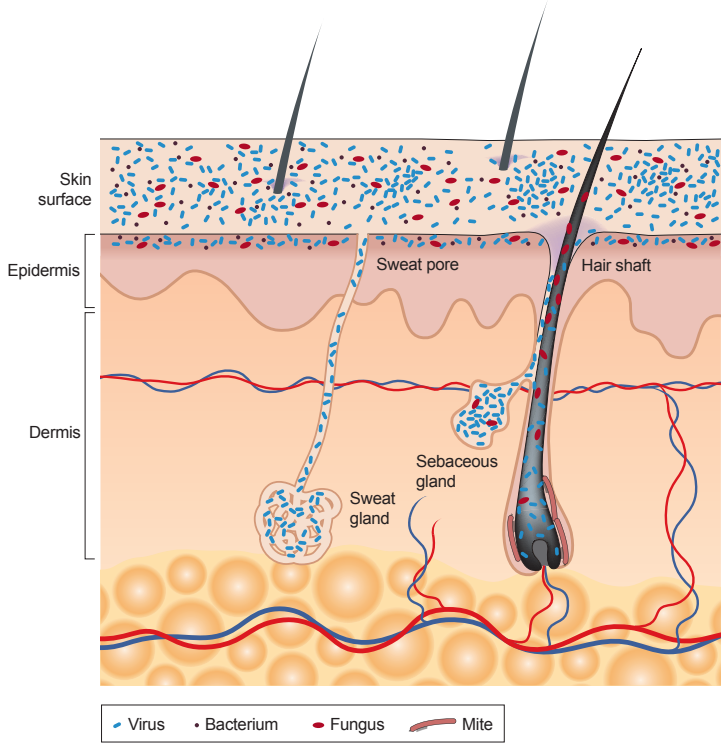
General introduction and aims of the thesis

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

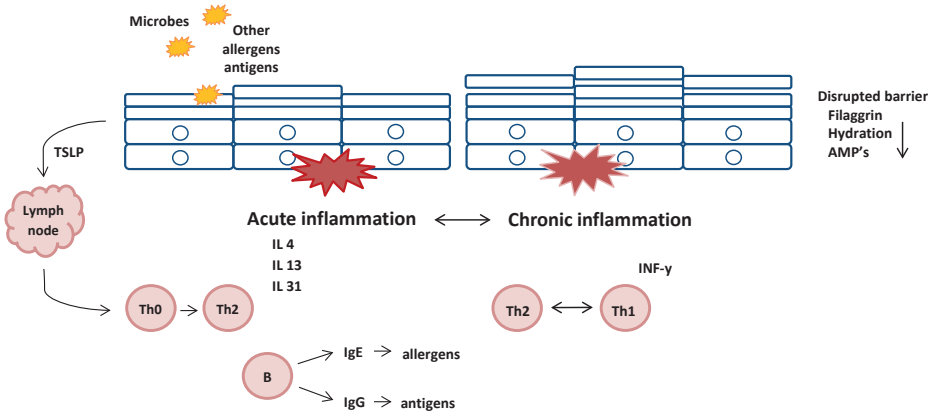
The healthy skin forms a strong barrier against harmful stimuli from the environment, including irritants, allergens, antigens and microorganisms. The outermost layer of the epidermis, the stratum corneum, consists of densely packed corneocytes (terminally differentiated keratinocytes) and proteins that 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]

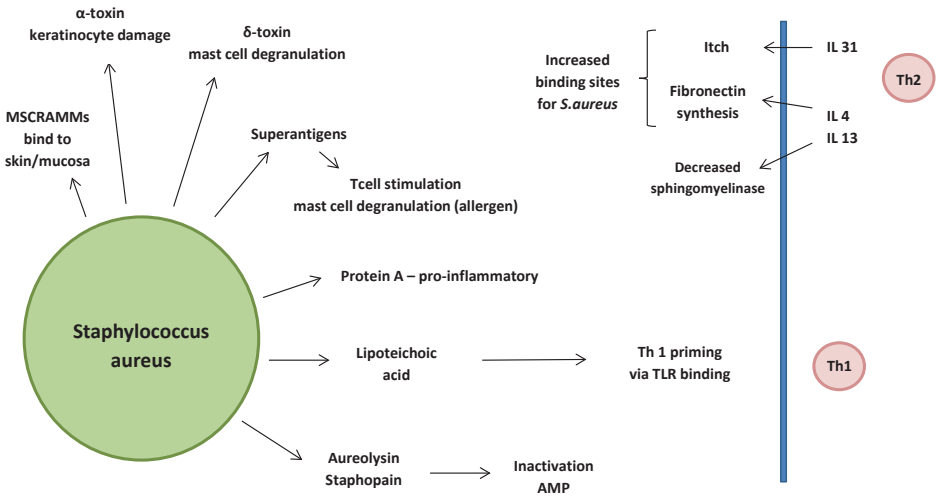
The gut microbiome is thought to play a role in shaping the immune system and it is speculated to influence the development of allergic diseases.[80] Gut microbes can modulate the direction of T-cell differentiation into T regulatory cells or effector T cells (Th1, Th2, Th17), which is important for 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]

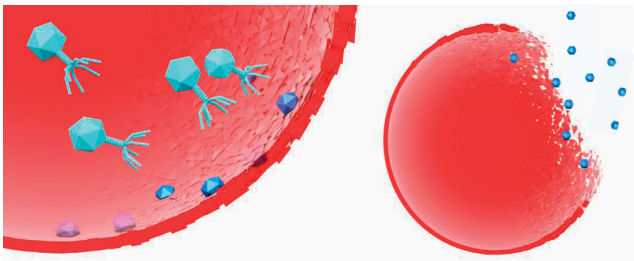
The microbiome as a therapeutic target

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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