic shock syndrome toxin-1. However, study heterogeneity and imprecision should be taken into consideration when interpreting the results. Data on IgG, IgM and IgA, as well as other antigens, are limited.

Conclusions

Patients with AD more often show an IgE antibody response directed against *S. aureus* superantigens than healthy controls, supporting a role for *S. aureus* in AD pathogenesis.

INTRODUCTION

Atopic dermatitis (AD) is a multifactorial disorder that arises from interactions between immune dysregulations, genetic predisposition, skin barrier defects and environmental factors.[1,2] Both the lesional and nonlesional skin and the noses of patients with AD are more likely to be colonized with *Staphylococcus* (*S.*) *aureus* compared with healthy controls.[3] Recent studies have shown that abundance of *S. aureus* is associated with AD severity, suggesting a causal role for *S. aureus* in the pathogenesis of AD.[2,4-9] However, the exact mechanisms by which *S. aureus* aggravates inflammation in AD are not fully understood.[10] *S. aureus* expresses a variety of virulence factors that could contribute to AD inflammation. Based on their biological function these antigens can be divided in four groups: (i) microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) such as clumping factor A (ClfA), which helps *S. aureus* adhere to the host cells, (ii) cell-membrane damaging molecules such as alpha toxin, which can induce keratinocyte cell death, (iii) household enzymes such as lipase, which provides cell nutrition and (iv) immunomodulatory proteins (superantigenic and nonsuperantigenic).[10-13] The latter include the group of staphylococcal superantigens, which have the ability to activate mast cells and T cells directly, resulting in the release of proinflammatory cytokines.[14-16] Expression of these *S. aureus* antigens varies between the different *S. aureus* isolates. However, it has been proven difficult to identify associations between the genetic composition of *S. aureus* strains and AD.[17-22]

Evaluation of the antibody response to these *S. aureus* antigens gives an indication of the antigens that are expressed by the bacterium *in vivo* and will give insight into how the immune system of patients with AD counteracts these antigens. This might help us to understand the role of *S. aureus* in AD pathogenesis as well as the mechanisms by which *S. aureus* causes inflammation. Since 1982 several studies have reported serum antibodies against *S. aureus* in patients with AD.[23-35] However, the prevalences of antistaphylococcal antibodies in these studies vary widely. This is probably due to low sample sizes and different methods used to detect antibodies [e.g. enzyme-linked immunosorbent assay (ELISA) or AlaSTAT]. Moreover, studies often focus on few antigens and/or antibody classes.

The aim of this systematic review was to provide an overview of the pooled prevalence and odds of antibodies (IgE, IgG, IgM and IgA) against *S. aureus* antigens in serum of patients with AD compared with healthy controls. Additionally, we reviewed the relationship between AD severity and anti-*S. aureus* antibodies.

MATERIALS AND METHODS

Study participants and outcomes

All original observational and experimental human studies were included. No restrictions were made on publication date or language. Case reports were excluded. Patients of all ages with AD, irrespectively of disease severity, in which anti-*S. aureus* antibodies were measured. Healthy controls were defined as persons who had neither AD nor an atopic constitution (food allergy, asthma, allergic rhinitis) nor another skin disease. The primary outcome was the proportion of patients with AD with antibodies (IgE, IgG, IgM, IgA) in serum against *S. aureus* antigens compared with healthy controls. The secondary outcome was the relationship between AD severity and antistaphylococcal antibodies.

Search strategy

The systematic electronic search was conducted in Embase, MEDLINE, Web of Science, Scopus, Cochrane, PubMed and Google Scholar up to 12 February 2016 (table S1). A cross-reference check was performed to identify other relevant studies.

Study selection and data extraction

Initially, all studies identified in the systematic search were screened for relevance by title and abstract. Duplicates and studies that did not meet our inclusion criteria were excluded (appendix S1, see Supporting information). The remaining articles were assessed for eligibility by full-text review. Translation of non-English studies was conducted officially. Study selection and data extraction were performed independently by two researchers (F.J.M.vB. and either J.E.E.T. or J.dW). Disagreements were resolved and consensus was reached. If one population was described in different articles, we included the study with the most detailed description of the results. The methodological quality of the articles was scored based on an adapted version of the Newcastle-Ottawa Scale (NOS).[3] Studies could reach a maximum score of nine points for case-control studies and five points for uncontrolled studies. Using a scoring algorithm, the controlled studies were classified as being of poor, fair or good methodological quality, based on their NOS scores (appendix S2, see Supporting information).[36] The overall quality of evidence was discussed according to the principles of the GRADE approach (i.e. limitations in study design or execution, inconsistency of results, indirectness of evidence, imprecision and publication bias).[37]

Statistical analysis

A meta-analysis was performed using a random-effects model in case of at least two available studies. We extracted the prevalences of antistaphylococcal antibodies in patients with AD and controls from the included studies. If required we calculated

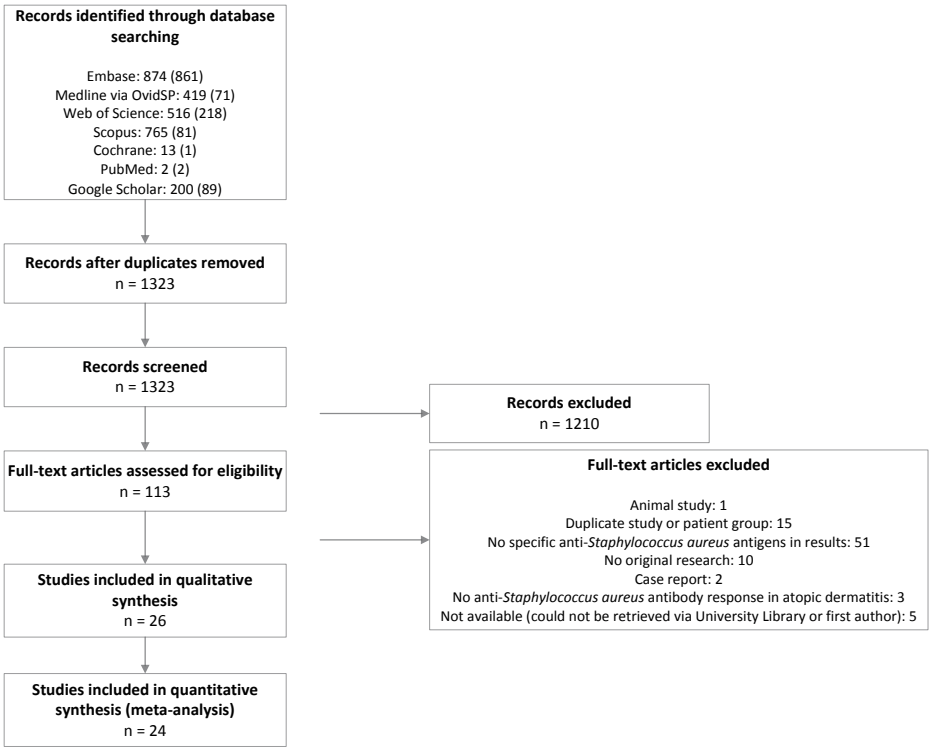
prevalences with the available raw data. The prevalences of antistaphylococcal antibodies were pooled. Furthermore, in controlled studies these prevalences in patients and controls were compared, expressed as ORs with a 95% confidence intervals (CIs). Antibody prevalences were descriptively presented for single studies. When the antibody prevalence in the control group was 0% an OR could not be calculated and a continuity correction factor using the Mantel-Haenszel method was added to both the patient and control groups (based on the unbalanced group ratio).[38,39] Heterogeneity was assessed using the Higgins I^2 test.[40,41] However, I^2-values should be interpreted cautiously in small meta-analyses.[42] In case of substantial (I^2 = 50-90%) or considerable (I^2 = 75-100%) heterogeneity, sources were explored using subgroup analyses for the variables age, method of antibody identification and geographical region of the study centres (≥10 available studies). Possible publication bias was assessed in case of ≥10 studies using funnel plots and Egger's test (*P*-value <0.05).[43,44] Analyses were performed using Comprehensive Meta-Analysis Version 2.2 (Biostat, Englewood, NJ, U.S.A.). This systematic review was conducted and reported according to the Meta-Analysis of Observational Studies in Epidemiology (MOOSE) guidelines.[45]

RESULTS

Study characteristics and quality

The literature search identified 2789 studies. After removal of duplicates, 1323 studies remained. Screening on title and abstract yielded 113 full-text articles. Finally, 26 articles with a total of 2369 patients were included for further analysis (figure 1).[24,25,27-35,46-60] Twenty-one articles reported the sex of the patients, with a mean percentage of males of 53.4% (range 28.1-81.8).[25,28-30,33-35,46-49,51-60] The mean age was 24.1 years (range 4.4-68.9), reported in 15 articles.[25,28-30,33,34,46-49,51,53-55,58] Thirteen articles scored the AD severity using the SCORing Atopic Dermatitis (SCORAD) with three articles reporting a mean of 33.6 (range 32.2-36.0), corresponding with moderate AD.[30,32-35,46,47,49-52,57,59,61] Nine articles used other scoring criteria for AD severity.[25,28,29,31,48,54-56,58] Most studies were conducted in Europe and Asia. Methods for identification of antistaphylococcal antibodies consist mainly of radioimmunoassay (RIA) tests, ELISA and AlaSTAT, an enzyme immunoassay method for the measurement of allergen-specific IgE (table S2). One study measuring multiple antibodies used both a RIA test for IgE and an ELISA for IgG.[31] NOS scores of the 11 controlled studies were rated as good (n=1), fair (n=5) and poor (n=5).[24,25,27-33,36,53,60] The main reason for downgrading the quality of these studies was incomparability of the patient and control groups. The quality scores of the 15 studies without a control group varied between 2 and 4 points out of 5 (table S2 and appendix S2).[34,35,46-52,54-59]

Figure 1. Flow chart of search strategy and study selection



Prevalence of antibodies against *Staphylococcus aureus*

IgE

Twenty-four studies including 2206 patients reported the prevalence of antistaphylococcal IgE. [24,25,27-31,33-35,46-59] These studies predominantly determine the antibody response against staphylococcal enterotoxin (SE)A, SEB, SEC, SED and toxic shock syndrome toxin (TSST)-1 (19, 23, seven, three and 10 studies, respectively) (table S3).[24,25,27-31,34,35,46-59] Pooled prevalences of antistaphylococcal IgE in patients were 33% for SEA (95% CI 23-45; I^2 94.23)[24,25,27-30,34,35,46-48,50,51,53,54,56-59], 35% for SEB (95% CI 27-43; I^2 91.36)[24,25,27-31,34,35,46-59], 14% for SEC (95% CI 8-22; I^2 78.26)[24,34,46,47,52,56,58], 5% for SED (95% CI 1-16; I^2 70.49)[24,34,47] and 16% for TSST-1 (95% CI 10-25; I^2 85.28)[24,27,34,35,46-48,52,56,58] (table 1 and figure S1a-e). There was a great variation in prevalence between studies (0.8-78.8% for SEA, 1.4-72.9% for SEB, 5.4-40.0% for SEC, 0.0-10.7% for SED and 1.4-53.3% for TSST-1), probably resulting in the substantial to considerable heterogeneity. One study showed a prevalence of 35.8% of fibronectin-binding protein (FBP)-specific IgE, while another study found a prevalence of 48.1% of IgE against lipoteichoic acid (LTA).[27,33] Undetectable to very low prevalences of IgE against the staphylococcal antigens SEE, SEI, SEH, SEK, SEJ, exfoliative toxin (ET)-1 and ETA were found in several single studies (table 2).[24,31,47]

Table 1. IgE against SEA, SEB, TSST-1, SEC and SED in patients with atopic dermatitis

***Staphylococcus aureus* antigen (subgroup)**	**Studies**	**Patients**	**Pooled proportion of patients with detectable antigens (95% CI)**	**Heterogeneity (I^2)**
SEA				
All studies	19	1852	0.33 (0.23-0.45)	94.23
Studies including age <18	6	507	0.31 (0.11-0.63)	96.56
Studies including age ≥18	7	859	0.27 (0.17-0.42)	88.22
Studies including RIA methods*	8	1139	0.19 (0.12-0.29)	86.36
Studies including ELISA method	3	169	0.61 (0.34-0.82)	86.83
Studies including AlaSTAT method	6	461	0.42 (0.28-0.57)	89.04
Studies including Immunoblot method	1	27	0.48 (0.30-0.66)	-
Studies performed in Europe	11	1220	0.24 (0.16-0.34)	87.87
Studies performed in Asia	7	576	0.51 (0.33-0.70)	93.75
Studies performed in USA	1	56	0.32 (0.21-0.45)	-
SEB				
All studies	23	2111	0.35 (0.27-0.43)	91.36
Studies including age <18	8	631	0.25 (0.13-0.43)	92.73
Studies including age ≥18	8	968	0.38 (0.29-0.48)	84.70
Studies including RIA methods*	11	1418	0.25 (0.18-0.34)	86.77
Studies including ELISA method	4	209	0.47 (0.24-0.72)	89.39
Studies including AlaSTAT method	6	461	0.48 (0.33-0.64)	90.42
Studies including Immunoblot method	1	27	0.63 (0.44-0.79)	-
Studies performed in Europe	12	1304	0.28 (0.21-0.36)	84.70
Studies performed in Asia	10	751	0.48 (0.36-0.61)	90.06
Studies performed in USA	1	56	0.18 (0.10-0.30)	-
SEC				
All studies	7	540	0.14 (0.08-0.22)	78.26
SED				
All studies	3	317	0.05 (0.01-0.16)	70.49
TSST-1				
All studies	10	1110	0.16 (0.10-0.25)	85.28
Studies including age <18	5	631	0.13 (0.05-0.28)	85.00
Studies including age ≥18	3	1039	0.12 (0.05-0.27)	84.65
Studies including RIA methods*	6	918	0.12 (0.05-0.25)	90.38
Studies including ELISA method	2	109	0.18 (0.11-0.26)	0.00
Studies including Immunoblot method	1	27	0.41 (0.24-0.60)	-
Studies performed in Europe	7	945	0.15 (0.07-0.28)	90.09
Studies performed in Asia	2	109	0.18 (0.11-0.26)	0.00
Studies performed in USA	1	56	0.21 (0.13-0.34)	-

SE, staphylococcal enterotoxin; TSST-1, toxic shock syndrome toxin; RIA, radioimmunoassay; ELISA, enzyme-linked immunosorbent assay; CI, confidence interval

* CAP fluorescent enzyme immunoassay, ImmunoCAP, and UniCAP

Table 2. IgE, IgG and IgM against *Staphylococcus aureus* antigens in patients with atopic dermatitis

Antibody and *Staphylococcus aureus* antigen	Studies	Patients	(Pooled) proportion of patients with detectable antigens (95% CI)
IgE			
SEE	1	140	0.01
SEI	1	140	0.01
SEH	1	140	0.00
SEK	1	140	0.00
SEJ	1	140	0.00
ETA	1	26	0.00
FBP	1	95	0.36
LTA	1	27	0.48
IgG			
SEA	1	74	0.77
SEB	2	114	0.64 (0.42-0.81)[a]
TSST-1	1	74	0.77
ETA	1	26	0.35
IgG_2			
SEB	1	77	0.87
SEC_1	1	78	0.62
IgM			
SEB	1	40	0.63

Ig, immunoglobulin; SE, staphylococcal enterotoxin; TSST-1, toxic shock syndrome toxin 1; ET, exfoliative toxin; FBP, fibronectin-binding protein; LTA, lipoteichoic acid; CI, confidence interval; [a] Heterogeneity: $I^2 = 78.8$

IgG, IgM and IgA

Prevalences of IgG against *S. aureus* antigens were determined in four studies.[31,32,55,60] The pooled prevalences of IgG against SEB, reported in two studies (114 patients), was 64% (95% CI 42-81; I^2 78.84) (figure S1f).[55,60] In single studies the IgG prevalences were 77.0% for SEA, 77.0% for TSST-1 and 34.6% for ETA.[31,60] IgG subclass 2 (IgG_2) was found in 87.0% of the patients with AD against SEB and in 61.5% against SEC_1.[32] Only one study determined antistaphylococcal IgM, and detect antibodies against SEB in 62.5% of the patients with AD (table 2).[55] None of the selected articles studied antistaphylococcal IgA.

Odds of antibodies against *S. aureus*

Of the 26 articles, 11 studies compared patients with AD against healthy controls (759 patients vs. 328 controls).[24,25,27-33,53,60] Nine studies reported the prevalence of antistaphylococcal IgE (596 patients vs. 189 controls).[24,25,27-31,33,53] These studies mainly described antibody responses against SEA and SEB (seven and eight articles, respectively).[24,25,27-31,53] Pooled analyses showed that antistaphylococcal IgE was found significantly more

Table 3. IgE against SEA, SEB and TSST-1 in patients with atopic dermatitis versus healthy controls

***Staphylococcus aureus* antigen**	**Studies**	**Patients**	**Controls**	**Pooled OR in patients vs controls (95% CI)**	**Heterogeneity (I^2)**
SEA	7	475	139	8.37 (2.93-23.92)*	0.00
SEB	8	501	172	9.34 (3.54-24.93)*	0.00
TSST-1	2	83	20	23.33 (0.47-1153.93)	0.00

OR, odds ratio; CI, confidence interval. * Significant result

often in the serum of patients compared with controls, with ORs of 8.37 for SEA (95% CI 2.93-23.92; $P<0.001$; I^2 0.00)[24,25,27-30,53] and 9.34 for SEB (95% CI 3.54-24.93; $P<0.001$; I^2 0.00)[24,25,27-31,53] (table 3 and figure S2a-b). A pooled OR of IgE against TSST-1, reported in two studies (83 patients vs. 20 controls), was 23.33 (95% CI 0.47-1153.93, $P=0.114$, I^2 0.00) (table 3 and figure S2c).[24,27] Prevalences of other antigens, including SEC, SED, ETA, ET-1, FBP and LTA, were described in single controlled studies and pooled estimates could not be provided.[24,27,31,33] The prevalences of all these *S. aureus* antigens were equal or increased in patients vs. controls (table 4). As most antibody prevalences in control groups were 0% the ORs could not be calculated. Prevalences of IgG in patients and controls were compared in three studies.[31,32,60] Compared with controls, patients were found to

Table 4. IgE and IgG against *Staphylococcus aureus* antigens in patients with atopic dermatitis versus healthy controls

Antibody and *Staphylococcus aureus* antigen	**Studies**	**Patients**	**Controls**	**Mean proportion of patients with detectable antigens**	**Mean proportion of controls with detectable antigens**
IgE					
SEC	1	56	15	0.05	0.0
SED	1	56	15	0.05	0.0
ETA	1	26	33	0.00	0.0
ET	1	56	15	0.02	0.0
FBP	1	95	17	0.36	0.0
IgG					
SEA	1	74	111	0.77	0.88
SEB	1	74	111	0.73	0.69
TSST-1	1	74	111	0.77	0.85
ETA	1	26	14	0.35	0.14
IgG_2					
SEB	1	77	27	0.87	0.78
SEC_1	1	78	28	0.62	0.86

SE, staphylococcal enterotoxin; TSST-1, toxic shock syndrome toxin 1; ET, exfoliative toxin; FBP, fibronectin-binding protein

have higher IgG prevalences to ETA and SEB and lower prevalences of IgG to SEA and TSST-1.[31,60] In patients, the IgG_2 prevalence to SEC_1 was lower and to SEB higher than in controls.[32] However, most differences in prevalences between patients and controls were small. No studies compared the antistaphylococcal IgM or IgA responses between patients and controls.

Subgroup analysis

Subgroup analyses of the variables age, method of antibody identification and geographical region of the study centres were performed to detect possible sources of heterogeneity. The prevalences of IgE against SEA, SEB and TSST-1 did not significantly differ between children and adults (31% vs. 27%, 25% vs. 38% and 13% vs. 12%, respectively). Studies using the ELISA method showed higher pooled prevalences of IgE against SEA, SEB and TSST-1 than studies using RIA tests (61% vs. 19%, 47% vs. 25% and 18% vs. 12%, respectively). Lastly, studies conducted in Asia showed higher pooled prevalences of IgE to SEA, SEB an TSST-1 compared to studies conducted in Europe (51% vs. 24%, 48% vs. 28% and 18% vs. 15%, respectively) (table 1).

Relationship between AD severity and antibodies against *S. aureus*

Considering the low number of studies reporting a mean SCORAD we could not calculate an overall association between AD severity and antistaphylococcal antibodies. However, several individual studies reported a significant association between superantigen-specific (e.g. SEA, SEB) IgE and AD severity, measured by SCORAD, the criteria of Rajka or the modified Leicester system.[29,32,55,58] This association could not be confirmed in four comparable studies.[25,28,46,58] Sohn *et al*. looked at IgG against SEB and did not find a relationship with AD severity.[55] However, Mrabet-Dahbi *et al*. found that patients with a deficiency of antistaphylococcal IgG_2 to SEC_1 had a more severe AD phenotype.[32] Based on these contradictory studies, no conclusions can be drawn about the association between the antistaphylococcal antibody response and severity of AD.

Publication bias

Funnel plots of the pooled prevalence of IgE against SEA, SEB and TSST-1 showed no asymmetry (figure S3). Egger's tests had intercepts of 0.52 for SEA (95% CI -4.40-5.44, P = 0.826), -0.44 for SEB (95% CI -3.78-2.91, P = 0.789) and -0.82 for TSST-1 (95% CI -4.40-2.76, P = 0.611), confirming no publication bias.

DISCUSSION

This systematic review includes 26 studies and 2352 patients with AD. IgE responses against SEA and SEB in serum were found more often in patients with AD than in healthy controls. IgE, IgG and IgM against a very limited panel of other antigens were reported in single studies. No data are available on antistaphylococcal IgA. Pooled prevalences of antistaphylococcal IgE in patients with AD are 33% for SEA, 35% for SEB and 16% for TSST-1. Substantial to considerable heterogeneity and imprecision (small studies) limit the quality of evidence and should be taken into consideration when interpreting the results. Subgroup analysis were performed to account for differences in outcome measures (indirectness). The quality of evidence was probably not influenced by publication bias.

Subgroup analyses suggest that the antibody prevalence is dependent on the method of antibody identification (ELISA vs. RIA) and the geographic region of the study centres (Asia vs. Europe). This is in accordance with the study of Taylor *et al*. that found ELISA more sensitive than RIA to detect IgG_1 in mouse.[62] It might also explain the higher prevalence of antibodies in Asia compared to Europe, as Asian studies use ELISA techniques more often. Furthermore, an ethnicity-dependent antibody response has been suggested, at least for TSST-1.[63] Because heterogeneity in subgroup analyses remains high, pooled prevalences and odds were probably also influenced by other variables, like AD severity. Unfortunately, we were not able to explore this as only a few studies reported a mean SCORAD. These individual studies showed contradictory results about the association between AD severity and IgE against predominantly superantigens.

The *S. aureus* antigens SEA and SEB belong to the group of immune modulators and act as superantigens. This indicates that they have the ability to stimulate T cells directly, resulting in T-cell proliferation and cytokine release, causing epithelial damage.[14-16] The increased anti-SEA and anti-SEB IgE responses could be the result of increased expression of these antigens by the *S. aureus* bacteria in patients with AD, indicating SEA and SEB as possible bacterial mechanisms to aggravate or even initiate inflammation in AD. However, the studies included in this systematic review predominantly examined the prevalence of antibodies against the superantigens SEA, SEB and TSST-1 and other common antigens such as clumping factor A and lipase were not tested. In addition, SEA, SEB and TSST-1 were present in only 14%, 24% and 14%, respectively, of the *S. aureus* isolates.[64] These data suggest a bias in the assessment of staphylococcal antigens and indicate also a large genetic diversity among the colonizing *S. aureus* strains. Furthermore, the increased IgE responses against these antigens may be the result of immunological cross-reactivity, where the corresponding antigen-coding genes of SEA, SEB and/or TSST-1 are not present in the isolate.[65-67]

This is the first systematic review summarising the available data on the prevalence of antistaphylococcal antibodies in patients with AD and the involved antigens. The broad selection criteria (e.g. all languages, exclusion only of case reports and nonoriginal studies) resulted in collecting the majority of articles about this subject and limiting selection bias. However, there are still some limitations in this study. Firstly, most articles did not report the AD treatment at the time of antibody measurement. The use of antimicrobial therapy might decrease the *S. aureus* load and *S. aureus* antibody titres.[4,68] In addition, the anti-inflammatory effect of systemic glucocorticosteroids could both cause a decrease in serum antibody concentrations and might also reduce *S. aureus*.[6,69,70] Even emollient monotherapy showed a decrease of *S. aureus* on the skin.[71,72] In the studies that did report the treatment at baseline, the therapies consisted mainly of topical corticosteroids or no treatment at all (n=9). Secondly, the cut-off values of antibody identification methods were highly variable, not mentioned or unclear in and between several methods. Through subgroup analysis we tried to correct for this variability partly. Last, mainly antistaphylococcal IgE was assessed, of which the choice for determination was often unsubstantiated or based on results of previous studies. Patients with AD have frequent high IgE responses to environmental antigens, for example *S. aureus*. In addition, IgG is the most common antibody in the extravascular fluid and among others it plays a role in the neutralization of toxins.[73,74]

To further investigate the role of (the immune response against) *S. aureus* in AD pathogenesis, future studies should focus on other antibody subtypes and other *S. aureus* antigens. IgG subclasses should be measured to detect possible biomarkers for AD severity, such as a selective deficiency in IgG_2 against SEC_1 in the study of Mrabet-Dahbi *et al*.[32] Furthermore, assessment of the antibody response against other *S. aureus* antigens, like MSCRAMMs, membrane-damaging molecules, housekeeping antigens and other types of immunomodulatory proteins, might give more insight in whether an increased IgE response is a secondary phenomenon of increased *S. aureus* colonization of AD skin.

CONCLUSION

In conclusion, this systematic review with meta-analysis shows that patients with AD have higher prevalences of IgE against the *S. aureus* antigens SEA and SEB than healthy controls, taking the large heterogeneity into consideration. These antigens, belonging to the group of immune modulators, are known as superantigens and have the ability to cause inflammation and epithelial damage. This supports a role for *S. aureus* in the pathogenesis of AD. IgE, IgG and IgM against a very limited panel of other antigens were studied in single studies. No data are available on antistaphylococcal IgA.

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SUPPORTING INFORMATION

Appendix S1. Inclusion criteria for selecting studies for this systematic review

Types of studies

- All original observational and experimental human studies which assess the presence of antistaphylococcal antibodies in the serum of patients with atopic dermatitis, reported per *Staphylococcus aureus* antigen.
- All study designs, except for case reports.

Participants

- Patients of all ages with atopic dermatitis irrespective of disease severity, and presence of antistaphylococcal antibodies. Atopic dermatitis diagnosed by a medical doctor.

Controls

- Persons who does not have atopic dermatitis neither an atopic constitution (asthma, allergic rhinitis, food allergy) or another skin disease.

Outcome measures

- Primary: Specific antibodies (IgE, IgG, IgM, IgA) against *Staphylococcus aureus* antigens.
- Secondary: The relationship between atopic dermatitis disease severity and specific antibodies (IgM, IgG, IgA, IgE) antibodies against *Staphylococcus aureus* antigens.

Appendix S2. Quality assessment score

Modified Newcastle-Ottawa quality assessment scale for cohort or cross sectional studies.

Stars indicate the points allocated if the item criterion is met. A maximum score of 9 can be allocated to each article. Uncontrolled studies can reach a maximum score of 5.

Selection

1. Representativeness of the exposed cohort

a) Truly representative of the general atopic dermatitis population *
b) Somewhat representative of the general atopic dermatitis population *
c) Selected group of atopic dermatitis patients (hospital based, tertiary center, inpatients, outpatients)
d) No description of the derivation of the cohort

2. Selection of the non-exposed cohort

a) Representative of the average community (healthy control, community control) *
b) Selected group of controls (hospital controls, other dermatological condition
c) No description of the derivation of the cohort

3. Ascertainment of atopic dermatitis

a) Diagnosed by dermatologist *
b) Diagnosed by physician other than dermatologist *
c) Diagnosed by clinical assessment*
d) Based on self-report
e) No description of atopic dermatitis case definition

4. Definition of the non-exposed cohort

a) No history of disease (endpoint) *
b) No description of source

Comparability

1. Comparability of exposed and non-exposed cohorts on the basis of design or analysis

a) Patients with AD and healthy controls are matched for age *
b) Patients with AD and healthy controls are matched for any additional factor *
c) No controlling for confounding or matching

Outcome

1. Assessment of outcome: measurement method of antibody response against *Staphylococcus aureus* antigens

a) Determined by ELISA, CAP, RAST, micro-array, AlaSTAT *
b) Not mentioned

2. Treatment during sampling

a) No treatment *
b) Systemic treatment
c) Topical treatment
d) Not mentioned

3. Missing data

a) Same rate for both patients with AD and healthy controls / no missing data *
b) Different rate for both patients with AD and healthy controls, but well described/ missing data, but well described *
c) Different rate for both patients with AD and healthy controls and not explained
d) Not mentioned

Modified Scoring algorithm controlled studies [36]

Quality rating	**Points in Selection Domain**	**Points in Comparability Domain**	**Points in Outcome domain**
Good	≥ 3	≥ 1	≥ 2
Fair	2	0	≥ 2
Poor	0-1	0	0-1

Appendix S3. Supplementary references

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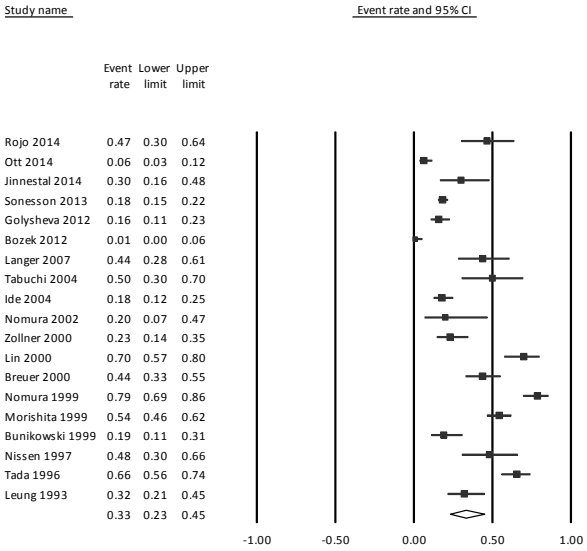
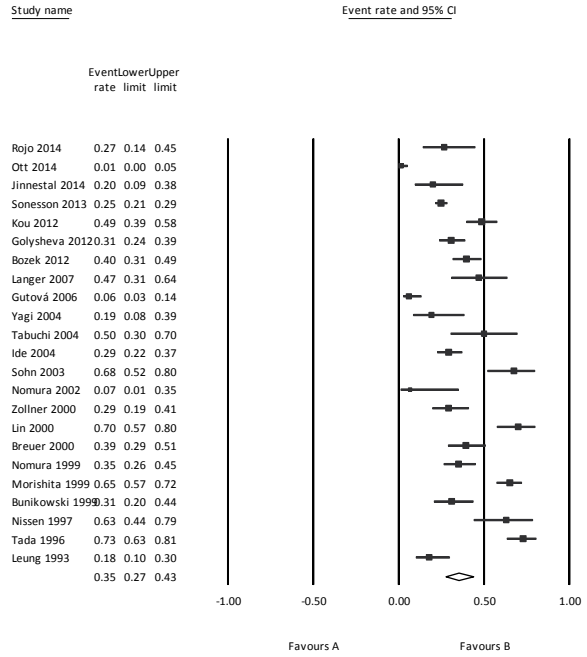
SUPPLEMENTARY FIGURES

Figure S1. Forest plots of prevalence meta-analyses

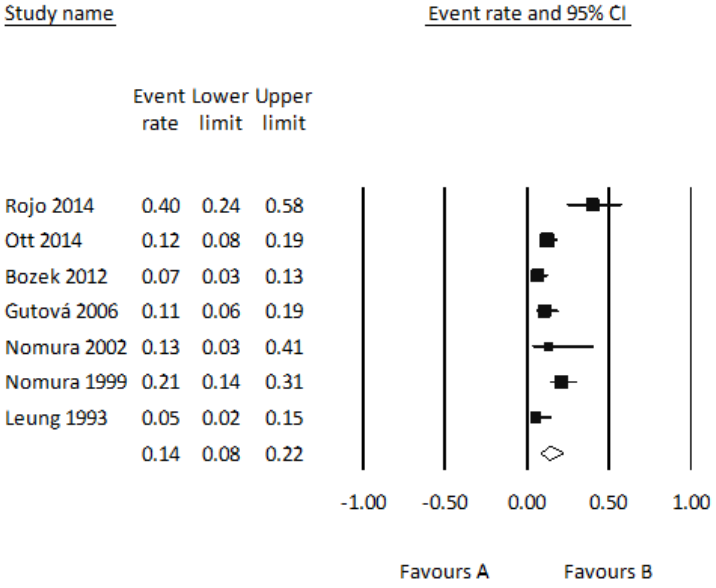
a. IgE against SEA in patients with atopic dermatitis



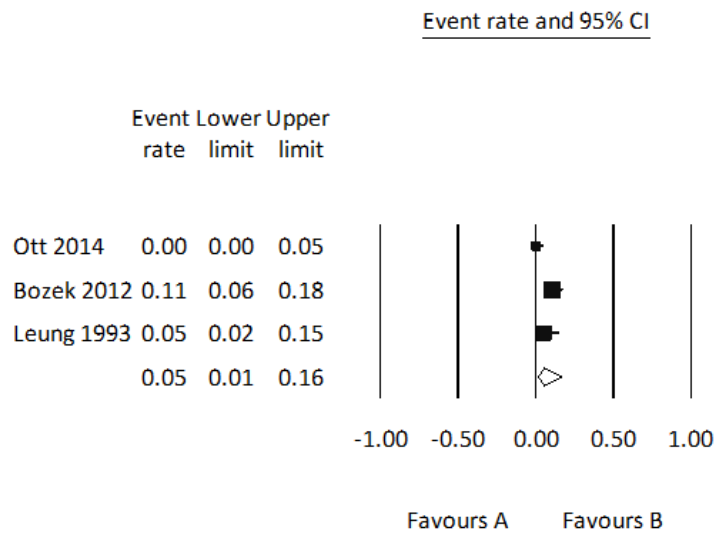
b. IgE against SEB in patients with atopic dermatitis



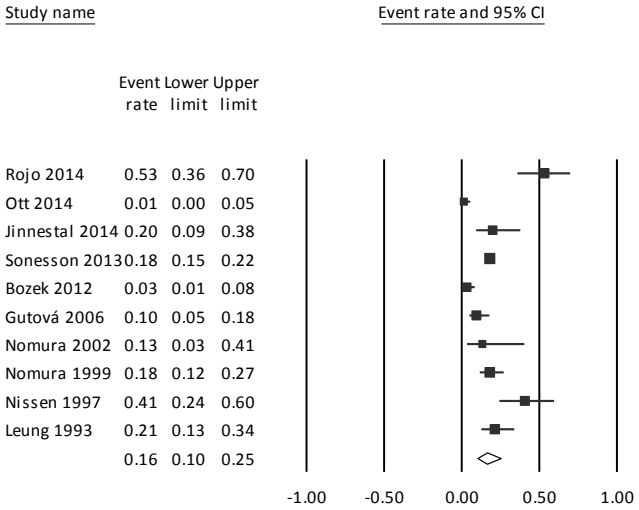
c. IgE against SEC in patients with atopic dermatitis



d. IgE against SED in patients with atopic dermatitis



e. IgE against TSST-1 in patients with atopic dermatitis



f. IgG against SEB in patients with atopic dermatitis

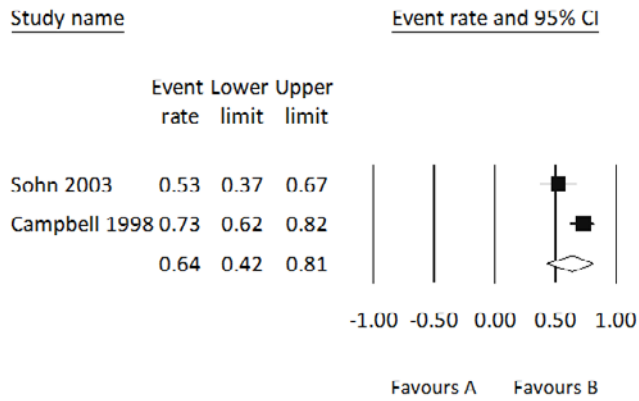
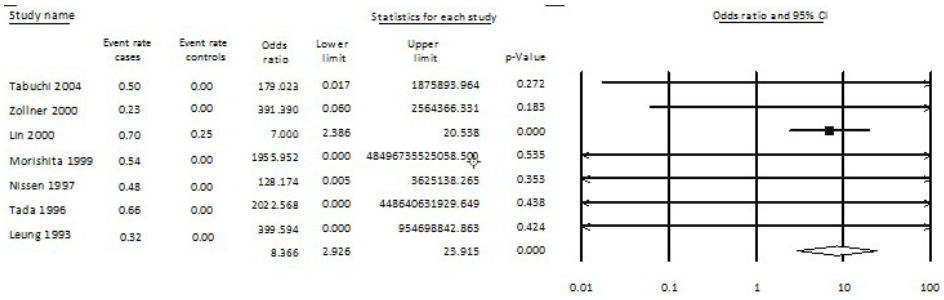
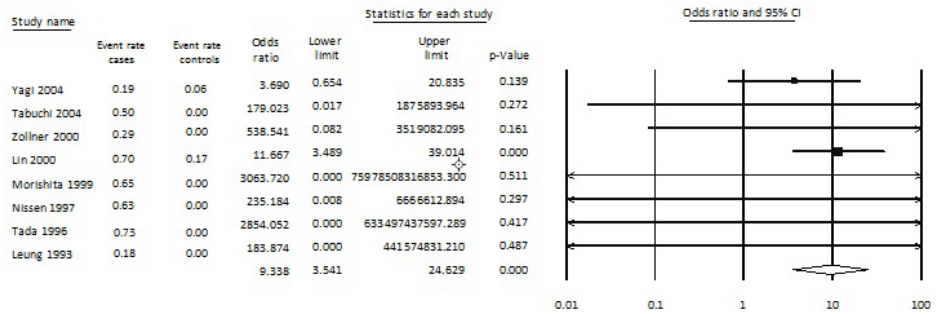


Figure S2. Forest plots of odds meta-analyses

a. IgE against SEA in patients with atopic dermatitis versus healthy controls



b. IgE against SEB in patients with atopic dermatitis versus healthy controls



c. IgE against TSST-1 in patients with atopic dermatitis versus healthy controls

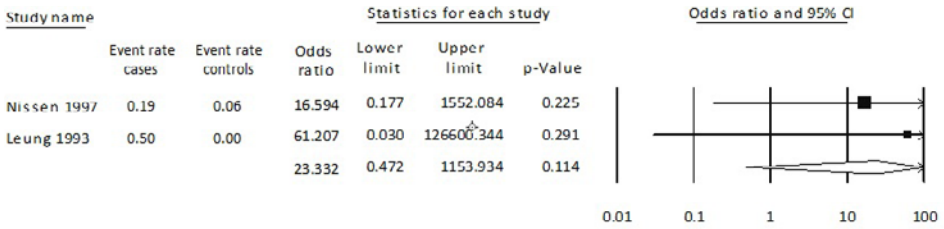
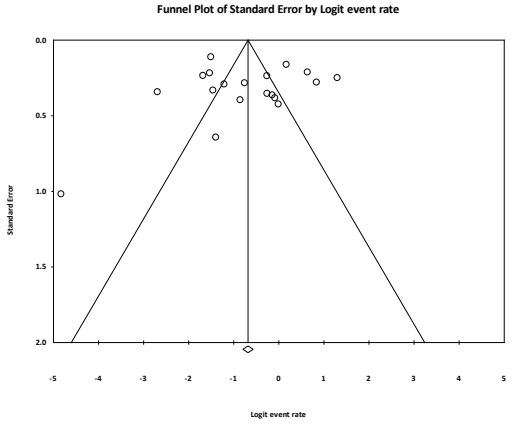
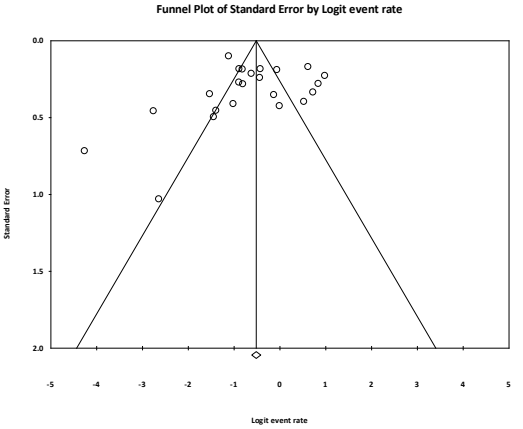


Figure S3. Funnel plots for publication bias

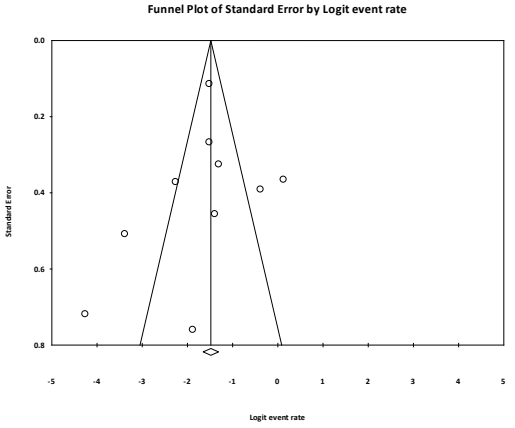
a. IgE against SEA in patients with atopic dermatitis



b. IgE against SEB in patients with atopic dermatitis



c. IgE against TSST-1 in patients with atopic dermatitis



SUPPLEMENTARY TABLES

Table S1. Electronic search

Database	Search
Embase	(eczema/exp OR 'atopic dermatitis'/exp OR (eczem* OR ((atopic OR constitutional*) NEAR/3 (dermatit* OR neurodermatit*))):ab,ti) AND ('Staphylococcus aureus'/de OR ('Staphylococcus aureus' OR 's aureus' OR 'Staph aureus'):ab,ti) AND (antibody/exp OR immunoglobulin/exp OR antigen/exp OR microbiome/de OR 'skin flora'/de OR (antibod* OR immunoglobulin* OR IGA OR IGE OR IGG OR IGM OR IGAs OR IGEs OR IGGs OR IGMs OR antigen* OR superantigen* OR microbiome* OR 'skin flora'):ab,ti)
Medline (OvidSP)	(eczema/ OR "Dermatitis, Atopic"/ OR (eczem* OR ((atopic OR constitutional*) ADJ3 (dermatit* OR neurodermatit*))).ab,ti.) AND ("Staphylococcus aureus"/ OR ("Staphylococcus aureus" OR "s aureus" OR "Staph aureus").ab,ti.) AND (exp immunoglobulin/ OR exp antigens/ OR microbiota/ OR (antibod* OR immunoglobulin* OR IGA OR IGE OR IGG OR IGM OR IGAs OR IGEs OR IGGs OR IGMs OR antigen* OR superantigen* OR microbiome* OR "skin flora").ab,ti.)
Pubmed	(eczema[mh] OR (eczem*[tiab] OR ((atopic OR constitutional*[tiab]) AND (dermatit*[tiab] OR neurodermatit*[tiab])))) AND (Staphylococcus aureus[mh] OR (Staphylococcus aureus*[tiab] OR s aureus*[tiab] OR Staph aureus*[tiab])) AND (immunoglobulin[mh] OR antigens[mh] OR microbiota[mh] OR (antibod*[tiab] OR immunoglobulin*[tiab] OR IGA[tiab] OR IGE[tiab] OR IGG[tiab] OR IGM[tiab] OR IGAs[tiab] OR IGEs[tiab] OR IGGs[tiab] OR IGMs[tiab] OR antigen*[tiab] OR superantigen*[tiab] OR microbiome*[tiab] OR skin flora*[tiab])) AND publisher[sb]
Web of Science	TS=(((eczem* OR ((atopic OR constitutional*) NEAR/3 (dermatit* OR neurodermatit*)))) AND (("Staphylococcus aureus" OR "s aureus" OR "Staph aureus")) AND ((antibod* OR immunoglobulin* OR IGA OR IGE OR IGG OR IGM OR IGAs OR IGEs OR IGGs OR IGMs OR antigen* OR superantigen* OR microbiome* OR "skin flora")))
Scopus	TITLE-ABS-KEY(((eczem* OR ((atopic OR constitutional*) W/3 (dermatit* OR neurodermatit*)))) AND (("Staphylococcus aureus" OR "s aureus" OR "Staph aureus")) AND ((antibod* OR immunoglobulin* OR IGA OR IGE OR IGG OR IGM OR IGAs OR IGEs OR IGGs OR IGMs OR antigen* OR superantigen* OR microbiome* OR "skin flora")))
Google scholar	Eczema|"atopic dermatitis""Staphylococcus|s|Staph aureus" antibody|antibodies|immunoglobulin|antigen|antigens|microbiome|"skin flora"|superantigen|superantigens
Cochrane Library	((eczem* OR ((atopic OR constitutional*) NEAR/3 (dermatit* OR neurodermatit*))):ab,ti) AND (('Staphylococcus aureus' OR 's aureus' OR 'Staph aureus'):ab,ti) AND ((antibod* OR immunoglobulin* OR IGA OR IGE OR IGG OR IGM OR IGAs OR IGEs OR IGGs OR IGMs OR antigen* OR superantigen* OR microbiome* OR 'skin flora'):ab,ti)

Table S2. Study characteristics per study
NOTE: Full reference details are provided in Appendix S3 (see Supporting Information)

	Country	Patients				
		N	% Male	Mean age (y)	Treatment at baseline	Mean AD severity score
Rojo 2014[43]	Spain	32/ 30[b]	65.6	23	-	SCORAD -
Ott 2014[44]	Germany	140	60.7	6.2	-	SCORAD 36
Jinnestål 2014[33]	Sweden	30	30.0	32.5 (med)	Topical treatment, no UV treatment	SCORAD -
Sonesson 2013[45]	Sweden	513	32.6	26.6	-	Rajka & Langeland 5.7
Kou 2012[46]	Japan	121/109[b]	57.0	35.7	Topical corticosteroid, 24 patients used cyclosporine	SCORAD 42 (med)
Golysheva[a] 2012[47]	Russia	133	-	Range 1-55	-	SCORAD -
Bozek 2012[32]	Poland	121	63.6	68.9	-	SCORAD 32.2
Reginald 2011[31]	Austria & Germany	95	47.4	34.4	-	SCORAD -
Langer 2007[48]	Germany	32	28.1	31.5	-	SCORAD 33.4
Gutová 2006[49]	Czech Republic	84	50.0	Range 4 mo – 9 y	-	SCORAD -
Mrabet-Dahbi 2005[30]	Germany	89	-	31 (med)	No corticosteroid and systemic or topical AB 4 weeks prior to the study	SCORAD 45 (med)

Controls			Antibody	Cut-off detection method	NOS	Patients	Controls
N	% Male	Mean age (y)				Prevalence positive anti-*S. aureus* antibodies	Prevalence positive anti-*S. aureus* antibodies
			IgE	UniCAP 10 kU/L	2	SEA: 0.47 SEB: 0.27 SEC: 0.40 TSST-1: 0.53	
			IgE	FEIA -	3	SEA: 0.06 SEB: 0.01 SEC: 0.12 SED: 0.00 SEE: 0.01 SEI: 0.01 SEH: 0.00 SEK: 0.00 SEJ: 0.00 TSST-1: 0.01	
10[c]	30.0	41 (med)	IgE	ImmunoCAP 0.35 kU/L	4	SEA: 0.30 SEB: 0.20 TSST-1: 0.20	
			IgE	ImmunoCAP 0.35 kU/L	3	SEA: 0.18 SEB: 0.25 TSST-1: 0.15	
50[c]	54.0	31.0	IgE	UniCAP 0.70 UA/mL	3	SEB: 0.49	
			IgE	ImmunoCAP -	2	SEA: 0.16 SEB: 0.31	
106[c]	-	68.1	IgE	CAP assay 0.35 kU/L	2	SEA: 0.01 SEB: 0.40 SEC: 0.07 SED: 0.11 TSST-1: 0.03	
17	29.4	36.2	IgE	ELISA -	3	FBP: 0.36	FBP: 0.00
9	-	-	IgE	CAP FEIA 0.35 kU/L	2	SEA: 0.44 SEB: 0.47	
10[c]	-	-	IgE	CAP (SEC, TSST-1) - Immulite 2000 (SEB) -	2	SEB: 0.06 SEC: 0.11 TSST-1: 0.10	
28	-	27 (med)	IgG_2	CAP (IgE) 0.35 kU/L ELISA (IgG_2) -	6	SEB: 0.87 (IgG_2) SEC_1: 0.62 (IgG_2)	SEB: 0.78 (IgG_2) SEC_1: 0.86 (IgG_2)

NOTE: Full reference details are provided in Appendix S3 (see Supporting Information) (continued)

	Country	Patients				
		N	% Male	Mean age (y)	Treatment at baseline	Mean AD severity score
Yagi 2004[29]	Japan	105/26[b]	-	-	-	Modified Leicester system -
Tabuchi 2004[50]	Japan	22	81.8	27.5	-	-
Ide 2004[51]	Japan	140	65.0	4.4	According to guidelines	1999 Japanese Therapeutic Guidelines for Atopic Dermatitis -
Sohn 2003[52]	South-Korea	40	45.0	5.2	-	Criteria of Rajka -
Nomura 2002[53]	Japan	15	73.3	6 mo (med)	Systematic washing of skin using soap and topical corticosteroid, antihistamines and some patients used systemtic AB	Modified Leicester system -
Zollner 2000[28]	Germany	65	41.5	41	No AB or systemic immunosuppressives 4 weeks prior to the study	SCORAD -
Lin 2000[27]	Taiwan	60	66.7	7.2	-	Criteria of Rajka -
Breuer 2000[54]	Germany	71	40.8	32 (med)	No treatment	SCORAD -
Nomura 1999[55]	Japan	94	59.6	7.8	-	Modified Leicester system 35.4
Morishita 1999[26]	Japan	149	44.3	21.4	-	Criteria of Rajka -
Bunikowski 1999[56]	Germany	58	65.5	30 mo (med)	No topical or systemic antimicrobial drugs 2 weeks prior to the study	SCORAD -
Campbell 1998[57]	Australia	74	59.5	-	-	-

Controls			Antibody	Cut-off detection method	NOS	Patients	Controls
N	% Male	Mean age (y)				Prevalence positive anti-*S. aureus* antibodies	Prevalence positive anti-*S. aureus* antibodies
33	-	-	IgE, IgG	UniCAP (IgE) 0.35 kU/L ELISA (IgG) -	3	SEB: 0.19 (IgE) ETA: 0.00 (IgE) ETA: 0.35 (IgG)	SEB: 0.06 (IgE) ETA: 0.00 (IgE) ETA: 0.15 (IgG)
8	50.0	31.9	IgE	AlaSTAT 0.10 IU/mL	3	SEA: 0.50 SEB: 0.50	SEA: 0.00 SEB: 0.00
			IgE	ImmunoCAP 0.7 U/mL	2	SEA: 0.18 SEB: 0.29	
40[c]	65.5	6.6	IgE, IgG, IgM	ELISA 12.11 U/mL (IgE) 26.11 U/mL (IgG) 19.83 U/mL (IgM)	3	SEB: 0.68 (IgE) SEB: 0.53 (IgG) SEB: 0.63 (IgM)	
			IgE	ELISA 1.07 U (SEA) 12.6 U (SEB) 7.4 U (SEC) 8.3 U (TSST-1)	2	SEA: 0.20 SEB: 0.07 SEC: 0.13 TSST-1: 0.13	
65	-	-	IgE	AlaSTAT 0.7 U/mL	5	SEA: 0.23 SEB: 0.29	SEA: 0.00 SEB: 0.00
24	41.7	8.4	IgE	ELISA 0.16 kU/L (SEA) 0.7 kU/L (SEB)	7	SEA: 0.70 SEB: 0.70	SEA: 0.25 SEB: 0.17
			IgE	AlaSTAT 0.35 kU/L	4	SEA: 0.44 SEB: 0.39	
			IgE	ELISA 1.07 U (SEA) 12.4 U (SEB) 7.8 U (SEC) 8.3 U (TSST-1)	3	SEA: 0.79 SEB: 0.35 SEC: 0.21 TSST-1: 0.18	
11	27.3	26.9	IgE	AlaSTAT 0.35 IU/mL	5	SEA: 0.54 SEB: 0.65	SEA: 0.00 SEB: 0.00
22[c]	-	73 mo (med)	IgE	AlaSTAT 0.7 kU/L	3	SEA: 0.19 SEB: 0.31	
111	-	-	IgG	ELISA 2.1 LU (SEA) 2.4 LU (SEB) 8.7 LU (TSST-1)	3	SEA: 0.77 SEB: 0.73 TSST-1: 0.77	SEA: 0.88 SEB: 0.69 TSST-1: 0.85

NOTE: Full reference details are provided in Appendix S3 (see Supporting Information) (continued)

	Country	Patients				
		N	% Male	Mean age (y)	Treatment at baseline	Mean AD severity score
Nissen 1997[25]	Denmark	34/ 27[b]	-	31 (med)	-	-
Tada 1996[23]	Japan	96	42.7	20.2	-	Criteria of Rajka -
Leung 1993[22]	USA	56	-	-	-	-

[a] Only abstract available

[b] Number of patients included in study (characteristics refer to this number) / number of patients included in the outcome

[c] Control group included in the study but the outcome was not reported

Abbreviations: N, number of patients or controls; y, year; mo, months; AD, atopic dermatitis; NOS, Newcastle-Ottawa Scale; *S. aureus*, *Staphylococcus aureus*, med, median; AB, antibiotics; UV, ultraviolet, SCORAD, SCORing Atopic Dermatitis; Ig, immunoglobulin; FEIA, fluorescent enzyme immunoassay; SE, staphylococcal enterotoxin; TSST-1, toxic shock syndrome toxin 1; FBP, fibronectin-binding protein; ET, exfoliative toxin; LTA, lipoteichoic acid

Controls			Antibody	Cut-off detection method	NOS	Patients	Controls
N	% Male	Mean age (y)				Prevalence positive anti-*S. aureus* antibodies	Prevalence positive anti-*S. aureus* antibodies
5	-	-	IgE	Immunoblot -	2	SEA: 0.48 SEB: 0.63 TSST-1: 0.41 LTA: 0.48	SEA: 0.00 SEB: 0.00 TSST-1: 0.00 LTA: 0.00
11	27.7	29.4	IgE	AlaSTAT 0.35 IU/L	5	SEA: 0.66 SEB: 0.73	SEA: 0.00 SEB: 0.00
15	-	-	IgE	ELISA -	9	SEA: 0.32 SEB: 0.18 SEC: 0.05 SED: 0.05 TSST-1: 0.21 ET: 0.02	SEA: 0.00 SEB: 0.00 SEC: 0.00 SED: 0.00 TSST-1: 0.00 ET: 0.00

Table S3. Studies reporting IgE antibodies against *Staphylococcus aureus* antigens in patients with atopic dermatitis

Staphylococcus aureus antigen	Number of studies	Number of controlled studies
SEA	19	7
SEB	23	8
SEC	7	1
SED	3	1
SEE	1	
SEI	1	
SEH	1	
SEK	1	
SEJ	1	
TSST-1	10	2
ETA	1	
FBP	1	
LTA	1	
ET1	1	1

Abbreviations: SE, staphylococcal enterotoxin; TSST-1, toxic shock syndrome toxin 1; ET, exfoliative toxin; FBP, fibronectin-binding protein; LTA, lipoteichoic acid

Chapter 6

The IgG response against *Staphylococcus aureus* is associated with severe atopic dermatitis in children

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ABSTRACT

Background

An altered immune response against *Staphylococcus (S.) aureus* might contribute to inflammation and barrier damage in atopic dermatitis (AD).

Objectives

To profile IgG antibodies against 55 *S. aureus* antigens in sera of children with mild-to-severe AD and to evaluate the association between IgG levels and disease severity.

Methods

In this cross-sectional study, we included children with AD from two interventional study cohorts, the Shared Medical Appointment (SMA) cohort (n = 131) and the older DAVOS cohort (n = 76). AD severity was assessed using the Self Administrated-Eczema Area and Severity Index (SA-EASI) and levels of thymus and activation-regulated chemokine (TARC) in serum. IgG antibody levels against 55 *S. aureus* antigens were quantified simultaneously using a Luminex assay. Pair-wise correlations were calculated between the 55 IgG levels using the Spearman rank correlation test. Linear regression analysis was performed to test for associations between 55 IgG levels and SA-EASI and TARC, adjusting for age, sex and *S. aureus* colonization.

Results

In the SMA cohort 16 antigens were associated with SA-EASI and 12 antigens were associated with TARC (10 overlapping antigens; *P*-values 0.001 to 0.044). The associated IgG antibodies targeted mainly secreted proteins with immunomodulatory functions. In the DAVOS study, IgG levels against only four and one *S. aureus* antigen(s) were associated with SA-EASI and TARC, respectively (no overlap).

Conclusions

In young children, severity of AD is associated with an IgG response directed against *S. aureus* antigens with mainly immunomodulatory functions. These findings encourage further evaluation of the role of *S. aureus* in the pathogenesis of AD.

INTRODUCTION

Staphylococcus (S.) aureus is involved in the multifactorial pathogenesis of atopic dermatitis (AD).[1] Approximately 70% of the skin lesions in AD are colonised with *S. aureus*, and bacterial density was found to be associated with the severity of AD.[2] The exact mechanisms through which *S. aureus* causes inflammation are not fully understood, but the bacterium expresses different virulence factors that can trigger T-cell immune responses in AD and contribute to the inflammatory response.[3] For example, staphylococcal enterotoxins (SE) have the ability to act as superantigens via direct stimulation of T cells.[4] Colonization with staphylococcal strains that produce these virulence factors, including SEA, SEB, SEC and SED, is thought to be related to AD severity.[5,6] To date, the role of other staphylococcal antigens in AD has barely been investigated.[7]

There is increasing interest in understanding the immune response against *S. aureus* in AD as an altered immune response might contribute to inflammation and barrier damage. The current literature focuses on IgE antibody titers directed against some of the *S. aureus* antigens. Increased IgE-specific antibodies against *S. aureus* antigens, mainly SEA and SEB, have been described in patients with AD vs. healthy controls. Furthermore, an association between IgE levels and AD severity has been confirmed in some studies.[8-10] Although IgG is known for its involvement in the neutralization and elimination of microbes, little is known about anti-*S. aureus* IgG antibody patterns in patients with AD.[11] Previous studies measured IgG against two antigens, exfoliative toxin A and SEB, and reported higher IgG levels in patients vs. controls (significant for SEB).[12,13] Other antigens were not studied. Two studies performed detailed IgG subclass analysis and found an IgG2 deficiency against SEC1 and an elevated IgG4 against SEB in patients with AD.[14,15] Although studies are limited in number and focus on a few single antigens, they emphasize the possible relevance of IgG in the response against *S. aureus* in AD.

To gain more insights into the IgG-mediated immune response against *S. aureus* in patients with AD, we profiled IgG antibodies against 55 *S. aureus* antigens in sera of children with mild-to-severe AD, using a Luminex assay.[16] Additionally, we evaluated the association between IgG levels and disease severity.

MATERIALS AND METHODS

Study design and population

This cross-sectional study was embedded in two interventional studies: the Shared Medical Appointment (SMA) study and the DAVOS study.[17,18] SMA included patients with mild-to-severe AD, aged between 0 and 18, between November 2009 and December 2011. The DAVOS study included children with difficult-to-treat eczema, aged 8-18 years,

between January 2011 and June 2015. Both studies were conducted at the Wilhelmina Children's Hospital in The Netherlands and were approved by the University Medical Centre Utrecht's medical and ethical review board (09-192/K, 08-368/K). Written informed consent was obtained from all patients. Serum samples, microbial swabs, eczema severity scores and patient characteristics, obtained at baseline in both the SMA and DAVOS studies, were analysed in this study. In both studies, AD was diagnosed according to the UK Working Party criteria.[19-21] Severity was assessed by the parents using the Self-Administrated Eczema Area and Severity Index (SA-EASI).[22] Apart from the SA-EASI, the levels of thymus and activation-regulated chemokine (TARC) in serum were used as a marker for AD severity.[23,24] Age of AD onset, treatment history and diagnosis of asthma and allergic rhinitis were based on clinical history. Food allergy was diagnosed based on convincing clinical history in the SMA study and/or double-blind food provocation test in the DAVOS study.

Microbial samples

Skin microbial samples were taken from the nose and lesional skin according to a standardized procedure using a sterile swab (Sterile Dryswab™) moistened with NaCl 0.9%. Skin samples were collected from lesions at the antecubital fold or the popliteal fossa. Bacterial cultures for *S. aureus* were performed using routine diagnostic culture procedures using *S. aureus*-specific Mannitol salt agar plates.

Measurement of antibodies against *S. aureus*

Antigens and coupling procedure

In a pilot experiment, the IgG antibody titers against a set of 57 *S. aureus* antigens were measured in sera of 23 AD patients. It was decided to include 55 antigens in the present study as the signals for two antibodies (ESAT-6 secretion system extracellular A and SA2097) were very low in the pilot study (data not shown). The 55 antigens used in this study were divided into four categories based on their biological function: household enzymes, immune modulators (superantigens and nonsuperantigens), cell membrane-damaging molecules and microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) (table S1). All used antigens were recombinant proteins, expressed with a histone tag in the *Escherichia coli* XL1-blue strain and purified under denaturing conditions with nickelnitrilotriacetic acid agarose. They were coupled to the SeroMAP carboxylated beads (Luminex Corporation) as described previously.[25,26] The final bead concentration was adjusted to 3000 beads/µL and they were stored at 6°C in the dark. As a negative control, the coupling procedure was performed in absence of any antigen.

Luminex assay

Serum samples were stored at -80°C until quantification of IgG antibody levels against *S. aureus* antigens with a fluorescent bead-based flow cytometry technique (xMapâ, Luminex).[16] In the wells of a 96-well filter microtiter plate (Millipore Corporation), 50 µL bead mix (containing the different antigen-coupled beads each at a concentration of 3000 beads/µL) was mixed with 50 µL 1:100 diluted patient serum. Follow-up steps have previously been described in detail.[27] Each measurement lasted 1 minute; during this time a minimum of 100 beads had to be analysed for each antigen-coupled bead, otherwise the data were excluded from further analysis. IgG antibody levels against *S. aureus* antigens were expressed as median fluorescence intensity (MFI). A without non-protein-coupled control bead was included to determine nonspecific antibody binding. The nonspecific MFI values were subtracted from the results.[26] The MFI values of the two independent experiments, reflecting semi-quantitative antibody levels, were averaged. The coefficient of variation (CV) was calculated for the duplicate experiments. Measurements were excluded if the CV value was >25% and average MFI values were >1000. Failures of the Luminex per well were defined as missing values.

Statistical analysis

For this study, a convenience sample was obtained from the SMA and the DAVOS studies. As the DAVOS and SMA studies used different inclusion criteria regarding AD severity and age, the study cohorts were analysed separately. For further analysis the IgG data were preprocessed by replacing negative and zero MFI values (resulting from correction for nonspecific binding) by 1. Absolute IgG levels per antigen are presented as median [interquartile range (IQR)]. The IgG data were log-transformed to obtain a parametric distribution, and standardised using a zero mean unit variance method. Pairwise correlations were calculated between the 55 IgG levels using the Spearman rank correlation test. A hierarchical clustering analysis with the 55 antigens was carried out to identify main antibody clusters, but no robust clusters were identified (data not shown). Therefore, for further analysis the 55 antibodies were analysed separately.

Linear regression analysis

Severity scores and patient age were tested for normal distributions with the one-sample Kolmogorov–Smirnov test and transformed when necessary, to obtain a normal distribution. Multivariable linear regression analyses were carried out using the standardized IgG levels (described above) for each of the 55 *S. aureus* antigens against the SA-EASI score as a main predictor, adjusted for age, sex and colonization with *S. aureus* on skin and/or in nose (*S. aureus* present, 'yes' or 'no'). The multivariable linear regression was repeated in a separate model using TARC as a main predictor instead of SA-EASI to validate our results with an intrinsic marker for AD severity. For antibodies that did

not follow linear distribution after transformation, bootstrapping (iteration 1000) was used to obtain regression coefficients and 95% confidence intervals (CIs). Given the exploratory nature of this study and the observed correlations between the 55 tested antibody levels, we used a *P*-value <0.05 to indicate significant associations between antibody levels and SA-EASI/ TARC. Analyses were performed using SPSS version 23.0 for Windows (IBM, Armonk, NY, U.S.A.).

RESULTS

Population characteristics

We included 136 and 76 children from the SMA and DAVOS studies, respectively (figure S1). The median age of the children from the SMA cohort was 2 years (IQR 1-5). The DAVOS population consisted of older children with a median age of 13 years (IQR 11-15). Eczema severity measured with the SA-EASI gave a median of 27 (IQR 16-42) in the SMA cohort and 24 (IQR 12-42) in the DAVOS cohort. Median TARC values were also higher in the SMA cohort [1441 in pg/ml (IQR 713-2794) vs. 1119 (IQR 696-2400). Skin and nasal colonization with *S. aureus* was found in 36% and 34% of the children in the SMA cohort, respectively, and in 47% and 67% of the children in the DAVOS cohort. Detailed baseline characteristics, including use of medication, are given in table 1.

Antibody characteristics

Median IgG levels against 55 *S. aureus* antigens measured in the sera of the children from both study cohorts are presented in table S1. In the DAVOS cohort, the medians of the absolute antibody levels were higher and showed less variation than the SMA study cohort. Figure 1 shows that the IgG antibody responses do not clearly differ between the four main biological groups of antigens (immune modulators (superantigens and non-superantigens), household enzymes, cell-membrane-damaging molecules and MSCRAMMs). A Spearman correlation test showed correlations between the IgG levels of the staphylococcal superantigen-like (SSL) proteins 3, 5, 9 and 10 (coefficients >0.7). High correlations (>0.7) were also identified between leukotoxin (Luk) E, LukD, LukS and between extracellular fibrinogen-binding protein and alanine transaminase 2. Additionally, some enterotoxins were correlated: SEB with SEC (>0.7), SEI with SEM (>0.8), SEN with SEI (0.69) and SEA with SEE (0.69) (figure 2).

Association between antistaphylococcal IgG levels and atopic dermatitis severity measured with SA-EASI and TARC

We found significant associations between IgG levels and AD severity in the SMA cohort. Sixteen antigens were associated with SA-EASI and 12 with TARC (table 2). Ten of the

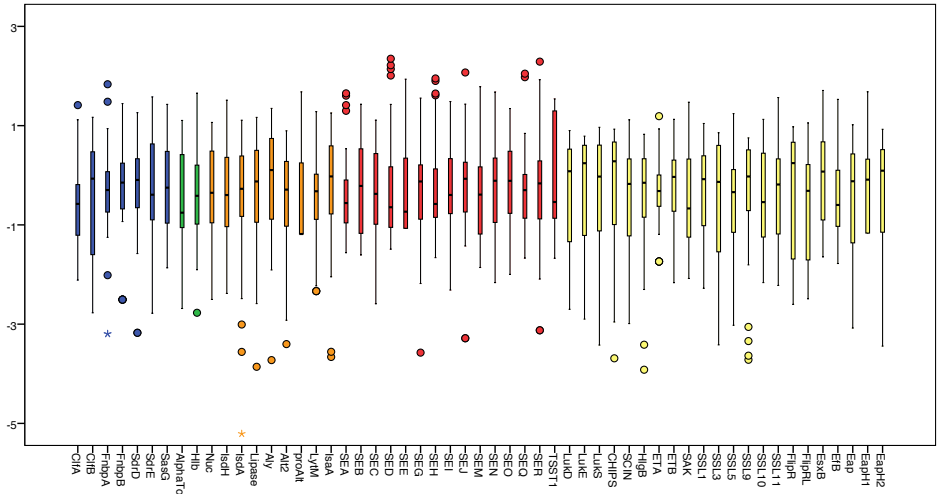
Table 1. Baseline characteristics

	SMA cohort (n=131)	DAVOS cohort (n=76)
Age in years; median (IQR)	2 (1-5)	13 (11-15)
Sex (male)	63 (48.1)	39 (51.3)
Ethnicity		
Dutch	95 (72.5)	54 (71.1)
Other ethnicity	19 (14.5)	22 (28.9)
Missing	17 (13.0)	0 (0)
Age of onset AD		
0-<2 years	106 (80.9)	66 (86.8)
2-<6 years	6 (4.6)	8 (10.5)
Missing	19 (14.5)	2 (2.6)
Atopy		
Food allergy	53 (40.4)[1]	49 (64.5)[1]
Allergic asthma	40 (30.5)[2]	59 (77.6)
Allergic rhinoconjunctivitis	36 (27.5)[2]	67 (88.2)
SA-EASI; median (IQR)	27.00 (16.00-42.20)[3]	24.00 (11.95-41.75)[2]
TARC pg/mL; median (IQR)	1441 (713-2794)	1119 (696-2400)[2]
Corticosteroid treatment		
Topical corticosteroid	101 (77.1)	70 (92.1)
Systemic corticosteroid	0 (0)	3 (3.9)
Neoral	0 (0)	7 (9.2)
Antibiotic treatment		
Topical antibiotic	11 (8.4)	2 (2.6)
Systemic antibiotic	3 (2.3)	1 (1.3)
***Staphylococcus aureus* positive** (> 10 CFU)		
Skin	47 (35.9)[4]	36 (47.4)[3]
Nose	45 (34.4)[2]	51 (67.1)[3]

Data are n (%) unless otherwise indicated. SMA, Shared Medical Appointment; IQR, interquartile range; AD, atopic dermatitis; SA-EASI, Self-Administered Eczema Area and Severity Index; TARC, thymus and activation-regulated chemokine; CFU, colony-forming unit.
Missings SMA: [1] = 4 (3.1%); [2] = 2 (1.5%); [3] = 41 (31.3%); [4] = 3 (2.3%)
Missings DAVOS: [1] = 2 (2.6%); [2] = 3 (3.9%); [3] = 6 (7.9%)

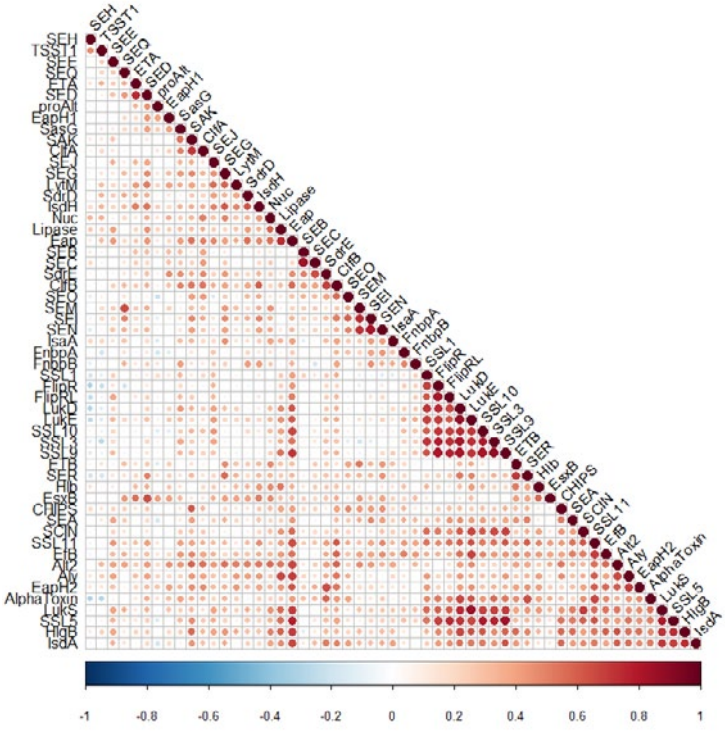
12 antigens associated with TARC were also associated with SA-EASI (*P*-values 0.001 to 0.044). The associated IgG antibodies targeted mainly secreted proteins with immunomodulatory functions (e.g. LukD and LukE; table 2). The described associations between antigen levels and AD severity were independent of age, sex and colonization of the skin and/or nose with *S. aureus*. In the DAVOS study, IgG levels against only four and one *S. aureus* antigen(s) were associated with SA-EASI and TARC, respectively, and there was no overlap between the two markers for AD (tables S2).

Figure 1. Boxplots showing the levels of IgG against 55 antigens in the SMA study (normalized data)



NOTE: Blue indicates MSCRAMMs, green the membrane-damaging molecules, orange the housekeeping antigens, red the superantigens and yellow the immunomodulating proteins.

Figure 2. Spearman's rank correlation coefficients of the IgG values (MFI) against 55 antigens (SMA cohort).



NOTE: The size and intensity of the red dots reflects the height of the correlation coefficients, identifying high correlations for example between the SSL 3, 5, 9 and 10 antigens.

Table 2. List of *S. aureus* antigens of which the IgG levels were significantly associated with patient eczema severity, according to SA-EASI and TARC, *P*-value < 0.05)

Antigens	SMA study (n=131)						DAVOS study (n=76)					
	SA-EASI ^			TARC			SA-EASI			TARC		
	Regression coefficient (SE)	95% CI	*P*-value	Regression coefficient (SE)	95% CI	*P*-value	Regression coefficient (SE)	95% CI	*P*-value	Regression coefficient (SE)	95% CI	*P*-value
LukD	0.134 (0.042)*	0.054-0.219	0.003	0.379 (0.160)*	0.080-0.714	0.018	-	-	-	-	-	-
LukE	0.111 (0.048)*	0.016-0.213	0.033	0.396 (0.141)*	0.108-0.672	0.005	-	-	-	-	-	-
SSL3	0.145 (0.052)*	0.045-0.258	0.008	0.404 (0.144)*	0.150-0.700	0.006	-	-	-	-	-	-
SSL5	0.153 (0.044)*	0.070-0.242	0.001	0.379 (0.179)	0.024-0.734	0.036	-	-	-	-	-	-
SSL9	0.149 (0.048)*	0.053-0.243	0.004	0.309 (0.143)*	0.036-0.617	0.035	-	-	-	-	-	-
SSL10	0.127 (0.047)*	0.036-0.224	0.009	0.432 (0.151)*	0.162-0.747	0.004	-	-	-	-	-	-
FlipRL	0.120 (0.057)*	0.018-0.243	0.043	0.398 (0.183)*	0.047-0.768	0.036	-	-	-	-	-	-
SEA	0.177 (0.057)*	0.062-0.292	0.002	0.613 (0.194)	0.230-0.997	0.002	-	-	-	-	-	-
IsdA	0.203 (0.045)*	0.115-0.294	0.001	0.346 (0.135)*	0.094-0.624	0.022	-	-	-	-	-	-
Eap	0.146 (0.048)*	0.047-0.236	0.002	0.286 (0.142)*	0.018-0.582	0.044	-	-	-	-	-	-
LukS	0.113 (0.058)*	0.002-0.223	0.047	-	-	-	-	-	-	-	-	-
HlgB	0.116 (0.047)*	0.022-0.214	0.019	-	-	-	-	-	-	-	-	-
SSL1	0.134 (0.050)*	0.045-0.238	0.004	-	-	-	-	-	-	-	-	-
FlipR	0.144 (0.059)*	0.026-0.262	0.020	-	-	-	-	-	-	-	-	-
SdrE	-0.140 (0.062)*	-0.271-(-0.022)	0.036	-	-	-	-	-	-	-	-	-
IsaA	0.125 (0.047)*	0.023-0.215	0.008	-	-	-	-	-	-	-	-	-
SEE	-	-	-	0.550 (0.212)*	0.135-0.980	0.011	-	-	-	-	-	-
EfB	-	-	-	0.450 (0.178)	0.097-0.803	0.013	-	-	-	-	-	-
Hlb	-	-	-	-	-	-	0.155 (0.058)*	0.041-0.262	0.010	-	-	-
SEG	-	-	-	-	-	-	0.165 (0.069)*	0.050-0.326	0.019	-	-	-
FnbpB	-	-	-	-	-	-	0.151 (0.060)*	0.029-0.261	0.015	-	-	-
ClfA	-	-	-	-	-	-	0.142 (0.067)*	0.023-0.291	0.040	-	-	-
SAK	-	-	-	-	-	-	-	-	-	0.695 (0.302)*	0.084-1.285	0.027

^ N = 90 for SA-EASI analysis due to missing SA-EASI scores
* Regression coefficients and CI were obtained using bootstrapping iteration 1000
- = no significant association between the antigen and the severity parameter.
SMA, Shared Medical Appointment; AD, atopic dermatitis; SA-EASI, Self-Administered Eczema Area and Severity Index; TARC, thymus and activationregulated chemokine; SE, standard error; CI, confidence interval. Please find abbreviations of the antigens in table S1.

DISCUSSION

For the first time, IgG immune responses against a large panel of 55 *S. aureus* antigens were profiled in children with AD, showing that the children are exposed to the antigens and develop an IgG-mediated humoral immune response towards them. Additionally, AD severity was found to be associated with IgG antibodies directed against *S. aureus* antigens with mainly immunomodulatory functions. LukD and LukE are commonly expressed by strains of *S. aureus* and are involved in cell lysis of neutrophils.[28] SSL3, SSL5, SSL9 and SSL10 are variably expressed and are all involved in immunomodulation, for example by inhibiting complement activation.[29] Iron-responsive surface determinants A is a cell-surface protein that may function in both iron acquisition and adhesion.[30] SEA is more rarely expressed and has a strong immunostimulatory function. As a superantigen it can cause cytokine release and epithelial damage, but the literature also describes (anti-inflammatory) cytokine downregulating functions (interleukin-4).[28,31] *Staphylococcus aureus* formyl peptide receptor-like 1 inhibitor (FLIPr) and its homologue FLIPr-like are potent FcyR antagonists that inhibit IgG-mediated effector functions.[32]

Only Sohn *et al.* have studied the staphylococcal IgG response in relation to AD severity. They measured SEB only and found no correlation with AD severity, which corresponds with our findings (table 2).[13] The finding that the antibodies that were associated with AD severity in this study are known to target antigens with an immunomodulatory function suggests that *S. aureus* downregulates the immune system locally to help it maintain its colonization on the skin, a theory that was recently suggested by Biedermann *et al.*[33]

In contrast to the associations between AD severity and IgG levels for specific antigens found in the SMA study, few associations were found in the DAVOS cohort. In addition, there was no overlap between the antibodies associated with the SA-EASI and these associated with TARC in the DAVOS cohort, which suggests that these associations were false-positive results. The lack of associations found in the DAVOS cohort could be the result of the older age of the DAVOS participants, who may have been more chronically exposed to *S. aureus* (see tables 1 and S1) and therefore exhibited higher levels of IgG.[12,34] Furthermore, IgG antibody levels are known to increase with age (which is also true for patients with AD), reaching a plateau around adulthood.[34-36] Indeed, comparison between IgG levels of the SMA cohort and a sample of healthy adult pooled serum (appendix S1), showed higher levels for most of the tested IgG antibodies in the healthy

pooled serum (figure S2). The DAVOS cohort had higher values than the healthy adult sample for a large part of the antibodies (figure S1). It could be argued that a plateau could have been reached in the older patients of the DAVOS cohort resulting in a lack of found associations. However, the DAVOS study included patients with difficult-to-treat (severe) eczema, most of whom were treated with topical corticosteroids or even systemic immunosuppressive therapy (table 1). Hence, the SA-EASI scores at baseline may have been biased towards lower scores, which could have hampered the associations. Most likely the lack of association is a combination of both biased scores and the older age of the cohort.

IgG levels against specific antigens showed correlations (Spearman's correlation test). SSL antigens and the superantigens SEI, SEM and SEN are known to be co-produced by *S. aureus*.[37,38] The combined presentation of these antigens to the immune system probably contributed to the observed correlations between IgG responses. However, other factors probably also drive the height and correlations of the IgG-specific immune responses.

This study has several limitations. Firstly, a control group of children without AD that would allow investigating the normal range of IgG antibodies was not available. Therefore, our conclusion focusses on a comparison between different severity phenotypes in a well-characterized cohort of children with AD. Due to the cross-sectional design we cannot conclude whether AD severity is the result of an altered IgG response. In addition, owing to the small sample size of this study, we could not perform multivariate analysis, such as unsupervised clustering, which would have allowed us to better understand the correlations found for different antibodies. Although we tested 55 different associations, we did not correct for multiple testing. Owing to the high correlations found between some of the antibodies, multiple testing correction is rather conservative. Because of this, and the hypothesis-generating nature of this study, our associations were kept nominally significant. Although cross-reactivity with other staphylococcal antigens cannot be completely ruled out, it is highly unlikely as *S. aureus* produces species-specific virulence factors that have not been found in *Staphylococcus epidermidis*.[39] Despite these limitations, the present study is the first to evaluate antibody responses against a broad panel of *S. aureus* antigens. Our study sheds light on the IgG-mediated immune response to *S. aureus* in children with AD and it highlights the relevance of other antigens (adhesins and immune modulators) next to the often studied superantigens. Further studies need to be conducted to validate the associations we found.

Interestingly, the association between IgG against *S. aureus* antigens and the severity of AD was independent of skin and nasal colonization with *S. aureus*. This suggests that the immune response against *S. aureus* might be altered, irrespective of the bacteria present on the skin at that time, and raises the question whether anti-*S. aureus* treatment should be guided by a positive culture. To further explore the significance of our

findings, future studies should relate our findings to IgE and IgG subclass responses to *S. aureus* antigens in AD. These findings could also be related to *S. aureus* strain differences as different strains might have different ability to elicit immunological alterations in the host.[40] *In vitro* experiments should reveal the functional effect of the relevant *S. aureus* antigens on T-cell differentiation.

CONCLUSION

In a cohort of young children with AD, we identified significant associations between disease severity and IgG antibodies directed against *S. aureus* antigens with mainly immunomodulatory functions. The results of this study encourage more detailed evaluation of the role of *S. aureus* in the pathogenesis of AD.

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SUPPLEMENTARY MATERIAL

Appendix S1. Measurement of antibodies against *S. aureus* in human pooled serum.

Human pooled serum (HPS) is a mix of serum samples from 35 healthy adults (25% male, age between 19 and 61 years). The serum of all the AD patients included in this study (SMA=137 and DAVOS=76) was analysed for detection of IgG antibodies against 55 *S. aureus* antigens by Luminex assay. Detailed description of the Luminex assay is described in the methods section of the main manuscript. This analysis included three samples of the HPS mix, resulting in three HPS measurements per *S. aureus* antigen. After quality control as described the methods section of the main manuscript, the HPS IgG measurements were averaged per antigen for further analysis.

Figure S1. Flowchart of the study population, SMA study and DAVOS study

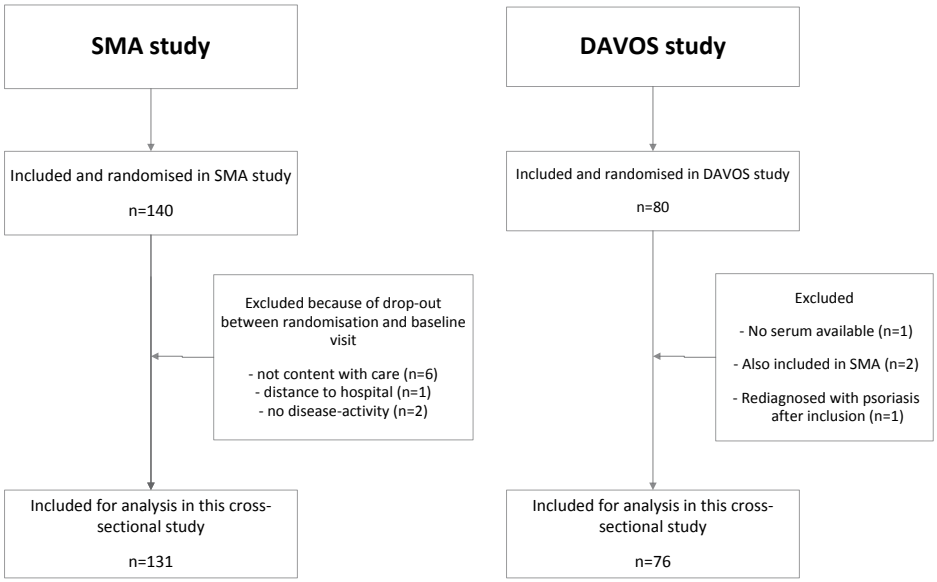

Figure S2. IgG levels of the 55 antigens in SMA, DAVOS and HPS

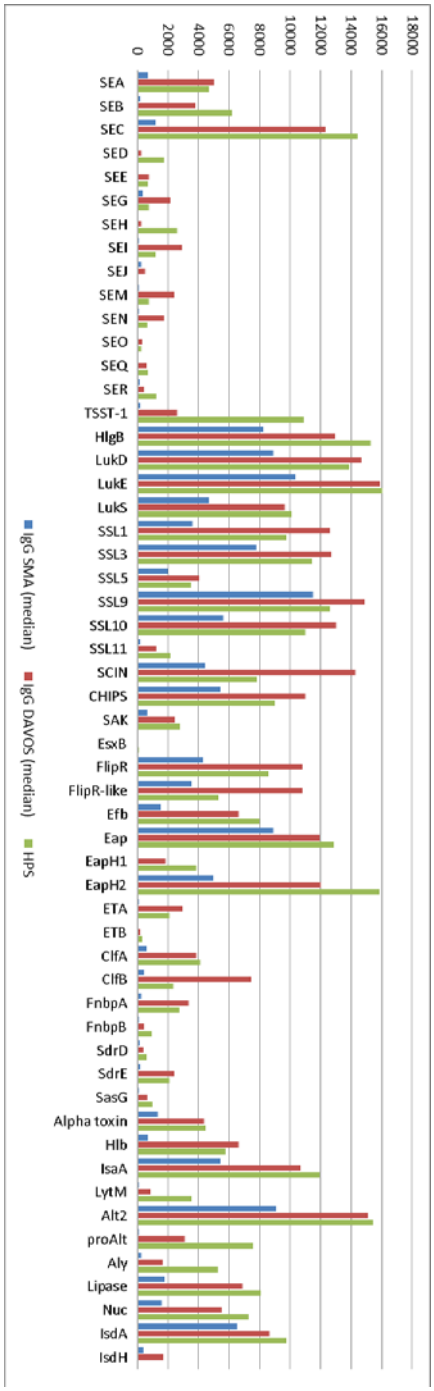

Figure S2 shows the absolute IgG levels of the 55 antigens for the SMA cohort (median), DAVOS cohort (median) and the human pooled serum (HPS). HPS signals for two antigens, SEJ and IsdH, were excluded according to the exclusion criteria described in the methods section of the main manuscript (e.g. coefficient of variation for duplicate experiment >25%). Overall, HPS levels are higher than the SMA levels for all 53 antigens. HPS levels are higher than the DAVOS samples for only 31 antigens. NOTE: as the IgG values are not standardized, the IgG levels between the different antigens cannot be compared. Figure 1 of the manuscript includes a boxplot with standardized IgG levels.

Table S1. Overview of 55 *S. aureus* antigens with function and MFI values

***S. aureus* antigen**	**Description**	**N**	**IgG SMA** (median, IQR)	**N**	**IgG DAVOS** (median, IQR)	***P*- value ***
Immune modulators (superantigens)						
SEA (Staphylococcal enterotoxin A)	Superantigen [1]	127	702.50 (228.50-4005.50)	63	5011.25 (931.00-11820.00)	< 0.001
SEB (Staphylococcal enterotoxin B)	Superantigen [1]	126	203.75 (9.25-3283.44)	62	3803.50 (780.25-13750.69)	< 0.001
SEC (Staphylococcal enterotoxin C)	Superantigen [1]	126	1179.38 (87.88-12018.94)	62	12330.63 (7764.56-14071.19)	< 0.001
SED (Staphylococcal enterotoxin D)	Superantigen [1]	119	24.00 (6.00-185.50)	68	222.50 (109.25-975.31)	< 0.001
SEE (Staphylococcal enterotoxin E)	Superantigeni [1]	119	12.50 (0.00-394.50)	67	764.00 (56.50-2279.00)	< 0.001
SEG (***St***aphylococcal enterotoxin G)	Superantigen [1]	125	323.00 (107.50-1410.13)	76	2156.5 (565.88-3468.13)	< 0.001
SEH (Staphylococcal enterotoxin H)	Superantigen [1]	120	30.50 (13.94-494.69)	69	232.75 (23.63-7071.25)	= 0.001
SEI (***St***aphylococcal enterotoxin I)	Superantigen [1]	120	95.63 (38.00-1012.75)	68	2920.38 (1034.13-5347.13)	< 0.001
SEJ (Staphylococcal enterotoxin J)	Superantigen [1]	119	223.50 (96.50-553.75)	68	490.50 (195.81-1071.88)	= 0.001
SEM (***St***aphylococcal enterotoxin M)	Superantigen [1]	127	72.50 (18.00-374.50)	63	2424.75 (480.75-4780.50)	< 0.001
SEN (Staphylococcal enterotoxin N)	Superantigen [1]	128	92.38 (28.81-328.06)	63	1769.00 (384.00-3827.50)	< 0.001
SEO (Staphylococcal enterotoxin O)	Superantigen [1]	127	34.00 (10.50-140.00)	63	305.25 (90.25-546.50)	< 0.001
SEQ (Staphylococcal enterotoxin Q)	Superantigen [1]	119	34.75 (12.75-130.50)	67	591.50 (47.00-4981.75)	< 0.001
SER (Staphylococcal enterotoxin R)	Superantigen [1]	123	147.75 (59.50-424.50)	49	429.00 (168.38-1488.75)	< 0.001
TSST-1 (Toxic shock syndrome toxin 1)	Superantigen [1]	125	163.75 (53.13-7202.00)	74	2626.00 (67.63-6578.75)	= 0.152
Immune modulators (non-superantigen)						
HlgB (Gamma-hemolysin component B)	Pore forming toxin [1]	128	8217.13 (4881.06-11112.38)	62	12963.00 (9359.13-14555.13)	< 0.001
LukD (Leukotoxin D)	Cell lysis of neutrophils [1]	127	8894.00 (3666.75-12978.50)	62	14705.13 (12837.31-15792.00)	< 0.001
LukE (Leukotoxin E)	Cell lysis of neutrophils [1]	127	10373.75 (5276.75-14596.75)	62	15897.13 (14282.13-16821.13)	< 0.001
LukS (Leukotoxin S)	Cell lysis of neutrophils [1]	126	4648.50 (2034.31-8396.38)	62	9663.00 (5065.19-12566.82)	< 0.001

Table S1. Overview of 55 *S. aureus* antigens with function and MFI values (continued)

***S. aureus* antigen**	**Description**	**N**	**IgG SMA** (median, IQR)	**N**	**IgG DAVOS** (median, IQR)	***P*- value ***
SSL1 (Staphylococcal superantigen like-protein 1)	Immune-modulation. Limits neutrophil chemotaxis[2]	125	3587.50 (491.38-13491.00)	75	12624.13 (6916.50-15991.94)	< 0.001
SSL3 (**S**taphylococcal superantigen like-protein 3)	TLR signalling inhibition[3]	125	7765.50 (1355.75-12847.38)	75	12696.00 (9673.75-15214.75)	< 0.001
SSL5 (Staphylococcal superantigen like-protein 5)	Prevents neutrophil rolling on activated endothelial cells[3]	125	1983.25 (891.63-4833.00)	75	4066.00 (2792.75-5798.00)	< 0.001
SSL9 (Staphylococcal superantigen like-protein 9)	Complement inhibitor[4]	125	11502.50 (5653.50-14845.38)	76	14879.38 (11952.56-15889.25)	< 0.001
SSL10 (Staphylococcal superantigen like-protein 10)	Phagocytosis inhibition[3]	124	5597.88 (2168.06-10963.44)	76	13037.25 (11159.19-14609.50)	< 0.001
SSL11 (Staphylococcal superantigen like-protein 11)	Chemotaxis inhibition[3]	125	179.50 (37.88-647.75)	75	1239.25 (480.75-2996.50)	< 0.001
SCIN (Staphylococcal complement inhibitor)	Chemotaxis inhibitory, inhibits C3 convertase [1]	123	4420.00 (1442.00-10108.50)	49	14291.00 (11844.75-15457.75)	< 0.001
CHIPS (Chemotaxis inhibitory protein of Staphylococci)	Chemotaxis inhibition[3]	122	5442.13 (621.44-8926.31)	60	11027.63 (7898.25-12846.00)	< 0.001
SAK (Staphylokinase)	Binding/ inactivate complement C3b and IgG bound to surface bacterial cells [5]	118	646.88 (75.94-3691.31)	63	2433.00 (936.25-5507.50)	< 0.001
EsxB (ESAT-6 secretion system extracellular B)	Interferes with host cell apoptotic pathways [6]	125	28.00 (6.25-71.38)	76	53.50 (25.75-97.00)	= 0.001
FlipR (Formyl peptide receptor-like 1 inhibitor)	Inhibition of opsonophagacytosis and killing by neutrophils[5] Chemotaxis inhibition[3]	122	4336.88 (499.81-9194.56)	66	10812.25 (7690.13-13461.31)	< 0.001

Table S1. Overview of 55 *S. aureus* antigens with function and MFI values (continued)

***S. aureus* antigen**	**Description**	**N**	**IgG SMA** (median, IQR)	**N**	**IgG DAVOS** (median, IQR)	***P*- value ***
FlipR-like (Formyl peptide receptor-like1 inhibitor like)	Chemotaxis inhibition[3]	123	3537.00 (748.00-8240.00)	74	10785.00 (6972.56-13626.94)	< 0.001
Efb (Extracellular fibrinogen-binding protein)	Inhibits complement activation and blocks opsonophagocytosis [3, 5]	119	1551.25 (688.50-3872.00)	67	6621.75 (3033.50-9304.75)	< 0.001
Eap (Extracellular adhesive protein)	Phagocytic killing inhibition[3] Prevent neutrophil attachment to and migration through endothelial cells [5]	128	8878.63 (3729.81-12629.06)	64	11957.38 (8675.69-14846.63)	< 0.001
EapH1 (Extracellular adherence protein homolog 1)	Phagocytic killing inhibition[3]	128	39.75 (0.00-552.38)	64	1853.00 (509.69-4266.69)	< 0.001
EapH2 (Extracellular adherence protein homolog 2)	Phagocytic killing inhibition[3]	117	4953.25 (646.13-13314.88)	61	11998.25 (8251.13-14424.38)	< 0.001
ETA (Exfoliative toxin A)	Serine proteases: hydrolyze desmosomal proteins in the skin [7]	126	68.25 (18.88-332.12)	62	2969.75 (164.75-10600.31)	< 0.001
ETB (Exfoliative toxin B)	Serine proteases: hydrolyze desmosomal proteins in the skin [7]	127	52.00 (20.75-127.75)	62	162.88 (42.38-5042.50)	< 0.001
MSCRAMMs						
ClfA (Clumping factor A)	Adhesin: (fibrinogen) [1]	120	572.75 (145.25-2259.31)	69	3876.50 (1739.38-6289.63)	< 0.001
ClfB (Clumping factor B)	Adhesin: (fibrinogen) [1]	101	417.75 (55.75-1104.75)	24	7453.25 (3470.50-9834.63)	< 0.001
FnbpA (Fibronectin-binding protein A)	Adhesin: (fibronectin) [1]	108	214.25 (90.75-1061.00)	64	3342.25 (854.50-4989.19)	< 0.001
FnbpB (Fibronectin-binding protein B)	Adhesin: (fibronectin) [1]	119	102.00 (48.25-346.50)	68	425.25 (194.75-1090.81)	< 0.001
SdrD (Serine-aspartate repeat protein D)	***S.aureus*** adhesion[8]	120	144.88 (63.38-288.94)	68	395.13 (138.63-659.38)	< 0.001
SdrE (Serine-aspartate repeat protein E)	***S.aureus*** adhesion[9]	120	177.25 (50.50-1186.38)	64	2414.63 (1144.19-5548.81)	< 0.001
SasG (***S. aureus*** surface protein G)	Biofilm formation [10]	120	101.63 (18.38-387.75)	69	620.00 (342.63-1548.00)	< 0.001

Table S1. Overview of 55 *S. aureus* antigens with function and MFI values (continued)

***S. aureus* antigen**	**Description**	**N**	**IgG SMA** (median, IQR)	**N**	**IgG DAVOS** (median, IQR)	***P*- value ***
Membrane damaging molecules						
Alpha toxin	Pore forming toxin [1]	128	1357.88 (348.06-3391.19)	62	4356.75 (2713.56-6074.63)	< 0.001
Hlb (Beta-hemolysin)	Pore forming toxin [1]	127	669.50 (181.00-4042.75)	64	6608.13 (2150.88-11467.31)	< 0.001
Housekeeping function						
IsaA (Immunodominant Staphylococcal antigen A)	Assist in cell wall expansion, turnover, growth, and cell separation [11]	117	5435.25 (2779.50-9894.38)	69	10696.75 (6047.63-14414.50)	< 0.001
LytM (Peptidoglycan hydrolase)	Assist in cell wall expansion, turnover, growth, and cell separation [12]	120	104.00 (40.50-877.13)	68	830.25 (80.81-4097.06)	< 0.001
Alt2 (alanine transaminase 2)	Unknown	128	9086.13 (4358.75-13817.69)	64	15067.00 (13257.38-16258.88)	< 0.001
proAlt	Unknown	128	72.25 (0.00-475.06)	76	3094.75 (404.38-10124.19)	< 0.001
Aly	Unknown	83	216.50 (76.75-506.75)	24	1662.13 (656.13-4573.75)	< 0.001
Lipase	Enzyme: lipase [13] (spreading, nutrition)	124	1815.63 (378.13-5336.38)	75	6898.00 (3489.50-11195.25)	< 0.001
Nuc (Nuclease)	Enzyme: nuclease (nutrition)[14]	126	1580.13 (447.81-6626.31)	63	5543.25 (2052.31-9983.75)	< 0.001
IsdA (Iron-responsive surface determinants A)	Ferritin uptake [1]	117	6535.25 (3178.00-9277.75)	65	8648.25 (4964.63-11689.13)	= 0.001
IsdH (Iron-responsive surface determinants H)	Ferritin uptake [1]	115	373.75 (39.50-1041.50)	53	1686.50 (664.88-2499.88)	< 0.001

N = number of patients of which the MFI values could be included for calculation of medians (see exclusion criteria for measurements in the methods section of the manuscript)

* *P*-value SMA vs. DAVOS, Mann-Whitney U test

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Table S2a. Results linear regression analysis GMA study – association with SA-EASI

Antigen	*Regression coefficient (SE)*	*95% CI*	*P-value*
CHIPS	0.015 (0.052)	-0.088-0.115	0.770
SKIN	0.080 (0.058)	-0.037-0.193	0.171
Alpha toxin	-0.031 (0.053)	-0.135-0.073	0.564
Hlb	-0.032 (0.065)	-0.156-0.106	0.608
Nuc	0.026 (0.065)	-0.118-0.139	0.693
ETA	0.017 (0.062)	-0.102-0.134	0.793
ETB	0.043 (0.060)	-0.076-0.167	0.484
SAK	0.035 (0.059)	-0.095-0.143	0.537
SSL11	0.063 (0.047)	-0.031-0.156	0.181
Lipase	0.021 (0.062)	-0.109-0.140	0.721
Aly	0.077 (0.092)	-0.108-0.244	0.418
Alt2	0.069 (0.055)	-0.045-0.166	0.214
ProAlt	-0.029 (0.064)	-0.152-0.105	0.634
EsxB	-0.062 (0.068)	-0.189-0.081	0.356
SEB	-0.043 (0.061)	-0.164-0.074	0.482
SEC	-0.060 (0.062)	-0.184-0.067	0.322
SED	-0.035 (0.071)	-0.169-0.114	0.633
SEE	0.121 (0.061)	-0.003-0.243	0.051
SEG	0.041 (0.048)	-0.053-0.142	0.405
SEH	0.028 (0.069)	-0.106-0.171	0.683
SEI	0.032 (0.046)	-0.057-0.125	0.473
SEJ	-0.105 (0.071)	-0.248-0.035	0.142
SEM	0.032 (0.045)	-0.053-0.126	0.497
SEN	0.104 (0.057)	-0.009-0.217	0.069
SEO	0.012 (0.049)	-0.079-0.112	0.802
SEQ	-0.041 (0.066)	-0.167-0.089	0.557
SER	0.089 (0.058)	-0.017-0.203	0.152
TSST-1	0.067 (0.067)	-0.071-0.187	0.322
FnbpA	0.007 (0.060)	-0.110-0.124	0.905
FnbpB	-0.059 (0.075)	-0.202-0.097	0.435
SdrD	-0.027 (0.078)	-0.179-0.117	0.723
ClfA	0.006 (0.059)	-0.110-0.115	0.914
ClfB	0.063 (0.077)	-0.095-0.217	0.397
IsdH	0.137 (0.068)	0.013-0.284	0.054
EfB	0.043 (0.047)	-0.042-0.148	0.368
SasG	-0.075 (0.062)	-0.204-0.051	0.062
LytM	-0.056 (0.066)	-0.178-0.078	0.398
EapH1	0.035 (0.066)	-0.090-0.172	0.586
EapH2	0.038 (0.066)	-0.092-0.166	0.568

Bootstrapping iter 1000 for all antigens, except SEN

Table S2b. Results linear regression analysis SMA study – association with TARC

Antigen	*Regression coefficient (SE)*	*95% CI*	*P-value*
LukS	0.259 (0.144)	-0.017-0.541	0.064
CHIPS	0.174 (0.196)	-0.207-0.572	0.382
SKIN	0.273 (0.165)	-0.052-0.596	0.099
Alpha toxin	0.075 (0.154)	-0.205-0.402	0.630
Hlb	-0.130 (0.217)	-0.563-0.311	0.559
HlgB	0.207 (0.109)	-0.009-0.424	0.059
Nuc	-0.097 (0.179)	-0.459-0.227	0.600
ETA	-0.106 (0.248)	-0.590-0.412	0.668
ETB	-0.070 (0.216)	-0.522-0.350	0.744
SAK	0.217 (0.196)	-0.190-0.589	0.268
SSL1	0.315 (0.196)	-0.047-0.734	0.113
SSL11	0.105 (0.155)	-0.177-0.411	0.504
FlipR	0.328 (0.190)	-0.031-0.725	0.088
Lipase	0.04 (0.168)	-0.283-0.329	0.981
Aly	0.480 (0.349)	-0.187-1.188	0.179
Alt2	0.237 (0.133)	-0.011-0.514	0.074
ProAlt	-0.051 (0.196)	-0.440-0.333	0.802
EsxB	-0.334 (0.201)	-0.740-0.064	0.096
SEB	0.069 (0.200)	-0.362-0.460	0.720
SEC	-0.106 (0.207)	-0.525-0.277	0.625
SED	-0.379 (0.200)	-0.805-0.020	0.062
SEG	-0.062 (0.203)	-0.452-0.344	0.765
SEH	0.205 (0.221)	-0.237-0.654	0.351
SEI	0.237 (0.186)	-0.100-0.643	0.193
SEJ	-0.233 (0.256)	-0.761-0.258	0.360
SEM	0.274 (0.206)	-0.100-0.703	0.183
SEN	0.131 (0.190)	-0.245-0.507	0.493
SEO	-0.169 (0.192)	-0.533-0.220	0.384
SEQ	0.238 (0.207)	-0.183-0.643	0.259
SER	-0.086 (0.224)	-0.545-0.351	0.707
TSST-1	0.090 (0.210)	-0.331-0.497	0.659
FnbpA	0.160 (0.205)	-0.224-0.581	0.448
FnbpB	-0.265 (0.215)	-0.708-0.154	0.233
SdrD	-0.402 (0.243)	-0.883-0.054	0.106
SdrE-	0.155 (0.244)	-0.633-0.309	0.522
ClfA	0.128 (0.197)	-0.249-0.518	0.507
ClfB	0.215 (0.217)	-0.170-0.678	0.307
IsdH	0.243 (0.199)	-0.132-0.629	0.233
SasG	-0.295 (0.231)	-0.734-0.153	0.203
IsaA	0.128 (0.167)	-0.201-0.456	0.457
LytM	-0.186 (0.242)	-0.684-0.266	0.450
EapH1	0.270 (0.200)	-0.127-0.664	0.179
EapH2	0.365 (0.255)	-0.066-0.950	0.156

Bootstrapping iter 1000 for all antigens, except SEN

Table S2c. Results linear regression analysis DAVOS study – association with SA-EASI

Antigen	*Regression coefficient (SE)*	*95% CI*	*P-value*
LukD	0.058 (0.067)	-0.060-0.197	0.413
LukE	0.107 (0.078)	-0.038-0.216	0.224
LukS	0.103 (0.064)	-0.014-0.233	0.118
CHIPS	-0.009 (0.071)	-0.162-0.109	0.898
SKIN	-0.034 (0.099)	-0.191-0.194	0.762
Alpha toxin	0.074 (0.095)	-0.116-0.277	0.462
Hlb	0.155 (0.058)	0.041-0.262	0.010 *
HlgB	0.022 (0.051)	-0.088-0.120	0.658
Nuc	0.030 (0.063)	-0.099-0.154	0.642
ETA	0.079 (0.059)	-0.049-0.180	0.168
ETB	0.029 (0.067)	-0.119-0.149	0.655
SAK	-0.005 (0.068)	-0.124-0.138	0.938
SSL1	0.080 (0.071)	-0.042-0.242	0.290
SSL3	0.080 (0.094)	-0.052-0.306	0.519
SSL5	0.085 (0.094)	-0.048-0.291	0.473
SSL9	0.095 (0.097)	-0.026-0.323	0.474
SSL10	0.090 (0.093)	-0.029-0.299	0.449
SSL11	0.058 (0.069)	-0.057-0.225	0.384
FlipR	0.085 (0.089)	-0.044-0.293	0.372
FlipRL	0.117 (0.083)	-0.006-0.308	0.299
Lipase	0.111 (0.087)	-0.039-0.298	0.257
Aly	0.149 (0.122)	-0.121-0.367	0.245
Alt2	0.015 (0.065)	-0.109-0.142	0.800
ProAlt	0.010 (0.049)	-0.087-0.111	0.842
EsxB	0.055 (0.064)	-0.066-0.201	0.393
SEA	0.078 (0.066)	-0.046-0.206	0.247
SEB	0.023 (0.072)	-0.122-0.161	0.760
SEC	0.068 (0.067)	-0.082-0.182	0.346
SED	0.074 (0.074)	-0.074-0.224	0.313
SEE	0.119 (0.069)	-0.033-0.248	0.102
SEG	0.165 (0.069)	0.050-0.326	0.019 *
SEH	-0.024 (0.063)	-0.152-0.091	0.703
SEI	0.054 (0.065)	-0.065-0.183	0.409
SEJ	0.067 (0.075)	-0.075-0.223	0.380
SEM	-0.017 (0.072)	-0.0156-0.120	0.819
SEN	0.009 (0.062)	-0.0113-0.133	0.902
SEO	0.098 (0.056)	-0.015-0.209	0.083
SEQ	0.010 (0.063)	-0.113-0.137	0.877
SER	0.023 (0.080)	-0.132-0.179	0.795
TSST-1	0.045 (0.064)	-0.079-0.176	0.479
FnbpA	0.050 (0.067)	-0.099-0.167	0.448
FnbpB	0.151 (0.060)	0.029-0.261	0.015 *
SdrD	-0.045 (0.065)	-0.166-0.091	0.470
SdrE	0.050 (0.064)	-0.086-0.160	0.418
ClfA	0.142 (0.067)	0.023-0.291	0.040 *
ClfB	-0.047 (0.094)	-0.246-0.131	0.618

Table S2d. Results linear regression analysis DAVOS study – association with TARC

Antigen	*Regression coefficient (SE)*	*95% CI*	*P-value*
LukD	0.022 (0.349)	-0.785-0.576	0.929
LukE	0.148 (0.538)	-0.573-0.871	0.665
LukS	0.400 (0.239)	-0.117-0.840	0.091
CHIPS	0.014 (0.379)	-0.810-0.704	0.965
SKIN	0.065 (0.335)	-0.592-0.755	0.865
Alpha toxin	0.593 (0.341)	-0.043-1.303	0.092
Hlb	0.437 (0.333)	-0.270-1.073	0.192
HlgB	0.054 (0.204)	-0.374-0.448	0.801
Nuc	0.335 (0.292)	-0.240-0.957	0.256
ETA	-0.233 (0.393)	-1.078-0.519	0.528
ETB	0.067 (0.381)	-0.755-0.754	0.850
SAK	0.695 (0.302)	0.084-1.285	0.027 *
SSL1	0.110 (0.392)	-0.882-0.658	0.795
SSL3	0.413 (0.374)	-0.061-1.310	0.435
SSL5	0.300 (0.358)	-0.257-1.071	0.495
SSL9	0.315 (0.358)	-0.207-1.197	0.480
SSL10	0.243 (0.355)	-0.273-1.071	0.557
SSL11	0.515 (0.347)	-0.109-1.212	0.155
FlipR	0.108 (0.395)	-0.654-0.888	0.818
FlipRL	0.375 (0.343)	-0.206-1.149	0.380
Lipase	0.661 (0.351)	0.094-1.454	0.058
Aly	0.485 (0.709)	-0.889-1.879	0.485
Alt2	0.405 (0.324)	-0.258-1.043	0.228
ProAlt	0.035 (0.315)	-0.598-0.646	0.906
EsxB	0.257 (0.297)	-0.363-0.858	0.363
SEA	0.154 (0.354)	-0.557-0.861	0.655
SEB	0.326 (0.297)	-0.203-0.923	0.286
SEC	0.167 (0.272)	-0.312-0.751	0.535
SED	-0.146 (0.267)	-0.685-0.360	0.576
SEE	-0.001 (0.341)	-0.724-0.605	0.998
SEG	0.138 (0.335)	-0.511-0.832	0.673
SEH	0.284 (0.338)	-0.417-0.892	0.402
SEI	0.375 (0.288)	-0.161-1.001	0.176
SEJ	0.215 (0.320)	-0.355-0.886	0.521
SEM	0.110 (0.356)	-0.617-0.749	0.756
SEN	0.123 (0.391)	-0.609-0.926	0.760
SEO	0.324 (0.364)	-0.427-1.085	0.353
SEQ	0.298 (0.336)	-0.372-0.944	0.385
SER	0.270 (0.441)	-0.664-1.128	0.536
TSST-1	0.074 (0.323)	-0.541-0.706	0.809
FnbpA	0.040 (0.340)	-0.659-0.723	0.910
FnbpB	0.522 (0.305)	-0.059-1.122	0.092
SdrD	0.387 (0.320)	-0.257-0.988	0.237
SdrE	0.000 (0.303)	-0.564-0.596	0.998
ClfA	0.355 (0.263)	-0.180-0.910	0.181
ClfB	-0.346 (0.506)	-0.1.328-0.627	0.508

Table S2c. Results linear regression analysis DAVOS study – association with SA-EASI ***(continued)***

Antigen	*Regression coefficient (SE)*	*95% CI*	*P-value*
IsdH	0.113 (0.076)	-0.028-0.260	0.138
EfB	-0.023 (0.062)	-0.139-0.101	0.707
IsdA	0.077 (0.082)	-0.040-0.270	0.428
SasG	0.065 (0.066)	-0.064-0.196	0.331
IsaA	-0.045 (0.073)	-0.184-0.105	0.513
LytM	0.026 (0.063)	-0.101-0.151	0.643
Eap	-0.006 (0.069)	-0.133-0.140	0.936
EapH1	0.073 (0.072)	-0.061-0.219	0.322
EapH2	0.026 (0.069)	-0.087-0.182	0.711

* = significant P-value, Bootstrapping iter 1000 for all antigens

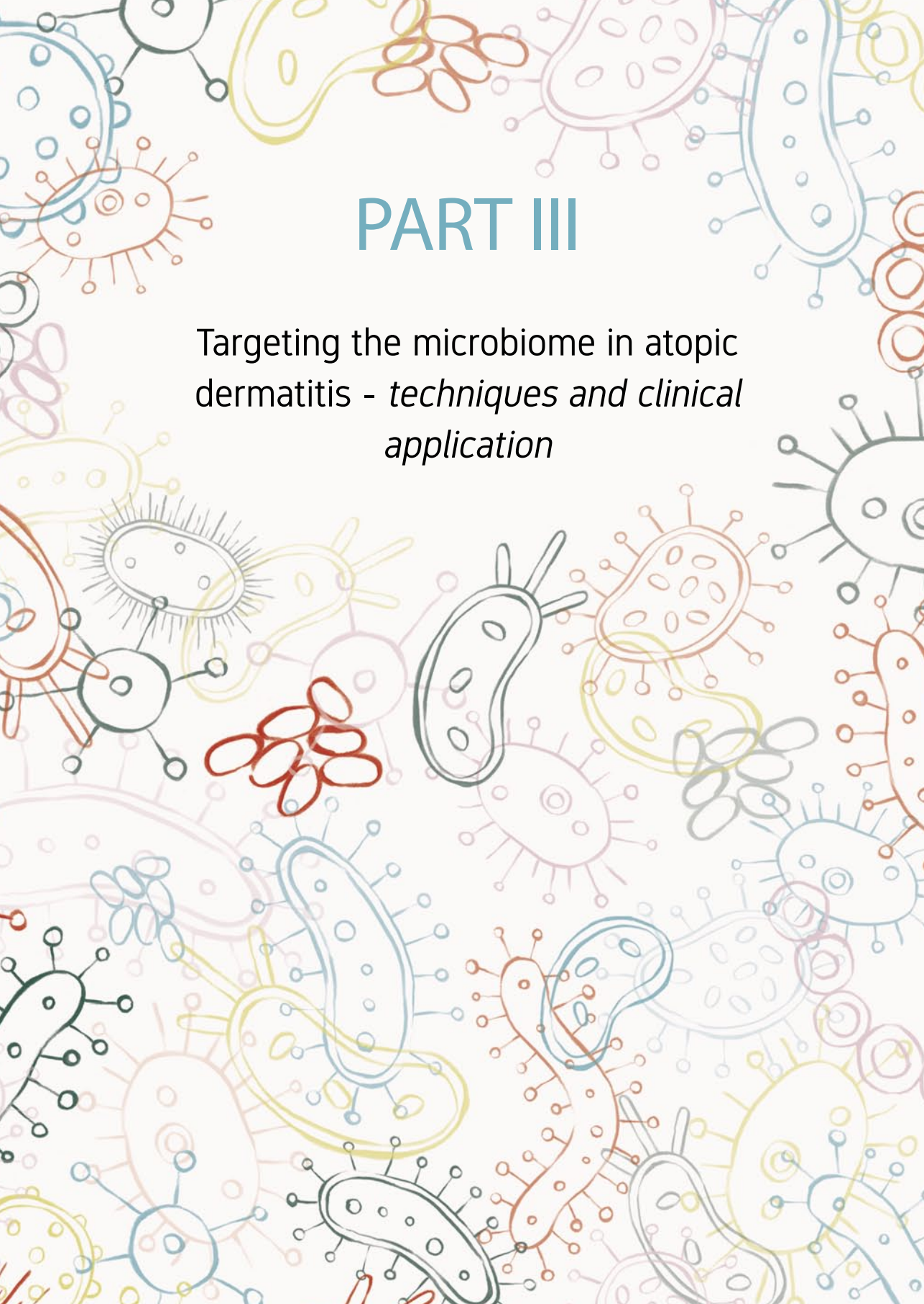
Table S2d. Results linear regression analysis DAVOS study – association with TARC ***(continued)***

Antigen	*Regression coefficient (SE)*	*95% CI*	*P-value*
IsdH	0.358 (0.329)	-0.240-1.065	0.279
EfB	-0.074 (0.384)	-0.843-0.692	0.858
IsdA	0.416 (0.271)	-0.008-1.031	0.223
SasG	0.215 (0.239)	-0.225-0.751	0.383
IsaA	0.074 (0.284)	-0.487-0.654	0.769
LytM	-0.164 (0.385)	-0.863-0.611	0.683
Eap	-0.229 (0.307)	-0.800-0.412	0.464
EapH1	0.625 (0.336)	-0.027-1.287	0.078
EapH2	0.079 (0.221)	-0.343-0.498	0.741

* = significant P-value, Bootstrapping iter 1000 for all antigens

PART III

Targeting the microbiome in atopic dermatitis - *techniques and clinical application*



Chapter 7

Skin microbiota sampling in atopic dermatitis: to swab or scrub?

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ABSTRACT

Background

Collecting high quality samples for skin microbial analysis is challenging due to the low number of microorganisms on the skin. Studies comparing different available techniques for skin sampling are scarce.

Objective

This study compares the detection of total bacterial DNA, *Staphylococcus (S.) aureus* DNA, microbial diversity and fungi by two different methods, namely dry flocked swabbing and scrubbing.

Methods

As part of an ongoing study in atopic dermatitis, we collected 39 swab and 39 scrub samples from 16 patients. *S. aureus* specific and total bacterial DNA were measured with quantitative (q)PCR. To identify bacteria and fungi we sequenced the 16S rRNA gene and the fungal internal transcribed spacer region 1.

Results

The qPCR showed a higher absolute amount of total bacterial DNA in the scrubs ($p<0.001$). Sequencing of 16S rRNA identified 323 and 318 different genera in the swabs scrubs, respectively. The majority was identified equally well with the two techniques and biodiversity was not significantly different. Interestingly, we found fungal DNA more often in the scrubs than in the swabs (36% versus 9%).

Conclusion

Scrubs result in a higher collection of bacterial and especially fungal DNA. Therefore, they are preferable for studying low-biomass skin areas or fungi.

INTRODUCTION

Changes in the cutaneous microbial ecosystem are associated with skin diseases. For example, atopic dermatitis (AD) is characterized by overgrowth of *Staphylococcus (S.) aureus*.[1] Advances in sequencing technologies and metagenomics have enabled more precise studies of the microbial composition of the skin, including the interactions between microbes.[2] Together with these advances, questions arose about the optimal approaches to study the skin microbiota.[3] Recent studies focused on primer selection and the quality control of sequencing runs.[3-6] In addition, consistency in the sampled skin site was shown to be important, as bacterial communities appear to be body-site specific.[7] However, collecting a valid sample of the skin still remains a challenge due to the low number of microorganisms on skin compared to other body sites.[8] There are different techniques to profile the skin microbiota, with skin punch biopsy, swabs, tape-stripping and scrubs being the most widely used. Punch biopsy gives a complete microbial profile of all the skin layers, but its use is limited by the invasiveness.[9] Superficial swabs are practical and the smooth surfaced cotton swabs (premoistened) are often used for skin sampling.[7] However, flocked (brush like) swabs could increase the detection of bacteria compared to smooth swabs. [10,11] The scrub was pioneered for culture-based studies and its use in microbiome studies has only been investigated in a single pilot study with three healthy volunteers.[7,12,13] Compared to the swab, this technique might profile microbes at a deeper level of the skin, where bacterial composition and behaviour could be different.[14-16] Each technique has its own specific sampling depth and usability characteristics, leading to possible variation in the type and density of the collected micro-organisms. The choice of sampling method depends on the research question, sampling site, the feasibility of the method and the amount of total DNA required for further analyses. Selecting the right technique is crucial. However, studies that compare sampling methods in relation to different skin microbial outcomes are scarce.

As part of an ongoing interventional study to understand the role of microbes in AD and changes in skin microbial populations after treatment, we compared total bacterial DNA, *S. aureus* DNA, microbial diversity and the presence of fungi between dry nylon-flocked swabs and scrubs.

METHODS

Study design and patients

The aim of the study was to investigate skin microbial collection methods for use in a future interventional study in patients with AD.[17,18] Patients were recruited between June 2014 and January 2015 in the Erasmus Medical Center and the Havenziekenhuis,

Rotterdam. Inclusion criteria were diagnosis of AD, age above 18 years and the ability to read the relevant patient information and provide informed consent. Criteria for exclusion were the use of systemic antibiotics, corticosteroids or immunosuppressive medication 6 months before the study and the use of topical antibiotics or steroids 7 days before the study. AD was diagnosed according to the UK Working Party's diagnostic criteria.[19] Severity of AD was scored by the patient using the self-administrated eczema area and severity index (SA-EASI).[20] Additional information about medication use and the presence of atopic diseases was retrieved via medical history. Swab- and scrub samples were collected from skin lesions in patients that met the criteria for inclusion (visit 1). A second, third and fourth swab- and scrub sample were taken one, two and three weeks after visit 1 (visit 2, 3, and 4), only in those patients that were positive for *S. aureus* at visit 1 (figure S1). The medical ethical committee of the Erasmus Medical Center approved the study and written informed consent was obtained from all participants.

Skin microbiome samples – swab and scrub

Microbial skin samples were collected from a skin lesion, preferably located at the antecubital fold. In case of absence of antecubital lesions, the most predominant skin lesion was chosen. Two types of samples were obtained, a swab and a scrub sample. These two samples were collected simultaneously from non-overlapping areas of the same skin lesion. First, a sterile dry nylon-flocked swab (Copan480CE) was rubbed along the antecubital crease for 30 seconds and put back in the tube containing liquid Amies Medium. The liquid medium was additionally extracted into an eppendorf. Second, a modified scrub method was used, based on the method of Williamson and Kligman.[12,21] A sterile PVC sampling ring with an internal diameter of 4 cm was placed just below the antecubital crease and 0.75 mL of sterile wash fluid (0,85% NaCl, 0.1% bacteriological peptone, 0.1% Tween 80) was added. After scrubbing the surface of the skin within the ring with a sterile swab for 1 min, the fluid was collected in a 2 ml eppendorf. Samples were stored at -80^{o}C until further processing for DNA based analysis. All samples were collected by a medical doctor wearing sterile gloves.

The patients were informed to avoid bathing or showering in the 12 hours before sampling and could not use personal antibacterial hygiene products from 48 hours before sampling.

DNA isolation and qPCR

For cutaneous DNA isolation approximately 150 uL material was directly transferred to a DNA isolation plate and 0.5 mL phenol pH8.0 (Phenol solution, catalogue P4557, Sigma-Aldrich, St Louis, MO) was added. The samples were mechanically disrupted by bead beating twice for 3 minutes with a 96-well plate Beadbeater (Biospec Products, Bartlesville). Following bead beating, samples were centrifuged at 1880 rcf (4000rpm)

for 10 minutes to separate the aqueous and phenolic phases. The aqueous phase was transferred to a new 96-well plate and DNA was purified with the AGOWA mag Mini DNA Isolation Kit (AGOWA, LGC genomics, Berlin, Germany) in accordance with the manufacturer's recommendations. After elution, the total bacterial load in each sample was assessed by quantitative (q)PCR using an universal bacterial primer-probe set.[22] The total load of *S. aureus* DNA was assessed by qPCR using the following primers : 16S-S. aur-F1 (5'-GCG AAG AAC CTT ACC AAA TCT TG-3') and 16S-S.aur-R1 (5'-TGC ACC ACC TGT CAC TTT GTC-3'), and the 16S-S.aur MGB Taqman® probe (5'-CAT CCT TTG ACA ACT CT-3') with a FAM label.

16S rRNA sequencing

Analysis of the microbiome composition was performed by massively parallel sequencing of the archaeal and bacterial V4 hypervariable region of the 16s rRNA gene on an Illumina MiSeq sequencer (Illumina, San Diego, CA). To prevent over-amplification, the barcoded DNA fragments spanning the V4 hypervariable region were amplified from a standardized level of template DNA (1 ng). Amplicons generated using adapter primers F515 and 806R (using 30 PCR cycles), were bidirectionally sequenced as described previously.[24] Pre-processing, analysis of unique sequences and classifications were performed using modules implemented in the Mothur software platform.[25] Standardized mock communities were included to check for technical performance of all experimental steps. Sequences were grouped with the OTU clustering module in MOTHUR with a 0% cut-off leading to separate clusters for each unique sequence. To assign taxonomic information to the different unique sequences, they were blasted in the Ribosomal Database Platform (RDP).[26] Negative control samples of the lysis buffer contained substantial amounts of *Schlegelella*, and *Enhydrobacter*, both described as contaminant genera before.[23] Therefore, these genera were excluded for further analysis. Shannon diversity index was determined to describe the microbial diversity for the swabs and the scrub samples.

Sequencing of Fungal ITS amplicons

Barcoded amplicons of fungal internal transcribed spacer regions (ITS) were generated using a two step PCR approach. Fungal ITS-1 regions were first amplified with the following primers: nex-ITS-BITS-F: TCGTCGGCAGCGTCACCTGCGGARGGATCA and nex-ITS-B58S3-R GTCTCGTGGGCTCGGGAGATCCRTTGYTRAAAGTT (adapted from Bokulich & Mills[27]). Each reaction contained 5ul undiluted stock DNA, 1 unit Phusion Hot start II (F-549L) enzyme, 1x High Fidelity buffer, 200nM deoxynucleotide triphosphates (Thermo Scientific) and nuclease free PCR grade water to a 25 µl final reaction volume. PCR reactions consisted of an initial denaturation step of 98 °C, for 5 min and 30 amplification cycles (98 °C for 10 sec, annealing 48 °C for 30 sec and elongation 72 °C for 30 sec and

final extension step (72 °C for 5 min) followed by cool down (10 min at 4 °C). A negative control (blank) was included for each set of 24 PCR reactions. Reactions were cleaned by solid-phase reversible immobilization (SPRI) using AMPure XP SPRI beads (Beckman Coulter, Inc.). Dual barcodes (8 bp) and Illumina Sequencing adapters were attached using the Nextera XT Index Kit (Illumina, San Diego, CA) according to the manufacturer's protocols. Barcoded amplicons were quantified using the Caliper LabChip GX II system (Perkin Elmer, Hopkinton, USA), normalized to the same concentrations, pooled, and gel purified using the Qiaquick spin kit (Qiagen) and AMPure XP SPRI beads. Pooled amplicons were 250-bp paired-end sequenced using the MiSeq (Illumina). Raw Illumina fastq files were demultiplexed, quality filtered, and analyzed using modules implemented in the Mothur software platform.[25] Unique sequences were taxonomically classified by the RDP-II Naïve Bayesian Classifier [26] using a 60% confidence threshold against the Mothur formated UNITE Database (Version No. 7).[25] The mock samples confirmed good technical performance.

Statistical analysis

Patient and sample characteristics are presented with median and interquartile range (IQR) for continuous data and counts and percentages for categorical data. Total bacterial DNA, *S. aureus* specific DNA and Shannon diversity were compared between baseline swabs and baseline scrubs, using a non-parametric Wilcoxon signed rank test for related samples. A linear mixed effects model was used to account for the repeated measurements (different time points) for each patient.[28] This mixed effects model determined if there was a significant difference between scrubs and swabs regarding uptake of *S. aureus* specific DNA, total bacterial DNA and Shannon diversity, adjusted for the use of antibiotics (during the study), use of antibacterial products (before/during the study) and the time variable. *P*-values were corrected for multiple testing (3 tests) and therefore a *P*-value of ≤0.0167 was considered significant. To display the microbial composition per sample, as measured using 16S rRNA, the different genera were expressed using the mean relative abundance per sample. SPSS version 21 was used for descriptive data analysis. Microsoft Excel 2010 was used to generate graphs and figures.

RESULTS

Patient characteristics

For this study, 21 patients with AD were screened for participation (visit 1). Of these, 17 patients were included for analysis as four patients did not meet the diagnostic criteria for AD. Ten of these 17 patients were positive for *S. aureus* at the screening. To mimic the longitudinal design of the intervention study and to take into account the

microbial variation over time, a follow-up including three measurements was performed in these ten patients (visits 2 to 4; one week between each visit; figure S1). The mean self-reported eczema severity at baseline, measured with the SA-EASI [20], was 23.2 (IQR 17.48; scale 0-96). Patient characteristics are shown in table 1.

Table 1. Patient characteristics (n=17)

Male : female	6 : 11
Age	
Median (IQR)	38 (26)
Atopy (n (%))	
Asthma	9 (52.9)
Rhinitis	13 (76.5)
Food allergy	9 (52.9)
Age of onset (n (%))	
Early childhood	14 (82.4)
Adult	3 (17.6)
SA-EASI baseline (range 0-96)†	
Median (IQR)	23.2 (17.48)
Use of medication at start of the study (n(%))	
Emollient	17 (100)
Topical calcineurin inhibitor	2 (11.8)
Topical steroids	14 (82.4)
Topical OTC antibacterial (shower) product	1 (5.9)
Use of medication during the study (other than emollient) (n(%))	
Topical calcineurin inhibitor	2 (11.8)
Topical steroids	5 (29.4)
Topical OTC antibacterial (shower) product	10 (58.9)
Systemic antibiotics	1 (5.9)

† n=15 due to missing data, OTC = over the counter

Sample characteristics

The screening of 17 patients and follow-up of ten patients yielded a total of 47 swab- and 42 scrub samples. For an optimal comparison between swab and scrub samples, we included only swab-scrub pairs that were taken at the same skin site at the same time. Thirty-nine swab-scrub pairs could be included, of which 37 were taken from the antecubital fold, one from the knee and one from the upper arm. Of the 39 paired swab-scrub samples, 38 and 35 (obtained from 16 patients) could be included for qPCR and sequencing analysis, respectively. One swab and four scrub samples did not contain sufficient bacterial DNA to generate a PCR amplicon for qPCR or sequencing respectively (figure S1).

Total bacterial DNA and *S. aureus* DNA – swab vs. scrub (qPCR)

Comparison of the scrubs and the swabs that were collected at baseline in 13 patients, showed that the absolute amount of both total bacterial DNA and *S. aureus* DNA was higher in the scrub samples, indicating a better resolution/sensitivity of the scrub measurement. However, when the variation at different time points and use of antiobiotics/antibacterial products (during the study) was taking into account (Methods section; 38 samples), only the total amount of bacterial DNA found with qPCR was significantly higher in the scrubs (log1.23fg/ul; p<0.001; table 2a-b).

Table 2a. Baseline scrubs versus baseline swabs

	Swab (median (IQR))	**Scrub** (median (IQR))	**P- value**
total bacterial DNA (fg/ul)	14888 (6355-26192)	144828 (79881-589027)	0.001*
***S. aureus* DNA** (fg/ul)	83 (0-453)	547 (107-9518)	0.008*
Shannon-diversity	2.93 (2.37-4.29)	2.31 (1.50-3.82)	0.019

The analysis includes 13 baseline samples from 13 individuals. All p values are calculated using Wilcoxon signed rank test for related samples. * significant P-value (<0.0167)
NOTE: The table presents absolute amounts. The relative amount of S. aureus compared to the complete microbiome cannot be calculated due to primer specific differences between the specific S. aureus primer and the universal primer for total bacterial DNA.

Table 2b. Mixed effects model scrub versus swab

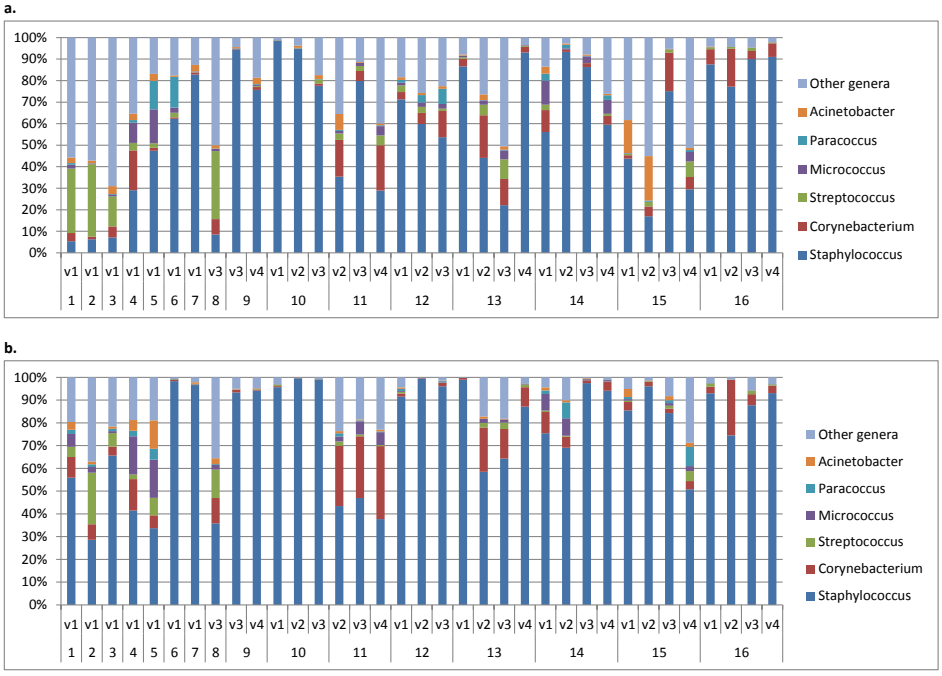
	Coefficient (95% CI)	**P- value**
Log total bacterial DNA (fg/ul)	1.23 (0.58-1.88)	< 0.001*
Log *S. aureus* DNA (fg/ul)	0.94 (-0.50-2.38)	0.195
Shannon-diversity	0.73 (-1.88-0.43)	0.210

The analysis includes 38 samples from 16 individuals. * significant P-value (<0.0167)

Microbial diversity – swab vs. scrub (16S rRNA sequencing)

In the 35 swabs that could be analysed with 16S rRNA sequencing, a total of 441044 reads could be assigned to 321 genera. These belonged to 20 different phyla of which Firmicutes, Proteobacteria, Actinobacteria and Bacteriodetes were most abundant. The five most predominant genera found at baseline (visit 1), available from 13 patients, were *Staphylococcus (60%)*, *Corynebacterium (6%), Streptococcus (5%), Micrococcus (2%)* and *Paracoccus(2%).* In the 35 scrub samples, 533004 high quality reads belonging to 316 different unique sequences and 15 different phyla were obtained. *Staphylococcus (76%), Corynebacterium (7%), Micrococcus (2%), Streptococcus (2%)* and *Acinetobacter (1%)* were the five most predominant genera. Figure 1 shows the mean relative abundances of the 5 predominant genera per sample per technique. Of the 356 detected genera, 281 were identified by both sampling methods (78.9%). These represented over 99% of the total reads, showing that dominant genera are detected equally well by the

Figure 1. Relative abundance of 5 most abundant genera in (a) swabs and in (b) scrubs



NOTE: Numbers 1 to 16 refer to the 16 different patients. v1-v4 refer to the 4 different visits, all with one week in between. Individual nr. 13 started oral AB during the study.

swab and the scrub method (figure 2). The Shannon diversity index indicated that bacterial diversity did not significantly differ between the swab and the scrub samples (table 2a-b; p=0.210).

Fungi

Fungal DNA was found in four of the 47 collected swabs (8.5%). These four swabs belonged to four different individuals. In the scrub samples, fungal DNA was present in 15 of the 42 samples (35.7%), belonging to 8 individuals. More different unique sequences were identified in the scrubs (285) than in the swabs (130). The fungal generum that was most abundant in both the swabs and scrubs was *Malassezia* (table S1).

Figure 2. Venn diagram illustrating the observed overlap of (a) the identified reads (sequences) and (b) the identified genera, using 36 swabs and scrubs

DISCUSSION

This study compares the use of dry flocked swabs and scrubs to measure the skin microbiota in patients with AD, adjusting for a time variable and the use of antibacterial products (during the study) in a mixed model. A higher absolute amount of both bacterial and fungal DNA was recovered from the scrub samples, compared to the swabs. The potential of the scrub method to increase bacterial discovery rates was described earlier based on culture methods.[29] Secondly, dry swabs and scrubs were found to detect the dominant genera equally well at a given time (figure 2). This confirms that dry swabs can be used for microbiome analysis of the skin. Finding the same bacterial species using a swab and a scrub method, suggests that the deeper level (reached by the scrub) harbours no different bacteria from the superficial layer. It also raises the question whether the scrub actually reaches much deeper than the swab. What technique should be used for microbiome experiments depends on the type of study.

The increased resolution of the scrub makes it the preferred method, especially for characterizing fungi, rare microorganisms and for low biomass areas of the skin. However, dry flocked swabs are more user-friendly, can be applied on all skin sites and identify the dominant sequences of the microbiome equally as well as scrubs. Therefore,

the swabs can be considered for large scale studies and for body sites that are difficult to sample with the scrub.

The four most abundant genera that we found on the skin with 16S rRNA sequencing were *Staphylococcus* (Firmicutes), *Corynebacteria* (Actinobacteria), *Streptococcus* (Firmicutes) and *Micrococcus* (Actinobacteria), representing common bacterial phyla of the normal skin microbiota.[30] A clear dominance of staphylococci was found, as expected in patients with AD.[31] We found a low abundance of fungi, with fungal DNA in only 36% of the scrub samples and in less than 10% of the swab samples. This indicates the importance of using adequate collection methods when sampling the skin for fungi. The skin fungal microbiota in healthy controls mainly consists of the genus *Malassezia*.[32] Patients with AD are thought to have a higher abundance and diversity of *Malassezia* species compared to controls, suggesting a contribution of fungal species to AD.[33] In our AD patients, *Malassezia* was the most abundant genus on the skin. However, we were not able to draw any conclusions about the relationships between fungi on the skin and AD.

Limitations of this study include the use of the 515F/806R primer for amplification of the V4 variable region, a standard primer at the time we performed our analysis. The 806R primer is now known for its poor coverage of Propionibacteria. Recent studies were published that recommend amplification of the V1-V3 region for the skin or a the use of a modified V4 primer.[4,5] In our samples Propionibacterium were low abundant. However, this could also be inherent to eczema lesions where staphylococci overgrow. We do not expect that the choice of sequence region influences our conclusions about the swab-scrub comparison. Our study included negative controls (lysis buffer), but contamination from other sources e.g. the air or the swab solution (prepared sterile) cannot be excluded. We used a cohort of AD patients in this study and the results could be specific for this group. The use of antibacterial products during the study could have influenced the bacterial composition on the skin. However, this effect is expected to be equally present in the swabs as in the scrubs. Finally, the scrub method itself has some practical limitations as the collection of scrub samples is restricted to areas of the body where the cup can be placed. Despite these limitations, this study provides insight into the specific characteristics of different skin microbiota collection methods which is important for designing future skin microbiome studies.

In conclusion, scrubs result in increased yields of bacterial and especially fungal DNA. This increases the resolution of the measurement and makes the scrub preferable when working with fungi or low- biomass skin areas. However, dry flocked swabs identify the dominant genera of the bacterial microbiota equally as well as scrubs. Therefore, swabs can be considered for large population studies that examine higher-biomass areas of the skin.

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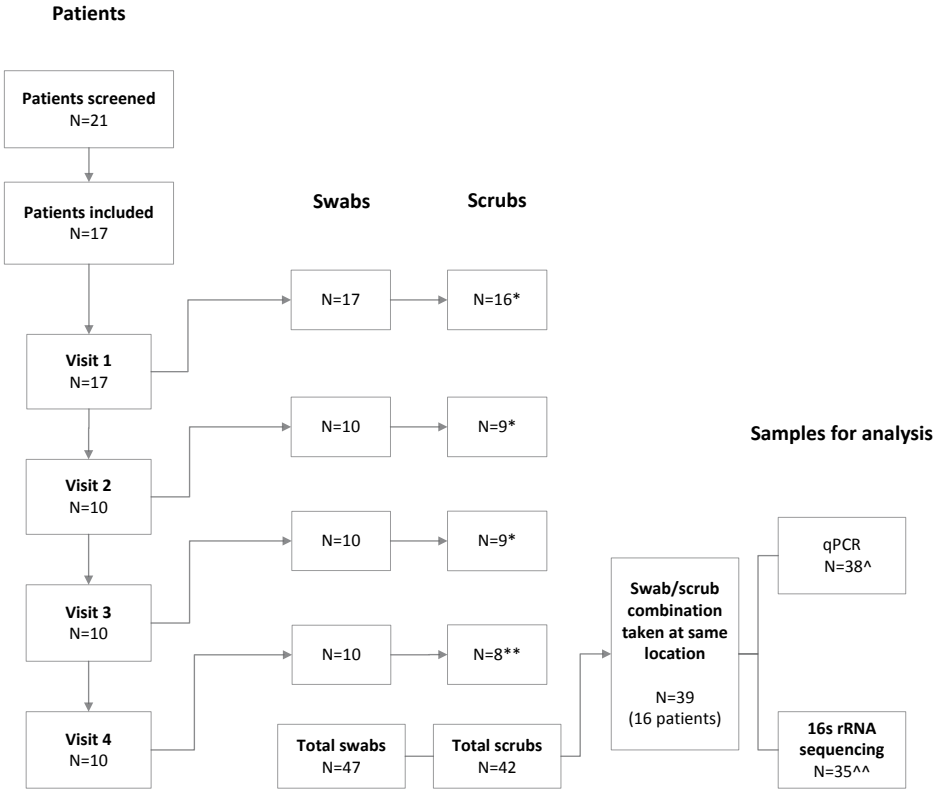
SUPPLEMENTARY MATERIAL

Figure S1. Flowchart of the included patients and collected samples



* 1 missing (eczema on the volar hand making scrub not feasible)
** 2 missings (eczema on the volar hand + logistic problem)

^ 1 missing samples (failure to collect DNA)
^^ 4 missing samples (bacterial DNA yield to low to generate a PCR amplicon)

Table S1. Presence of fungal genera in the swab and the scrub samples

	Swab **Number of sequences per sample (n=4)** Mean (range)	**Scrub** **Number of sequences per sample (n=15)** Mean (range)
Total fungal DNA	31173 (22780-40086)	37663 (20511-85031)
Malassezia	7554 (2172-13555)	13620 (112-43939)
Unclassified	6263 (982-14500)	1365 (0-4519)
unclassified_Malasseziales	5477 (3655-7118)	3623 (118-8994)
Candida	4036 (0-10823)	626 (0-4056)
Unclassified	2830 (1308-3490)	2566 (150-8160)
Cladosporium	821 (43-1825)	1289 (0-4099)
Cryptococcus	317 (0-919)	664 (0-3954)
Sporobolomyces	127 (31-267)	5305 (0-74381)

Chapter 8

Targeted antistaphylococcal 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).

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Trials. 2017 Aug 31;18(1):404

ABSTRACT

Background

Atopic dermatitis (AD) is associated with a reduced skin microbial diversity and overgrowth of *Staphylococcus (S.) aureus*. However, the importance of *S. aureus* colonization in the complex pathogenesis remains unclear and studies on the effect of antistaphylococcal therapy in non-infected AD show contradictory results. Long-term anti-*S. aureus* interventions might be needed to restore the microbial balance, but carry the risk of bacterial resistance induction. Staphefekt, an engineered bacteriophage endolysin, specifically kills *S. aureus* leaving other skin commensals unharmed. Bacterial resistance towards endolysins has not been reported, nor is it expected, which allows us to study its effect as long-term antistaphylococcal treatment in non-infected AD.

Methods

This is a multi-center, placebo controlled, double blinded and randomized superiority trial with a parallel group design. A total of 100 participants, aged 18 years or older, diagnosed with moderate to severe AD and using a topical corticosteroid in the weeks before enrolment are included in the study. The study is executed in the Erasmus MC University Medical Centre Rotterdam in collaboration with the Havenziekenhuis Rotterdam. After a 2-week run-in period to standardize the corticosteroid use with triamcinolone acetonide 0.1% cream, participants will be randomized to either treatment with Staphefekt in a cetomacrogol-based cream or a placebo for 12 weeks, followed by an 8-week follow-up period. The primary objective is to assess the difference in the need for corticosteroid co-therapy between the Staphefekt and the placebo group, measuring the number of days per week of corticosteroid cream (triamcinolone) use. Secondary outcomes include the difference in use of corticosteroid cream measured in grams, differences in clinical efficacy, quality of life (QoL), microbial composition (including *S. aureus*) between the Staphefekt and the placebo group, and the safety and tolerability.

Discussion

The results of this trial will provide data about the effect of long-term antistaphylococcal therapy with Staphefekt on corticosteroid use, clinical symptoms and QoL in patients with moderate to severe AD. Additional data about growth characteristics of the skin microbiome, including *S. aureus*, will give insight in the role of the microbiome as a factor in the pathophysiology of AD.

Trial registration ClinicalTrials.gov, NCT02840955. Registered on 11 July 2016.

BACKGROUND

Atopic dermatitis (AD) is a chronic inflammatory skin disease that is associated with reduced quality of life (QoL), primarily due to an itchy skin.[1-3] The disease is characterised by reduced skin microbial diversity and overgrowth of *Staphylococcus (S.) aureus,* a bacterium that can aggravate skin inflammation via the production of staphylococcal enterotoxins that stimulate the release of pro-inflammatory cytokines.[4-7] However, the importance of *S. aureus* colonization in the complex pathogenesis, compared to the other involved genetic and immunologic factors involved, remains unclear.

Current treatment approaches for AD include topical treatment with emollients and anti-inflammatory therapy with (topical) immunosuppressive agents (corticosteroids and calcineurin inhibitors), according to the international guidelines.[8,9] Antistaphylococcal therapy is only recommended in cases of fever or clinically infected skin.[8,9] Clinical studies that evaluated the added value of antistaphylococcal therapy in non-infected AD, have shown contradictory results. Bath Hextall *et al.* performed a systematic review of 26 studies and showed that antistaphylococcal agents reduced the amount of *S. aureus* on the skin in AD. However, the bacteriological reduction did not translate into a decrease in clinical symptoms.[10] These studies mainly investigated short-term therapies of less than one month duration and comprised small and poor-quality studies. As discontinuation of therapy after a short treatment period can result in quick regrowth of *S. aureus*[11], the results of this systematic review do not necessarily mean that antistaphylococcal agents do not work. A more recent review of Brüssow et al. summarizes two intervention trials that reported significant improvement of disease severity in non-infected AD after two and three months of therapy with antistaphylococcal therapy (bleach baths).[12-14] We hypothesize that long-term therapy may be needed to reduce to *S. aureus* overgrowth and maintain a stable and balanced skin microbial composition. Ultimately, this could result in disease improvement, prevention of AD flares and less need for (topical) immune suppression. However, long-term use of antibiotics can induce bacterial resistance,[15] and both the use of antibiotics and dilute bleach baths can cause unnecessary harm to the commensal flora, that is hypothesized to have antistaphylococcal properties.[16]

In the context of the increasing incidence of bacterial resistance, the interest in bacteriophages and their endolysins as antibacterial therapy has been renewed.[17] Staphefekt SA.100 is an engineered chimeric endolysin that specifically lyses the cell membrane of *S. aureus* via endopeptidase and putative amidase activities.[18-20] Long-term application of Staphefekt on the skin, targeting only *S. aureus* and leaving skin commensals unharmed, may improve long-term AD outcomes, such as the number of disease flares, and may reduce the use of topical corticosteroids. Bacterial resistance to Staphefekt or other endolysins has not been observed and could not be induced, which enables us

to study the effect of long-term antistaphylococcal treatment in non-infected AD using this endolysin-based agent.[19,21,22]

The aim of this randomized controlled trial, the MAAS trial, is to evaluate the effect of a 3-month antistaphylococcal therapy with Staphefekt on the frequency and quantity of topical corticosteroid use, clinical symptoms and QoL in patients with moderate to severe AD. In addition, data on the growth characteristics of the skin microbiome, including *S. aureus*, will be collected, which will gain insight in the role of the microbiome as a factor in the pathophysiology of AD.

METHODS/ DESIGN

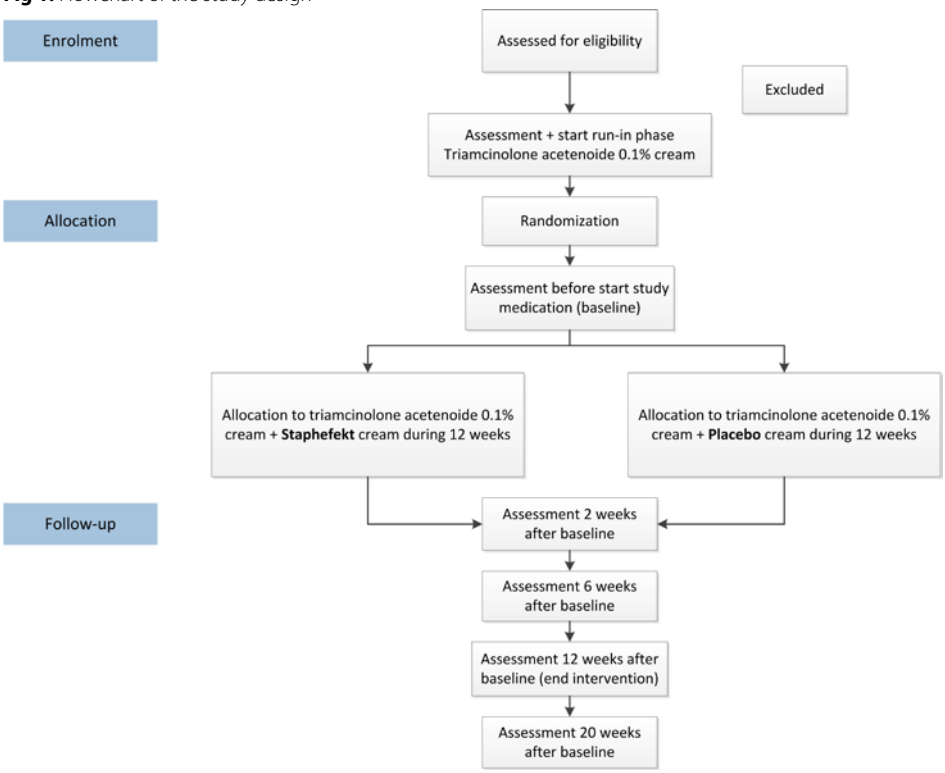
Design and setting

The MAAS trial (Microbiome in atopic dermatitis during antistaphylococcal therapy and the effect on steroid use), is a multi-centre, randomized, double-blinded, placebo-controlled superiority trial with a parallel group design (figure 1). The study aims to evaluate the effect of Staphefekt on the use of corticosteroids, disease severity, QoL and composition of the microbiome in patients with AD**.** The study was designed by the Department of Dermatology of the Erasmus MC University Medical Centre Rotterdam and will be executed in collaboration with the Havenziekenhuis Rotterdam. Enrolment and follow-up visits take place at these two locations. Participants who comply with the criteria for in- and exclusion will start with a 2-week run-in period to standardize the corticosteroid use with triamcinolone acetonide 0.1% cream. After completion of the run-in phase, participants will be randomized to either treatment with Staphefekt or a placebo for 12 weeks, followed by an 8-week follow-up period. An Eczema Area Severity Index (EASI) over 50 after the run-in phase is a contraindication for further participation. During the course of the study, participants visit the outpatient clinic six times (visit 1 through 6) and data will be collected on corticosteroid use, disease severity, QoL, skin microbiome and adverse events. See table 2 for the SPIRIT diagram of the trial procedures.

Ethical considerations

This study follows the Dutch Medical Research Involving Human Subjects Act 1998 (WMO) and the Helsinki Declaration principles 2008. All study procedures have been reviewed and approved by the Medical Ethics Committee of the Erasmus MC University Medical Center Rotterdam, the Netherlands (reference 2016-233). Protocol amendments will be submitted for review at the Medical Ethics Committee.

Fig 1. Flowchart of the study design



Participants

This study will enrol adults (18 years or older) diagnosed with AD according to the UK working party diagnostic criteria for AD.[23-25] Participants are eligible for enrolment if they have a score between 7.1 and 50.0 on the EASI for disease severity. Topical corticosteroids must have been prescribed before enrolment. All patients must be able to read and understand the patient information and provide written informed consent. Patients are not eligible for enrolment if they used: (1) systemic antibiotics or corticosteroid in the two months prior to enrolment, (2) oral immunosuppressive agents or UV therapy in the three months before enrolment or (3) local antibiotics or Staphefekt (from commercial sources) one week before enrolment. Other criteria for exclusion are a known contact allergy to any of the components of the study drug (e.g. propylene glycol), clinically infected AD or the existence of other skin condition(s) that could interfere with the assessment of the AD severity.

Recruitment, inclusion and consent

Participants with AD will be recruited from the dermatology outpatient clinic of the Erasmus MC and the Havenziekenhuis Rotterdam. Furthermore, Dutch dermatologists are informed about the study via the Dutch Trial Network and via scientific conferences. Patients with AD are informed via the patient support group and via online media, such as DermHome (www.huidhuis.nl). In addition, recruiting advertisements will be placed on student forums and in local newspapers. Patients who are interested in participation in the trial can contact the researcher directly via email or phone. After a first screening with regard to the inclusion and exclusion criteria via email or phone, potentially eligible participants receive an information letter and will be invited to the dermatology outpatient clinic to further assess eligibility. Patients who fulfil the inclusion criteria and are willing to participate, will be included in the study after providing written informed consent.

Sample size

The sample size for this study was calculated based on the primary outcome, namely the difference in mean days per week corticosteroid use over 12 weeks between the Staphefekt arm and the placebo arm, in patients who are positive for *S. aureus* on the skin at baseline. This is the first study measuring clinical outcomes of Staphefekt in patients with AD. We expect to find a mean topical corticosteroid use of 5 days/week in the placebo group. This was based on a study of Hon *et al.* that showed decreased use of topical corticosteroids when taking bleach baths, an antistaphylococcal therapy.[26] Based on the results of this study, we anticipate an effect size of 1.25 day/week reduction of topical corticosteroid use in the Staphefekt arm. A sample size was calculated using an unpaired *t*-test to compare means in a superiority trial design. With a power of 0.80, alpha of 0.05 and SD of 2.0, 40 patients are needed per treatment arm. Assuming 10% drop out and 90% of the patients being positive for *S. aureus* on the skin lesions, 50 patients will be assigned to each of the two treatment arms.

Randomization and blinding

The participants are randomly assigned, in a 1:1 fashion to either treatment with Staphefekt or placebo. Stratified block randomization for AD severity is performed to ensure equal distribution of patients with moderate and severe AD over the treatment arms (EASI 7.1-21 and EASI 21.1-50). Randomization is done by an independent biostatistician of the Erasmus MC, using the statistical software package R version 3.2.2. The participants, the researchers and laboratory analyst are blinded for the intervention. The pharmacy manages the randomisation list and provides blinded study medication.

Table 1. Corticosteroid dosing regimen

	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9
Saturday	X X	X	X	X					
Sunday	X X	X			X	X	X	X	
Monday	X X	X	X	X					
Tuesday	X X	X			X	X			
Wednesday	X X	X	X	X					
Thursday	X X	X			X	X	X	X	
Friday	X X	X	X	X					

Start in week 1 or 2 depending on severity of the AD. If the symptoms allow, reduce the use of corticosteroid cream weekly according to the scheme. Return to week 1 or 2 in case of an exacerbation. Based on patient' assessment.

Intervention

After enrolment, all participants start a run-in phase of two weeks in which they receive a standardized dosing regimen of topically applied triamcinolone acetonide 0.1% cream (table 1). After the run-in period, the patient and the researcher evaluate further participation, with very severe AD (EASI>50) as a contraindication for continuation. The run-in period and randomization is followed by a 12-week treatment period and an 8-week follow-up period. During the treatment period, Staphefekt or placebo cream will be applied on the total skin surface twice daily to reach optimal reduction of *S. aureus,* as both lesional and non-lesional skin are often colonized.[4] The Staphefekt endolysin is made available in a cetomacrogol-based cream. The placebo is composed of the same cetomacrogol-based cream, without Staphefekt. During the treatment and follow-up period triamcinolone will be used according to the corticosteroid dosing regimen (table 1). Measurements and assessments will be performed at enrolment (start of the run-in phase, visit 1), baseline (start treatment with Staphefekt/placebo, visit 2a), 0.5 hours after baseline and 2, 6, 12 and 20 weeks after baseline (visit 2b to 6). Table 2 provides an overview of the measurements per visit. Unless it is in the best interest of the patients (for example in case of an eczema flare), patients are not allowed to use systemic or topical immunosuppressive medication (including calcineurin inhibitors), antibiotics or antiseptics during the study. Escape medication will be prescribed according to current treatment guidelines and its use will be registered. At start of the study, patients receive an emollient according to patient's preference for use during the course of the study. The use of this emollient will be registered by weighing the tubes at each visit.

Detailed sample and laboratory procedures

Sampling procedures are based on the 'Manual of Procedures' for microbiome sampling of the Human Microbiome Project.[27] All samples are obtained by one of the researchers

wearing gloves (sterile for the skin scrub). Sterile Copan 490CE.A swabs are used to sample the skin, nasal cavity and pharynx. Skin samples are taken from lesional skin, preferably located at the antecubital folds or the popliteal fold. The skin surface is swabbed during 30 seconds. The mucosal surfaces of both the anterior nares are gently rubbed going round the area during 10 seconds. The rear of the oropharynx is swabbed for 5 seconds, using a tongue depressor. For the skin scrub sample, a ring with an internal diameter of 4cm will be placed on the same skin lesion where the swab was collected, but on a non-overlapping area. 1 ml of swab solution (0,85% NaCl, 0.1% bacteriological peptone, 0.1% Tween 80) is pipetted in the ring. After rubbing over the skin with a Copan 480CE swab during 1 minute, the swab-solution will be pipetted out the ring into an Eppendorf tube. The swabs will be sent to the laboratory at the day of collection using mail. A semi-quantitative culture technique and MALDI-TOFF for identification of *S. aureus* will be performed. The scrub samples will be stored at -80 degrees Celsius at the Erasmus MC Rotterdam until 16S rRNA-sequencing and quantitative *S. aureus* analysis.

Primary and secondary outcomes

The primary outcome of this study is the days per week of corticosteroid use, compared between the Staphefekt and the placebo group over 12 weeks. Patients report their triamcinolone use daily in a secured digital platform, 'DermHome'.* Additionally, the use of triamcinolone cream will be measured in grams by weighing the study medication at time of issue and return (each visit). Secondary outcomes include clinical efficacy and QoL from baseline through week 12 and week 20, change of the microbial composition (including *S. aureus)* and safety. Clinical efficacy is measured using the EASI, the Investigators Global Assessment (IGA) and registration of the number of flares.[28] A flare is defined as an exacerbation that requires the need to intensify treatment, from a doctor or patient's perspective. This implies stronger topical therapy or the need for systemic treatment. A 50% increase in the EASI score compared to baseline is used as an indication to intensify treatment. The Pruritus Numerical Rating Scale (Pruritus NRS) and the Patient Orientated Eczema Measure (POEM) are included as patient-reported efficacy outcomes.[29,30] QoL is measured using the Skindex-29.[31,32] Changes in the microbiome are evaluated by comparing the changes in bacterial composition between the treatment groups, determined by 16S rRNA sequencing of the skin scrub samples. Reduction of *S. aureus* is determined by quantitative PCR (and culture for the comparison between visit 2a and visit 2b). Safety and tolerability is assessed by monitoring the incidence of (serious) adverse device events through the end of the study, evaluated by medical check-ups that include evaluation of vital signs. Reportable adverse events will be reported within the set timelines to the competent authorities. Table 2 gives a detailed overview of the measurements per visit.

Table 2. SPIRIT diagram of study procedures

		Study period						
		Run-in phase	Baseline/Allocation		Intervention			Follow-up
Timepoint/visit		**Visit 1**	**Visit 2a**	**Visit 2b**	**Visit 3**	**Visit 4**	**Visit 5**	**Visit 6**
ENROLMENT								
Eligibility screen		X						
Informed consent		X						
Baseline questionnaire		X						
Allocation			X					
INTERVENTION								
ASSESSMENTS								
Efficacy	Questionnaire triamcinolone use (primary outcome)							
	Weight triamcinolone tube at issue and return	X	X		X	X	X	X
	EASI[28]	X	X		X	X	X	X
	IGA		X		X	X	X	X
	Pruritis NRS[29]							
	POEM[30]		X		X	X	X	X
Quality of life	Skindex-29[31,32]		X		X		X	X
Microbiome	Swab skin	X	X	X	X		X	X
	Scrub skin		X		X		X	X
	Swab nose	X	X				X	X
	Swab throat	X	X				X	X
Safety	Medical check-up	X	X		X	X	X	X
Other	Photograph (overview + close up sampled lesion)		X		X	X	X	X
	Questionnaire use of emollients and escape medication		X		X	X	X	X

EASI, eczema area and severity index; IGA, investigators global assessment; Pruritus NRS, pruritus numerical rating scale; POEM, patient orientated eczema measure. Visit 1, enrolment in the trial and start of a two weeks run-in phase; Visit 2a, start of the intervention (baseline); Visit 2b, 0.5 hours after baseline; Visit 3, 2 weeks after baseline; Visit 4, 6 weeks after baseline; Visit 5, 12 weeks after baseline and end of the intervention; Visit 6, follow-up visit 20 weeks after baseline. All visits take place plus or minus two days from the indicated timeframe.

* 'DermHome' is a secured digital treatment and research platform, developed in collaboration with Patient 1 BV, Almere.[33] The platform provides an user-friendly individual account that allows patients to report their pruritus score and triamcinolone use daily. Thereby the platform provides digital information about the study, including the use of

the study medication, and an option to contact the researcher and to upload photos in case of questions. After every visit the researcher can make notes in the digital file about findings, agreements and future appointments.

Data collection, monitoring and data analysis

Data collected during the visits are entered in Open Clinica. This data management system allows direct data entry. Data entry is monitored by an independent researcher according to a predefined monitoring plan. Triamcinolone use and itch scores filled in daily by the patients in 'Dermhome' will be extracted in an SPSS format and combined with the Open Clinica database. Patients confidentiality will be ensured by using identification numbers. Data will be analysed on an intention-to-treat basis. A mixed linear regression model will be used to examine if there is a significant difference in corticosteroid use over 12 weeks between the intervention and the placebo group.[34] This model accounts for repeated measurements for each patient and is valid in the case of missing data. Covariates that could influence the outcome variable will be included in the model. Subgroup analysis will be performed to analyse patients that are positive for *S. aureus* on the skin versus patients that are negative for *S. aureus* before start of the intervention. Positive patients are defined as having positive cultures both at visit 1 and 2a. Negative patients must have two negative cultures. Patients that have one positive and one negative culture will not be included in the subgroup analysis. Secondary outcomes will also be analysed using a mixed model analysis (linear or logistic according to the type of data). The findings of this study will be published in national and international journals (according CONSORT 2010 Statement) and will be communicated to the relevant patient associations.

DISCUSSION

The MAAS trial is a randomised, placebo-controlled trial that investigates the effect of a 3-month antistaphylococcal therapy with Staphefekt on topical corticosteroid use, clinical symptoms and QoL in adults with moderate to severe AD. Additionally, data will be collected about the growth characteristics of the skin microbiome, including *S. aureus*. Taking in consideration the current literature on antistaphylococcal therapy, a study design using a long-term antistaphylococcal intervention, measuring long term outcomes was chosen.

Evidence for the clinical efficacy of Staphefekt, registered as a class 1 medical device in Europe, is based on in vitro studies and a case series.[18-20] These *in vitro* studies showed that Staphefekt kills different strains of *S. aureus* (also methicillin-resistant strains), without harming the commensal flora or inducing bacterial resistance.[19] A case series

describes clinical improvement of *S. aureus* related symptoms, such as folliculitis and superinfected dermatitis, and no development of resistance during long-term daily treatment with Staphefekt based on the minimal inhibitory concentrations of the cultured *S. aureus* strains over time.[35] The lack of resistance induction can be expected, as bacterial killing by an endolysin is independent of the involvement of the bacterial metabolism. The co-evolution of bacteriophages and their host bacteria over millions of years, ensures that phage endolysins attack essential bonds in the bacterial cell wall that cannot be adapted by the host.[22] Thereby, the lytic activity of exogenously applied endolysins results in lysis of the target cells within seconds, restricting the possibility to adapt and develop resistance. Furthermore, attacking several bonds of the bacterial wall simultaneously by the use of more than one enzymatically active domain in the Staphefekt molecule, makes resistance development even less likely to occur.[18]

Because of the proteinaceous nature of endolysins, immunogenicity can be of concern. The literature shows the possibility of the formation of non-neutralizing antibodies against lysins other than Staphefekt.[22] In a study in which the presence of anti-Staphefekt IgG was evaluated in serum from 21 Staphefekt-naive healthy human donors, pre-existent IgG antibodies recognizing Staphefekt epitopes were detected in all the donors (unpublished data). This can be explained, as humans are exposed daily to *S. aureus* and therefore to bacteriophages and their lysins. However, Staphefekt is a large size protein molecule (>50kDa), making penetration through the skin and mucosa and subsequent antibody reactions unlikely.[36]

Calculation of the sample size for this study was hampered as no information was available about the effect of Staphefekt on corticosteroid use and clinical efficacy in AD. Therefore, the study should be considered as hypothesis generating, giving insight into effect sizes and distributions of clinical outcomes. Our expected effect size was based on a study of Hon *et al*. that studies the effect of bleach on corticosteroid use in AD. We chose a slightly higher effect size, because we expect the effect of Staphefekt that specifically targets *S. aureus* to be more efficacious than bleach. We consider this effect size, a reduction in corticosteroid use of more than one day a week over 12 weeks, as clinically relevant because of the (low) risk of side effects and a general reluctance of patients to use corticosteroids, resulting in poor compliance and a lack of treatment efficacy.[37,38]

No consensus has been reached yet on a standardized outcome for long-term AD control, the primary goal of our study. The Harmonising Outcome Measures for Eczema (HOME) initiative reached consensus on the use of EASI and POEM as doctor-based and patient-based measures of AD severity, both of which are included as secondary outcomes in this trial.[39] According to the authors of HOME, measures of long-term control could include time to flare and the use of rescue medicine.[39] Next to corticosteroid use, both these study outcomes were included in this study as secondary parameters.

In conclusion, this study will evaluate the effects of a 3-month targeted antistaphylococcal therapy with Staphefekt in moderate to severe AD. The lack of resistance induction allows long-term treatment with this antistaphylococcal agent. This study will provide the first data on the use of antistaphylococcal therapy with Staphefekt in AD and may provide new insights in the role of *S. aureus* in the pathophysiology of AD.

TRIAL STATUS

The first patient was included in the study in July 2016. Patient recruitment is currently ongoing and the inclusion is expected to be completed by August 2017.

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Chapter 9

General discussion

MAIN FINDINGS OF THIS THESIS

The aim of this thesis was to characterize the microbial composition of the skin, nose and gut in patients with mild to severe atopic dermatitis (AD) We focused on *S. aureus* and the humoral immune response towards this bacterium, and we designed a clinical study to test the effect of a new endolysin-based therapy that specifically targets *S. aureus* in AD. In the following paragraphs we discuss the main findings of this thesis.

The skin and nasal microbiome are associated with AD severity

In Chapter 2 of this thesis we found an association between the microbial composition of both the nose and skin and AD severity (R^2 = 2.6%; p=0.017 and R^2 =7%; p=0.004). The skin microbiome has been associated with AD severity before in children.[1] However, as far as we know we were the first to evaluate both the skin and nasal microbiome of children in a multivariate model, adjusting for covariates including age, use of antibiotics and the location of sample collection on the skin. We found that staphylococci highly contribute to the association with AD severity in both the nose and skin. Although we could not differentiate between the different staphylococcal species, our results are in line with studies that showed higher density of *S. aureus* in more severe AD (Chapter 4).[1,2] In an additional analysis to characterize *S. aureus,* we found that children with severe AD were positive for *S. aureus* on lesional skin more often than children with mild AD (58% vs 39%). However, this difference was not significant. In the nose, next to staphylococci, also *Moraxella* were positively associated with AD severity. Although the presence of this species in the nose has not been associated with AD severity before, different studies have found that *Moraxella* is associated with asthma and asthma development.[3,4] We retrieved information on the diagnosis of asthma or bronchial hyperactivity (28% of the children) from patient records, and the diagnosis did not influence our results when adjusting for it. For some species, a decreased abundance contributed to the association between the microbiome and AD severity in our study, for example *Dolosigranulum* in the nose and *Streptococcus* on the skin. High abundance of *Dolosigranulum* was suggested to be beneficial for respiratory health in a study of Biesbroek *et al*.[5] *Streptococcus* was observed before in lower relative abundance in skin lesions compared to nonlesional skin in young patients.[6] Although the cross-sectional design of our study precludes evaluating cause-event relationships, our results indicate that both the nasal and skin microbiome might play a role in the severity of inflammation in pediatric AD. Staphylococcal species seem important drivers for the association between both skin and nasal microbiome and AD severity. Prospective and controlled cohort studies are needed to validate our results and determine which species contribute to (or protect for) AD and its severity.

The skin and nose in AD harbor distinct microbial communities, but correlations exist between the two niches.

In Chapter 2 we found that microbial communities in the skin and nose are significantly different from each other (R^2=26.8, p<0.001). This is in line with studies in healthy subjects showing that each body site is characterized by its own microbial community harboring dominant signature taxa.[7] Nevertheless, some species such as staphylococci, were present in both niches in our patients. However, where most skin samples were dominated by staphyloccoci, only a few nasal samples showed dominance of staphylococcal species. Looking at abundance patterns of species in the nose compared with (other species) on the skin, we found that many species in the nose and skin showed similar patterns of species abundance. Staphylococci on the skin formed an exception as they were negatively correlated with many other species in the nose and skin. The exact meaning of these relations should be further explored as different biological and/or immunological mechanisms might underlie our observation. A possible underlying mechanism might be cross-transmission of bacteria between the nose and skin.[8-10] Prospective large cohort studies should further evaluate the presence of cross-transmission between the nasal and skin microbiome, as it might be of relevance for determining treatment strategies for AD. Furthermore, it might help to determine if a diagnosis of AD influences the persistence of nasal carriage of *S. aureus,* a risk factor for infections with the bacterium.[11-13]

The gut microbiome differs between children with AD, with and without a food allergy

Severe AD is associated with food allergy.[14] Although several studies have shown associations between the intestinal microbiome and development of atopic diseases, the link between intestinal microbiota and food allergy has rarely been studied.[15-17] In Chapter 3 of this thesis we described the gut microbiome in a group of 82 children with mild to severe AD. Of these children, 20 were diagnosed with food allergy mainly for peanut and cow's milk. We aimed to identify gut microbial characteristics associated with food allergy, using the gold standard for diagnosing food allergy (double blind placebo controlled food challenge), which has rarely been done before. Six bacterial species from the gut microbiome were identified that when combined discriminate between children with and without food allergy: *Bifidobacterium breve, Bifidobacterium pseudocatenulatum, Bifidobacterium adolescentis, Escherichia coli, Faecalibacterium prausnitzii* and *Akkermansia muciniphila (*AUC 0.83, sensitivity 0.77, specificity 0.80). Our pilot results are based on a small cross-sectional study and should be confirmed in future prospective studies and further adjusted for confounders, such as diet and AD severity. The exact mechanisms through which the intestinal microbiome influences food allergy are not elucidated yet and it is not clear whether a change in microbiome precedes

or follows the development of food allergy. A possible mechanism is disruption of the gut microbiome that alters the gut epithelial integrity, thereby increasing the risk of allergic sensitization through direct uptake of allergens, a hypothesis that is mainly based on animal studies.[17] Another possible mechanism could be the immune stimulatory capacity of gut microbes (via secretion of molecules).[18-20] In our study, the children with a food allergy were also found to have higher thymus and activation-regulated chemokine (TARC) levels, a serum biomarker for AD severity, suggesting increased AD severity compared to the non-allergic group.[21,22] This raises the possibility that the six bacterial species also correlate with AD severity. Furthermore, we cannot exclude that elimination diets in the food allergic group have led to changes in the gut microbial composition. However, one third of the children in the non-food allergic group also reported an elimination diet for a specific food, which makes it unlikely that our findings are solely attributed to differences in diets. Our results need to be confirmed in larger studies that enable stratification on specific food allergies and adjustment for AD severity and dietary factors.

Patients with AD have an increased risk of colonization with *S. aureus*

In Chapter 4 we quantified the prevalence of *S. aureus* in AD patients compared to controls and concluded that patients with AD are significantly more likely to carry *S. aureus* than healthy controls on both the lesional and nonlesional skin. *S. aureus* was identified on lesional skin in 70% of the patients, with a higher prevalence of *S. aureus* in patients with severe AD and in patients under 18 years old. We showed that heterogeneity among the included studies was substantial, ranging from 63% to 88% for the pooled outcomes, which can be partly explained by the quality of the studies and disease severity of the included patients. We also detected publication bias causing an overestimation of the pooled outcomes. Although different studies calculated prevalence rates of *S. aureus* in AD, with this meta-analysis we were the first to systematically summarize the data of the different studies. Our meta-analysis indicates the importance of *S. aureus* in AD, not only in skin lesions but also in nonlesional skin and the nose. A positive association between AD and the presence of *S. aureus* both on the skin and nose suggest a possible role of the bacterium in aggravation of AD inflammation and encourages further research into exact mechanisms.

S. aureus evokes an IgE based immune response in a subgroup of patients with AD

In Chapter 5 of this thesis we described the first systematic review that summarizes data on antibody prevalence against *S. aureus* in AD. In a pooled analysis we found that IgE against SEA and SEB (two staphylococcal superantigens) is present significantly more often in patients with AD compared to healthy controls. Pooled analysis of IgE

against TSST-1 included only two studies and showed the same trend (not statistically significant). Pooled prevalence estimates of antistaphylococcal IgE patients were 33% for SEA, 35% for SEB, 14% for SEC, 5% for SED and 16% for TSST-1. Data on other antigens were insufficient for a pooled analysis. The observed high heterogeneity in the pooled analysis could be partly explained by the variety in IgE detection methods used. We did not find a difference in IgE levels between children and adults. Other variables such as AD severity and treatment are also likely to contribute to the observed heterogeneity, but could not be explored as this information was not available for the included studies. The increased IgE in patients compared to controls indicates that *S. aureus* might stimulate AD inflammation via IgE mediated mechanisms, such as mast-cell degranulation, suggesting a role of *S. aureus* as an allergen. It is unclear whether the increased IgE is the result of increased skin barrier permeability in AD, predominance of SEB and SEA carrying strains in AD skin and/or an inappropriate immune response towards the bacterium. It is probably a combination of these factors. Notably, only a subgroup of patients show elevated IgE, suggesting that only a part of the AD patients reacts in an IgE dependent matter towards *S. aureus*. On the other hand, only 14% and 24% of the *S. aureus* isolates carry the genes to express SEA and SEB, so a group of AD patients might not have encountered the allergen yet.[23] Further research is needed to clarify the clinical relevance of IgE responses against *S. aureus* in patients. Our results also indicate a lack of studies that evaluate immune responses other than the IgE mediated response against staphylococcal superantigens.

Children with AD develop an IgG dependent immune response against *S. aureus* and the bacterium might use immune-modulatory antigens to persist on the skin in AD

As outlined in Chapter 5, most research has focused on IgE humoral responses towards a limited panel of *S. aureus* antigens. As IgG is known for its involvement in the neutralization and elimination of microbes, we profiled IgG antibodies against 55 *S. aureus* antigens in two cohorts of children with AD (Chapter 6). Our results showed that the children are exposed to a wide range of antigens and develop an IgG mediated humoral immune response towards them. In one of the cohorts, the IgG response against antigens with mainly immune-modulatory functions, (e.g. Leukotoxin (Luk) D and E) was associated with the severity of the AD. The exact pathophysiological mechanism that explains this finding is unclear, although it can be argued that children with more severe AD might have an altered immune response against staphylococcal antigens. The association could also be a reflection of the higher *S. aureus* load on the skin of children with more severe AD, that IgG tries to counteract. However, in the latter case one would expect increased IgG against all *S. aureus* antigens rather than a subset. On the other hand, *S. aureus* might express more immune-modulatory antigens, which may lead to a more se-

vere AD phenotype. By down regulating the immune system locally, *S. aureus* can more easily maintain its colonization on the skin, which can cause chronic inflammation in AD, characterized by Th1 cells in the skin. *In vitro* studies showed that activation of dentritic cells by *S. aureus* lipoteichoic acid and the Th2 cytokine IL-4 (combined) can result in enhanced Th1 and Th17 priming.[24,25] In acute AD inflammation, where IL-4 is present, additional presence of *S. aureus* on the skin can cause a state of persistent and chronic AD. The exact role of *S. aureus* specific IgG in the inflammatory response in AD should be further investigated. A study of Meulenbroek *et al*. found that the presence of IgG affects the binding of food allergen-IgG complexes to B-cells. This mechanism was also seen for birch pollen, suggesting a role for IgG also in the allergic response.[26] Determination of IgG subtypes combined with IgE can help clarify the role of IgG in AD, as different IgG subtypes might have different characteristics relating to other AD phenotypes. For example in AD, tolerance to cow's milk was associated with increased IgG4 in combination with low specific IgE.[27] A control group of children is needed to investigate the normal range of IgG antibodies in children without AD. Nevertheless, the results of our study shed light onto the IgG mediated immune response to *S. aureus* in children with AD and highlight the relevance of other antigens (adhesins and immune modulators) next to the often studied superantigens. Further studies, including IgG subtype responses, need to be conducted to validate our results and will help us understand how microbes interact with the immune system and possibly induce inflammation.

Techniques for skin microbiome research: scrub results in higher collection of bacterial and fungal DNA compared to swab

As mentioned in the introduction of this thesis, investigating the skin microbiome imposes some challenges. For example, the low biomass of microbial DNA on the skin requires adequate sampling that produces sufficient yield for reliable analysis. In Chapter 7 we compared dry nylon-flocked eSwabs versus a scrub method to collect skin microbial samples. These eSwabs are thought to enhance bacterial absorption and release compared to traditional cotton or rayon swabs, which could be attributed to the flocked structure of the swab.[28] The scrub sample was collected by placing a sterile sampling ring on the skin and adding sterile wash fluid. After scrubbing the skin within the ring with a sterile swab, the fluid was collected and analyzed. [29,30] We showed with quantitative qPCR that scrubs result in significantly higher amounts of total bacterial DNA compared to the swab. However, with 16S rRNA sequencing we showed that both methods identify the major genera equally well. The potential of the scrub method to increase bacterial discovery rates was described earlier, but based on culture methods.[31] Fungal DNA was found more often in scrub samples compared to swabs (36% versus 9%). Increased yields of bacterial and fungal DNA make scrubs the preferred method, especially for characterizing fungi, rare microorganisms and for low biomass areas of

the skin. However, dry eSwabs are more user-friendly and can be applied on all skin sites. Also, eSwabs identify the dominant sequences of the microbiome equally well as scrubs, at least in the sites sampled in our study (mainly the antecubital folds). Therefore, the swabs can be considered for large scale studies and for body sites that are difficult to sample with the scrub. A comparison of the eSwab and the scrub with premoistened cotton swabs, a method that is commonly used now for microbiome analysis, might further help identify the preferred method for microbial analysis.

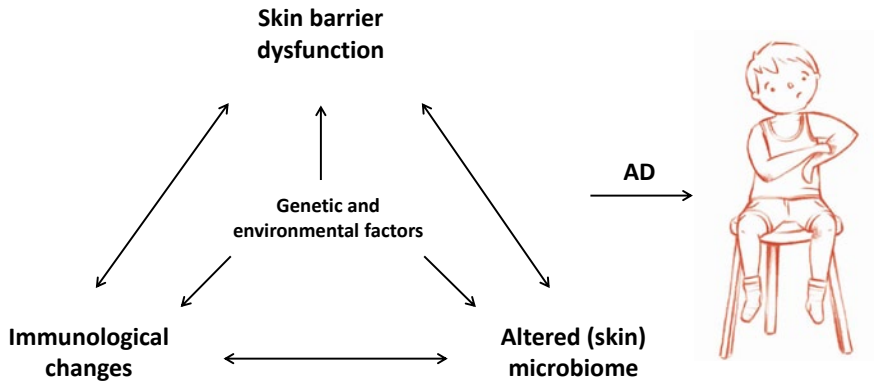
PLACE OF THE MICROBIOME IN THE ATOPIC DERMATITIS DISEASE MODEL

A recent review on atopic dermatitis describes two main pathophysiological landmarks in AD, namely (1) abnormalities of the skin barrier and (2) changes in the immune response, with a close interaction between skin barrier biology and immune mechanisms. *S. aureus* is shortly mentioned as a possible contributor to AD exacerbations and chronic inflammation.[32] We propose a model for AD pathophysiology that includes a third main component, namely alterations in the microbiome (figure 1). This model incorporates several findings of this thesis that include an association between microbial composition and AD severity (Chapter 2 and 4) and the interaction of *S. aureus* with the immune system (Chapter 5 and 6).

The model in figure 1 shows three main components: the skin barrier, the immune system and the (skin) microbiome. Together they contribute to a balanced ecosystem which is important for a healthy skin, influenced by genes and environment (including environmental microbes). Bidirectional interactions between the components contribute to the mechanical and immunological barrier function of the skin. A healthy microbiome strengthens the mechanical skin barrier as commensals occupy space that could otherwise be colonized by pathogens. Microbes such as *S. epidermidis* support the local immune system by producing antimicrobial peptides that suppress *S. aureus*.[33,34] Inflammation in AD is probably the result of a disbalance in this skin ecosystem, resulting in an altered barrier, immunological changes and an altered skin microbiome (figure 1). The alterations in all three components together contribute to AD etiology. The initial trigger that causes the disbalanced ecosystem can affect each of the components, which might result in a vicious circle where the components constantly aggravate each other, leading to a situation of chronic inflammation. It is likely that the contribution of each of the three components to the inflammation differs per patient and even within patients over time.

The model in figure 1 includes microbiome alterations as a basis for AD pathogenesis, and questions the relevance of the debate on whether alterations in the microbiome are cause or consequence of AD. It is likely that microbial dysbiosis and *S. aureus* overgrowth

Figure 1. Disease model of AD including the (skin) microbiome. *(Illustration by Marloes van Loon)*



are not the primary events that cause (flares in) AD. A pre-existent barrier defect and immunological changes are probably a prerequisite for *S. aureus* to overgrow. The skin barrier defect in AD provides a suitable environment for *S. aureus* to grow and allows contact with immune cells in the skin. Also, presence of Th2 cells is needed for the production of IgE against *S. aureus* antigens.

Once present on the skin, *S. aureus* can induce inflammation via different mechanisms, namely excretion of virulence factors and the activation of both IgG and IgE mediated immune pathways. Via these mechanisms *S. aureus* has the ability to further aggravate inflammation or induce flares, even if the microbial dysbiosis and overgrowth is primarily caused by other factors, such as a skin barrier defect. Theoretically, this makes the microbiome a possible target for treatment of AD (flares). Although we know that it is theoretically possible, evidence that proves that *S. aureus* is actually aggravating the inflammation during a flare is still scarce. Large prospective cohort studies are needed that further clarify the role of the microbiome in AD, including *S. aureus,* with sampling specifically around disease flares. The above mentioned 'cause or consequence' discussion is also outlined in a letter published by our group in the British Journal of Dermatology (Totté *et al*. 2017).[35]

CLINICAL IMPLICATIONS OF THE RESULTS PRESENTED IN THIS THESIS

The skin microbiome as a therapeutic target in AD

Currently, there is no place for antimicrobial therapy in the treatment of AD without signs of infection (fever, high staphylococcal load or clinically infected AD (impetiginization of the lesions)).[36] Based on our model for AD pathogenesis (figure 1), we hypothesize that a

combined treatment strategy targeting all three components, including the alterations in the microbiome, could optimize the treatment of AD. To determine the right strategy to target the microbiome, we need to know which alterations in the microbiome are associated with AD and AD flares. The established association between *S. aureus* colonization and AD, makes *S. aureus* the first target of interest (Chapter 4). In Chapter 2 of this thesis we identified other microorganisms that were associated with AD severity. Their role as potential treatment targets, next to *S. aureus,* in AD should be further explored. The added value of antistaphylococcal therapy in AD without symptoms of infection could not be confirmed before in a published review of clinical studies.[37] However, the studies that were assessed in this review included approaches that have been tested on their ability to treat an exacerbation, in trials with relatively short follow-up periods, not showing whether the treatment can provide long-term control of the disease activity. We hypothesize that long-term modulation of the microbiome may be needed to maintain a stable and balanced skin microbial composition. This can be argued as *S. aureus,* combined with a Th2 cell acute inflammatory environment, can contribute to a state of persistent AD.[24] Antistaphylococcal treatment could temporarily reduce the 'pressure' of *S. aureus* and relieve symptoms, but after stopping the treatment the bacterium is likely to regrow quickly. Especially, in the presence of an underlying genetic barrier effect in part of the AD patients, which might facilitate colonization with *S. aureus*.[38] Studies did describe before that mutations in the gene encoding filaggrin, an important protein for skin barrier homeostasis, were associated with the microbial composition in nonlesional AD skin.[10] Also gram-positive anaerobe cocci were found underrepresented in the microbiome filaggrin-deficient human skin, which was speculated to favour growth of *S. aureus*.[39]

Results of two more recent intervention trials that reported significant improvement of disease severity in non-infected AD after two and three months of therapy with antistaphylococcal therapy (bleach baths) support the hypothesis that long-term treatment is necessary for better disease control.[16,40-42] We conclude that long-term studies are needed to determine if treatment that aims to restore microbial alterations might have a place in the treatment of non-infected AD.

Strategies for long term modulation of the skin microbiome

Currently available treatment strategies to reduce *S. aureus*, include antibiotics, bleach baths and Povidon-iodine (Betadine) scrubs. These agents have a broad-spectrum activity, indicating that they also affect beneficial microbes on the skin and other body sites.[43] In addition, long-term treatment with antibiotics is undesirable as it can induce *S. aureus* resistance to antibiotics, causing more severe disease, prolonged hospitalization and increased mortality.[44-46] New treatment strategies that specifically target only the microbe of interest and that allow long-term treatment is therefore urgently needed. New tar-

geted strategies have been developed, including anti-*S. aureus* vaccines (unsuccessful in clinical testing until now) and topical endolysin-based treatment against *S. aureus* that recently became available for clinical use.[38,47] Next to reducing the load of certain target species, alterations in the behavior of pathogens changing from virulent to commensal have been studied. In an *in vitro* study, *S. aureus* was found to change from virulent to commensal when exposed to the commensal *Corynebacterium striatum*.[48] A third option to reduce the overgrowth of *S. aureus* includes topical probiotic strategies that involve application of species that inhibit growth of *S. aureus*, for example *S. epidermidis* and *S. hominis.*[33]

Next to therapy directed to *S. aureus,* treatment with an emollient has also been found to reduce AD symptoms with subsequent reduction of *Staphylococcus* species load.[49] Also, emollient treatment from birth was considered an effective approach for atopic dermatitis prevention in a group of high-risk infants.[50] These results make treatment with an emollient an important first step to optimize the skin microbial composition. The precise mechanisms through which emollients have beneficial effects are still poorly understood and need further exploration, but they are probably related to improved barrier integrity.[32] Furthermore, Czarnowicki *et al.* identified alterations in the expression of antimicrobial peptides with the application of petrolatum, an over-the-counter moisturizer.[51]

Influence of extra-cutaneous microbial niches

In Chapter 4 we found an increased risk for *S. aureus* colonization in the nose of AD patients compared to healthy controls. These results raise the question whether the nose should be included when applying topical therapy to modulate the skin microbiome. Current American treatment guidelines also recommend combined intranasal mupirocin and bleach baths for infections in AD.[52] Studies that evaluate the effect of new topical antimicrobials should incorporate measurements of extra-cutaneous microbial niches. This will help determine whether a certain extra-cutaneous microbial profile influences treatment effects. In general, niches that can be carriage sites are the nares, the oropharynx, the axillae, groin, the perineum, and the vagina.[53]

The microbiome and diagnostics

In current clinical practice, swabs of AD lesions are rarely collected when the lesion does not show signs of clinical infection. In this thesis we discussed that *S. aureus* on the skin and in the nose likely plays a role in aggravating inflammation in non-infected AD as well, particular in severe AD. This raises the question whether collection of skin and nasal swabs to determine *S. aureus* load in non-infected AD should be considered standard practice. Having information about the presence or absence of *S. aureus* can help determining whether targeting the microbiome should be considered as part of the treatment

strategy, next to skin barrier and anti-inflammatory treatment. Chapter 6 of this thesis supports that *S. aureus* might also contribute to development of chronic AD lesions, also previously suggested by Biedermann *et al*.[24] These results should encourage clinicians to also consider *S. aureus* culture in chronic dermatitis where the role of *S. aureus* seems less obvious compared to the more fierce red and oozing acute lesions. Future studies should investigate how *S. aureus* behaves on/in skin with chronic dermatitis compared to skin with acute inflammation.

Although the data are very preliminary, the results of Chapter 3 suggest possibilities to use the microbial composition as a diagnostic tool. Our pilot data indicated that microbial patterns in the gut are associated with food allergy in children with AD and that it is possible to distinguish non-food allergic children from food allergic children using fecal samples. The use of fecal samples would allow for a simple and cheap method to distinguish children without a food allergy from children with a food allergy as a first step in the diagnostic process, as the current gold standard for diagnosis of food allergy, a double blind placebo controlled food challenge, is (time) invasive, costly and can be difficult in young children. As discussed in Chapter 3, this was a pilot study and the findings need to be validated in other (clinical) studies, including adjustment for important confounders.

Design for an intervention study to target atopic dermatitis - anti-*S. aureus* endolysins

When the first targeted anti-*S. aureus* treatment strategy, based on endolysins, became available on the market, we decided to study its effect in a clinical setting and were the first to report on this.[47,54] The endolysin Staphefekt SA.100™ (Staphefekt) is an engineered chimeric endolysin that specifically lyses the cell membrane of *S. aureus* via endopeptidase and putative amidase activities. Incorporated in a cetamacrogol based cream, the endolysin is registered as a medical device class 1 in Europe and available for topical application. In vitro studies tested the activity of Staphefekt in phosphate buffered saline against 28 clinical strains of MSSA, 8 strains of MRSA, and four other staphylococcal strains (*S. epidermidis, S. hominis, S. haemolyticus* and *S. lugdunensis)*.[47] These results indicate that Staphefekt kills different types of *S. aureus* including methicillin resistant strains (mean reduction in OD of 58%), while causing little harm to the four other staphylococci (mean reduction in OD of 4.3%). The effect on other commensal species needs further investigation.

We tested Staphefekt in a study of 3 cases with *S. aureus*-related skin conditions, folliculitis and impetiginized dermatitis, and found that Staphefekt led to a reduction of clinical symptoms (Totté et al. CR in Dermatology 2017).[54] Bacterial resistance to Staphefekt was not found in our case study during 4 months of treatment (assessed in one of the three patients). We hypothesized that targeting *S. aureus* with Staphefekt can

restore the microbial balance, which might relieve AD inflammation or even prevent flares and reduce the use of (topical) steroids. The low risk of resistance should enable us to study the effect of long-term microbiome modulation in non-infected AD. Also, the targeted mechanism of action enables us to study specifically the role of *S. aureus* in AD pathogenesis. In Chapter 8 we described a protocol to carry-out a randomized controlled trial to evaluate the effect of a three months antistaphylococcal therapy with Staphefekt. The clinical trial started in 2016 and data collection just finished at the time of writing this thesis.

In the trial design, we incorporated the knowledge gained from the studies in this thesis. For example, only patients with moderate to severe AD were included, as they have more overgrowth of *S. aureus* and are therefore more likely to benefit from the anti-microbial therapy (Chapter 4). Also, we choose to collect additional scrubs of the skin for optimal collection of microbial biomass and resolution for sequencing (Chapter 7). The decision to test the efficacy of the endolysin directly in patients, has its pros and cons. The AD skin is subject to many factors that influence microbial growth due to interaction with the skin barrier and host immune system and its direct exposure to the environment. This hampers measuring the exact effect of Staphefekt on *S. aureus* growth. An experimental *in vitro* set up, or a model using the human nares, would enable more stable conditions and a better estimation of the direct effect of the endolysin on the bacterium. However, the patient-based setting and pragmatic approach in which we compared the add-on of the endolysin to standard care with standard care alone, gives results that are directly relevant to practice and will help making decision about treatment options. The relevance of microbial outcomes is hard to interpret without being able to relate them to clinical outcomes. For example, we do not know yet whether a complete elimination of *S. aureus* needs to be achieved for clinical improvement, or if decreasing the bacterial load will also be sufficient. Based on our case series, where we found a clinical improvement while *S. aureus* could still be cultured from the skin, we hypothesize that a reduction of *S. aureus* can already initiate rebalancing of the microbial dysbiosis. The extent of that reduction is difficult to determine and probably differs per patient and disease episode, skin barrier status and host-immune factors. We expect that the trial will increase our understanding about how antimicrobial therapies affect the microbiome and how this affects diseases states.

METHODOLOGICAL CONSIDERATIONS

In part 1 and 2 of this thesis we used two pediatric AD patient cohorts to characterize the microbiome, with a focus on staphylococci, and the antibody response against it. To investigate how specific the results are for AD inflammation, healthy control groups and

patients with other chronic inflammatory skin diseases, such as psoriasis, are needed. However, our conclusion focusses on AD severity in well characterized cohorts of children with AD. The lack of controls does not influence this particular comparison. Another limitation might be the cross-sectional design of our studies which does not allow the investigation of cause–effect relationships between the microbiome and AD severity and between anti-*S. aureus* IgG levels and AD severity. Furthermore, we conducted our cohort studies in academic centers and our results might not be representative for the general AD population.

In our microbiome studies, we determined the bacterial microbiome by sequencing of the 16S rRNA gene. We amplified the V4 variable region of the gene. This region was often used in studies characterizing the gut microbiome. In more recent studies it was shown that sequencing of this region does not allow classification of staphylococci at the species level, which is important when characterizing the skin microbiome. To further specify staphylococci in our studies, we performed additional qPCR on *S. aureus* and S. epidermidis. Because we were not able to identify staphylococcal species or even strains, we might have missed existing correlations between staphylococcal species and AD severity. Additionally, the V4 region in combination with the reverse primer that we used (806R) is known for poor coverage of Propionibacteria. Of note, in our samples Propionibacterium were indeed low in presence in both the skin and nasal samples, while they are described as part of the healthy microbial communities.[55] However, the low Propionibacteria can still be a true reflection of the AD lesions as staphylococci are known to overgrow other species. Thereby, we sampled the antecubital folds which are known for a low abundance of Propionibacteria that prefer more sebaceous environments.[56] Current studies recommend amplification of the V1-V3 region or the use of a modified V4 primer for skin microbiome research. This results in better coverage of Propionibacterium and better classification of S. epidermidis. Classifying S. aureus remains difficult with current 16S rRNA sequencing approaches. [57,58] It should be noted that amplification of long regions (such as V1-3) also imposes some challenges with regard to generating good quality sequences. New recommendations for sequencing will be taken forward for analyzing samples of the clinical trial (Chapter 8).

FUTURE PERSPECTIVES

Aims for future studies

This thesis and previously published literature have shown the complexity of the microbial ecosystem and its interaction with the skin barrier and immune system. It also shows the relevance of the microbiome in AD pathogenesis and encourages further research on the topic as many aspects still need to be elucidated, such as 1) which microbes are

implied in AD pathogenesis, 2) by which mechanisms do they interact with the skin barrier and immune system and cause inflammation and 3) what is the effect of microbial modulation on clinical disease.

In this thesis we confirmed the role of *S. aureus* in AD and we identified other microorganisms that might be involved in AD inflammation. Future studies should confirm the associations found in this thesis and determine associations on the species level. A comparison of microbial communities between patients and healthy controls is crucial to generate hypotheses around possible drivers of AD disease. Explorative research to identify microorganisms of relevance, should not only focus on bacteria but also on other microorganisms including fungi and yeasts. Chapter 3 of this thesis highlights the relevance of looking at the effects of groups of bacteria when performing this kind of studies, instead of single species. Furthermore, the influence of communities on other body sites apart from the skin should be part of the focus. Once species are identified, experimental *in vitro* models and animal challenge models could help to understand by which mechanisms bacteria influence inflammation and interact within the skin barrier and local immune system. Although there is great interest in new omics techniques, culturing of species of interest from humans remains very important to perform these experiments. With respect to *S. aureus,* it would be particularly interesting to study which mechanisms or triggers change the behavior of *S. aureus* into a disease causing pathogen, as we know the bacteria can also colonize the skin and mucosa of healthy individuals.[13] As *in vitro* and animal models lack generalizability to the complex skin ecosystem *in vivo*, additional patient-based studies are needed to further clarify the role of microbial dysbiosis in AD and untangle interactions between the microbiome and the host skin barrier and immune system. The still developing AD and immune-system makes the pediatric population of interest for studying these interactions. After identifying mechanisms that are of possible relevance in AD inflammation, well-designed clinical trials are needed to determine the added value of new treatment strategies.

Longitudinal studies will allow the investigation of cause-effect relationships between AD, the microbiome, and the humoral immune response. Longitudinal population based studies from birth to disease development are needed to clarify the role of the microbiome in AD development. Furthermore, it might be important to sample at multiple time points during a child's development. Some studies suggest that the microbiome might only influence the development of AD in certain 'critical' timeframes during infancy.[59-61] As evidence that proves that *S. aureus* is truly aggravating the inflammation is still scarce, longitudinal sampling around the period of a disease flare is also still needed to identify whether changes in the microbiome and increased *Staphylococcus* levels precede flares. This would support a contribution of the microbiome in the onset of flares.

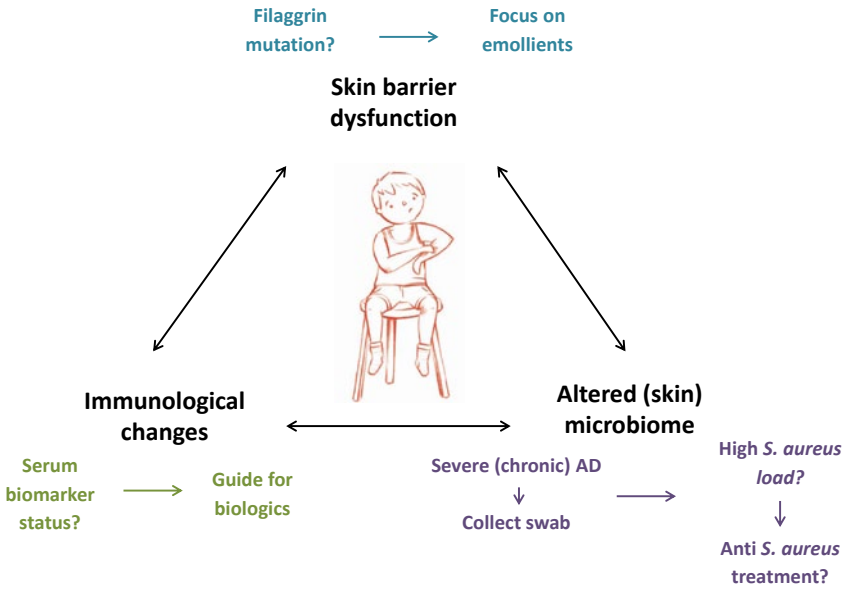
Techniques and standardization

A complex ecosystem requires complex analysis techniques to enable analysis of all species and their interactions with each other and the host. The upcoming omics techniques already incorporate a broader perspective of the microbiome. Eventually, these new techniques that allow functional analysis next to association analysis, will be the key to a better understanding of the relations between microbes and the host immune system, the skin barrier and the other microbes and antimicrobial peptides. Especially in AD research, where it is crucial to specify staphylococci at the species level, metagenomics sequencing has advantages as it provides sufficient resolution to differentiate species and even strains.[62] This has been proven difficult with different 16S rRNA sequencing approaches, but seems important as studies showed that functional differences of staphylococcal strains exist and probably contribute to the complexity of AD.[2,57] However, due to the rapid development of the field, methods are not standardized yet. In general, differences in study population, sample preparation, sequencing methodology and use of bioinformatics tools including reference databases probably cause variation in outcomes. Specifically in skin microbiome research, we have to consider the unique features of the skin. The low biomass (compared to for example the gut and nose), the site specific microenvironments, the distinct local immune system and the high risk of contamination require specific approaches. Standardized methods and bioinformatics protocols are needed to gain more robust results. Transparency and sharing of sequencing and bioinformatics methodology in international databases would help standardize microbiome research and improvement of methods.

Personalized treatment: identifying phenotypes for atopic dermatitis

The diverse symptoms, different ages of onset, varying natural disease courses and comorbidities illustrate the heterogeneity of AD. Due to this heterogeneity, it can be expected that patients do not respond equally well to standardized 'one size fits all' treatment regimens. Also, systemic treatment and upcoming biologics are costly. Therefore, it is important to decide which patient will benefit from which type of treatment. Characterizing patients based on biomarkers of genetic, immunological and microbiological origin (the three components of figure 1), will support clinical decision-making and lead to a more personalized treatment of AD. By measuring specific biomarkers related to these three components, a balanced treatment strategy with more or less focus on certain components can be designed. Likely a mix of multiple biomarkers will be needed to define subpopulations and predict treatment response (figure 2).[63] Some research has been done on characterization of barrier and immunological biomarkers. Skin barrier integrity can be measured via filaggrin mutation status amongst others.[64] In case of a genetic defect, focus should be on treatment with emollients to enhance the barrier function of the skin. A recent study among patients with moderate to severe AD already

Figure 2. possible biomarkers of interest for research on AD treatment personalization. *(Illustration by Marloes van Loon)*



identified distinct clusters of patients based on serum biomarker profiles, illustrating biological differences between patients, which will help guide the use of biologics.[65] With respect to the microbial component, it is also likely that the role of the microbiome in AD pathogenesis differs within subsets of patients. We found that *S. aureus* was not dominating the lesional skin in all children and every child with severe AD had a unique skin microbial composition (Chapter 2). Treatment targeting the microbiome might therefore not be preferred for all AD patients at all times. Patients with severe AD are more likely to benefit from anti- *S. aureus* treatment, while in mild AD a skin (and nasal) swab can be collected to guide decisions.

Next to determining biomarkers to guide AD treatment, it is also important to identify biomarkers in young patients that predict the development towards severe disease. We know that early treatment of high risk patients has been shown to prevent AD.[50] Currently, family history is often used to identify high risk patients.[66] Also the filaggrin mutation status can be used as a biomarker, because a mutation in this gene strongly predicts a worse prognosis with more severe AD and atopic comorbidities.[64,67]

It should be clear that first attempts have been made towards personalizing treatment for AD. However, further characterization of patients and development of biomarkers is needed. Future cohort studies and trials should incorporate an endotyping approach in their design, including assessment of the epidermal, immunological and microbial bio-

markers, such as filaggrin mutation status and *S. aureus* presence, and sufficient power to eventually determine which type of patients will benefit from which therapy.[63]

FINAL CONCLUSION

This thesis shows the relevance of the microbiome, in particular *S. aureus*, in the pathogenesis of AD. *S. aureus* seems to evoke immune responses through different mechanisms, as a directly stimulating antigen and as an allergen. We found that next to *S. aureus,* other microbes on the skin and also microbial communities in the nose might be involved in AD inflammation. Our results may contribute to the development of treatment strategies that target the microbiome in AD. Further prospective cohort studies and experimental research are needed to clarify the role of the microbiome in AD and its role in AD treatment. As the role of the microbiome likely differs between patients, probably as a result of various genetic and environmental factors, further stratification of patients is needed to better guide therapeutic approaches. Eventually this will lead to more personalized treatment of AD.

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Chapter 10

Summaries

SUMMARY

Atopic dermatitis (AD) is a common chronic inflammatory skin disease affecting up to 25% of the children and 7% of adults. The disease pathogenesis of AD is complex and an impaired skin barrier and altered immune mechanisms are considered the two major players in AD inflammation. Also, changes in the skin microbiome are well described in AD. The microbiome is the collection of all the microorganisms living in and on the human body. Different body sites, such as the gut, nose and skin, harbor distinct microbial communities that are essential for maintaining health. For example, the skin microbiome is important for the mechanical and the immunological barrier function of the skin. It is still poorly understood how the altered microbial composition that is seen in patients with AD plays a role in the pathogenesis of the disease. Insight in the role of the microbiome in the pathogenesis of AD may help determine the added value of treatment strategies that target the microbiome in AD. It will also guide the development of new treatment strategies to modulate the microbiome.

Chapter 1 is the general introduction and provides the main objectives of this thesis. We first aimed to characterize the microbial composition of the skin, nose and gut in pediatric patients with mild to severe AD. Our second aim was to estimate the prevalence of *S. aureus* in patients with AD and to study the humoral immune response against *S. aureus*. Additionally, we aimed to design a clinical study to test the effect of a new endolysin-based therapy that specifically targets *S. aureus* in AD.

PART I of this thesis describes the microbiome in patients with AD, including associations with disease severity and food allergy**.** In ***Chapter 2*** we characterized the bacterial microbiota of the skin and nose using 16S rRNA sequencing in a cohort of children with AD. We concluded that both the skin and nasal microbiome are associated with the severity of AD. Results were independent of age, use of medication and location of sampling and suggest that both the skin and nasal microbiome influence the severity of the inflammation in AD. In both the skin and nose an increased abundance of the genus *Staphylococcus* was an important contributor to the association. However, increased or decreased abundance of other species was also found to contribute. We additionally concluded that the nose and skin harbor distinct bacterial communities, but observed that correlations exists between species in the nose and (other) species on the skin. The underlying biological mechanisms of these correlations need to be further explored. In ***Chapter 3*** we described a proof of concept study in which we characterized the gut microbiome in pediatric patients with AD, with and without a food allergy. We found that six bacterial species, when combined, discriminate between children with

and without food allergy. Our results need to be confirmed in larger studies using well-defined patient populations.

PART II describes the role of *S. aureus* in atopic dermatitis and the human immune response towards it. In ***Chapter 4*** we presented the results of a systematic review and meta-analysis and concluded that patients with AD have a higher risk of having *S. aureus* present on the skin (lesional and non-lesional) and in the nose compared to controls. This risk increases with disease severity for lesional skin. Based on the results of this study we encourage further evaluation of the role of *S. aureus* and antistaphylococcal therapy in AD. ***Chapter 5*** outlines a systematic review and meta-analysis that shows that IgE against staphylococcal enterotoxin (SE) A and SEB in the serum is found more often in patients with AD compared to healthy controls. Data on IgG, IgM and IgA as well as other antigens were limited. The increased IgE response against *S. aureus* superantigens in certain patients indicates that *S. aureus* might stimulate AD inflammation via IgE mediated mechanisms, such as mast-cell degranulation, in a subgroup of patients. In ***Chapter 6*** we profiled IgG antibodies against 55 *S. aureus* antigens in sera of two pediatric cohorts with mild to severe AD. We found that children develop an IgG mediated humoral immune response towards the different antigens, showing that the children are exposed to a wide range of antigens. In one of the cohorts, the response against a group of mainly secreted proteins with immune-modulatory functions was associated with AD severity. The exact pathophysiological mechanism that explains our findings has to be further explored. It could be that *S. aureus* uses immune-modulatory mechanisms to maintain its colonization on the skin, which can lead to persistence of AD inflammation.

PART III describes methods to study the effect of a newly developed targeted therapy directed against *S. aureus* in AD. In ***Chapter 7*** we compared a dry flocked swab with a scrub to collect skin microbiome samples as a pilot for a clinical trial. We concluded that scrubs result in a higher collection of bacterial and especially fungal DNA. However, dry flocked swabs identify the dominant sequences of the bacterial microbiota equally well as scrubs. The results suggest that scrubs are preferable when working with fungi or low- biomass skin areas. ***Chapter 8*** describes the study protocol of a randomized controlled trial that studies the effect of local application of a new endolysin-based therapy that specifically targets *S. aureus* on the corticosteroid use and symptoms in AD. Methodological considerations and lessons learned from the former chapters, such as the choice for a scrub sample method, are outlined in the study design.

Finally, in ***Chapter 9***, we provided a general overview of the main findings, discussed the clinical implications of these findings, considered the limitations of our research and suggested directions for future research. This thesis shows the relevance of the

microbiome, in particular *S. aureus*, in the pathogenesis of AD. We proposed a model for AD pathogenesis that includes the (skin) microbiome, next to the skin barrier and immune system, as a main component involved in AD etiology. Together, the components contribute to a balanced ecosystem important for a healthy skin, influenced by genes and environment. A disturbance in one of the components can lead to a disbalance of the ecosystem and eventually chronic inflammation. We found that *S. aureus,* but also other microbes on the skin and microbial communities in the nose, might be involved in AD inflammation. *S. aureus* seems to evoke immune responses via different mechanisms, as a directly stimulating antigen and as an allergen. The results of this thesis may contribute to the development of treatment strategies that target the microbiome in AD. Further prospective cohort studies and experimental research is needed to clarify the role of the microbiome in AD and study how the microbiome may be modulated to improve AD symptoms. As the role of the microbiome likely differs between patients, it is important to define subpopulations of patients, based on epidermal, immunological and microbial biomarkers, to predict treatment response and better guide therapeutic approaches. Eventually this will lead to more personalized treatment in AD.

SAMENVATTING

Constitutioneel eczeem (CE) is de meest voorkomende chronische inflammatoire huidaandoening. Tot 25% van de kinderen en 7% van de volwassenen lijdt aan CE. De pathogenese van de aandoening is complex, maar de belangrijkste factoren die bijdragen aan de inflammatie bij CE zijn een defect van de huidbarrière en een veranderde activiteit van het immuunsysteem. Daarnaast zijn er bij CE ook veranderingen in de samenstelling van het microbioom beschreven, met name van de huid. Het microbioom is de verzameling van alle micro-organismen in en op ons lichaam. Micro-organismen bevinden zich op verschillende plekken in en op ons lichaam, waaronder de huid, de neus en de darmen. In de gezonde situatie vormen de micro-organismen een ecosysteem met hun gastheer en vervullen ze belangrijke functies, zoals bijvoorbeeld het versterken van de mechanische een immunologische barrière van de huid. Het is nog onduidelijk wat de precieze rol is van de veranderingen in het microbioom in de pathogenese van CE. Meer inzicht in de rol van het microbioom van de huid, neus en darmen bij CE helpt bepalen of therapie gericht op het microbioom van toegevoegde waarde kan zijn en is van belang voor het ontwikkelen van nieuwe gerichte behandelstrategieën op dit vlak.

Hoofdstuk 1 is een algemene introductie en beschrijft de belangrijkste doelstellingen van dit proefschrift. We stelden ons ten doel om het microbioom van de huid, neus en darmen in kaart te brengen bij kinderen met mild tot ernstig CE. Daarna bepaalden we de aanwezigheid van *S. aureus* bij patiënten met CE en de humorale immuunrespons tegen *S. aureus*. Een laatste doel was het opzetten van een klinische trial om het effect van een nieuwe behandelstrategie, gebaseerd op endolysinen technologie en specifiek gericht tegen *S. aureus,* te testen bij CE.

Deel I van dit proefschrift beschrijft de samenstelling van het microbioom in patiënten met CE in relatie tot de ernst van de ziekte en het hebben van een voedselallergie. In ***Hoofdstuk 2*** karakteriseerden we het bacteriële microbioom van de huid en neus middels 16S rRNA sequencing in een cohort van kinderen met CE. We concludeerden hieruit dat de samenstelling van het microbioom van de huid en neus beiden geassocieerd zijn met de ernst van het CE. Deze resultaten bleken onafhankelijk te zijn van de leeftijd van het kind, het gebruik van antibiotica en de locatie waar het huidsample was afgenomen. De resultaten suggereren dat het microbioom van de huid en neus beiden een rol spelen bij de ernst van de inflammatie in CE. Een verhoogde aanwezigheid van *Staphylococcen* leverde een belangrijke bijdrage aan de associatie tussen het huid- en neusmicrobioom en de ernst van het CE. Echter, verhoogde of verlaagde aanwezigheid van andere bacteriesoorten bleek ook een rol te spelen. Als laatste concludeerden we dat het microbioom van de neus en de huid beiden een unieke samenstelling hebben,

maar dat er wel correlaties bestaan tussen soorten in de neus en (andere) species op de huid. De onderliggende mechanismen voor deze correlaties dienen verder geëvalueerd te worden. In ***Hoofdstuk 3*** beschreven we het microbioom van de darm bij kinderen met eczeem met en zonder een voedselallergie in een pilotstudie. We vonden dat de combinatie van zes bacteriesoorten discrimineert tussen kinderen met voedselallergie en CE en kinderen zonder een voedselallergie en CE. Deze resultaten moeten verder gevalideerd worden in grotere cohort studies met goed gekarakteriseerde patiëntgroepen.

In **deel II** van dit proefschrift werd specifieker naar de rol van *S. aureus* gekeken en naar de reactie van het humane immuunsysteem op de bacterie. In ***Hoofdstuk 4*** presenteerden we resultaten van een systematisch literatuur onderzoek en meta-analyse waaruit we concluderen dat bij patiënten met CE vaker *S. aureus* gevonden wordt in de neus en op de huid, zowel lesionaal als niet-lesionaal. Ernstiger CE gaat samen op met een verhoogde aanwezigheid van *S. aureus* op de lesionale huid. Deze resultaten moedigen aan tot meer onderzoek naar de precieze rol van *S. aureus* bij eczeem en de toegevoegde waarde van therapie gericht tegen deze bacterie. In ***Hoofdstuk 5*** beschreven we een systematisch literatuur onderzoek met meta-analyse welke laat zien dat bij patiënten met CE vaker IgE tegen Staphylococcus enterotoxinen A en B wordt gevonden in het bloed dan bij gezonde controles. Studies die rapporteerden over IgE tegen andere antigenen en over IgG, IgM en IgA, waren zeer schaars. De verhoogde aanwezigheid van IgE tegen *S. aureus* antigenen in bepaalde patiënten suggereert dat *S. aureus* de inflammatie in CE stimuleert via IgE gemedieerde mechanismen, zoals mestcel degranulatie. In ***Hoofdstuk 6*** bepaalden we de serum IgG antilichaam respons tegen 55 *S. aureus* antigenen in twee cohorten bestaande uit kinderen met CE. We vonden een IgG gemedieerde immuunrespons tegen veel antigenen in beide cohorten wat suggereert dat kinderen met CE worden blootgesteld aan een breed scala aan *S. aureus* antigenen. In een van de cohorten vonden we dat de IgG respons tegen vooral immuun-modulerende antigenen geassocieerd was met de ernst van het CE. Verklarende mechanismen voor deze observatie zouden verder onderzocht moeten worden. Mogelijk kan de *S. aureus* bacterie door middel van het moduleren van het humane immuun systeem zijn positie op de huid behouden, wat kan leiden tot chronische inflammatie in CE.

Deel III van dit proefschrift beschrijft methoden om het effect van een nieuwe interventie gericht tegen *S. aureus* bij CE te testen. In ***Hoofdstuk 7*** vergeleken we een droge 'flocked' swab met een scrub voor afname van huidmateriaal voor microbioom onderzoek, als een pilot voor een klinische studie. De scrubs resulteerden in meer opbrengst van bacterieel en schimmel DNA, wat suggereert dat het gebruik van scrubs van toegevoegde waarde is, met name wanneer men geïnteresseerd is in schimmels en gisten en in gebieden van

de huid die bekend staan om hun lage microbiële biomassa. Echter, de swab methode presteerde even goed met betrekking tot het identificeren van de meest voorkomende bacteriën. ***Hoofdstuk 8*** beschrijft een studieprotocol voor een gerandomiseerde en gecontroleerde klinische trial waarbij gekeken wordt naar het effect van een nieuwe interventie, gebaseerd op endolysinen technologie en specifiek gericht tegen *S. aureus*, op het corticosteroïd gebruik en de symptomen bij CE. Leerpunten uit de voorgaande hoofdstukken werden meegenomen bij het ontwerpen van de trial, zoals de keuze voor de scrub sample methode.

Ten slotte werd in ***Hoofdstuk 9*** een overzicht gegeven van de belangrijkste bevindingen uit dit proefschrift. We benoemden de methodologische beperkingen van de beschreven studies, bediscussieerden de implicaties van de bevindingen voor de kliniek en deden aanbevelingen voor toekomstig onderzoek. In deze thesis beschreven we dat het microbioom en met name *S. aureus*, een relevante rol speelt in de pathogenese van CE. We stelden een ziekte model voor waarbij we beschreven dat het microbioom en belangrijke component vormt, naast de huid barrière en het immuun systeem. De drie componenten vormen samen een gebalanceerd ecosysteem, wat interacteert met genetische en omgevingsfactoren, en van belang is voor een gezonde huid. Een verstoring in een van de componenten kan tot een disbalans in het hele ecosysteem leiden, met chronische inflammatie tot gevolg. We vonden dat *S. aureus*, maar ook andere micro-organismen op de huid en in de neus een rol zouden kunnen spelen in de inflammatie bij CE. *S. aureus* lijkt via verschillende mechanismen met het immuun systeem te interacteren, enerzijds als antigeen en anderzijds als allergeen. De resultaten van dit proefschrift dragen mogelijk bij aan de ontwikkeling van behandelstrategieën voor CE die gericht zijn op modulatie van het microbioom. Toekomstige prospectieve studies, op cohort- en experimentele basis, zijn nodig om de rol van het microbioom bij CE beter te begrijpen en nieuwe behandelstrategieën te onderzoeken. De rol van het microbioom in de pathogenese verschilt waarschijnlijk tussen patiënten. Het identificeren van subgroepen van patiënten met CE, op basis van epidermale, immunologische en microbiële biomarkers, is belangrijk om gerichtere behandelstrategieën in te kunnen zetten. Dit zal uiteindelijk leiden tot meer gepersonaliseerde behandeling van CE.

Appendices

List of publications

List of contributing authors

Portfolio

Curriculum vitae

Dankwoord

LIST OF PUBLICATIONS

Publications in this thesis

Totté JEE, Pardo LM, Fieten KB, Vos MC, van den Broek TJ, Schuren FHJ, Pasmans SGMA.
The nasal and skin microbiome are associated with severity in pediatric atopic dermatitis – exploring relations between the skin and nasal microbiome.
Submitted

Fieten FB, **Totté JEE**, Levin E, Reyman M, Meijer Y, Knulst AC, Schuren F, Pasmans SGMA.
Fecal microbiome and food allergy in pediatric atopic dermatitis: a cross-sectional pilot study.
Int Arch Allergy Immunol. *2018 Jan 25;175(1-2):77-84*

Totté JE, van der Feltz WT, Hennekam M, van Belkum A, van Zuuren EJ, Pasmans SG.
Prevalence and odds of S*taphylococcus aureus* carriage in atopic dermatitis: a systematic review and meta-analysis.
Br J Dermatol. *2016 Oct;175(4):687-95*

de Wit J, **Totté JEE**, van Buchem FJM, Pasmans SGMA.
The prevalence of antibody responses against *Staphylococcus aureus* antigens in patients with atopic dermatitis: a systematic review and meta-analysis.
Br J Dermatol. *2017 Dec 16. [Epub ahead of print]*

Totté JEE, Pardo LM, Fieten KB, de Wit J, de Boer DV, van Wamel WJ, Pasmans SGMA.
The IgG response against *Staphylococcus aureus* is associated with severe atopic dermatitis in young children.
Br J Dermatol. *2017 Nov 30. [Epub ahead of print]*

Totté JEE, Pardo LM, Ouwens AMT, Herpers BL, Pasmans SGMA, Schuren FHJ.
Skin microbiota sampling in atopic dermatitis: to swab or scrub?
Submitted

Totté J, de Wit J, Pardo L, Schuren F, van Doorn M, Pasmans S.
Targeted antistaphylococcal 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).
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Other publications

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Successful Treatment of Chronic *Staphylococcus aureus*-related dermatoses with the topical endolysin Staphefekt SA.100: a report of 3 cases.
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PhD period	August 2013 – June 2018
Promotor	Prof. dr. S.G.M.A. Pasmans, Prof. dr. M.C. Vos
Copromotor	dr. L.M. Pardo

	Year	Workload (Hours/ECTS)
1. PhD training		
Courses		
• PCDI course 'Employability outside Academia', Utrecht	2017	20 hours
• BLS and AED training	2017	2 hours
• Herregistratie Basiscursus Regelgeving en Organisatie voor Klinische Onderzoekers (BROK)	2017	8 hours
• Erasmus summer course 'Diagnostic data for dummies'	2017	0.7 ECTS
• Molmed: Basic course on 'R'	2017	1.8 ECTS
• Molmed: the Course on Microbiomics 1	2016	0.6 ECTS
• NIHES: Biostatistical Methods 1: basic principles part A	2016	2.0 ECTS
• ABCDE skills training	2016	6 hours
• PBLS, BLS and AED training	2016	3 hours
• NIHES: Biostatistics for clinicians (EWP)	2014	1.0 ECTS
• NIHES: Advanced topics in clinical trials (EWP)	2014	1.0 ECTS
• Research Integrity	2014	0.3 ECTS
• Molmed: Biomedical English Writing Course	2014	2.0 ECTS
• Open Clinica	2014	8 hours
• Basiscursus Regelgeving en Organisatie voor Klinische Onderzoekers (BROK)	2013	1.0 ECTS
Workshops		
• Molmed: Photoshop and Illustrator workshop	2015	0.3 ECTS
• Systematic literature retrieval in Pubmed part 1 & 2	2015	8 hours
• Workshop Road to the clinic for biologic therapeutics, London	2015	3 hours
• Media contact for researchers	2015	2.5 hours
• EADV presentation skills, Utrecht	2014	6 hours
Oral Presentations		
• 19th Annual meeting NVED, Lunteren, The Netherlands. ***Sampling the skin microbiome, swab or scrub?***	2018	1.0 ECTS
• Labmeeting, Grice Laboratory, Dermatology Department, University of Pennsylvania, Philadelphia, USA. ***The microbiome in atopic dermatitis.***	2017	1.0 ECTS
• Skintermezzo, Rotterdam, The Netherlands. ***Microbioom van de huid.***	2017	1.0 ECTS

• 5th Exploring Human Host-Microbiome Interactions in Health and Disease meeting, Cambridge, UK. ***Superficial swab versus scrub method to sample the skin microbiome in atopic dermatitis.***	2016	1.0 ECTS
• 13th ESPD Congress, Paris, France. ***Staphylococcus aureus carriage in atopic dermatitis – systematic review and meta-analysis.***	2016	1.0 ECTS
• 23rd EADV Congress, Amsterdam, The Netherlands. ***Transmural care for infantile hemangioma using eHealth.***	2014	1.0 ECTS
Poster Presentations		
• 17th Gut Day, Rotterdam, The Netherlands. ***Microbial species in the gut identify food allergic children with atopic dermatitis.***	2015	1.0 ECTS
• 16the Annual meeting NVED, Lunteren, The Netherlands. ***A systematic review and meta-analysis: Staphylococcus aureus and chronic inflammatory diseases.***	2014	1.0 ECTS
• 24th EADV Congress, Copenhagen, Denmark. ***Targeted endolysin therapy against Staphylococcus aureus in rosacea and acne.***	2014	1.0 ECTS
Conferences (attending)		
• Radboud New Frontiers in the Microbiome symposium, Nijmegen, The Netherlands.	2017	1.0 ECTS
• 'Microbiome and skin', the 24th annual meeting of the Fondation René Touraine, Paris, France.	2016	1.0 ECTS
• 45th Annual ESDR meeting, Rotterdam, The Netherlands.	2015	1.0 ECTS
Committees		
• Organizing 3th PhD weekend, Wassenaar	2015	1.0 ECTS
Awards		
• Award for best poster presentation Gut day	2015	
Other		
• Annual PhD weekend Dermatology (attending)	2014-2017	
• Weekly 'Methodenuur Dermatology and Journal Club', Erasmus MC, Rotterdam	2013-2017	2.0 ECTS
2. Teaching		
Supervising practicals and excursions, Tutoring		
• Supervising SPSS practical Erasmus summer programme	2016	0.5 ECTS
Supervising Master's thesis		
• Supervising master theses of Danique de Boer	2015	2.0 ECTS
Other		
• Supervising research projects Anouk Nouwen	2017	1.0 ECTS
• Supervising research projects Jill de Wit	2015-2016	2.0 ECTS
• Supervising research projects Minke van Mierlo	2017-2018	2.0 ECTS

CURRICULUM VITAE

Joan Totté was born on August 1st 1988 in Hulst, The Netherlands. She grew up in Graauw, a small village located in Zeeuws-Vlaanderen. In 2006 she completed high school at Reynaert College Hulst and started her BSc in Medicine at Utrecht University. After obtaining her bachelor's degree she continued her MSc in Medicine. During her research projects as a master student her interest in research started, resulting in multiple scientific publications on the treatment of infantile hemangioma. After her graduation in 2013 she participated in the development of www.huidhuis.nl, an online treatment and research platform for patients with dermatologic disorders and their health care professionals, at Patient1 in Almere. In 2013 she started her PhD 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 M.D. PhD, prof. M.C. Vos M.D. PhD and co-supervision of L.M. Pardo M.C. PhD. Currently, she works as a researcher at the Department of Dermatology at Erasmus MC.

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