TESSA NIEMEYER-VAN DER KOLK

INVESTIGATIONS OF SKIN INFLAMMATION

WITH A NOVEL DERMATOLOGY TOOLBOX FOR EARLY PHASE CLINICAL DRUG DEVELOPMENT

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'Pappa, ik lijk steeds meer op jou.'

Opgedragen aan mijn vader
Gerard van der Kolk
1962 – 2011

En schoonvader
Dr. Menco Niemeyer
1956 – 2020

INVESTIGATIONS OF SKIN INFLAMMATION WITH A NOVEL DERMATOLOGY TOOLBOX FOR EARLY PHASE CLINICAL DRUG DEVELOPMENT

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CHAPTER I

INTRODUCTION

Chronic immune-mediated inflammatory skin diseases are defined as disorders of one of the layers of the host defense system of the skin in the absence of an infectious agent or internal/external injury and affect a large proportion of the population worldwide, and have a significant impact on the physical, social and psychological aspects of a patient's life.[1-3] Most patients (around 80%) suffer from mild-to-moderate disease and are treated with topical drugs. Of these topical drugs, corticosteroids remain the commonly used treatment for most of the mild-to-moderate chronic inflammatory skin diseases. However, although corticosteroids are highly effective, they are associated with significant side effects such as skin atrophy and striae, and can be become less effective (tachyphylaxis) when used extensively for a longer period of time. Alternative topical anti-inflammatory drugs like calcineurin inhibitors (e.g. tacrolimus) are usually less potent and suffer from other relevant side-effects such as a burning sensation, limiting their application in daily clinical practice. Therefore, there is a high medical need for new and effective local therapies with a favorable safety profile. As there is a growing number of therapeutic candidates in this field, innovative strategies are required to optimize the process of dermatological drug development. Clinical scores still dominate the core outcome sets in drug development programs for inflammatory skin diseases, but these obviously have many disadvantages i.e. limited objectivity since the physician that performs the assessment might introduce a response quantification bias and the lack of sensitivity needed to quantify smaller effects of novel drug candidates, which are important parameters in early clinical phase research. Novel, more objective endpoints are needed to support more rational and efficient evaluation of drug effects in early phase clinical development. However, the pertaining literature with regard to rational drug development in chronic immune-mediated skin diseases is sparse.

The scope of this thesis is to support the development of tools and treatments for chronic inflammatory skin diseases, in particular atopic dermatitis (AD), by

1. developing skin inflammation models in healthy volunteers,
2. to investigate novel biomarkers and methods for AD studies and
3. to explore effects of novel treatments for AD.

The introduction of this thesis provides a general outline of the skin and its functions, followed by a summary of the pathophysiology of AD. Subsequently, an alternative, new multimodal approach for early phase dermatological drug development is introduced. Finally, the aims and outlines of this thesis are discussed.

THE SKIN AND ITS FUNCTIONS

The skin is the largest organ of the human body and accounts for 15% of the human body weight.[4] The epidermis, the top layer of the skin, can be divided from outside to inside into stratum corneum (SC), i.e. the actual physical barrier, the stratum granulosum, the stratum spinosum and the stratum basale. Below the basement membrane and epidermis is a layer of connective tissue situated, i.e. the dermis, which can be divided in the papillary and reticular dermis. Many resident cells reside in the skins' epidermis and dermis fulfilling multiple functions (*Figure 1*).

The primary function of the skin is to protect the internal organs from external influences such as micro-organisms, UV-radiation and physical stimuli. Moreover, the skin is involved in regulation of temperature and water release, sensation of touch, and has an endocrine function (e.g. vitamin D synthesis). Last, but not least the skin fulfills an important social function.

SKIN IMMUNITY ❊ Besides acting as physical barrier, the skin also functions as an immunological barrier and forms the first line of defense against infectious agents. Although the complex machinery is not completely elucidated, the following mechanisms have been identified in the sentinel defense.

When a pathogen is detected by the Langerhans cells (epidermal dendritic cells, key immunological sentinels of the skin) or keratinocytes, these react by producing several pro-inflammatory cytokines (activation of the innate immune system, a nonspecific anti-pathogen response), including interleukin-1β (IL-1β), IL-6, IL-18 and tumour necrosis factor alpha (TNF-α). These pro-inflammatory cytokines enable the activation of dermal dendritic cells (DCs). Furthermore, plasmacytoid DCs (pDCs) will be attracted to the skin, activated by stress signals from keratinocytes. pDCs will in turn produce interferon (IFN)-α that also contributes to the activation of dermal DCs. In addition, fibroblasts and natural killer T cells in the skin enhance this inflammatory response by producing IL-6, TNF-α and IFN-γ and consequently activate dermal DCs. By means of antigen presentation the dermal DCs activate skin-resident memory CD4+ T helper (TH) cells or CD8+ T cells, and promote clonal expansion (activation of the adaptive immune system, specific response against the pathogen). Under the influence of multiple pro-inflammatory cytokines, the dermal DCs push the naïve CD4+ T cells to different TH phenotypes, *Figure 2*.[5-8] Dysregulated skin immune responses contribute to the pathogenesis of many chronic inflammatory skin diseases, such as psoriasis (dysregulated TH17/TH1 response) and AD (primarily dysregulated TH2 response).[7,9]

THE SKIN MICROBIOME ❋ The skin surface and appendages are colonized by a host of microorganisms, which together constitute the skin microbiome.[10] Topographical body location, host specific factors (e.g. sex, age) and environmental factors (e.g. climate, occupation, hygiene) all influence the variation in microbiome profiles. In general, Actinobacteria (52%), Firmicutes (24%), Proteobacteria (17%) and Bacteroidetes (7%) are the four most abundant phyla present in the microbiota of the skin.[11] Regarding different topographical locations, sebaceous locations such as the face and back have higher abundances of the genera *Cutibacterium* (formerly *Proprionibacterium*)[12] and *Staphylococcus*. Moist regions such as the antecubital and popliteal fossa are dominated by *Corynebacterium* and *Staphylococcus*.[11] Most of the microorganisms that reside on the skin (commensals) are harmless for the host. Some members of the skin microbiota even have an educative function regarding maturation and homeostasis of the cutaneous immunity.[13,14] In the last years multiple new techniques such as sequencing of the 16S ribosomal RNA gene and whole genome shotgun metagenomics (WGS) have been developed to identify and display the composition of bacteria on the skin, which has contributed to many new discoveries regarding microbiome alterations (dysbiosis) and skin disease.[15,16]

CROSSTALK BETWEEN THE SKIN MICROBIOME AND THE IMMUNE SYSTEM ❋ Not only the communication between the immune cells facilitates homeostasis, but also the crosstalk between immune cells, keratinocytes and components of the skin microbiome contribute to this complex interaction. This way, the skin has the important ability to protect the body from pathogen invasion.[17] An example of this crosstalk is the production of antimicrobial peptides (AMPs) by certain microbes next to the production by keratinocytes. AMPs can directly inhibit pathogen colonization and control the production of immune mediators such as IL-1 and complement, which can enhance inflammation (*Figure 3*).[18,19] Although fundamental mechanisms underlying the immune-commensal crosstalk are only beginning to unfold it is clear that this axis might provide new therapeutic options for inflammatory skin diseases.[20] A disturbance of the skin microbiome is associated with multiple chronic inflammatory skin diseases such as acne vulgaris, psoriasis vulgaris, hidradenitis suppurativa and atopic dermatitis.[21-24] For atopic dermatitis (AD), this disturbance mainly consists of a higher abundance of the bacterium *Staphylococcus aureus* (*S. aureus*). *S. aureus* may directly cause or increase ongoing inflammation by binding of its superantigens (SAGS) to MHCII molecules which induces excessive production of T cell cytokines.[25]

ATOPIC DERMATITIS

Atopic dermatitis is a common inflammatory skin disorder with an estimated prevalence of 3% in adults and up to 25% in children of the Western world.[26-28] Clinical features include a xerotic erythematous skin with a varying degree of edema, papulation, oozing, crusting and lichenification. However, the clinical characteristics can be highly heterogeneous between patients and different age groups.[29,30] Severe pruritus is the main and most bothersome symptom for most patients, and can lead to a substantial impairment of disease-related quality of life.[31] Often patients with AD also exhibit other atopic traits such as (food) allergies, pollinosis and asthma. The pathogenesis of AD is not completely understood but genetic susceptibility, environmental factors, epidermal barrier abnormalities, immunological disturbances and dysbiosis of the skin microbiota all appear to play an important role. The variability of these different factors may explain the heterogeneous character of AD. Overall, it remains hard to determine which of the mechanisms involved are primary events (causing AD) or secondary events (resulting from AD).[32] Two of the major contributors, i.e. immunological disturbances and dysbiosis, are highlighted in the following paragraph as these are important for this thesis.

IMMUNOLOGICAL DISTURBANCES ❋ At present, AD is considered to be a primarily T-cell driven disease, with a strong TH2 activation in both lesional and non-lesional skin.[30,33,34] Because of the impaired skin barrier caused by genetic factors (e.g. filaggrin deficiency) and inflammation, irritants, microbes and (auto)allergens can penetrate the skin and trigger the keratinocytes to produce thymic stromal lymphopoietin (TSLP), which in turn stimulates the dermal dendritic cells to produce cytokines that induce TH2 cell polarization.[35] The chemokine thymus and activated regulated chemokine (TARC, also known as CCL17), a downstream marker of TSLP, guides the TH2 cells during their migration across the endothelium to the skin. TARC was found to be one of the best correlating serum biomarkers with AD clinical severity.[36] Once arrived in the skin, the TH2 cells produce many pro-inflammatory cytokines including IL-4, IL-5, IL-13 and IL-31.[33] These cytokines further amplify the ongoing inflammation, especially IL-4 that upregulates the production of TARC, attracting even more TH2 cells to the skin.[37] Moreover, TH2 cytokines are known to reduce the expression of filaggrin and other structural proteins by keratinocytes. This inflammatory cascade eventually leads to a further impairment of the skin barrier. In addition, TH2 cytokines are known to suppress the production of AMPs in the skin, which leads

to an increased susceptibility for bacterial colonization, e.g. with *S. aureus*.[34,38] Also, a higher number of other immune cell types, such as mast cells, can be found in lesional skin. Mast cells can be activated when two immunoglobulin E (IgE) molecules on the cell surface are cross-linked by extrinsic allergens, such as house dust mite, grass pollen, or certain food allergens. Activated TH2 cells will in turn activate B cells, which will differentiate in plasma cells that produce high levels of antigen specific IgE. Therefore, many AD patients exhibit high levels of circulating IgE.

DYSBIOSIS ❋ New sequencing techniques provided greater insight in the microbiome and microbiome alterations in AD. From traditional culturing it was already known that the skin in up to 90% of AD patients are colonized with *S. aureus*, compared to only 5-30% of the general population.[39-41] Utilization of new techniques confirmed this disturbance of the microbiome ('dysbiosis'). Lesional skin shows higher abundances of the genus *Staphylococcus* and a decrease of the overall diversity of the microbial flora.[21] Many factors are involved in this higher *Staphylococcus* colonization rate. A deficiency in AMPs, which play an important role in the host defence system of the skin, may partially account for the susceptibility to *S. aureus* colonization.[38]

MANAGEMENT OF AD ❋ Daily application of bland emollients is the primary therapeutic intervention for patients with mild AD in combination with topical anti-inflammatory agents such as corticosteroids and calcineurin inhibitors when needed.[42] Phototherapy and systemic immunosuppressive drugs are therapeutic options for the moderate to severe patient group.[43] Dupilumab, the first monoclonal anti-IL4/IL-13 antibody treatment for patients with highly resistant disease was registered in 2019, and it is likely that many other targeted treatments will follow.[44,45] This is however unlikely to be a viable or sustainable treatment option for patients with mild to moderate disease activity. For these patients novel anti-inflammatory agents with a more favorable side effect profile compared to topical corticosteroids seems to be a more realistic option. Though frequently used, use of topical corticosteroids should not be considered harmless. Dose-dependent serious side effects are not rare and include both local (skin atrophy, irreversible striae) and systemic (suppression of the hypothalamic-pituitary-adrenal (HPA)-axis) unwanted effects.[46] It is proposed that novel drugs designed to target the microbiome and the skin microbiome-immune axis in AD (antimicrobial peptides) may be alternative treatments for patients with mild to moderate atopic dermatitis.[47]

DEVELOPMENT OF NOVEL THERAPEUTICS IN DERMATOLOGY USING NEW METHODOLOGIES AND BIOMARKERS

The escalating number of therapeutic candidates requires new strategies to optimize the process of clinical development, as the traditional 4-phased process of clinical drug development is an extensive and expensive process. The use of validated biomarkers is a common approach to guide a new drug through the development process. A biomarker is defined as a characteristic that can be objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention.[48] Usability of a biomarker can be assessed by the following general criteria:

1. there must be a consistent response of the biomarker across studies (preferably from different research groups) and drugs from the same mechanistic class,
2. the biomarker must respond clearly to therapeutic (not supratherapeutic) doses,
3. there must be a clear dose- or concentration-response relationship and
4. there must a plausible relationship between the biomarker, pharmacology of the drug class, and disease pathophysiology.[49]

Depending on the specific biomarker, some criteria may be more important than others. With a well-chosen package of biomarkers, *proof-of-pharmacology* can be obtained at an early stage of drug development, which can save time and money. Moreover, there will be less unnecessary exposure of the compound to subjects participating in trials. Ideally, a biomarker can function as 'surrogate endpoint' e.g. as a substitute and support for a clinical endpoint.

SKIN INFLAMMATION CHALLENGE MODELS ❋ The absence of a disorder in healthy volunteers may hamper investigation of the above-mentioned hallmarks of dermatological drug development. Inflammation, for example, plays an important role in many skin diseases. Difficulties arise when investigating anti-inflammatory drugs in healthy volunteers who obviously do not have this disease. In this case, pharmacological challenge models that mimic the pathophysiological characteristics of skin inflammation can be applied. These models become more and commonly applied to obtain early insight into target engagement. Ultimately, the information gained with a pharmacological challenge model study will help to answer important questions for the future

development of a new drug. For example, questions regarding drug profiling, dose or drug regimen selection and possibly refining the right target indication for the compound. The answers to these questions would directly influence the next steps of the development process. As different components of the skin are involved in inflammation (e.g. the skin barrier, microbiome and immune system), ideally a multimodal profile of the pharmacodynamic effects of the compound is constructed that shows the effects on the different modalities that are involved. Currently, there is a high need for novel biomarkers and methodologies to facilitate this multimodal approach.

MULTIMODAL PROFILING IN INFLAMMATORY SKIN DISEASE ❋ At present, most biomarkers and endpoints for clinical trials in dermatology are clinical scores as assessed by physicians, e.g. the Eczema Area and Severity Index (EASI) and Scoring Atopic Dermatitis (SCORAD) for AD or patients, e.g. Patient Oriented Outcome Measures (POEM), numeric rating scale (NRS) for itch and the Dermatology Life Quality Index (DLQI).[50] As disease clearance is often lengthy, clinical trials are commonly long and intensive and thus the demand for informative and efficient trials is high. To address this need, different approaches can be taken. Surrogate endpoints would have a great added value, particularly for early phase clinical trials where mechanistic, pharmacodynamic exploration of the drug candidate is the main objective. Detection of the absence of pharmacodynamic effects of a novel drug at an early stage of development would influence the following steps directly. For example, for AD one target lesion could be characterized with novel biomarkers and followed up during a clinical trial to explore the pharmacodynamics of the compound locally in addition to the total body scoring systems (e.g. EASI). Finally, evaluating patient reported outcomes (PROs) would complete the patient profile, *Figure 4*.

CHARACTERIZATION OF SKIN INFLAMMATION WITH THE DERMATOLOGY TOOLBOX ❋ The 'dermatology toolbox' for characterization of skin inflammation consists of multiple novel applications on all aspects of skin inflammation to facilitate a multimodal (patient) approach (*Figure 4*). Inflammation is characterized by dolor (pain), calor (heat), rubor (erythema), tumor (swelling) and function laesa (loss of function). With the toolbox we introduce a new multidimensional approach on different domains, i.e. clinical, imaging, biophysical, molecular, cellular, microbiome and patient reported biomarkers. The goal of this approach is to have a more complete (patient) profile regarding skin inflammation that can be used to characterize drug specific effects in early phase clinical trials.

AIMS AND OUTLINE OF THIS THESIS

The main objective of this thesis was to develop and characterize skin inflammation models in healthy volunteers, and to characterize AD with novel biomarkers using a newwly constructed 'dermatology toolbox' that contains an array of innovative methodologies that can be applied in early phase development trials. The thesis entails investigations of the development and application of section 1) skin inflammation models in healthy volunteers, section 2) novel biomarkers and methodologies for AD studies and finally section 3) novel pharmacological treatments for AD.

SECTION 1

The first section of this thesis describes the development, characterization and application of a novel local skin inflammation model in healthy volunteers.

Chapter 2 describes the development of a rapid, temporary skin inflammation model in healthy volunteers. Cutaneous inflammation in this model was induced by the topical TLR-7 agonist imiquimod (Aldara®). After clinical validation, the model was applied in a topical drug-drug interaction study (chapter 3). In this chapter we performed the pharmacodynamic profiling of a new compound omiganan using the imiquimod-induced skin inflammation model.

SECTION 2

In this section the development and selection of novel biomarkers as tools for *proof-of-concept* and *proof-of-pharmacology* trials in dermatology are reported.

Development and application of integrating an electronic e-diary, i.e. a mobile application, for treatment compliance in clinical trials is reported in chapter 4.

In chapter 5, a literature review on the suitability of the human skin microbiome as biomarker to add to the dermatology toolbox for clinical trials in different inflammatory skin diseases is reported. The stability of the skin microbiota of atopic dermatitis patients over time determined with different methods and an advice on the use of these comprehensive data in clinical trials is described in chapter 6.

SECTION 3

In the last section of this thesis, the *proof-of-pharmacology* studies with omiganan in atopic dermatitis are described.

The results of phase 2 trial investigating the effects of new topical formulation with the compound omiganan applied daily on a single target lesion in patients

with AD are described in chapter 7. Subsequently, in chapter 8 the results of a study investigating the twice daily application of omiganan on all lesions of AD patients are described.

Lastly, chapter 9 provides a synthesis of the main findings of this thesis, accompanied by a general discussion and recommendations regarding suitability of the different applications of omiganan, the skin inflammation model and the dermatology toolbox for early drug development trials in dermatology.

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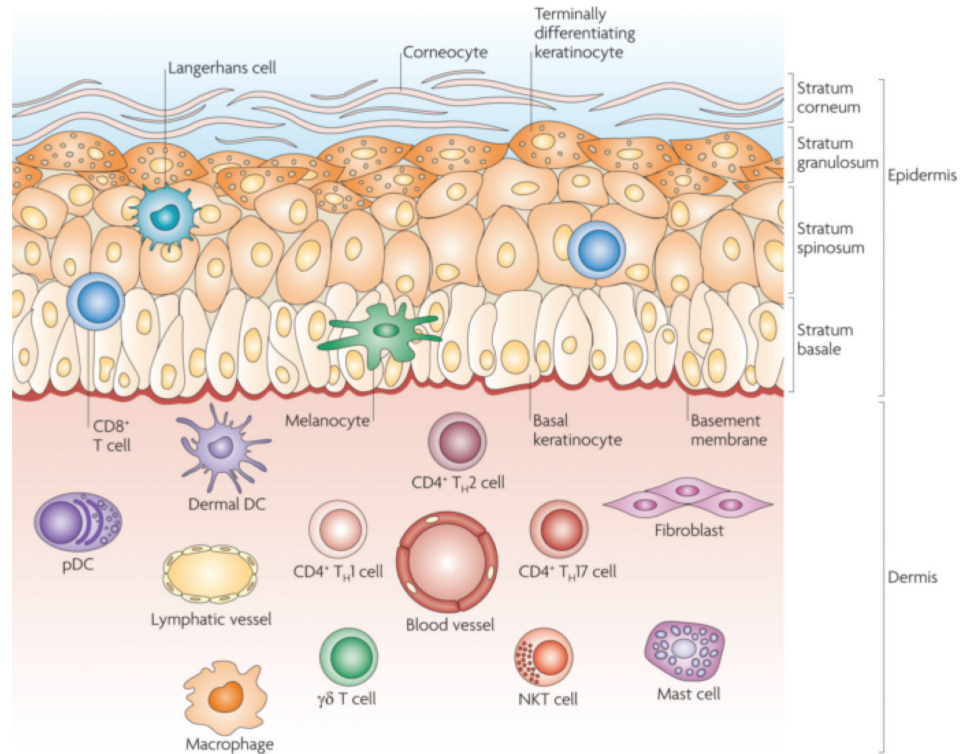
FIGURE 1 The structure and cells of the skin.



Published with permission Nestle et al Nat Rev Immunol. 2009.[5]

FIGURE 2 The differentiation of naive CD4+ cells to effector cells.

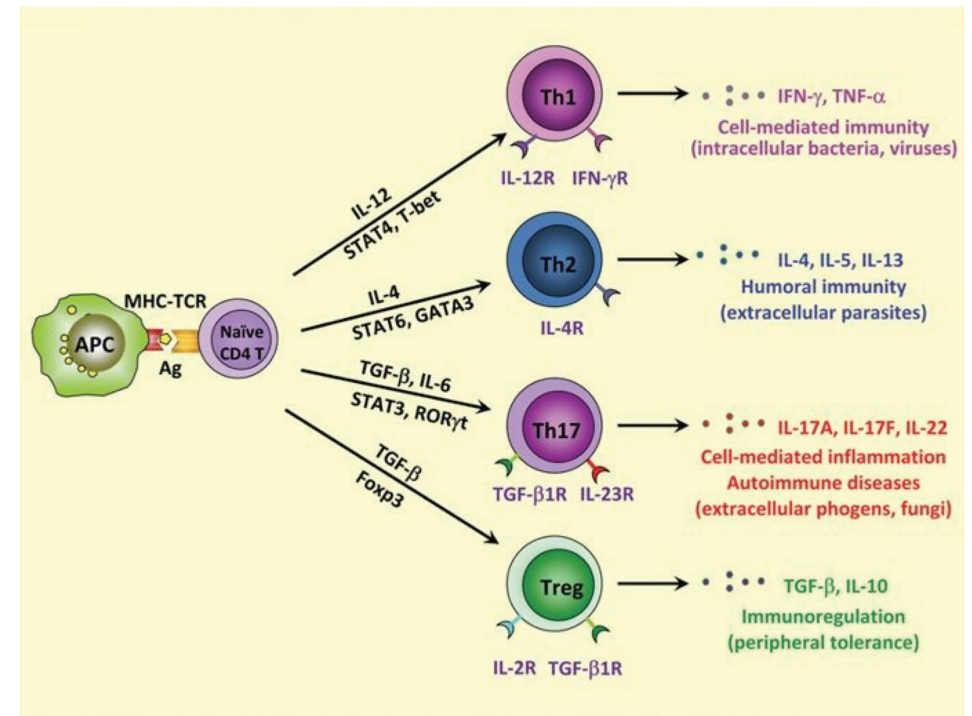


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FIGURE 3 Crosstalk between the skin and the microbiome.

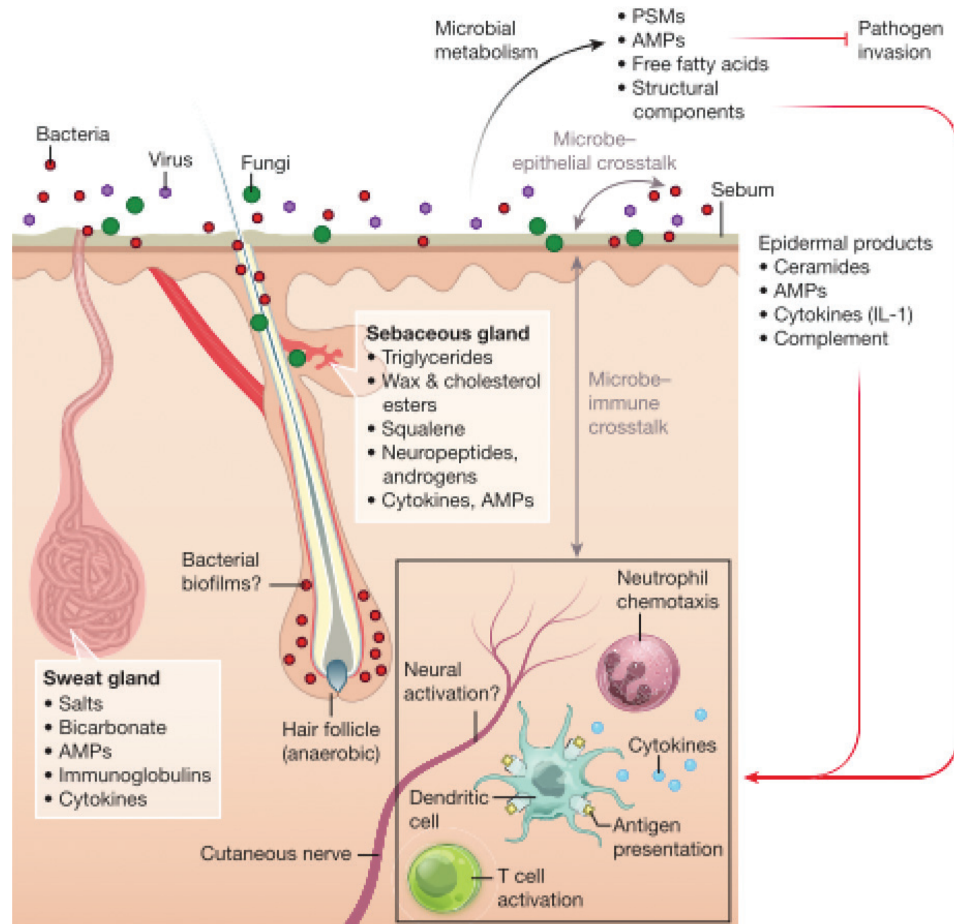

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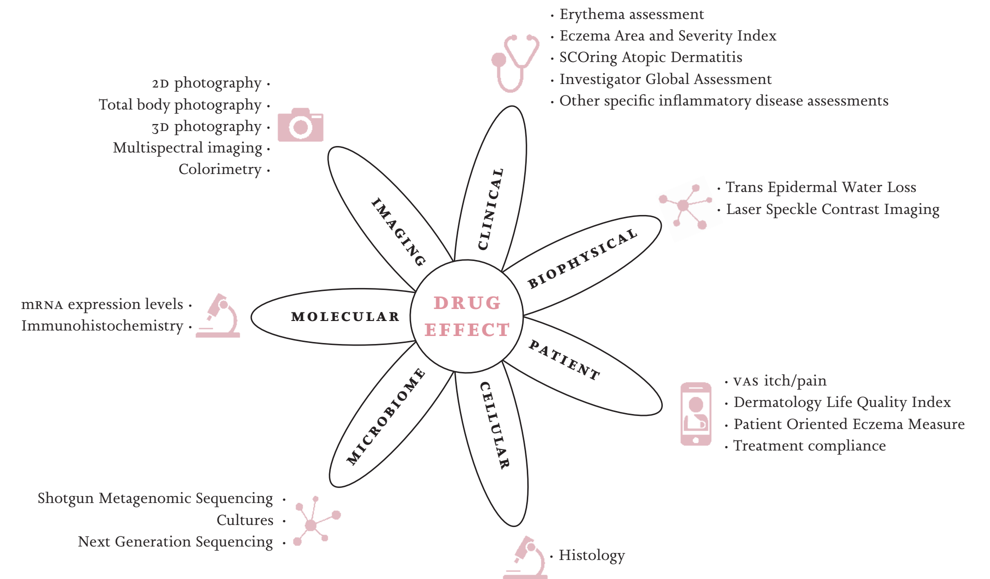
FIGURE 4 Multimodal profiling in chronic inflammatory skin disease.



SECTION I

HUMAN INFLAMMATION MODELS

CHAPTER II

COMPREHENSIVE, MULTI-MODAL CHARACTERIZATION OF AN IMIQUIMOD-INDUCED HUMAN SKIN INFLAMMATION MODEL FOR DRUG DEVELOPMENT

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ABSTRACT

Imiquimod (IMQ) is often used as topical challenge agent to provoke local skin inflammation. The objective of this study was to develop and refine a rapid, temporary and reversible human skin inflammation model with IMQ for application in clinical drug development. A randomized, vehicle-controlled, open-label, dose-ranging study was conducted in 16 healthy male subjects. IMQ (5 mg) was applied once daily for 72h under occlusion to intact skin (n=8) or tape stripped (TS) skin (n=8). Although IMQ alone induced limited effects, TS+IMQ treatment showed larger responses in several domains including erythema and perfusion ($p<0.0001$), mRNA expression of inflammatory markers ($p<0.01$) and inflammatory cell influx compared to vehicle. In conclusion, a rapid, human IMQ skin inflammation challenge model was successfully developed with a clear benefit of TS prior to IMQ application. Future interaction studies will enable *proof-of-pharmacology* of novel compounds targeting the innate immune system.

INTRODUCTION

Skin inflammation is a physiological immune response to various stimuli including skin trauma, physical challenge, exposure to xenobiotics, microbes and parasites. Dysregulation of this immune response is involved in chronic inflammatory skin diseases e.g. psoriasis vulgaris, acne vulgaris and atopic dermatitis.[1,2] Although much mechanistic insight has been gained, including involvement of the innate immune system via Toll-like receptors (TLRS) and the adaptive immune system, the pathophysiology of skin inflammation is complex and remains to be elucidated further.[3]

Different models that mirror aspects of chronic inflammation have been developed to study skin inflammation. For instance, rapid, acute skin inflammation can be induced by topical, cutaneous application of imiquimod cream (IMQ; Aldara®). IMQ application leads to agonistic activation of TLR7 and TLR8-mediated MYD88 signalling, activation of NF-κB and the induction and release of pro-inflammatory cytokines, type-I interferons, chemokines and other mediators. Ultimately, this leads to an innate and TH1 and TH17-weighted cellular immune activation and enhancement of pro-inflammatory effects.[4]

Although the use of IMQ appears to be safe and reasonably tolerated, disease exacerbation can occur in psoriasis patients and even the development of psoriasis in individuals without a prior history of the disease is reported.[5-10] Based on the initial findings, the first IMQ-induced skin inflammation mouse model was successfully developed.[11] This model has become widely accepted for preclinical studies of psoriasis because of its straightforward approach, the inexpensiveness and the fast acute inflammatory response.[11,12] Nevertheless the murine model has some crucial disadvantages the major ones being immediate systemic effects and the limited extrapolation of murine findings to humans due to differences in the immunology and TLRS.[12,13]

Therefore, several skin inflammation models with IMQ have been evaluated in humans. A short, 7-day model in psoriasis patients using IMQ and tape stripping (TS), showed that psoriasis-like skin inflammation occurred but typical psoriasis did not develop.[5] Contact dermatitis driven by plasmacytoid dendritic cells (pDC) was observed in an extended model of 4 weeks, both in healthy volunteers and in patients with inflammatory skin diseases. Only limited aspects of the molecular signature of psoriasis were observed.[14] Other studies in healthy volunteers characterized the model with either solely focussing on biopsy biomarkers or only systemic effects after high topical doses of IMQ.[15,16] Acute and rapid IMQ-induced inflammation models in healthy volunteers with detailed characterization have not yet been reported. A well-characterized, comprehensive human model to study skin inflammation would open opportunities for understanding the pathogenesis of several skin diseases and for the profiling of novel drugs in development.
With this study we aimed to develop a skin inflammation challenge model with

1. topical IMQ application for 24, 48 and 72 hours on a fully competent skin barrier, and
2. topical IMQ application for 24, 48 and 72 hours on TS-perturbed skin barrier to enhance drug delivery.

Cetomacrogol cream, an indifferent neutral emollient, was used as control. Skin inflammation was assessed by measurement of erythema, perfusion and using biopsy material (mRNA expression, histology, immunohistochemistry). In the future, these models may be used in drug development programs for *proof-of-pharmacology*, drug profiling or interactions studies of novel compounds targeting the innate immune system and translational research of inflammatory skin diseases.

MATERIALS AND METHODS

The protocol of this randomized, open-label, vehicle-controlled, parallel-cohort, dose ranging study was approved by the independent Medical Ethics Committee 'Medisch Ethische Toetsingscommissie van de Stichting Beoordeling Ethiek Biomedisch Onderzoek' (Assen, the Netherlands). The study was conducted

according to the Dutch Act on Medical Research involving Human Subjects (WMO). Subjects were recruited throughout the Netherlands via advertisement campaigns on the internet and in the newspaper. All subjects gave informed consent prior to any study procedure. The study was conducted from May 2016 to June 2016 at the Centre for Human Drug Research, Leiden, The Netherlands.

STUDY POPULATION ❋ Sixteen (16) healthy male Caucasian (Fitzpatrick skin type I-II) volunteers, aged 18-45 years, were included in the study. Health status was verified with a medical history, physical examination, laboratory tests and 12-lead electrocardiodiagrams (ECG). Subjects with a medical history or family history of psoriasis or any disease associated with immune system impairment were excluded from the study. Previous use of imiquimod, resiquimod or gardiquimod was not allowed.

IMQ TREATMENT ❋ Prior to the first dose administration, 4 treatment areas (squares) were identified on the upper back and marked with a dermatological marker. During the treatment period a standard daily dosage containing 5 mg IMQ (100 mg Aldara®) was applied under occlusion by a 12 mm Finn chamber (Smart Practice, Phoenix, USA), meaning the Finn chamber was replaced with a new dose after 24h. Treatment area 1 was treated 1 day (5mg IMQ, 24h), treatment area 2 was treated 2 days (cumulative 10mg IMQ, 48h) and treatment area 3 was treated for 3 days (cumulative 15mg IMQ, 72h). Treatment area 4 was treated with 100 mg cetomacrogol (indifferent) cream for 72h (negative control), *Figure 1A*. The sample size and dose were selected based on a previous study with imiquimod in healthy volunteers. Given the exploratory character of the study no formal power calculation was performed. Subjects were randomized 1:1 to receive these treatments either over a fully competent skin barrier or over a disrupted skin barrier by tape stripping (TS) of the skin. Tape stripping enhances drug delivery over the skin barrier. It is known from literature that only a limited amount of drug can be delivered over a fully competent barrier. Given the short treatment duration in this study it was decided to tape strip one group before application to ensure drug delivery. The tape stripping procedure was performed as follows: tape (D-Squame, CUDERM, Dallas, US) was applied to the marked treatment area and a roller was used to press the tape to the skin to avoid furrows and wrinkles by a single operator. After this the tape was removed at a constant velocity. The procedure was repeated for at least 15 times until the trans epidermal water loss (TEWL) by Aquaflux (BioX, London, UK) was between 20-25 g/m²h (partial

removal of the stratum corneum). Safety and tolerability were monitored by recording adverse events.

CLINICAL SCORES ❋ Clinical assessments were performed daily of treatment area 3 and 4, and included visual erythema grading (Clinician Erythema Assessment (CEA) scale, 0 represents absence of erythema, 4 very severe), colorimetry (a value (DSM II ColorMeter, Cortex Technology, Hadslund, Denmark) with a total of 3 repeats on each treatment area, 2D photography erythema index analysis and perfusion by laser speckle contrast imaging (LSCI; PeriCam PSI System, Perimed Jäfälla, Sweden).

TRANSDERMAL ANALYSIS PATCH (TAP) ❋ Skin surface biomarkers were collected pre-dose and after 1, 2 and 3 days of treatment by with TAP (FibroTx, Estonia). TAP consists of a multiplex capture-antibody micro-array that is supported by a dermal adhesive bandage for fixture to skin and can measure up to 6 markers per TAP. IFN-β, IFN-γ, IL-8, IL-6, HBD-2, IL-1β were chosen and captured from skin were qualitatively and quantitatively analysed by spot-ELISA.

SKIN PUNCH BIOPSIES ❋ Three millimetre skin biopsies were collected pre-dose (of tape stripped skin in the TS cohort), 24h after end-of-treatment of each treatment area and from a distant site with a total of six biopsies per subject. After harvest, the biopsies were placed in RNA later medium and stored at 4°C. The biopsy samples were analysed at the Immunology Laboratory at Erasmus MC, Rotterdam, The Netherlands. RNA extraction and real-time quantitative PCR analysis was performed for the following biomarkers: IP10/CXCL10, IFN-β, IFN-γ, TNF-α, IL-1β, IL-6, HBD-2, MX1, MXA and ICAM-1, that were chosen based on the murine model. Additionally, a histopathological score was obtained of haematoxylin and eosin (H&E) stained tissue by two blinded persons for the following characteristics of psoriasis and dermatitis: general infiltration (all type of inflammatory cells present), parakeratosis, acanthosis, papillomatosis and spongiosis. The histopathological score for each characteristic was graded based on fold increase or decrease compared to a reference biopsy of a healthy subject not related to the clinical trial (1; equal to the reference biopsy, 2; 2-fold increase compared to the reference biopsy etc.). Furthermore, immunohistochemical staining was performed to obtain scoring of markers CD11C, CD14, CD1a, CD4, CD8 and HLA-DR. This was also performed by two blinded persons and graded the same way as for the histopathological characteristics.

STATISTICS ❃ All calculations were performed using SAS for windows V9.4 (SAS Institute, INC., Cary, NC, USA). Treatments effects were analysed with a mixed model analysis of variance with fixed factors treatment, cohort, time, treatment by cohort, treatment by time, cohort by time and treatment by cohort by time, random factors subject, subject by treatment and subject by time and the baseline measurement as covariate. To determine the differences among the treatments, contrasts on all measurements were calculated. Analysis results per variable were generated with estimates of the difference of the different contrasts and a back transformed estimate of the difference in percentage for log transformed parameters, 95% confidence intervals (in percentage for log-transformed parameters) and Least Square Means (geometric means for log transformed parameters), and the p-value of the contrasts.

RESULTS

In total thirty-five (35) subjects were screened of whom twenty-six (26) subjects were eligible for participation in the study. All of the sixteen (16) included subjects completed the study and the treatments of IMQ or TS+IMQ were administered as depicted in *Figure 1a*. The study participants were all Caucasian and had a mean age of 22.3 (18-33) years. The treatments were in general well-tolerated. The most frequent occurring treatment- emergent adverse event was application site pruritus, observed in 25% of the participants of both IMQ-treatments. No increase in IFN-α or IFN-γ was detected in the systemic circulation.

IMQ APPLICATION INDUCED REVERSIBLE ERYTHEMATOUS HYPERPERFUSED SKIN LESIONS 3 ❃ A clear exposure-dependent increase in erythema was observed with IMQ and TS+IMQ for all erythema measurements compared to the control as determined by erythema index (EI) photo analysis, erythema by colorimetry and erythema by visual grading (*Figure 1b*, *Figure 2*). Upon 48h IMQ treatment, the difference with vehicle was statistically significant ($p<0.05$) (EI; 11.55, 95%CI 1.00-22.10, p=0.03; colorimetry; 2.16, 95%CI 0.66-3.65, p=0.006, *Table 1*). TS+IMQ resulted in more significant contrasts ($p<0.01$) compared to vehicle and these were already achieved after 24h (EI; 18.64, 95%CI 7.89-29.38, p = 0.001, colorimetry; 3.22, 95%CI 1.72-4.73, p = 0.0001, *Table 1*). An exposure-dependent increase in perfusion that plateaued after 48h was only observed with TS+IMQ (*Figure 2c*). Concordant with the erythema, this increase was already statistically significant 24h after application (21%, 95%CI 7.0%-36.9%, p=0.003, *Table 1*). TS itself did not induce significant changes in erythema and perfusion (Figure S1). The skin clinically recovered after end-of-treatment (not shown).

IMQ-INDUCED ACTIVATION OF INNATE IMMUNE SYSTEM ❃ Expression of CXCL10, HBD-2, ICAM-1, IFN-β, IFN-γ, IL-1β, IL-6, MX-1, MX-A and TNF-α in punch biopsies was investigated by real-time quantitative QPCR analysis, and normalized for the housekeeping gene ABL. As shown in *Figure 3*, CXCL10, MX-A, ICAM-1 and HBD-2 showed a statistically significant increase after 48h and 72h in the TS+IMQ treatment group compared to the untreated area ($p<0.01$). This was only observed for HBD-2 in the IMQ treatment group versus untreated. In addition, an increased expression of both TNF-α and IL-1β was observed with 48h and 72h treatment, compared to the untreated site, this was significant for IL-1β ($p<0.05$), and only observed for the conditions without tape stripping, *Figure 3*. TAP data was highly variable and not significantly different from untreated skin (data not shown).

INFILTRATION AND ACANTHOSIS INDUCED BY IMQ TREATMENT ❃ H&E stained skin punch biopsies were independently analysed by two investigators blinded to treatment. In the IMQ treatment group, no changes compared to the reference biopsy were observed. TS+IMQ treatment demonstrated ≥ 2-fold increase in general infiltration in 31% and 44% of the subjects after 48h and 72h, respectively, compared to the reference. Acanthosis was compared to the reference two times more frequent in 19% of these subjects after 48h (*Figure 3*, *Figure 4*). Moreover, parakeratosis, papillomatosis and spongiosis were scored. These parameters were not different from the reference biopsy in both cohorts. No histological skin changes were noted in the vehicle controls.

CD4+, CD8+, CD11+ AND HLA-DR+ CELLS INFILTRATED THE DERMIS FOLLOWING IMQ TREATMENT ❃ Immunohistochemical staining was performed to further explore cell infiltration and showed an infiltration of CD4+ T-cells, CD8+ T-cells, CD11+ dendritic cells and HLA-DR+ macrophages, mostly in the TS+IMQ treatment group. CD8+ cells were ≥1.5 times more present in 56% of the subjects in the TS+IMQ treatment group after 48h and 72h, compared to the reference biopsy (*Figure 4*, *Figure 5*). In 37.5% of the subjects CD4+ cells were ≥1.5 times more present examined after 48h and 72h of treatment in the TS+IMQ treatment group. Moreover, CD11C+ infiltration was slightly apparent (2 times more present in 21.4% of the subjects after 48h, 12.5% of the subjects after 72h in TS). HLA-DR+ cells 12.5% of the tape stripped subjects developed a 3-fold increase of HLA-DR+ cells after 48h of IMQ treatment, while 18.8% was observed after 72h of IMQ treatment, compared to the reference biopsy. However, HLA-DR was already more present at baseline in this group of subjects, compared to the reference biopsy (Figure 5).

DISCUSSION

COMPREHENSIVE CHARACTERIZATION OF THE SKIN INFLAMMATION MODEL ✻ This is the first study that comprehensively characterized an acute inflammatory model combining IMQ and TS in healthy volunteers with the purpose to apply in drug development programs. We utilized different, complementary assessment modalites to monitor the effects thouroughly, including clinical (physician scoring), biophysical (LSCI), imaging (colorimetry), molecular (mRNA expression) and cellular (IHC) aspects. The synthesis of this multi-modal assessment is presented in *Figure* 6 and clearly shows concordant effects on complementary modalities and a clear dose-dependency, exposure-response relationship. While previously models were developed in psoriasis patients or with a lengthy treatment-period of 28 days in healthy volunteers without TS,[5,14] our study shows a rapid and reproducible way of inducing short-term inflammatory skin lesions with effects on all the different domains as discussed in detail in the next paragraphs.

STRONG AGREEMENT OF MEASUREMENTS ASSESSING THE CLINICAL PHENOTYPE ✻ IMQ induced a dose-dependent increase in erythema in all measurements, which occured much quicker and more pronouced when combined with TS. Statistically significant effects on EI, colorimetry and LSCI were already observed 24h after TS+IMQ treatment, versus 48h without TS. There were no clear differences in erythema intensity between 48h and 72h of treatment for the TS+IMQ treatment which is also seen in the murine model.[11] Increased skin perfusion as a result of IMQ application was only observed when it was combined with TS and also no clear differences between 48h and 72h appeared. In a recent study where a human model with 4 weeks of IMQ treatment was developed to study psoriasis, maximal effects occurred at day 4 or later, but TS of the skin was not performed to enhance drug delivery of IMQ.[14] Importantly, in our study we showed that within 24-48h of the last dose, the skin fully recovered clinically (data not shown), which is in agreement with previous reports.[5] The similar clinical observations with different methods and reversibility of effects are two strong points of our approach.

FULLY COMPREHENSIVE MECHANISTIC INSIGHTS OF MOLECULAR PATTERNS ✻ CXCL10, MX-A, ICAM-1 and HBD-2 were all statistically significant upregulated in the skin of subjects treated with IMQ for 48h and 72h in combination with TS and to a lesser extent in the non-TS cohort (only for HBD-2) compared to vehicle, which is concordant with the molecular findings of *Dickson et al.*[15] This reflects the intermediate phase response of IMQ (24-72h) where activation of the innate as well as adaptive immune system occurs, featuring infiltration of neutrophils, lymphocytes and macrophages, as described in a recent review of the murine translational IMQ skin inflammation models.[17] CXCL10, a chemokine which is highly expressed when keratinocytes are activated in inflamed skin, is regulated by T-cells and found in psoriasis and other autoimmune diseases, which corresponds with the findings in our clinically induced skin inflammation.[1,15,18] Upregulation of MX-A, a down-stream mediator of interferons, reflects the activation of plasmacytoid dendritic cells (pDCs), which play a major role in the pathophysiology of psoriasis (1, 19, 20) . Furthermore, this refects the anti-viral response by IMQ, which was expected since IMQ is effective against several HPV-induced skin diseases.[21,22] The mRNA expression of adhesion molecule ICAM-1 was observed to be upregulated which corroborates previous findings showing induction by TNF-α.[23] ICAM-1 facilitates leukocyte endothelial transmigration and enhancement of skin inflammation. Upon IMQ treatment alone no statistically significant differences in upregulation were observed between 48h and 72h in the biopsy markers which confirms the better suitability of TS+IMQ combination for further application in drug development.

Interestingly, in the biopsies of the non-TS cohort, we found an upregulation of TNF- α and IL-1β compared to the untreated site while this was not present in the TS+IMQ cohort. Presumably, the initial phase (within 24h), where the innate immune system is activated as a consequence of release of inflammatory mediators including IFN-α, IL-1α, IL-1β, IL-6 and TNF-α and where cellular changes such as accumulation of neutrophils and proliferation of keratinocytes occur, is at a later time point because of less drug delivery without tape stripping.[17] This would also explain why TNF-α and IL-1β are not upregulated in the TS cohort after 48h; due to enhanced penetration by TS of the skin, the initial upregulation might already be passed. This matches with the fact that erythema was observed in the TS cohort already after 24h, but not in the non-TS cohort, where it appeared after 48h. Moreover, the presence of ICAM-1 in the TS cohort confirms the presence of TNF-α in an earlier stage, and therewith the initial phase as also seen in the murine model, since it is a downstream marker of TNF- α.[23] Vinter *et al.* did still find TNF-α and IL-1β after 48h, but tape stripped less extensively (10 times *vs.* ± 15 times in our study). Hereafter, both markers also normalized while the downstream markers increased.[5]

CONCORDANT CELLULAR OBSERVATIONS ✻ Histologically, a general infiltration with little acanthosis was seen only in the TS cohort. Classical dermatitis and psoriasis characteristics such as spongiosis, acanthosis and parakeratosis

were not observed. Although some of those characteristics were reported in literature after longer treatment, the treatment duration in this study of maximally 72h is presumably too short.[5,14] In psoriasis patients, exacerbations after IMQ treatment also occur only after a prolonged period of application (average 9 weeks).[7] In addition, CD11C+, HLA-DR, CD4+ and CD8+ cells infiltrated the dermis, more in the TS cohort than in the non-TS cohort and with no clear difference between 48h and 72h. CD11C+ cells reflect the inflammatory myeloid DCs. These are highly increased in the psoriatic dermis and are known to stimulate the production of type 1 helper (TH1) cytokines [1]. Likewise, the macrophages (HLA-DR) are involved in this process which are mediated via the TLR-7 response.[24-26] Infiltration of DCs, macrophages and T-cells indicate activation of both an innate and adaptive immune response. All histological effects are consistent with the intermediate and late phase response of IMQ. The late phase is characterized by expression of both IL-17 and IL-22 as a result of IL-23 production, and infiltration of T-cells. It normally occurs after 72h[17]. However, due to the enhanced drug delivery by TS of the skin this was already observed in the 48h biopsies.

TRANSLATIONAL VALUE OF THE INFLAMMATION MODEL FOR DRUG DEVELOPMENT ✻ Taken together, the IMQ-induced histological changes are highly similar to those that were observed in the murine model and have features of both psoriasis and contact dermatitis with activation of the innate and adaptive immunesystem. Concordant with findings of others no complete phenotype induction such as psoriasiform histology was observed.[5,14] However, since the purpose of the study was to develop a model for drug profiling and interaction studies, and not primarily to study disease pathophysiology this is not considered a limitation. A limitation of the study is the open-label design which could have lead to an observer bias of the clinical erythema grading. However, since clinical scores are highly concordant with the objective erythema measures, EI and colorimetry, the bias is presumably negligible.

In conclusion, we succesfully translated the murine IMQ skin inflammation model to a fully characterized safe, rapid and reversible human model in healthy volunteers. Clinical and histological phenotypes were fully concordant in the TS+IMQ cohort. Therefore, TS of the skin to enhance drug delivery of IMQ is required to induce a quicker and stronger inflammatory response. No significant differences in effects of IMQ were observed between 48h and 72h of application suggesting that 48h of treatment is the most suitable for this model. Future interaction studies with the model in drug development programs will enable *proof of pharmacology* of novel compounds targeting the innate immune system.

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TABLE 1 Analysis results of erythema and perfusion measurements. The differences between IMQ vs vehicle and TS + IMQ vs. TS + vehicle are shown. Data is presented as mean, 95% confidence interval and p-value.

	IMQ vs vehicle			**TS + IMQ vs TS + vehicle**		
	24h	**48h**	**72h**	**24h**	**48h**	**72h**
Erythema index	9.18 (-1.37, 19.73) p=0.09	11.55 (1.00, 22.10) p=0.03	16.84 (6.29, 27.38) p=0.003	18.64 (7.89, 29.38) p=0.001	29.76 (19.01, 40.50) p=<.0001	36.53 (25.78, 47.28) p=<.0001
Colorimetry	1.16 (-0.34, 2.66) p=0.12	2.16 (0.66, 3.65) p=0.006	3.66 (2.16, 5.16) p=<.0001	3.22 (1.72, 4.73) p=0.0001	5.13 (3.6, 6.64) p=<.0001	5.64 (4.13, 7.15) p=<.0001
Basal Flow (% change)	-3.1% (-13.5%, 8.5%) p=0.57	1.2% (-9.6%, 13.4%) p=0.83	6.6% (-4.9%, 19.4%) p=0.26	21.0% (7.0%, 36.9%) p=0.003	44.0% (28.5%, 61.4%) p=<.0001	38.8% (23.8%, 55.5%) p=<.0001

FIGURE 1A **Treatment schedule of the study**. Treatment area 1 and 2 served as biopsy sites for biopsies after 1 (24h) and 2 (48h) days of treatment. Treatment site 3 and 4 served as sites for all erythema and perfusion measurements and biopsies after the longest treatment duration (3 days, 72h, 5mg imiquimod (IMQ) application at 0h, 24h and 48h). In 8 subjects the local treatment area was tape stripped before IMQ application. All treatments were applied under occlusion by a 12 mm Finn chamber.

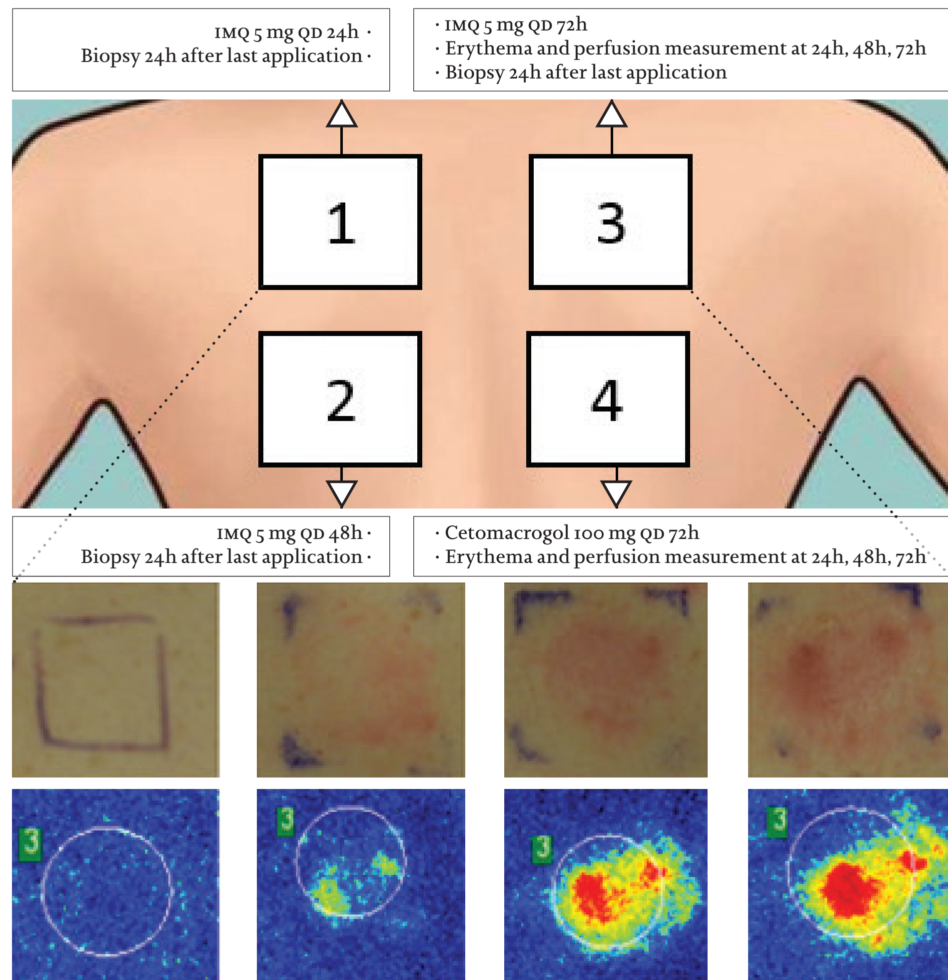


FIGURE 1B **Clinical impression of site 3, tape stripping +IMQ 72h**. An increase of erythema and perfusion is observed.

FIGURE 2 **Erythema and perfusion induced by imiquimod (IMQ) and tape stripping +IMQ application**. Error bars are defined as LSM +/- upper and lower limit. A) LSM change from baseline in erythema index, B) LSM change from baseline in erythema by colorimetry, C) LSM change from baseline in % by laser speckle contrast imaging, D) erythema by visual grading displayed as % presence per time point.

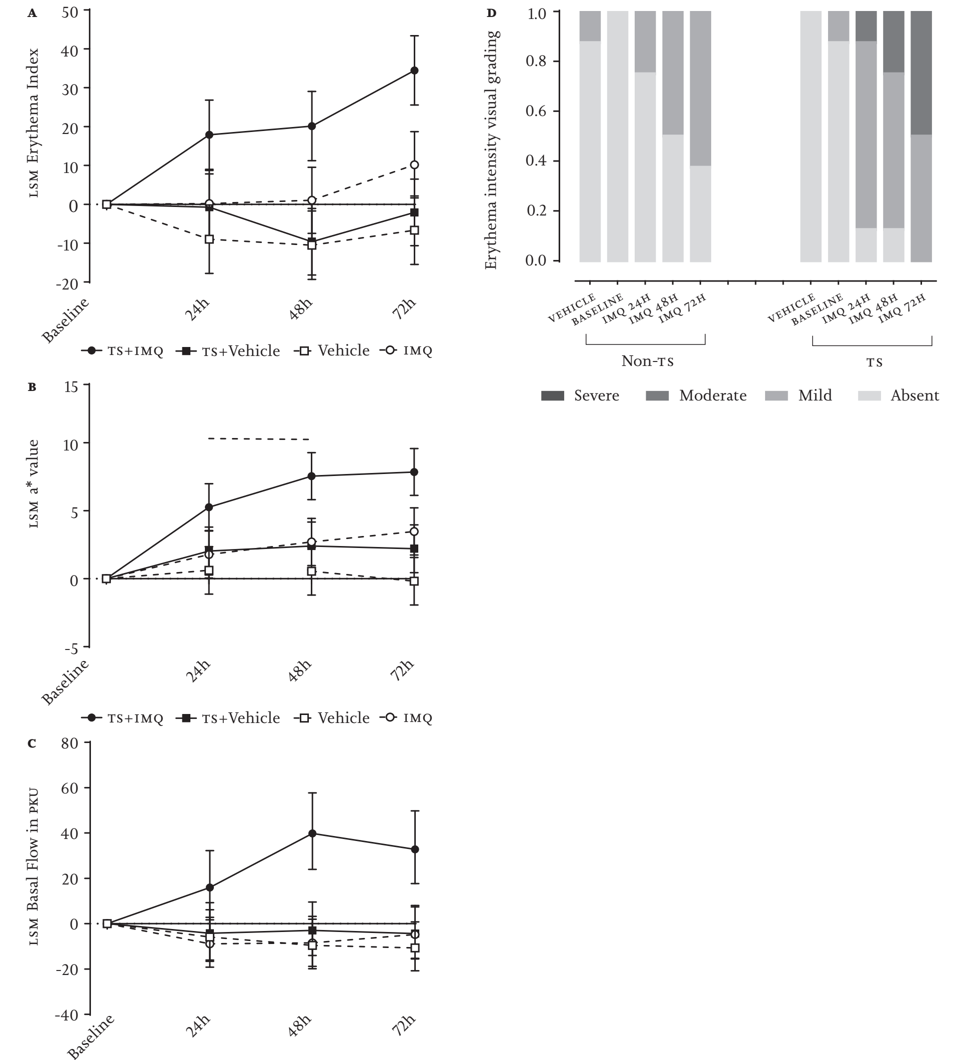
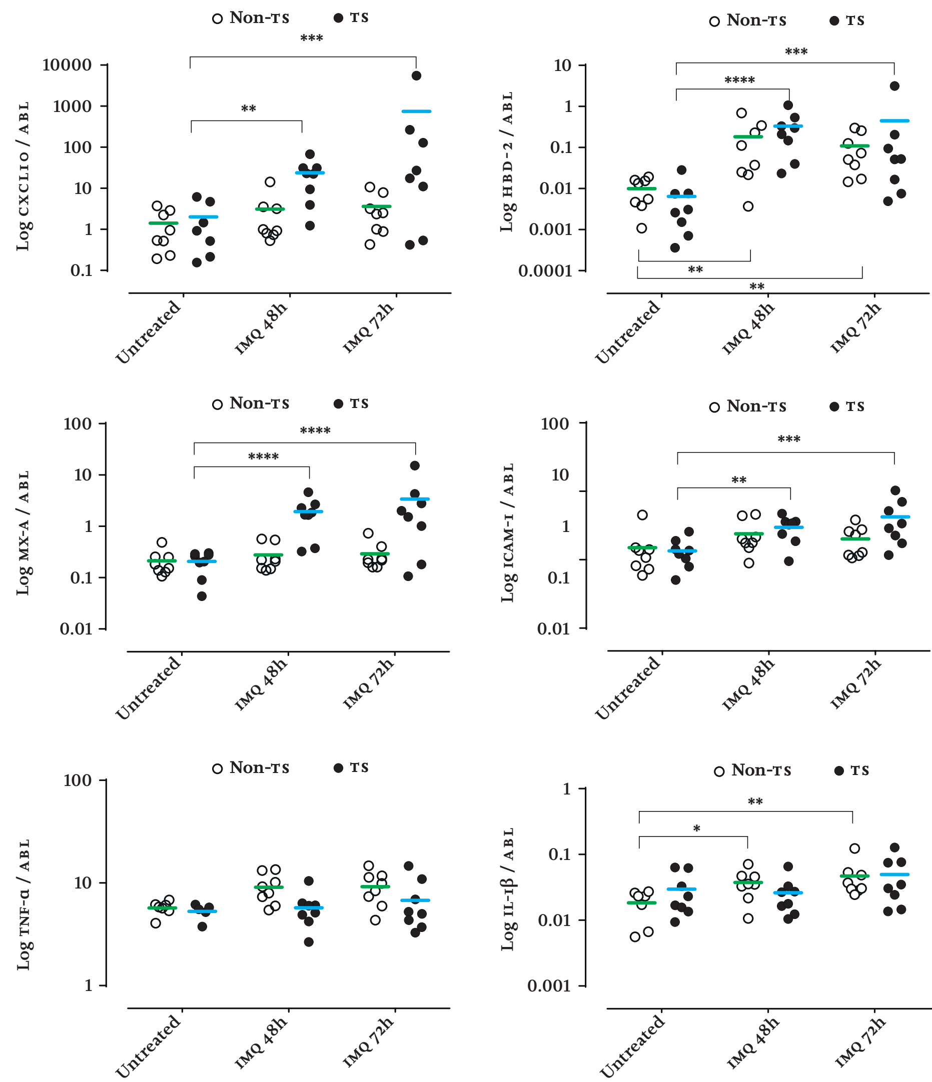
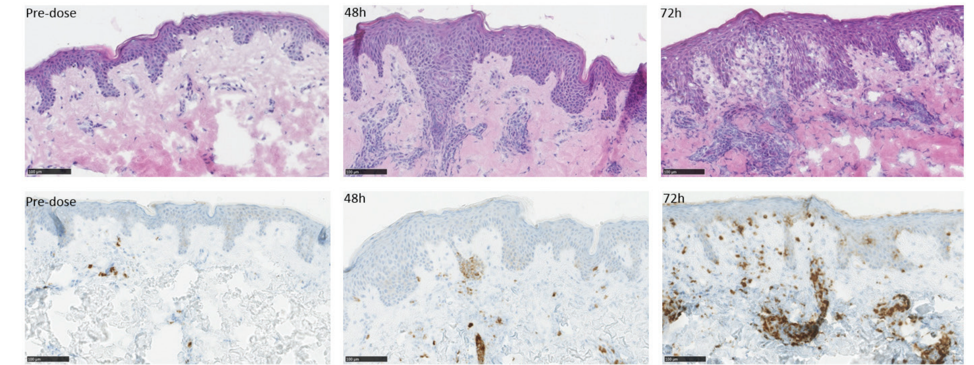


FIGURE 3 mRNA expression in skin over time in imiquimod (IMQ) and tape stripping +IMQ treated skin of CXCL10, HBD-2, MXA, ICAM1, TNF-α and IL-1 β.



*Statistical significance is indicated as follows: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

FIGURE 4 H&E (upper row) and CD8+ staining (lower row) over time of subject 15 treated with TS+IMQ.



Scale bars indicate 100μm.

FIGURE 5 Histology and immunohistochemistry in skin punch biopsies of imiquimod (IMQ) and tape stripping +IMQ treated skin compared to untreated skin, displayed in % fold increase compared to the reference biopsy. A) general infiltration, B) acanthosis, C) CD4+ and CD8+ infiltration, D) CD11C+ and HLA-DR infiltration. Fraction of subjects is depicted.

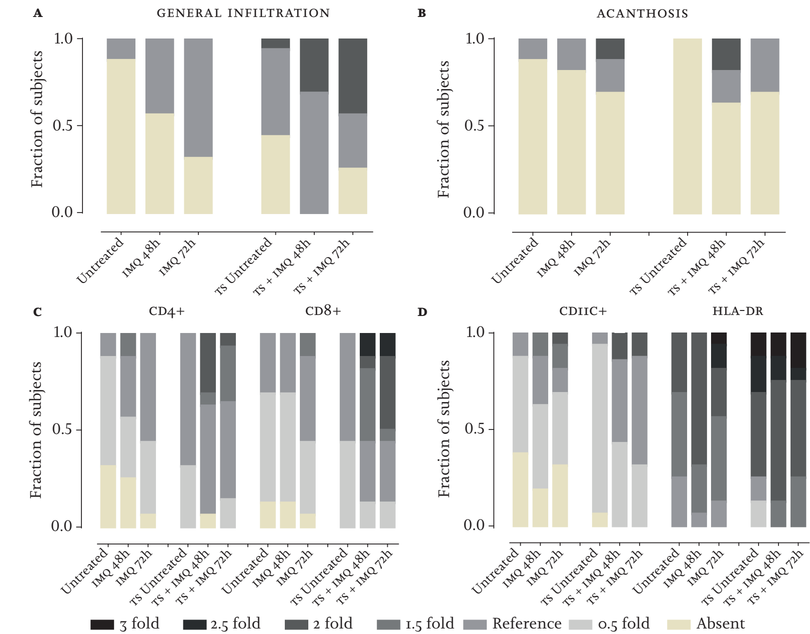


FIGURE 6 **Multi-modal assessment of the model including clinical (physician scoring), biophysical (laser speckle contrast imaging), imaging (colorimetry), molecular (mRNA expression, hBD2) and cellular (IHC, CD8+) aspects.** The observed maximal effect is used for normalization of the respective axes. For the imiquimod alone group, only increased erythema by colorimetry is observed without a dose dependent relationship. In the tape stripping+imiquimod group it is clearly visible that the effects on all domains spread over the spiderplot in a dose dependent manner.

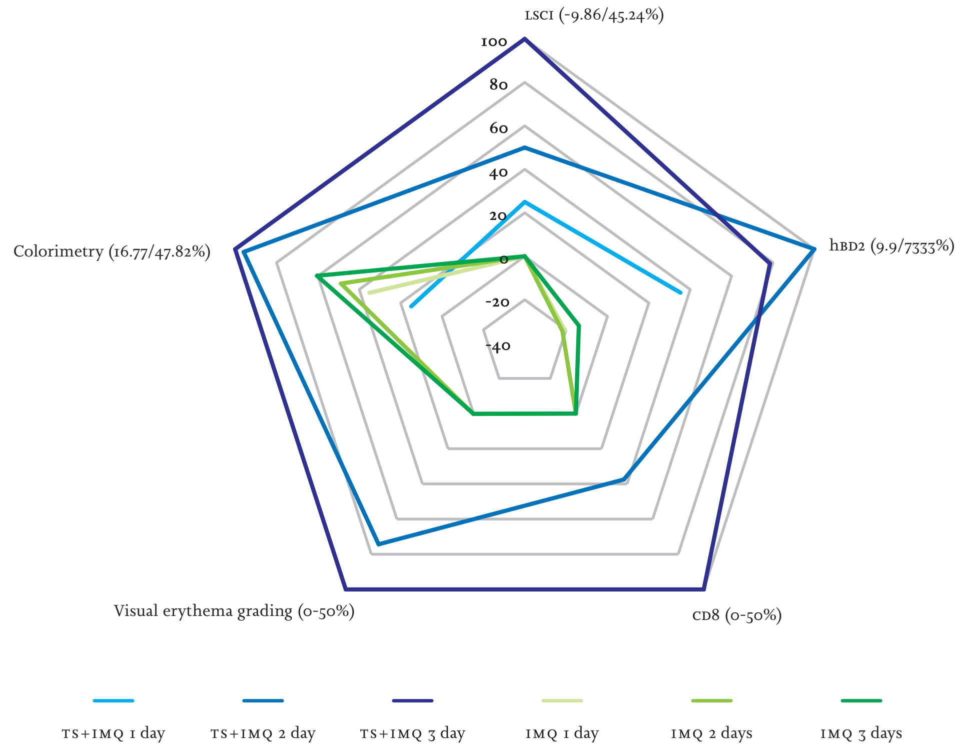
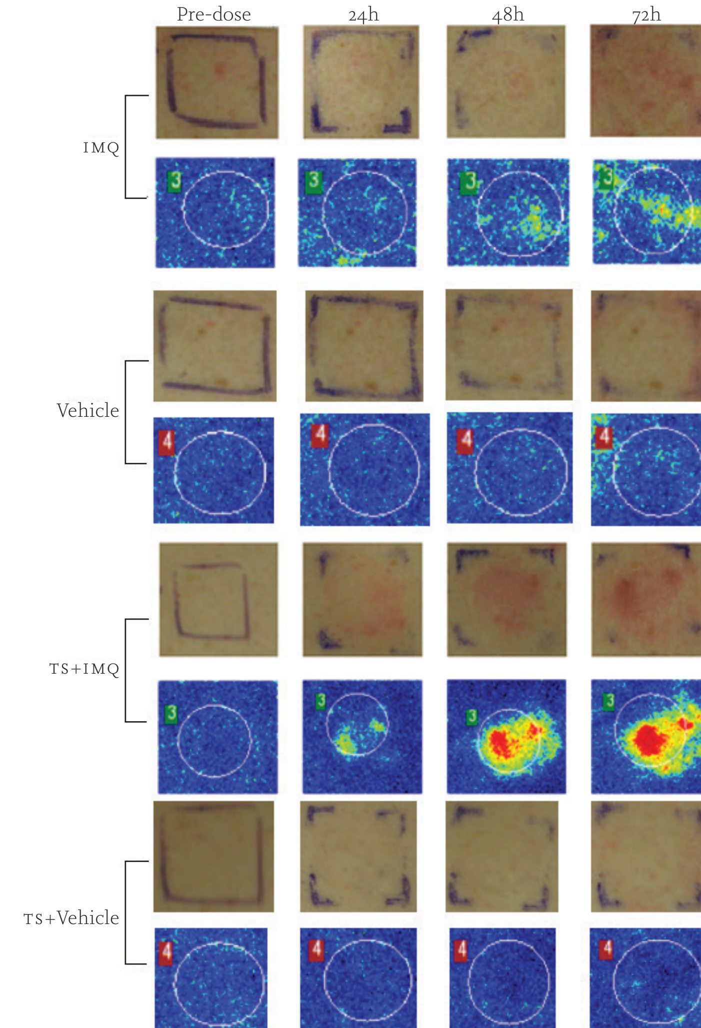


FIGURE S1 **Clinical impression and heat maps of laser speckle contrast imaging of the skin inflammation models in one subject with IMQ treatment and another subject upon TS+IMQ treatment.** Topical application of IMQ induces mild erythema and a slight increase in perfusion. When combined with TS prior to dose administration, moderate erythema with papules and marked increase in perfusion is observed. No increase in erythema and perfusion occurred in the vehicle controls.



CHAPTER III

OMIGANAN ENHANCES IMIQUIMOD-INDUCED INFLAMMATORY RESPONSES IN SKIN OF HEALTHY VOLUNTEERS

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ABSTRACT

Omiganan (omiganan, a synthetic cationic peptide) and imiquimod (imiquimod, a TLR7 agonist) have synergistic effects on interferon responses *in vitro*. The objective of this study was to translate this to a human model for *proof-of-concept*, and to explore the potential of omiganan add-on treatment for viral skin diseases. Sixteen (16) healthy volunteers received topical imiquimod, omiganan or a combination of both for up to 4 days on tape stripped skin. Skin inflammation was quantified by laser speckle contrast imaging and 2D photography, and molecular and cellular responses were analyzed in biopsies. Imiquimod treatment induced an inflammatory response of the skin. Co-treatment with omiganan enhanced this inflammatory response to imiquimod, with increases in perfusion (+17.1%, 95% CI 5.6-30%, $P<0.01$) and erythema (+1.5, 95% CI 0.25-2.83, $P=0.02$). IRF- and NFκβ-driven responses following TLR7 stimulation were enhanced by omiganan (increases in IL-6, IL-10, MXA, and IFNɣ), and more immune cell infiltration was observed (in particular CD4+, CD8+ and CD14+ cells). These findings are in line with the earlier mechanistic *in vitro* data, and support evaluation of imiquimod/omiganan combination therapy in HPV-induced skin diseases.

INTRODUCTION

Cathelicidins are a family of antimicrobial (cationic) peptides that play an important role in the first line immune defence of the skin, related to their broad antimicrobial activity against bacteria, viruses and fungi.[1] LL-37 is the only human member of the cathelicidin family.[1] Besides its antimicrobial effects, this peptide also has direct immunomodulatory activity. LL-37 affects the response of neutrophils to viruses, and modulates interferon (IFN) responses induced by viral triggers.[2] LL-37 converts self-RNA into a ligand for Toll Like Receptor (TLR) 7 and TLR8 in human dendritic cells, thereby enhancing IFNα production in human skin.[3]

Omiganan is a synthetic indolicidin (a cathelicidin isolated from bovine neutrophils), currently under development as topical gel for several clinical indications. Omiganan is known to have activity against a wide variety of microorganisms such as gram-positive and gram-negative bacteria and fungi.[4,5] Moreover, omiganan enhances IFN responses induced by TLR3 (POLY:IC), TLR7 (imiquimod), TLR8 (SSRNA) and TLR9 (CPG) in human immune cells, comparable but not similar to the effects observed for LL-37 (unpublished data, Grievink *et al.*). These observations support the future application of omiganan as co-treatment with endosomal TLR ligands for viral skin disease in humans.

Imiquimod is the only registered endosomal TLR ligand, as Aldara® topical cream. The mechanism of action of imiquimod is based on TLR7-dependent MYD88-signalling.[6,7] This results in two responses: a tumoricidal effect by the release of several pro-inflammatory cytokines (e.g. TNF-α, IL-6 and IL-8, via NFκβ) and an anti-viral response by the induction of IFNα and IFN-inducible genes (e.g. MXI and MXA, via IRF7).[8] Based on these mechanisms imiquimod is widely used in clinical practice for human papilloma virus (HPV)-induced anogenital warts and high grade squamous intraepithelial lesions of the vulva (vulvar HSIL), actinic keratosis (AK), and basal cell carcinoma (BCC).[9] In most of these conditions, drug efficacy is suboptimal, and lesions may reoccur after treatment discontinuation.[10] Therefore, a treatment enhancing the efficacy of imiquimod in these dermatological conditions would be of great benefit. Based on its observed preclinical activity, omiganan may be a good candidate for combination treatment with imiquimod.

We recently developed an *in vivo* challenge model with transient local skin inflammation, induced by 48h imiquimod (Aldara® cream) application under occlusion by a 12mm Finn Chamber to tape stripped skin.[11] This model was used in the current study to explore the potential of combined imiquimod and omiganan treatment as novel therapeutic modality for HPV-induced skin diseases, e.g. genital warts and vulvar HSIL. Omiganan was applied topically to imiquimod-primed skin, and the clinical, biophysical, cellular and molecular responses to this combined treatment were investigated.

METHODS

STUDY DESIGN AND SUBJECTS ✻ This was a randomized, open-label, evaluator-blinded, vehicle controlled, parallel-cohort, dose ranging study. The study was conducted from February 2017 to March 2017 at the Centre for Human Drug Research, Leiden, The Netherlands, and was approved by the independent Medical Ethics Committee 'Medisch Ethische Toetsingscommissie van de Stichting Beoordeling Ethiek Biomedisch Onderzoek' (Assen, the Netherlands). The study was conducted according to the Dutch Act on Medical Research involving Human Subjects (WMO). Before study procedures started, all subjects gave informed consent. Sixteen (16) healthy male and female Caucasian (Fitzpatrick skin type I-II) volunteers, aged 18 to 45 years, were included. Subjects with a (family) history of psoriasis or any disease associated with immune system impairment were excluded.

TREATMENTS AND RANDOMIZATION ❋ To explore the effect of omiganan and the combination of omiganan and imiquimod on tape stripped skin, treatment combinations were applied and randomized over different treatment sites on the back (*Table 1*). All 4 treatment combinations were explored in each study participant. A standard daily dosage containing either 100 mg Aldara® 5% (5 mg imiquimod, IMQ), 100 mg omiganan 1% (1 mg omiganan, OMN), 100 mg omiganan 2.5%, 100 mg omiganan vehicle (VehO) or cetomacrogol (which served as imiquimod vehicle, VehI) was applied under occlusion by a 12 mm Finn chamber (Smart Practice, Phoenix, U.S.A.). The tape stripping procedure included 20 times stripping with tape (D-Squame, CuDerm, Dallas, US) to induce mild barrier disruption.

It should be noted that within the same clinical study, alternative regimens and control conditions were explored, within the same group of 16 volunteers. These additional conditions included the reverse treatment sequence (first imiquimod, then omiganan) and partial control groups vehicle/imiquimod or vehicle/omiganan (1% or 2.5%). To increase the readability of this manuscript, it was decided to not present data related to these conditions.

SKIN ASSESSMENTS ❋ The skin was assessed daily for 5 days for signs of inflammation (erythema and hyper perfusion) by 2D photography erythema index analysis, visual erythema grading (Clinician Erythema Assessment (CEA) scale; 0 represents absence of erythema, 4 very severe), colorimetry (a value; DSM II ColorMeter, Cortex Technology, Hadslund, Denmark), and perfusion by laser speckle contrast imaging (LSCI; PeriCam PSI System, Perimed Jäfälla, Sweden). TAP (FibroTx, Estonia) were used to quantify skin surface biomarkers (IL-8, IFNα, IL-6, IL-10, CCL20 and HBD-2) by spot-ELISA at pre-dose and after end-of-treatment. Skin swabs were collected for microbiome analysis.

Three-millimetre punch biopsies were collected pre-dose (after tape stripping) and at end-of-treatment. For all 16 subjects, a biopsy of the VehO+VehI, IMQ+OMN1% and IMQ+OMN2.5% treated areas was collected. For only 8 subjects the IMQ+VehO treated area was biopsied, to limit the number of biopsies per subject. Biopsies were snap frozen using liquid nitrogen and stored at -80°C until analysis at the Immunology Laboratory of Erasmus Medical Center, Rotterdam, The Netherlands for determination of IFNα, IFN-γ, IL-1β, IL-6, IL-8, HBD-2, MX1, MXA, CCL20 and IL-10 mRNA expression relative to the housekeeping gene ABL by quantitative PCR. In addition, all biopsies were haematoxylin and eosin (H&E) stained to obtain histopathological scores of psoriasis and dermatitis; general infiltration, parakeratosis, acanthosis, papillomatosis and spongiosis. The histopathological score for each characteristic was graded based on fold increase or decrease compared to a reference biopsy of a healthy subject not participating in the clinical trial (1; equal to the reference biopsy, 2; 2-fold increase compared to the reference biopsy etc.). Furthermore, immunohistochemical staining was performed to obtain scoring of markers CD11C (Clone 5D11, Cell Marque), CD14 (Clone EPR3653, Cell Marque), CD1a (Clone EP3622, Cell Marque), CD4 (Clone SP35, Ventana), CD8 (Clone SP57, Ventana) and HLA-DR (CR3/43, Dako).

SAFETY ENDPOINTS ❋ Safety and tolerability were monitored by tracking adverse events, performing physical examination, measuring vital signs, 12-lead electrocardiograms, and laboratory tests (i.e. hematology, chemistry and urinalysis) at multiple time points throughout the study. IFNα, IFN-β and IFNγ were measured in blood samples to detect a possible systemic effect of the interventions.

STATISTICS ❋ Treatment effects were analysed with a mixed model analysis of variance with the baseline measurement as covariate. To determine the differences between the treatments, contrasts were calculated for all measurements. All calculations were performed using SAS for windows V9.4 (SAS Institute, Inc., Cary, NC, USA). Evaluation window for non-invasive measures was 0-96 hours (day 4), whereas biopsies were collected at 120 hours (day 5).

RESULTS

12 female (75%) and 4 male (25%) Caucasian subjects participated in the study. All 16 included subjects completed the study according to the schedule in *Table 1*. The mean age was 24.6 (SD ±5.8 years). Application site pruritus was the most frequent occurring Adverse Event (AE) in 14/16 subjects (87.5%). This can be related to the tape stripping procedure, occlusion procedure or one of the treatments or vehicles. No serious adverse events (SAEs) or discontinuations due to AEs occurred. No systemic effects of any of the treatment in terms of elevated circulating cytokines (serum IFNα, IFN-β of IFNγ) were observed (data not shown).

Imiquimod treatment resulted in a modest inflammatory response, observed as enhanced erythema (quantified by 2D photograph, Figure 1 top panel) and perfusion (quantified by laser speckle contrast imaging, Figure 1 bottom panel). The maximal imiquimod response was reached after 1-2 days treatment (*Figure 2*). After 48 hours of imiquimod/vehicle exposure, the target areas were treated with omiganan (or vehicle) for an additional 2 days. Omiganan treatment enhanced

the imiquimod-driven increase in skin perfusion and erythema, without an indication of omiganan dose-dependency (*Figure* 2). Omiganan treatment significantly enhanced perfusion (profile 0-96h) (*Figure* 2A: imiquimod+vehicle versus imiquimod+omiganan; +17.1%, 95% CI 5.6-30%, p<0.01 and +15.1%, 95% CI 3.8-27.7%, p<0.01, for 1% and 2.5% omiganan, respectively). For erythema, a statistically significant omiganan effect was observed (profile 0-96h;for colorimetry, but only at the 1% omiganan dose (*Figure* 2b: imiquimod+vehicle versus imiquimod+omiganan +1.5, 95% CI 0.25-2.83, p=0.02 and +0.92, 95% CI 0.37-2.21, p=0.16, for 1% and 2.5% omiganan, respectively). Omiganan treatment did not significantly alter imiquimod-related increases in erythema index (profile 0-96h; +0.8, 95% CI -1.62-3.25, p=0.51 and +2.21, 95% CI -0.23-4.64, p=0.08 for 1% and 2.5% omiganan, respectively). The enhanced inflammatory responses were observed during the omiganan treatment period (day 3 and 4, 48-96h). Hereafter, perfusion and erythema returned within one day to levels as observed for the imiquimod + vehicle treatment within one day (*Figure* 2, 120h).

In addition to the above non-invasive assessments, skin punch biopsies were taken from the target areas. Biopsies were stained for dermal immune cell infiltration, and independently analyzed by two investigators blinded to treatment compared to a reference biopsy (healthy unaffected skin). Imiquimod treatment resulted in an influx of immune cells in the skin, reflected by an increase in macrophages, HLA-DR cells, myeloid dendritic cells, Langerhans cells, and CD4+ and CD8+ T cells (*Figure* 3A-F, second bars versus first bars). Consistent with the observations for perfusion and erythema, omiganan treatment enhanced the imiquimod-driven inflammatory response as quantified in skin punch biopsies. When imiquimod-exposed skin was treated with omiganan, this resulted in a strong increase of infiltrating immune cells (*Figure* 3A-F, third and fourth bars versus second bars). There was no indication of a clear omiganan dose-dependency, although the response to the 1% omiganan formulation appeared slightly higher.

Subsequently, the effects of imiquimod and omiganan add-on treatment on local cytokine responses were investigated. As expected, imiquimod treatment resulted in an NFκβ-driven increase in IL-6 and IL-10 (*Figure* 4A, IL-6 imiquimod/vehicle versus vehicle/vehicle +120.9%, 95% CI 2.6%-375.6%, p=0.04, IL-10 imiquimod/vehicle versus vehicle/vehicle +132.1%, 95% CI 40.8%-282.8%, p=0.001). In line with this, imiquimod increased the expression of type I interferon-driven MXA (*Figure* 4B left panel, imiquimod/vehicle versus vehicle/vehicle +213.3%, 95% CI 50.7%-551%, p=0.002) and IFNɣ (*Figure* 4B right panel, imiquimod/vehicle versus vehicle/vehicle +542.4%, 95% CI 132.1%-1678.3%, p<0.001). No treatment effect was observed for MX1 expression. Subsequently, omiganan was applied for

two days to the target areas. Though omiganan did not significantly alter any of the imiquimod-driven responses, a higher level of cytokines was consistently found in the imiquimod/omiganan treatment group when compared with the imiquimod/vehicle treatment group (*Figure* 4A, IL-6 and IL-10: for 1% omiganan +26.3%, 95% CI -41.6%-173.1%, p=0.55, and +36.1%, 95%CI -17.7%-125.1%, p=0.23, for IL-6 and IL-10 respectively; *Figure* 4B: +88.4%, 95% CI -9.4%-291.5%, p=0.09, and +44.4%, 95%CI -48.1%-302.4%, p=0.48, for MXA and IFNɣ, respectively). Overall, the response induced by 1% omiganan was more outspoken than the response to 2.5% omiganan. IL-8 was induced by imiquimod but no enhancement was seen with omiganan addition (data not shown). No effects of imiquimod and omiganan add-on treatment were observed for the skin surface biomarkers by transdermal analysis patch (TAP), or on skin microbiome (data not shown).

DISCUSSION

In human peripheral blood mononuclear cells, omiganan enhances inflammatory responses driven by endosomal TLRS (unpublished data, Grievink *et al.*). Omiganan strongly increased type I IFN responses when cells were incubated with ligands for TLR3 (Poly:IC), TLR7 (imiquimod), TLR8 (SSRNA) or TLR9 (CPG). IRF (interferon regulatory factor) and NFκβ pathways, induced by these endosomal TLRS, drive tumoricidal and antiviral responses. Therefore, enhancement of endosomal TLR signalling in the skin may be of therapeutic interest for a variety of pathophysiological conditions. To investigate the clinical translation of omiganan's enhancement of endosomal TLR signalling, a healthy volunteer study was designed exploring the effects of imiquimod combined with omiganan add-on treatment. This combination was well tolerated by the study participants, the main adverse event being mild application site pruritus which was equal to the imiquimod alone and omiganan alone treatment groups. The clinical skin response was evaluated with laser speckle contrast imaging (perfusion) and erythema assessments (colorimetry, erythema, and visual grading by the physician). Two days of imiquimod treatment induced an inflammatory response similar as previously described,[11] with erythema, increased perfusion and increased inflammatory cell infiltration on histopathology lasting for at least 5 days. This effect was enhanced when imiquimod was combined with omiganan treatment. The influx of immune cells coincided with an increased cytokine response. Imiquimod induces an inflammatory response via TLR7-driven IRF and NFκβ signaling (Guiducci et al, 2009), which plays a role in a variety of dermal cells (T cells, keratinocytes, macrophages, Langerhans cells, dendritic cells). In this study, omiganan treatment

increased the imiquimod-driven production of IL-6 and IL-10, reflecting NFκβ activity. Also IRF-driven pathways were enhanced: after application of omiganan, elevated expression levels MXA were observed. MXA is a downstream mediator of interferons; its expression indicates an IFNα response.[12] Moreover, omiganan treatment increased type II interferon (IFNɣ) levels, which is mainly produced by T cells. Importantly, cellular and molecular responses were quantified in skin biopsies collected at day 5 (120 hours), where omiganan (or vehicle) was applied at 0, 24, 48 and 72 hours. It could be contemplated that at earlier (uninvestigated) time points, the additive effect of omiganan on immune responses was more outspoken, as observed for laser speckle and 2D photography data at time points 72 en 96 hours.

Our results relate to experimental conditions where skin of healthy human volunteers was first primed with imiquimod, and subsequently treated with omiganan. The reverse sequence was also studied, with omiganan pretreatment for 2 days followed by 2 days application of imiquimod. With this treatment sequence, the enhenced effects of omiganan on imiquimod responses were not observed (data not shown). This is in line with mechanistic in vitro experiments on human PBMCs, which suggest that coinciding exposure to omiganan and endosomal TLR ligands result in the strongest immune response (unpublished data, Grievink *et al.*). Furthermore, omiganan treatment alone did not induce any clinical, molecular or cellular immune response (data not shown), which also corroborates with earlier PBMC-based experiments. It is hypothesized that the immune enhancing effects of omiganan on endosomal TLR signaling requires a complex formation between the cationic peptide and the TLR ligand. Such complex formation has been demonstrated earlier, for example between TLR9 ligand CPG and the bovine host defense peptide indolicidin, thereby enhancing innate and adaptive immune responses.[13]

The potentiating effect of omiganan on imiquimod induced responses, and potentially on the effect of other endosomal TLR ligands that are currently under development as immunostimulatory compounds, may be interesting from a drug development perspective. The effectiveness of imiquimod treatment for HPV-induced skin disease is suboptimal. In anogenital warts for example, the estimated complete clearance is approximately 50%, with a recurrence rate of 13-19%. For HSIL, effectiveness of imiquimod is estimated to be 58% with a 16% recurrence rate (10, 14-16). These data underline the need for enhanced treatment modalities. The combination treatment of imiquimod with omiganan may be considered as such. Although omiganan's effect size on top of imiquimod-induced responses was relatively small in our study, and no clear dose-dependency for omiganan was observed, our findings support the mechanistic concept of omiganan-dependent enhancement of endosomal TLR signaling. Thus, optimization of combined omiganan/imiquimod treatment appears to be a rational way forward.

For practical reasons, imiquimod and omiganan could only be administered as alternating treatments. Since a plausible mechanistic basis for omiganan-enhanced imiquimod effects is the complex formation between TLR ligand and cationic peptide, it is not likely that pharmaceutical adjustments can be made to increase the desired effects. This may consist of optimization of the formulation containing a mixture of both compounds, or application of treatment regimens with rapid alternation of omiganan and imiquimod. Importantly, the observed enhanced imiquimod responses by omiganan co-treatment also support further exploration of treatments combining omiganan with other endosomal TLR ligands. The limitation is that currently no other endosomal TLR ligands besides imiquimod are available for clinical application in the EU. Rintatolimod, a TLR3 ligand, is only accessible via an Early Access Program for chronic fatigue syndrome. Other interesting candidates for combined treatment with omiganan include resiquimod, a TLR7/8 agonist, or one of the TLR9 agonists that are currently being evaluated in phase III clinical programs.

In summary, omiganan enhanced the inflammatory skin response to imiquimod, as studied in healthy volunteers with laser speckle contrast imaging (perfusion), 2D photography (colorimetry, erythema, visual grading), and analysis of molecular and cellular responses in skin biopsies. *Figure* 5 provides a graphical summary of key biomarkers, and underlines the omiganan-induced increase of imiquimod-driven responses. These findings are in line with the observations of enhanced endosomal TLR responses by omiganan in *in vitro* experiments on primary human immune cells, and are supporting evaluation of imiquimod/omiganan combination therapy in HPV-induced skin diseases such as anogenital warts or HSIL.

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TABLE 1 Treatment combinations.

	DAY 0	DAY 1	DAY 2	DAY 3
1	imiquimod	imiquimod	vehicle (omiganan)	vehicle (omiganan)
2	imiquimod	imiquimod	omiganan 1%	omiganan 1%
3	imiquimod	imiquimod	omiganan 2.5%	omiganan 2.5%
4	vehicle (omiganan)	vehicle (omiganan)	vehicle (imiquimod)	vehicle (imiquimod)

FIGURE 1 Clinical impression of imiquimod (IMQ) response (left panel) and imiquimod + omiganan (OMN, middle and right panel) of one subject at day 4, 24 hours after the last application of omiganan or vehicle.

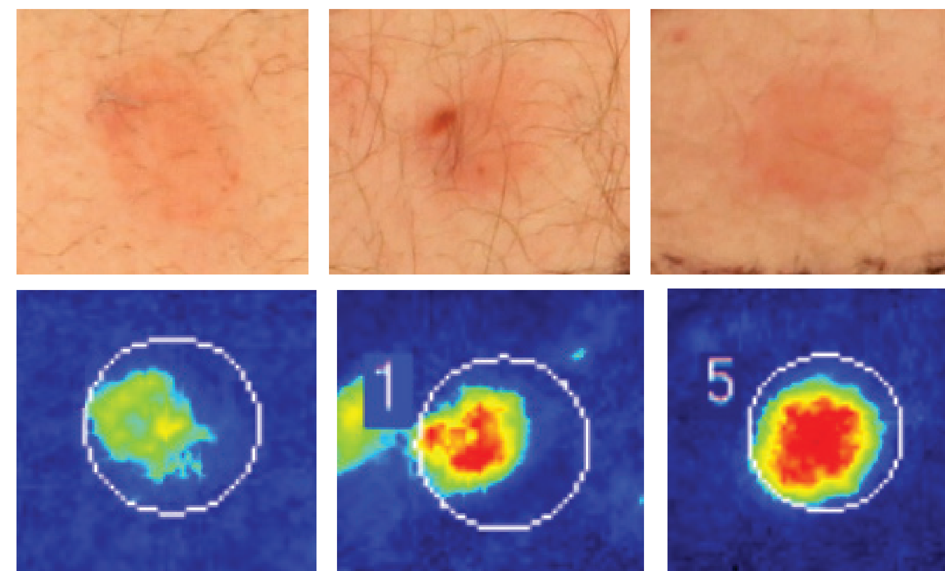


FIGURE 2 Skin inflammation induced by imiquimod (IMQ) and omiganan (OMN), as quantified by LSCI (perfusion/basal flow, A), and erythema assessments (B: colorimetry, C: erythema, D: visual grading).

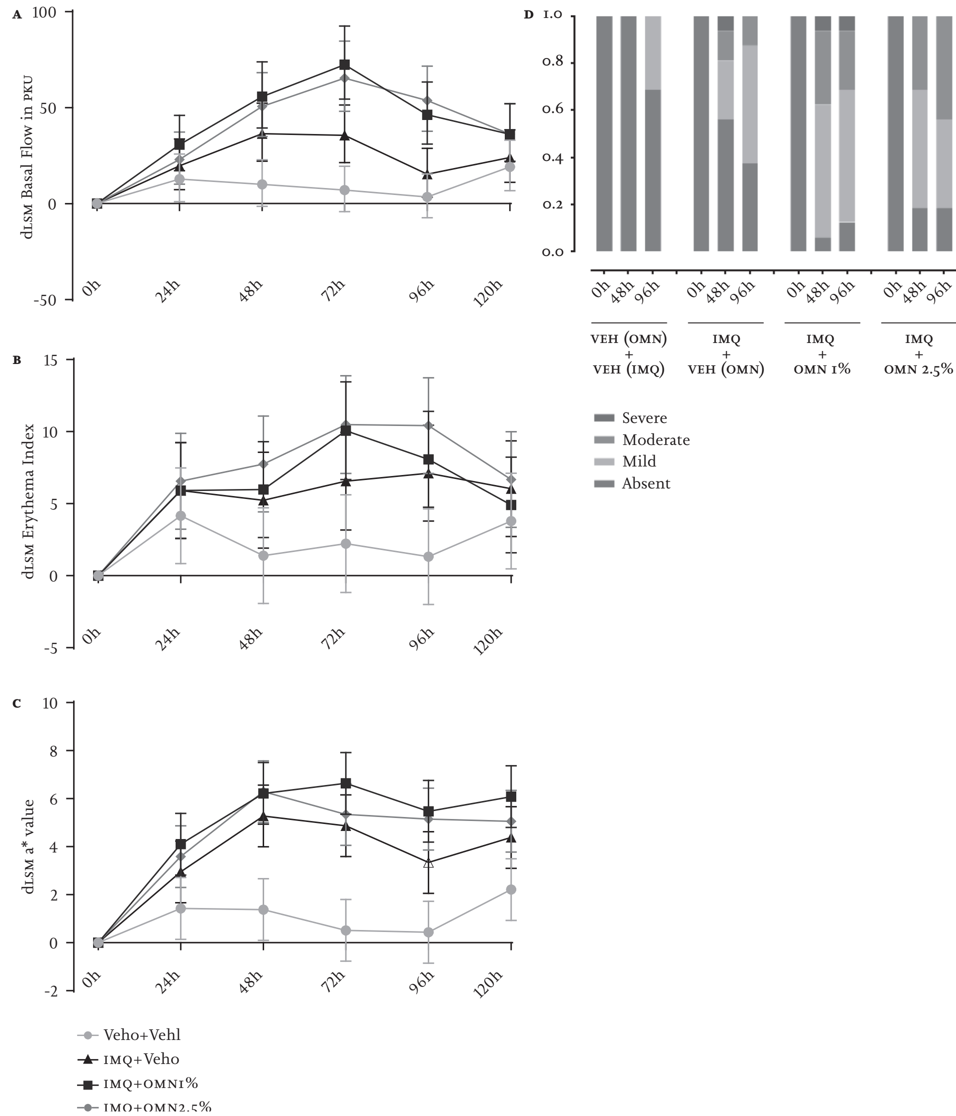


FIGURE 3 Skin inflammation induced by imiquimod (IMQ) and omiganan (OMN) on day 5 (scored compared to a reference biopsy), as quantified by immune cell influx. A: CD14+ macrophages, B: HLA-DR cells, C: CD11C+ myeloid dendritic cells, D: CD1A+ Langerhans cells, E: CD4+ T cells, F: CD8+ T cells.

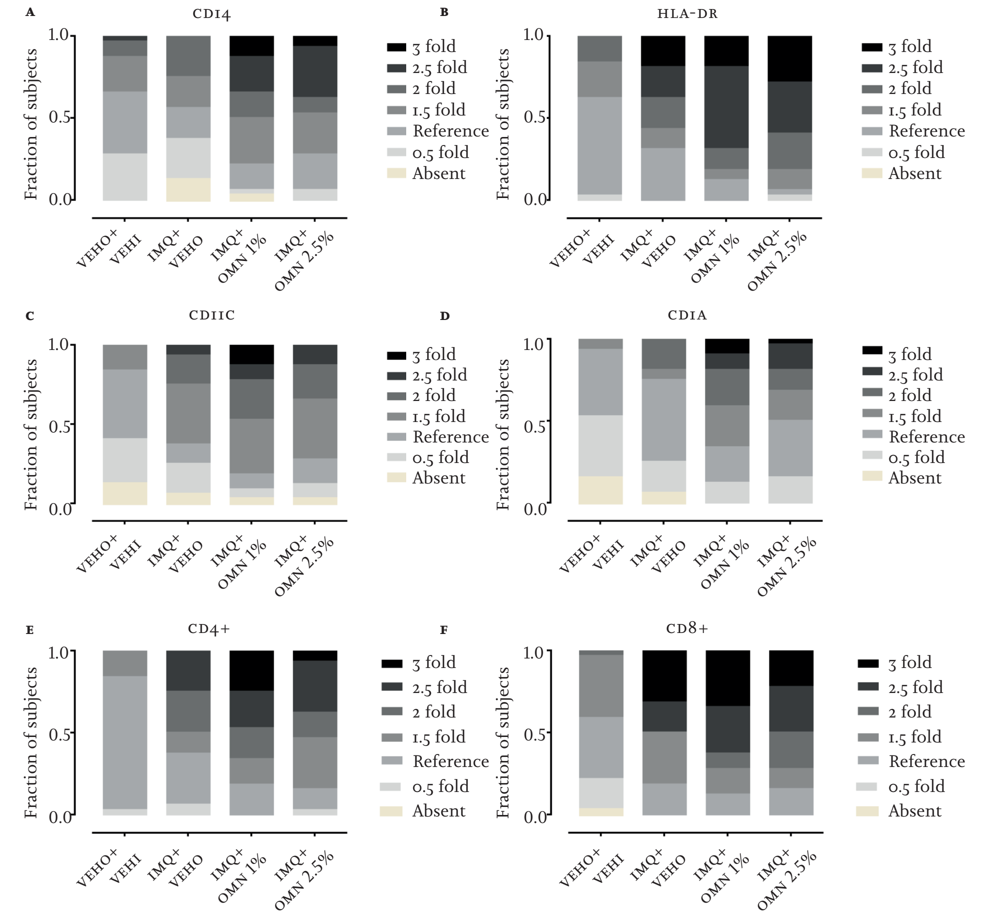


FIGURE 4 Skin inflammation induced by imiquimod (IMQ), omiganan (OMN), vehicle imiquimod (VI) and vehicle omiganan (VO) on day 5, as quantified by cytokine production (qPCR) relative to ABL. A: IL-6 (left panel) and IL-10 (right panel), B: MXA (left panel) and IFNγ (right panel). N=8 for the IMQ+veho contrast and N=16 for the other contrasts.

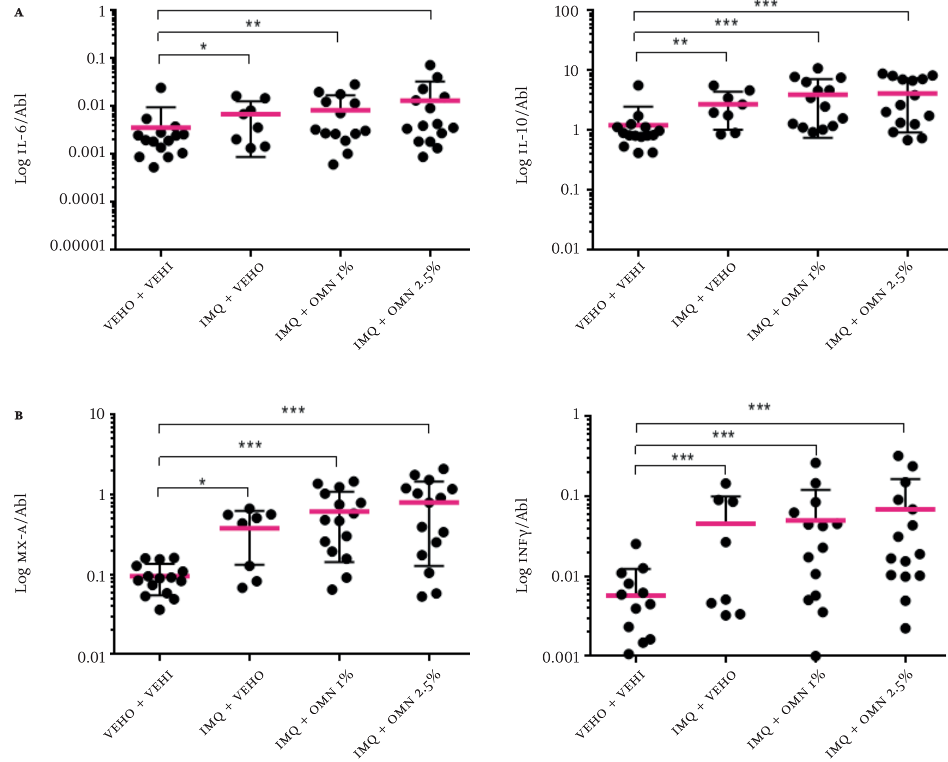
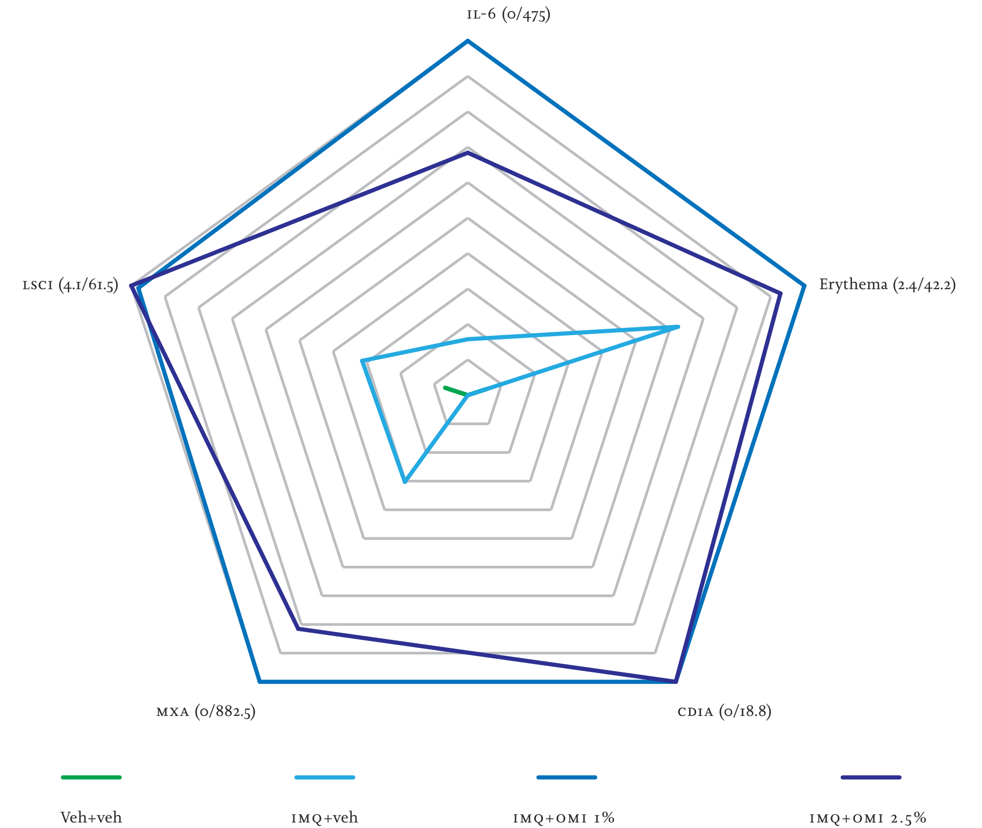


FIGURE 5 Graphical summary of key biomarkers. NF-KB-driven immune response (IL-6), IRF-driven immune response (MXA), perfusion (LSCI), colorimetry (erythema), and immune cell infiltration (CD1a Langerhans cells). Responses were normalized to the maximal effects. Category labels indicate the actual minimum and maximum response.



SECTION II

THE MODULAR DERMATOLOGY TOOLBOX

CHAPTER IV

MOBILE E-DIARY APPLICATION FACILITATES THE MONITORING OF PATIENT-REPORTED OUTCOMES AND A HIGH TREATMENT ADHERENCE FOR CLINICAL TRIALS IN DERMATOLOGY

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ABSTRACT

BACKGROUND Assessment of treatment effects in clinical trials requires valid information on treatment adherence, adverse events and symptoms. Paper-based diaries are often inconvenient and have limited reliability, particularly for outpatient trials.

OBJECTIVES To investigate the utility of an electronic diary (e-diary) application for patients with skin diseases in outpatient clinical trials.

METHODS An e-diary application was developed and technically validated. Treatment adherence as defined as topical administration by the patient and patient-reported outcomes, i.e. pain and itch, were evaluated by the e-diary in six clinical trials on newly tested topical drugs. Additionally, the proportion of patients capturing the applied topical drug by camera and filling in the pain and itch scores as defined as e-diary adherence, patients' perception of usefulness and acceptability of the e-diary were evaluated.

RESULTS Treatment adherence rates of the included 256 patients were high (median 98%, range 97-99%). E-diary adherence was also high with a median of 93% (range 87-97%) for capturing the applied drug by camera, 89% (range 87-96%) and 94% (range 87-96%) for entering respectively the itch and pain score. Daily symptom scores provided good insights in the disease burden and patients rated the e-diary as good to excellent with respect to user acceptability.

CONCLUSIONS The results suggest that the e-diary is an excellent way to ensure proper treatment administration, indicated by both the high user acceptability scores and high treatment adherence. Moreover, the e-diary may also be valuable for frequent and reliable monitoring of patient-reported outcomes in daily clinical practice.

INTRODUCTION

Treatment adherence is the degree to which patients take their medications as prescribed or as instructed by their treating physician[1] and is defined as taking ≥80% of the prescribed medicines.[2-7] It is known that adherence to long-term therapy for chronic illnesses in developed countries is only approximately 50%[5] and adherence to topical treatments is even poorer than oral treatments.[8] To

estimate the clinical efficacy of drugs and to examine new drugs in clinical trials, treatment adherence is of main importance. Safety, pharmacodynamics and efficacy can only be adequately assessed and interpreted if patient data on treatment adherence is available. The impact of poor adherence varies across numerous chronic skin disorders.[9,10] For instance, non-adherence to topical regimens leads to increased scores on the six area six sign atopic dermatitis (SASSAD) severity scale, indicating the disease severity in patients with atopic dermatitis.[11] For this reason, increasing adherence may even have a larger impact on patient-reported outcomes than the improvement of the treatment itself.[5]

Whereas good insight in the treatment adherence and symptoms of the patient is essential, patient-reported outcome measures are often recorded during visits and by use of paper diaries. This requires a good memory of the patient and depends on translation by the doctor / researcher which can both lead to erroneous interpretation and over or underreporting of medication use or symptoms. Paper diaries have a high recall bias [12], a low-to-moderate adherence rate and a limited reliability and are therefore considered as inappropriate to reliably measure treatment adherence.[13-16] Advancements in technology have enabled the widespread use of electronic diaries (e-diaries) for both the monitoring of patient outcomes and the improvement of treatment adherence in clinical trials.[13,17] In 2018, Svendsen *et al.* performed a randomized, controlled trial with a smartphone application for currently used topical treatment in patients with psoriasis and showed an improved short-term treatment adherence of 27% more adherence than the non-intervention group.[3]

The purpose of this study was to investigate the utility of an e-diary in 256 patients with various skin diseases participating in six clinical trials. In this study, treatment adherence and patient-reported outcomes were measured by an e-diary in six clinical trials on newly investigated topical drugs. Additionally, patient perception of usefulness and acceptability of the e-diary was evaluated.

MATERIALS AND METHODS

SUBJECTS AND DESIGN ❋ From December 2014 to March 2018 six randomized, double-blind, placebo-controlled clinical trials were performed including various skin diseases. Two different topical formulations were examined in cutaneous warts (CW), atopic dermatitis (AD), genital warts (GW) and vulvar high-grade squamous intraepithelial lesions (HSIL). The Declaration of Helsinki was the guiding principle for trial execution and all subjects gave informed consent before any procedure. The studies were approved by the Dutch Medical

Ethics Committee ('Stichting Beoordeling Ethiek Biomedisch Onderzoek', Assen, the Netherlands). The clinical efficacy and safety results of these studies have been or will be reported elsewhere.[18-21]

E-DIARY APPLICATION ❃ An IOS application was developed using Xcode 7 and Objective-C according to pre-defined *User Requirement Specifications* and subsequently technically validated using pertaining guidelines (see supplemental Figure). The application was installed on an iPod Touch or iPhone. The patients received oral, paper and digital (in the e-diary) instructions regarding the use of the e-diary. The subjects were instructed to take pictures of the amount of the topical drug applied using the integrated camera. A maximum of four scheduled e-diary notifications were repeated every 30 minutes until the picture was taken. Subjects were instructed to apply the drug daily and to directly answer questions about patient-reported outcomes. Data was saved and securely transferred to the on-site server using encryption the following day.

TREATMENT ADHERENCE ❃ Treatment adherence (i.e. actual administrations divided by the expected administrations) was measured by evaluating whether a patient had applied the topical drug, based on the presence of a picture in the e-diary or if absent (i.e. when for instance a technical issue occurred) after consultation of the patient. Expected entries were based on the number of patients and treatment days and calculated with the formula: number of patients times the amount of entries per day times treatment period in days.

E-DIARY ADHERENCE ❃ E-diary adherence was positive if the e-diary was used as intended, i.e. a picture and symptom scores were entered in the e-diary for one specific day. E-diary adherence was expressed as a percentage and was measured by dividing the total number of actual entries (present pictures and/or NRS scores) by the expected entries in the entire treatment period as defined per protocol.

PATIENT-REPORTED OUTCOMES ❃ Severity ratings of the disease or treatment-related symptoms pain and itch were assessed daily by a numeric rating scale (NRS) in the e-diary. The NRS was selected to assess pain and itch intensity once daily on a scale from 0 to 100 (0: no pain/itch and 100: worst pain/itch possible), if applicable, see *Table 1*. The symptom assessments were used to visualize the course of symptoms during the diseases and only patients who received placebo treatment were included in these analyses.

USER ACCEPTABILITY OF THE E-DIARY ❃ At the end of the treatment period, all patients were asked to fill out a 14-item questionnaire (in Dutch) regarding their experience using the e-diary (Supplemental Information, questionnaire translated to English). The questionnaire consisted of multiple-choice questions and Likert-type scales regarding general user experience, technical aspects of the e-diary and adherence. Two open-ended questions allowed patients to report the strengths and weaknesses of the e-diary and to fill in any comments or suggestions.

DATA ANALYSIS ❃ Descriptive analyses and visualization were performed using IBM SPSS (version 23, IBM Corporation, Armonk, New York, USA) and GraphPad Prism (version 6.05 for Windows, GraphPad Software, La Jolla, California, USA). Adherence was described in percentage and as the median percentage for all studies together.

RESULTS

PATIENT CHARACTERISTICS ❃ The use of the e-diary was evaluated in 256 patients in all treatment arms, including placebo (*Table 1*). The patient population in this study was the sum of patients enrolled and analyzed in the six trials, as there were no patients loss to follow up. Patients included in the trials received financial incentives.

TREATMENT AND E-DIARY ADHERENCE ❃ The overall median treatment adherence, i.e. the proportion of patients applying the topical drug, was 98% (*Table 2*). This was very consistent in the different trials indicated by a narrow range of mean adherence of 97-99%. The median e-diary adherence, i.e. the proportion of patients capturing the applied topical drug by camera, was 93% (range 87%-97%), see *Table 3*. The main reasons for not filling in the e-diary were either technical (empty device battery, no possibility of data entry after midnight) or patients forgot to take the photograph before application of the topical drug. The mean overall adherence of filling in the NRS for itch and pain was 90% for all trials together, see *Table 4*.

PATIENT-REPORTED OUTCOMES ❃ Patients with AD experienced more severe itch with an higher inter-patient variability compared to patients with GW and vulvar HSIL (Fig 1A). The inter-patient variability of pain in the GW and vulvar HSIL trials was also minimal and most patients (10/14 and 2/4, respectively)

experienced no pain (Fig. 1B). When examining the intra-patient variability of itch in the AD patients, there was an extensive variability in itch scores in course of disease during the 4 weeks but also between the morning and evening scores (data not shown). There was a minimal intra-patient variability of pain and itch in the GW and vulvar HSIL trials (data not shown).

USER ACCEPTABILITY OF THE E-DIARY ❋ A total of 249 (97%) patients completed the evaluation form (*Table 5*). In general, the e-diary was rated good to excellent by 89% of the patients and the user-friendliness was experienced as being good to excellent by 94% of the patients. Most patients (84%) reported that it took less than 5 minutes per day to use the e-diary. Of all patients, 67% never experienced any error and 23% of the patients reported a technical problem once or twice, i.e. empty device battery. In the open-ended questions regarding the strengths and weaknesses of the e-diary, most patients commented that they found the e-diary user friendly mainly because of its simplicity. Some patients experienced problems with filling in the e-diary before midnight and also suggested to consider developing the e-diary also for android-based operating systems.

DISCUSSION

This study is the first to show that a mobile e-diary application enhances the monitoring of patient-reported outcomes and is associated with a high treatment adherence in patients with skin disorders in an outpatient clinical trial setting. Overall, patients appreciated the e-diary and reported that the application was easy to use.

The observed treatment adherence in the current study was high compared to previously reported low adherence rates for topical treatment, i.e. up to 80% of psoriasis patients are classified as non-adherent and also adherence in atopic dermatitis patients is very poor.[5,8,22] However, before we draw convincing conclusions, there are a number of considerations that should be taken into account. At first, patients might have felt more responsible or obliged to be adherent due to a combination of our reminder strategy (i.e. patients received a second reminder when they did not correctly fill in the e-diary) and the financial incentive received. Second, we did not take the efficacy or tolerability of the drug into account, which could have influenced the adherence rate.

An additional limitation of our study is the lack of a head-to-head comparison with a paper diary. However, previous studies have already shown that paper diaries yield a much lower adherence, for instance Stone *et al.* found that the actual adherence of filling in pain scores with a paper diary was only 11% while adherence with an e-diary was as high as 94%.[13]

When interpreting the treatment adherence rates, it is important to additionally consider the trial protocol guidelines and their relation with real world clinical practice. The e-diary adherence in trial 4 (GW) was lowest with 87%, as patients experienced problems when applying the topical drug on a specific calendar day. As demanded by the study protocol of a well-controlled trial the time window for application was set at midnight, which was unfeasible for some patients. Therefore the time window in the study protocol in trial 5 (GW) was extended, which resulted in an improvement of e-diary adherence from 87% to 96%. The e-diary adherence in the trial involving patients with vulvar HSIL was marginally lower (89%) than in other trials, mainly caused by one subject who showed a very low treatment adherence of 30% due to not understanding the e-diary and device. It should be noted that the higher age of this population and lack of experience with mobile applications might have been a limiting factor. This is a clear indication that mobile apps do not provide a one-fits-all solution but that the use of an application needs to be carefully considered per specific age group and additional training may be required.

Altogether, we believe that our results indicate that this mobile e-diary platform can be used for the assessment of safety, efficacy and patient-reported outcomes in clinical trials in the future. We hypothesize that the reminder function of the e-diary does improve treatment adherence of patients in the six trials and can be applied to prevent under- and overdosing of topical treatments, as previously published results indicate that 67%-95% of the patients using topical treatments underdose their medication.[23,24] The e-diary will also enable the monitoring of disease-specific patient-reported outcomes and adverse events and this will support the clinician in daily clinical practice. In research settings, remote visits and monitoring could enhance recruitment and lower the burden for participants.[25] Despite of the promising features of the e-diary platform, mobile apps generally do not provide a one-fits-all solution. We should take notion of the age of future user groups, as our results also demonstrated that older patients experienced difficulties while using the application. Additional training may be required.

In conclusion, this study shows that a mobile e-diary application can be used to remotely monitor patient outcomes and treatment adherence in clinical trials with various skin disorders. Therefore, its use for personalized monitoring in the outpatient setting should be further explored. Further development of e-diaries may improve the collection of real-life patient-reported outcomes and treatment adherence, which may also lead to the improvement of disease outcomes in clinical practice.

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TABLE 1 Clinical characteristics of patients participating in the six clinical trials. Age is shown as mean in years. Sex is described as number of patients. Treatment period is described in weeks. The e-diary was filled in during the entire treatment period.

Trial number	1	2	3	4	5	6
Trial ID (NCT)	02333643	02456480	03091426	02849262	03334240	02596074
Disease	Cutaneous warts	Atopic dermatitis	Atopic dermatitis	Genital warts	Genital warts	Vulvar HSIL
N	80	36	80	24	24	12
Age (SD)	25.8 (10.6)	24.9 (7.8)	24.4 (6.5)	34.4 (11.6)	30.8 (10.6)	49.8 (11.0)
Female	49 (61%)	27 (75%)	44 (55%)	9 (38%)	5 (20.8%)	12 (100%)
Male	31 (39%)	9 (25%)	36 (45%)	15 (63%)	19 (79.2%)	0 (0%)
Treatment	ICVT	Omiganan	Omiganan	Omiganan	ICVT	Omiganan
Dose strength	Digoxin+ furosemide, digoxin, furosemide	1% 2.5%	1% 1.75% 2.5%	2.5%	Digoxin+ furosemide	2.5%
Active: placebo	1:1:1:1	1:1:1	1:1:1:1	2:1	3:1	2:1
Treatment period (weeks)	6	4	4	12	6	12
Regimen treatment	Once daily	Once daily	Twice daily	Once daily	Once daily	Once daily
NRS pain	-	-	-	Once daily	Once daily	Once daily
NRS itch	-	Twice daily	Twice daily	Once daily	Once daily	Once daily

NRS= numeric rating scale, ICVT= ionic contra-viral therapy, HSIL= high-grade squamous intraepithelial lesion

TABLE 2 Treatment adherence.

Trial	Expected admins[1]	Actual admins[2]	Overall treatment adherence[3]	Number of subjects with ≥80% treatment adherence
1 (CW)	3280	3187	97%	79/80 (99%)
2 (AD)	1013	993	98%	35/36 (97%)
3 (AD)	4318	4233	98%	79/80 (99%)
4 (GW)	1960	1942	99%	24/24 (100%)
5 (GW)	1008	998	99%	24/24 (100%)
6 (vulvar HSIL)	1020	1009	99%	12/12 (100%)
Overall mean	12599	12360	98%	253/256 (99%)
Median (range)			98% (97-99%)	100% (97-100%)

1: Expected administrations of study drugs based on number of patients and treatment days (Number of patients x treatment period in days)./2: Actual administrations based on photographs imported via the e-diary and recall of administration asked via mail or phone./3: Treatment adherence is the percentage of actual admins divided by the expected admins./CW= cutaneous warts, AD= atopic dermatitis, GW= genital warts, HSIL= high-grade squamous intraepithelial lesion

TABLE 3 E-diary adherence.

Trial	Expected entries[1]	Actual entries[2]	e-diary adherence[3]	Number of subjects with ≥80% e-diary adherence
1 (CW)	3280	3187	97%	79/80 (99%)
2 (AD)	1013	963	95%	35/36 (97%)
3 (AD)	4318	3958	92%	72/80 (90%)
4 (GW)	1960	1710	87%	17/24 (71%)
5 (GW)	1008	963	96%	23/24 (96%)
6 (vulvar HSIL)	1020	907	89%	11/12 (92%)
Overall mean	12599	11695	93%	237/256 (93%)
Median (range)			93% (87-97%)	94% (71-98%)

1: Expected entries of images in e-diary based on number of patients and treatment days (Number of patients x treatment period in days)/2: Actual entries are the imported images of topical drug amount/3: e-diary treatment adherence is the percentage of actual entries divided by the expected entries/CW= cutaneous warts, AD= atopic dermatitis, GW= genital warts, HSIL= high-grade squamous intraepithelial lesion

TABLE 4. Adherence of NRS of itch and pain. In patients with atopic dermatitis, itch was assessed twice daily.

	Itch			Pain		
Trial	Expected entries[1]	Actual entries[2]	NRS adherence[3]	Expected entries[1]	Actual entries[2]	NRS adherence[3]
2 (AD)	3192	2845	89%	N.A.	N.A.	N.A.
3 (AD)	4480	3909	87%	N.A.	N.A.	N.A.
4 (GW)	2016	1759	87%	2016	1760	87%
5 (GW)	999	962	96%	999	962	96%
6 (vulvar HSIL)	1020	957	94%	1020	957	94%
All studies	11707	10432	89%	4035	3679	91%
Median (range)	2016	1759	89% (87-96%)	1020	962	94% (87-96%)

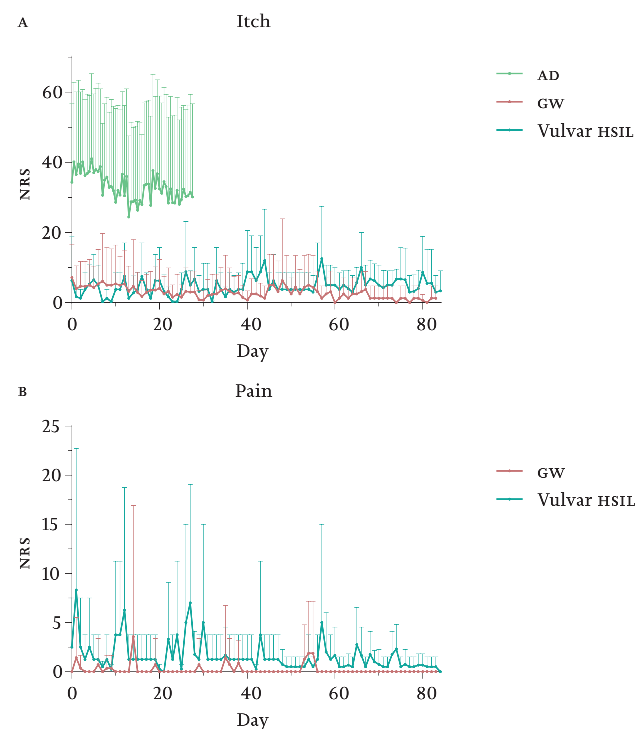
1: Expected entries pain/itch scores based on patients and treatment days (Number of patients x treatment period in days)/ 2: Actual pain/itch scores entered in the e-diary/3: NRS pain/itch adherence is the percentage of actual entries divided by the expected entries/CW= cutaneous warts, AD= atopic dermatitis, GW= genital warts, HSIL= high-grade squamous intraepithelial lesion, N.A.= not applicable

TABLE 5. Evaluation of e-diary.

General user experience		N	%
How user-friendly was the app?	Excellent	108	43%
	Good	126	51%
	Average	11	4%
	Fair	2	1%
	Poor	2	1%
In general, how would you rate the app?	Excellent	63	25%
	Good	159	64%
	Average	20	8%
	Fair	5	2%
	Poor	1	0%
How much time did it take to use the app each day?	1-5 min	209	84%
	5-10 min	37	15%
	10-15 min	2	1%
	15-20 min	0	0%
	>20 min	1	0%
How were the instructions given?	Excellent	130	52%
	Good	110	44%
	Average	9	4%
	Fair	0	0%
	Poor	0	0%
Technical aspects		**N**	**%**
How often did technical problems occur? (iPod, App or Camera)	Never	165	67%
	1-2 times	57	23%
	3-4 times	12	5%
	5-10 times	9	4%
	>10 times	5	1%
How would you rate the photo function of the app?	Excellent	67	27%
	Good	117	47%
	Average	53	21%
	Fair	8	3%
	Poor	2	1%
How would you rate the reminder-function on the app?	Excellent	46	19%
	Good	80	33%
	Average	79	32%
	Fair	39	16%
	Poor	2	1%
Did the reminder function support you to apply the gel on time?	Definitely	105	43%
	Maybe	52	21%
	No	90	36%
Adherence		**N**	**%**
How do you estimate the burden of using the app compared to a paper diary? The app is…	Much less work	146	59%
	Less work	56	23%
	Similar work	15	6%
	More work	8	3%
	Much more work	8	3%
	I do not know	13	5%
What do you prefer to use for subsequent studies?	E-diary	229	93%
	Paper diary	4	2%
	I do not know	13	5%

N= sum of all patients of all studies.

FIGURE 1 Symptoms itch (a) and pain (b) over time as monitored with the e-diary of patients in the placebo group. The symptoms itch and pain are monitored by using a numerical rating scale (NRS) from 0 to 100 (0 no pain/itch and 100 worst pain/itch). Per study day the mean itch of all subjects is shown +SD.



CHAPTER V

A SYSTEMATIC LITERATURE REVIEW OF THE HUMAN SKIN MICROBIOME AS BIOMARKER FOR DERMATOLOGICAL DRUG DEVELOPMENT

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ABSTRACT

AIMS To explore the potential of the skin microbiome as biomarker in six dermatological conditions i.e. atopic dermatitis (AD), acne vulgaris (AV), psoriasis vulgaris (PV), hidradenitis suppurativa (HS), seborrheic dermatitis/pityriasis capitis (SD/PC) and ulcus cruris (UC).

METHODS A systematic literature review was conducted according to the PRISMA guidelines. Two investigators independently reviewed the included studies and ranked the suitability microbiome implementation for early phase clinical studies in an adapted GRADE method.

RESULTS In total 841 papers were identified and after screening of titles and abstracts for eligibility we identified 42 manuscripts that could be included in the review. Eleven studies were included for AD, 5 for AV, 10 for PV, 2 for HS, 4 for SD and 10 for UC. For AD and AV, multiple studies report the relationship between the skin microbiome, disease severity and clinical response to treatment. This is currently lacking for the remaining conditions.

CONCLUSION For two indications, i.e. AD and AV, there is preliminary evidence to support implementation of the skin microbiome as biomarkers in early phase clinical trials. For PV, UC, SD and HS there is insufficient evidence from the literature. More microbiome-directed prospective studies studying the effect of current treatments on the microbiome with special attention for patient meta-data, sampling methods and analysis methods are needed to draw more substantial conclusions.

INTRODUCTION

The escalating number of therapeutic candidates in drug development programs require strategies that optimize the process of clinical development. A common approach is the use of biomarkers in clinical trials. A biomarker is defined as a characteristic that can be objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention.[1,2] Clinical biomarkers are thought to reflect disease activity and pathophysiology.[3,4] A useful biomarker in any class has to comply with the following general criteria: I) there must be a consistent response of the biomarker across studies (preferably from different research groups) and drugs from the same mechanistic class, II) the biomarker must respond clearly to therapeutic (not supratherapeutic) doses, III) there must be a clear dose- or concentration-response relationship and IV) there must a plausible relationship between the biomarker, pharmacology of the drug class, and disease pathophysiology.[4] Validated biomarkers are often being used to guide drug development programs from human pharmacology studies, i.e. phase 1 trials, to confirmatory trials, i.e. phase 3 studies.[2] For dermatological diseases the drug developers often rely on clinical efficacy scores, e.g. the Eczema Area and Severity Index (EASI) for atopic dermatitis, Psoriasis Area and Severity Index (PASI) for psoriasis vulgaris and inflammatory lesion count for acne vulgaris or investigator global assessments. However, more objective outcome measures including validated biomarkers would have great added value in this field. One of these potential new biomarkers is the human skin microbiome, which has the potential to monitor disease activity and drug specific (mechanistic) effects.

The human microbiome refers to the combined genomic information of all microbial communities living on or in the human body. Collectively, this encompasses fungi (mycobiota), bacteria (microbiota), viruses, bacteriophage, archaea and protozoa. This, along with the human genome, completes what is now termed the human microbial superorganism.[5] The skin microbiome harbors vast microbial communities living in a range of both physiologically and topographically distinct niches and microenvironments.[6,7] Actinobacteria (52%), Firmicutes (24%), Proteobacteria (17%) and Bacteroidetes (7%) are the four most abundant species identified on the skin.[8] Previous studies have shown that it is not only skin topography which influences microbial colonization, but also a vast range of host specific factors including age and sex, and environmental factors such as occupation, clothing choice, antibiotic use, cosmetics, soaps, environmental temperature, humidity and longitudinal and/or latitudinal variation in UV exposure, which can all contribute to the variability seen in the microbial flora of the skin.[9–15] Moreover, changes or aberrations in the skin microbiome have been implicated in the pathophysiology of numerous skin diseases such as atopic dermatitis (AD) and acne vulgaris (AV).[16]

Several reviews have described the role and impact of skin microbiome on disease.[17–22] However, to date, no structured review has been conducted to evaluate the feasibility, suitability and potential use of the skin microbiome as biomarker for early phase clinical drug development. Therefore, we conducted a systemic literature review with pre-defined search terms according to the PRISMA guidelines, with focus on six relevant disorders, i.e. atopic dermatitis (AD), seborrheic dermatitis and pityriasis capitis (dandruff) (SD/PC), acne vulgaris (AV), hidradenitis suppurativa (HS), psoriasis vulgaris (PV) and ulcus cruris / chronic wounds (UC). In

addition, we evaluated and ranked the conditions regarding the potential as clinical biomarker. Lastly, we provided recommendations for prospective microbiome investigations in clinical drug development programs.

METHODS

We followed the 'Preferred Reporting Items for Systematic Reviews and Meta-analysis' (PRISMA).[23] In collaboration with a trained librarian from the Leiden University Medical Centre, a structured electronic literature search was composed, using a combination of two main search criteria: microbiome and the targeted skin condition (i.e. AD, SD/PC, HS, AV, UC and PV). For each search term, all relevant keyword variations were used in conjunction with free text word variations. The search strategy was optimized for all consulted databases, taking into account the differences of the various controlled vocabularies, as well as the differences of database-specific technical variations (e.g. the use of quotation marks). The final search was performed on the 29 September 2017, using bibliographic databases including PubMed (incl. MEDLINE), Embase (OVID-version), Web of Science, Cochrane Library, CENTRAL, Academic Search Premier, and ScienceDirect. Animal-only studies, reviews without original data, non-English studies, and case studies were excluded. Moreover, culture based methods were excluded since the objective of this review was to explore the full microbiome profile and relative abundances compared to other genus as biomarker. The remaining studies were fully reviewed. The overall quality of evidence was rated using pre-defined criteria (group size, type of control, method of sampling, serial sampling available, well defined metadata, analysis method). 'Grading of Recommendations Assessment, Development and Evaluation' (GRADE) guidelines were used as guidance for rating the quality of evidence.[24] This was done by two investigators independently and the final outcome was determined by discussion once discrepancies occurred.

RESULTS

The search resulted in 841 titles. After duplicates were removed, 443 papers were screened for inclusion. Four-hundred-and-one manuscripts were excluded based on the exclusion criteria with mostly culture based studies that were not eligible. The remaining 42 studies were identified as using non-culture based methods to analyze microbiome populations in one of the targeted skin conditions and fully reviewed, *Figure 1*. All 42 were included in the review, the study characteristics can be found in *Table 1*.

PSORIASIS VULGARIS ❋ In 10 studies, the cutaneous microbiome in psoriasis vulgaris patients was investigated, *Table 1*.[25-34] Next to microbiota, these studies have focused also on the mycobiota. An increased diversity in the fungal flora in psoriatic skin lesions, compared to healthy skin was reported by Paulino *et al.*.[25] and Amaya *et al.*[26] No differences in the abundance of specific species was observed. Controversially, a significant dichotomy between the relative abundances of specific *Malassezia* species between healthy skin, and psoriatic skin lesions was found by Takemoto *et al.*.[32] Similar inconsistencies in findings were also observed in those studies assessing the microbiota.[28-31,34]

HIDRADENITIS SUPPURATIVA ❋ To date, only two studies were published that investigated the skin microbiome in HS, *Table 1*.[35,36] Both studies report a significant dysbiosis in HS lesional skin with more abundance of anaerobic genera. Five lesional microbiome types were identified of which type I (*Corynebacterium* species) and type IV (*Porphyromonas* and *Peptoniphilus* species) were most prevalent.[35] *Porphyromonas* was also found as predominantly abundant on lesional skin by Guet-Revillet *et al.*.[36], together with *Prevotella* species. In addition, clinical severity significantly correlated with *Fusobacterium* and *Parvimonas* species variation in this study.

ULCUS CRURIS ❋ The role of the skin microbiome in ulcus cruris was explored in 10 different studies, *Table 1*.[37-46] Current research into UC microbiome, comprises larger, longitudinal studies, compared to those in PV and HS. The skin mycobiota of diabetic foot ulcers was longitudinally assessed and was observed to be highly heterogeneous over time and between subjects while the diversity increased upon antibiotic treatment.[45] There have been similar efforts to reveal correlations between patient metadata, treatment and/or clinical outcomes and the cutaneous microbiome in studies investigating the microbiota in ulcus cruris.[38,42-44,46] Overall, the most common found genus in these studies was *Staphylococcus*, with *S. aureus* the most common species. Ulcer closing in diabetic patients was found positively correlated with higher microbial diversity and relative abundance of *Proteobacteria*, while a relative abundance of *Staphylococcus* was correlated negatively in a study by Gardner *et al.*.[42] Although *Staphylococcus* was consistently reported to be the most common genus, inconsistencies exist regarding other genus that are important in CU.

SEBORRHEIC DERMATITIS AND PITYRIASIS CAPITIS ❋ Four case-control studies investigated the microbiome in SD patients.[47-50] *Table 1*. In general,

Malassezia spp. were found to be more abundant on dandruff scalp compared to healthy scalp.[47,48,50] In addition to the mycobiota, a dysbiosis in *Staphylococcus* and *Propionibacterium* spp. was described in microbiota analysis.[48,50] One of the four studies did not find a general association between *Malassezia spp.* and SD but did find a higher abundance of *M. globate* in severe SD patients.[49]

ACNE VULGARIS ❋ Five studies investigated the skin microbiome in patients with AV, *Table 1*.[51–55] Three (3) were case-control studies and two (2) were small single-center, controlled studies, of whom one was a double-blind, randomized-controlled trial. In general, all case-control studies demonstrated similarly an increased microbial abundance of *P. acnes* in the skin microbiome of patients with AV, compared to healthy.[51–53] In addition, an association between a specific *P. acnes* strains and acne affected skin, and healthy skin respectively was demonstrated.[51,52] Acne improved and *Propionibacterium* abundance decreased after various treatments, together with an increase of microbial diversity in the two controlled studies. Moreover, a positive correlation between *Propionibacterium* abundance and acne severity grade was found.[54,55]

ATOPIC DERMATITIS ❋ The skin microbiome in patients with AD was assessed in 11 studies, *Table 1*.[56–66] A greater proportion of longitudinal studies and 2 completed randomized controlled trials were performed in AD patients. There is general consensus across studies, that skin affected by AD exhibits decreased bacterial diversity, as a result of an increased abundance of *S. aureus*.[60–64,66] In particular, AD flare ups were associated with an increased proportion of *Staphyloccocus* sequences, and *S. aureus* abundance correlated with disease severity.[67] In line with these results, microbial diversity in AD lesions was inversely correlated with overall eczema severity as observed by the EASI,[63,] with several further studies also reporting taxonomic normalization and increased bacterial diversity in AD lesional skin, following various treatments.[60,61,63,66]

DISCUSSION

This systematic review provides an overview of the clinical studies that have investigated non-culture skin microbiome associated outcomes in AD, SD, AV, HS, PV and UC with the goal to explore its potential as biomarker in early phase clinical drug development with drug specific or disease specific application, as also referred to as type 3 or type 6 biomarker according to the classic definition of Danhof *et al.*.[68]

POTENTIAL FOR MICROBIOME AS BIOMARKER: ATOPIC DERMATITIS AND ACNE VULGARIS ❋ From our analysis there is some preliminary evidence that the skin microbiota may be a suitable disease specific biomarker for clinical trials of AD. This is due to the correlation between *Staphylococcus* abundance, microbiome diversity profile and disease severity that seems to exist in multiple trials, therewith complying with most of the criteria for a useful biomarker, *Table 2*.[4] Objective data on the change of the microbiota may be valuable to support subjective AD efficacy scores in early phase clinical trials. However, it must be noted that the cause and effect relationship between skin microbiota dysbiosis and AD remains incompletely elucidated.[69] Currently no evidence of benefit of antimicrobial interventions directed at reduction of staphylococcus in patients with AD exists, only in secondarily impetiginized AD.[70–72] As multiple studies included in this review indicate that the skin microbiota within an individual patient varies over time,[60,61,63,64,] there is need for longitudinal, frequent sampling and standard analysis studies. Nevertheless, it has proven its potential value and is recommended to apply in AD clinical trials, in particular when microbiota can serve also as drug-specific biomarker, i.e. for drugs with antimicrobial activity such as antimicrobial peptides that are currently in clinical trials for AD.

In AV, a strong, positive correlation between *Propionibacterium* and acne severity grade is reported.[55] Moreover, acne improved and *Propionibacterium* decreased after treatment, while the microbial diversity increased.[54,55] Taken into account that a clear pathophysiological role of *P. acnes* exists and antimicrobial interventions are effective in AV,[73,74,] the adoption of the skin microbiome as biomarker in acne drug development programs is, although still in its infancy, suggested by our review, *Table 2*. Lesion clearance often takes a long time, therefore the inclusion of microbiota is a valid option to monitor subclinical treatment effects and restoration of normal bacterial profile, i.e. rebiosis. Although a small uncertainty remains regarding the exact relationship between aberrations in the skin microbiome and acne,[75,] we conclude that there is definitely a potential for the microbiota as biomarker in clinical trials (*Table 2*). Another option would be to culture *P. acnes* instead of profiling the whole skin microbiota in a clinical trials, however with this approach a comprehensive overview and insight in the diversity will be missed.

PSORIASIS VULGARIS, ULCUS CRURIS, HIDRADENITIS AND SEBORRHEIC DERMATITIS ARE LACKING EVIDENCE ❋ Although dysbiosis in psoriasis seems to exist in the micro- as well as the mycobiota, study findings are heterogeneous. Wide variability in study design, sampling methods, controllable factors, and sequencing techniques between groups, in conjunction

with small sample populations, could provide a possible explanation for this. Therefore, no clear recommendations can be made at this time. Future work focusing on serial sampling and longitudinal studying of skin microbiome populations it psoriasis vulgaris patients, may provide information on its potential applicability as biomarker, *Table* 2. From a clinical perspective we know that antimicrobial and antifungal agents are not successful in the treatment of psoriasis, which suggests it is less attractive to explore.[76, 77] However, since immune dysregulation is the key of psoriasis and recent investigations describe the extensive cross talk between the immune system and the microbiome, there may still be potential which should be explored.[78] For UC inconsistencies in study design, sampling methods and the heterogeneity of the disease group also limit the comparability of study findings. There appears to be a relation between certain species, types of ulcers and ulcer duration.[42,46] However, longitudinal studies with frequent standard sampling and standard analysis procedures are necessary to make a recommendation. The finding of dysbiosis in HS skin microbiome mostly regarding anaerobic species that is mostly consistent in two different studies opens up opportunities for the skin microbiome as biomarker in this field, *Table* 2.[35,36] However, future studies will have to confirm this potential. In SD, three different sequencing methods were used in the 3 different studies.[47,49,50] This, together with the small sample populations, single time point sampling and poor study designs, might explain the heterogeneity in findings. Since there is a clear evidence that anti-fungal agents such as ketoconazole are effective in SD,[79,] it is recommended to further explore the skin microbiome's potential in this disease in future clinical trials.

LIMITATIONS AND CONSIDERATIONS ❋ It is important to note that in all included studies, there was a high variability in study design and sampling methods between groups, which makes comparisons of specific findings difficult. Case-control studies (25/42, 60%) dominate research into the skin microbiome and skin disease. Patients are compared with healthy controls, capturing microbial profiles at a particular time, but have little predictive value in determining functionality, looking more at associations, and not causation. The small patient sample sizes across all studies may fail to account for inter-individual differences within the study population. The poorly defined inclusion and exclusion criteria, with certain studies including actively treated patients in their sample population, could also confound potential findings. The standardization of controllable factors to reduce confounders, was not well documented or maybe not performed in most of the included studies. As simple factors including but not limiting of age, ethnicity, environmental factors, soap use, hand-washing and the use of topical (antimicrobial) agents before sampling have been shown to alter microbial skin communities, documentation of this meta-data is essential to draw valid conclusions..[5,8,12,60,61,80–82] Multiple methods were used for skin microbiome sampling across the studies (i.e. swabs, biopsies, tape strips, wound curettes). Interestingly, all have been shown to exhibit a wide variation in biomass yield, microbial profile, human DNA contribution/contamination, sampling depth, and discomfort level for the test subject.[19,62,83–88] In addition to the sampling method, the selection of sampling sites and sampling frequency are important factors that were not always considered in the included studies. Consistent sampling of the same anatomical area of skin in all individuals in study cohorts is essential in order to limit confounders, and allow for the accurate comparison of skin microbiome populations. Moreover, regarding analysis, only consistent use of specific primers to target specific hypervariable V regions, will allow for collation of data and comparison between multiple studies. It is clear that broadly used analysis methods in this review as shown in *Table* 1 count as a limitation for comparison. Taken all the above together, based on the level of evidence it is clear that our recommendations should be made with some caution. A standard approach for skin microbiome study design, collection, storage, processing and analysis as proposed by Kong *et al.*, should be followed in future studies.[17] However, although the list of limitations and sometimes poor evidence might be assessed as a weak recommendation for the inclusion of cutaneous microbiome in dermatological trials, the recent finding that the gut microbiome partially explains the response/non-response to PD-1 immunotherapy in different cancer patients will foster research into microbiome in general.[89,90] In addition, lately also the relation between the gut microbiome in inflammatory bowel disease and response to infliximab was highlighted.[91] In particular, when considering the reports about the role of the gut-skin axis that might influence many diseases including the here investigated skin disorders.[92–94]

CONCLUSION

Only a small number of studies have consistently reported the cutaneous microbiome for skin diseases and chronic wounds. Our findings reveal that for two indications, i.e. AD and AV, there is preliminary evidence to support implementation of the skin microbiome as biomarker in early phase clinical trials. For PV, UC, SD and HS there is insufficient evidence. More standardized microbiome-directed studies studying the effect of current treatments on the microbiome are needed to draw conclusions.

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TABLE 1 Summary table of the studies included in the review.

First author yearref	Study design No. of patients	Sample collection methods	Analysis	Key findings	Weaknesses	Level evidence
PSORIASIS VULGARIS						
Paulino et al. 2006[18]	Case control 3 PV/5 HV	• Sterile swabs • Lesional and non-lesional skin Multiple sampling in one PV and 2 HV	18S rRNA 5.8S rDNA	• *Malassezia* mycobiota substantially different PV vs HV	• Small cohort	Low
Amaya et al. 2007[19]	Case control 22 PV/36 AD/30 HV	• OpSite® transparent adhesive dressings • Lesional and non-lesional skin	5.8S rDNA	• *Malassezia* species detected in overall sites higher in PV and AD compared to HV	• Small cohort • PV patients on treatment • Limited analysis • Different skin site collection PV vs AD & HV	Low
Paulino et al. 2008[20]	Case control 1 PV/1 HV	• Sterile swabs • Lesional and non-lesional skin • Multiple time points	5.8S rDNA	• Mycobiota relatively stable over time • No significant dichotomy between PV and HV	• Small cohort • Limited analysis	Low
Gao et al. 2008[21]	Case control 6 PV/6 HV	• Sterile swabs • Lesional and non-lesional skin	16S rRNA V1-V9	• Firmucutes more abundant in lesional skin PV vs non-lesional skin and HV • Actinobacteria less abundant in lesional skin PV vs non-lesional skin and HV	• Small cohort • No serial sampling	Low
Fahlen et al. 2011[22]	Case control 10 PV/12 HV	• 2mm skin punch biopsies	16S rRNA V3-V4	• Most common phyla in PV and HV: Firmicutis, Proteobacteria, Actinobacteria. • Staphylococci and Propionibacteria were less common in psoriatic lesions	• Small cohort • No serial sampling • Variation in skin sample sites	Low
Alekseyenko et al. 2013[23]	Case control & Prospective longitudinal cohort study CC: 54 PV/37 HV PC: 17 PV/15 HV	• Sterile swabs • Lesional and non-lesional skin • HV matched sites • Multiple sampling	16S rRNA V1-V3	• Most common phyla in PV and HV: Firmicutis, Proteobacteria, Actinobacteria. • Combined relative abundance of *Corynebacterium*, *Streptococcus* and *Staphylococcus* was increased in psoriatic skin, compared to unaffected skin and healthy control skin	• Some patients on active treatment • Mainly severe patients	Low to moderate
Statnikov et al. 2013[24]	Case control 54 PV/37 HV	• Sterile swabs • Lesional and non-lesional skin • HV matched sites	16S rRNA V1-V3 and V3-V5	• Microbiome signatures could be used to diagnose psoriasis	• No serial sampling	Low to moderate
Takemoto et al. 2015[25]	Case control 12 PV/12 HV	• PV: psoriatic scales by tweezer • HV: OpSite® transparent adhesive dressings	26S rRNA D1 - D2	• Psoriatic lesions exhibited significantly greater diversity compared to HV • *M. restricta* levels were significantly higher in psoriatic lesions, compared to healthy controls	• Small cohort • No serial sampling • Only male patients • Different sample method PV and HV	Low

TABLE 1 *(Continuation of previous pages)*

First author yearref	Study design No. of patients	Sample collection methods	Analysis	Key findings	Weaknesses	Level evidence
PSORIASIS VULGARIS						
Salava et al. 2017[26]	Case control 13 PV	• Sterile swabs • Lesional and non-lesional skin	16S rRNA V1-V3	• No significant differences microbial diversity between lesional and non-lesional skin	• Small cohort • No serial sampling • Variation in skin sample sites	Low
Tett et al. 2017[27]	Case control 28 PV	• Sterile swabs • Lesional and non-lesional skin	WMS sequencing	• Plaques at the ear had a significant decrease in microbial diversity, and increase in *Staphylococcus* abundance • At species level, no differences between lesional and non-lesional skin were observed	• Small cohort • No serial sampling • Some patients on active treatment	Low
HIDRADENITIS SUPPURATIVA						
Ring et al. 2017[28]	Case control 30 HS/24 HV	• Biopsies • Lesional and non-lesional skin	16S rRNA V3-V4 18S rDNA V3-V4	• Microbiome in HS significantly different from HV in lesional and non-lesional skin • 5 microbiome types identified • Lesional skin consisted predominantly of *Corynebacterium* species (type I) and *Peptoniphilus* species (type IV) • *Propionibacterium* showed a significant higher abundance in HV	• Small cohort • No serial sampling	Low
Guet-Revillet et al. 2017[29]	Prospective cohort 65 HS	• Sterile swabs • Lesional and non-lesional skin	16S rRNA V1-V2	• Lesional skin consisted predominantly of anaerobes (*Porphyromonas* and *Prevotella* species) • Clinical severity significantly associated with variations in lesional microbiota • Fusobacterium associated with severe HS	• Small cohort	Low
ULCUS CRURIS						
Dowd et al. 2008[30]	Prospective cohort 10 VLU/10 DFU/10 PU	• Debridement samples	16S rRNA V4	• Major populations include of all wound include: *Staphylococcus*, *Pseudomonas*, *Peptoniphilus*, *Enterobacter*, *Strenotrophomonas*, *Finegoldia* and *Serratia* species • Each wound type different profile, dependent on oxygen tolerance of the bacterial population	• Small study • No serial sampling	Low
Price et al. 2009[31]	Prospective cohort 7 DFU/7 NU/3 VLU/3 PSU/4 OTH	• Wound base curette • Multiple time points	16S rRNA V3	• Fastidious anaerobic bacteria of the Clostridiales family XI were the most prevalent bacteria in wounds • Wound microbiota from antibiotic treated patients were significantly different from untreated patients • In diabetic patients, *Streptococcus* was more abundant	• Small study • Sampling time point variable • Patients on wide variety of treatments	Low
Price et al. 2011[32]	Cross-sectional 4 DFU/3 NU/3 VLU/2 OTH	• Wound base curette • Multiple samples taken	16S rRNA V3-V4	• The ten most common genera included *Staphylococcus*, *Pseudomonas*, *Streptococcus*, *Anaerococcus*, *Ralstonia*, *Morganella*, *Porphyromonas*, *Peptoniphilus*, *Janthinobacterium* and *Corynebacterium* • Samples from different sites within individual wounds shared similarities in bacterial community compositions • Samples taken from different wounds were less similar than those taken from different sites within the same wound	• Small cohort • Patients on active treatment • No serial sampling	Low
Rhoads et al. 2012[33]	Cross-sectional 4 DFU/3 NU/3 VLU/2 OTH	• Wound base curette	16S rRNA V1-V3	• The ten most common genera included *Staphylococcus*, *Pseudomonas*, *Streptococcus*, *Anaerococcus*, *Ralstonia*, *Morganella*, *Porphyromonas*, *Peptoniphilus*, *Janthinobacterium* and *Corynebacterium* • Samples from different sites within individual wounds shared similarities in bacterial community compositions • Samples taken from different wounds were less similar than those taken from different sites within the same wound	• Small cohort • Patients on active treatment • No serial sampling	Low
Gjodsbol et al. 2012[34]	Comparative 46 VLU	• Filter paper pad & punch biopsies	16S rRNA V1-V3	• *S. aureus* most found species • Multiple sampling over time lead to identification of additional species • No difference in outcomes different sample techniques	• No controls	Low
Gardner et al. 2013[35]	Cross-sectional 52 DFU	• Sterile swabs	16S rRNA V1-V3	• The most abundant OTU was *Staphylococcus*, with *S. aureus* the most common species • Ulcer closing was positively correlated with number of species level OTUs, higher microbial diversity, relative abundance of Proteobacteria, and negatively correlated with relative abundance of *Staphylococcus* • Ulcer depth was negatively associated with *Staphylococcus* abundance and positively associated with anaerobic bacteria relative abundance	• No serial sampling • No controls	Low

TABLE 1 (Continuation of previous pages)

First author yearref	Study design No. of patients	Sample collection methods	Analysis	Key findings	Weaknesses	Level evidence
ULCUS CRURIS						
Wolcott et al. 2016[37]	Cohort 2963 910 DFU/916 VLU/676 DU/370 PSU	• Sharp debridement at surface wound bed	16S rRNA V1-V3	• Neither patient demographics (age, gender, race, diabetes status) nor wound type influenced the bacterial composition of the chronic wound microbiome • *Staphylococcus* and *Pseudomonas* comprise the most prevalent genera present in the microbiota of chronic wounds, with *S. aureus* and *S. epidermidis* the most predominant species • Chronic wounds are frequently colonized by communalistic and anaerobic bacteria, including coag-negatice *Staphylococcus*, *Corynebacterium*, and *Propionibacterium* species	• Unclear whether patients were on treatment	Low to moderate
Smith et al. 2016[36]	Cohort 20 DFU	• Sterile swabs	16S rRNA V4	• The most commonly detected bacteria in all ulcers were *Peptoniphilus*, *Anaerococcus*, and *Corynebacterium* species • In new ulcers, the most commonly detected bacteria were the above and *Staphylococcus* species • The majority of OTUs residing in both new and recurrent ulcers (>67%) were mostly gram positive cocci (*Staphylococcus*, *Streptococcus*, *Anaerococcus*, *Peptoniphilus* and *Finegoldia* • Lower HbA1c values and shorter duration of diabetes correlated with higher diversity within the ulcer	• Small cohort • No serial sampling • No controls	Low
Kalan et al. 2016[38]	Prospective longitudinal cohort 100 DFU	• Sterile swabs • Multiple time point sampling	ITS1 rRNA	• Fungal microbiome was highly heterogeneous over time and between subjects • Fungal diversity increased with antibiotic administration • The proportion of the phylum Ascomycota were significantly greater at the beginning of the study in wounds that took >8 weeks to heal	• No controls • Most patients on active treatment	Low to moderate
Loesche et al. 2017[39]	Prospective longitudinal cohort 100 DFU	• Sterile swabs • Multiple time point sampling	16S rRNA V1-V3	• The most abundant genus identified was *Staphylococcus*, followed by *Streptococcus*, *Corynebacterium* and *Anaerococcus* • The major OTU attributed to *Staphylococcus* was *S. aureus* • Ulcer microbiota was highly dynamic, with community type transitions occurring approximately every 3.52 weeks • Microbiota community instability was associated with faster healing and improved outcomes • Exposure to systemic antibiotics destabilize wound microbiota, rather than altering overall diversity or relative abundance of specific taxa	• No controls • Most patients on active treatment	Low to moderate
SEBORRHEIC DERMATITIS / PITYRIASIS CAPITIS						
Kuk Park et al. 2012[40]	Case control 4 PC 3 HV	• Sterile swabs	26S rRNA D1-D2	• *P. meleagrinum* and *P. chrusogenum* detected on dandruff scalp • *Malassezia* spp. 2 times more abundant on dandruff scalp	• Small cohort • No serial sampling	Low
Clavaud et al. 2013.[41]	Case-control 29 PC 20 HV	• Sterile swabs • In 20 PC patients lesional and non-lesional sampling	16S 28S-ITS	• *M. restricta* major fungal species on scalp PC and HV • *M. restricta and s. epidermidis* significantly more abundant on PC scalp • *P. acnes* significantly less abundant on PC scalp • *M. restricta/ P. acnes* ratio significantly higher in PC scalp	• Small cohort • No serial sampling	Low
Soares et al. 2015[42]	Case control 9 SD (5 mild, 4 severe) 5 HV	• Sterile swabs • Scalp, forehead chin, shoulder and interface samples	5.8S/ITS2 rDNA	• In general, no association between *Malassezia* mycobiota and SD was found • Higher *m. globosa* abundance was found in non-scalp lesions of severe SD patients	• Small cohort • No serial sampling	Low
Park et al. 2017[43]	Case control 29 SD/28 PC/45 HV	• Sterile swabs • Scalp samples	16S rRNA V4-V5 ITS1 rDNA	• Higher abundance of *Staphylococcus* sp. and *m. restricta*, and lower abundance of *Propionibacterium* associated with scalp disease	• No serial sampling	Low
ACNE VULGARIS						
Bek-Thomsen et al. 2008[44]	Case control 5 AV/3 HV	• Cyanoacrylate biopsy • AV acne lesion face • HV nose area	16S rRNA V1-V9	• Acne skin higher diversity, *p. acnes* and *s. epidermid-* is most common species	• Small cohort • Only moderate to severe patients • No serial sampling • No non-lesional patient sampling	Low
Fitz-Gibbon et al. 2013[45]	Case control 49 AV/52 HV	• Bioré® Deep Cleansing Pore strips • Nose area	16S rRNA V1-V9	• No difference relative abundance *p. acnes* AV and HV. • Association specific *p. acnes* strain and acne.	• Some patients on active treatment • No serial sampling • No non-lesional patient sampling	Low

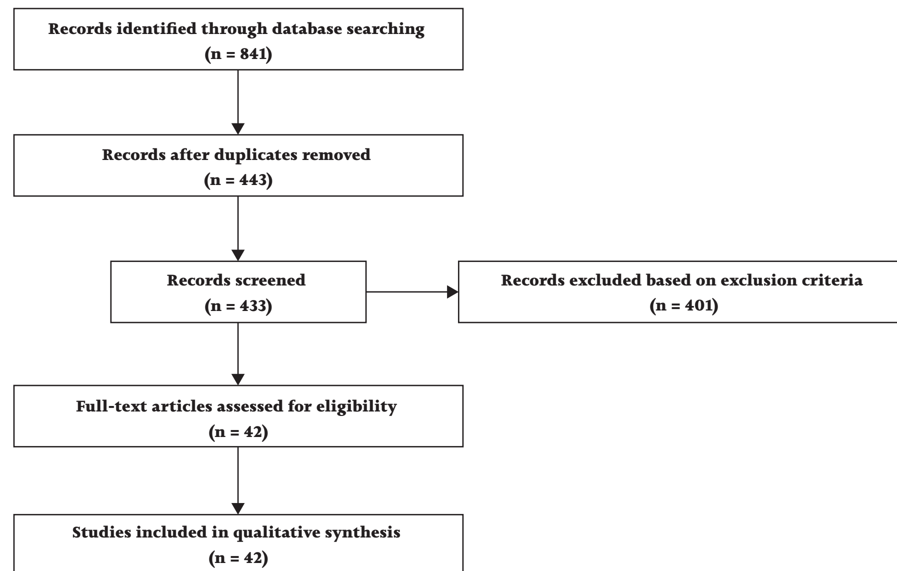
TABLE 1 *(Continuation of previous pages)*

First author yearref	Study design No. of patients	Sample collection methods	Analysis	Key findings	Weaknesses	Level evidence
ACNE VULGARIS						
Barnard et al. 2016[46]	Case control 38 AV/34 HV	• Bioré® Deep Cleansing Pore strips • Nose area	WMS sequencing	• Association specific *p. acnes* strain and acne.	• Some patients on active treatment • No serial sampling • No non-lesional patient sampling	Low
Dreno et al. 2017[47]	Single-center, randomized-controlled, double-blind Erythromycin 4% or Dermato-cosmetic 26 AV	• Sterile swabs • Lesional and non-lesional skin • Multiple time points	16S rRNA V4	• Different microbiota profiles on different sites. • Erythromycin treatment reduced the number of Actinobacteria, and dermocosmetic reduced Actinobacteria and *Staphylococcus* spp.	• Small cohort • Multiple samples excluded due to insufficient bacterial material	Moderate
Kelhala et al. 2017[48]	Single-center, controlled study Isotretinoin 0.4-0.6mg/kg orLymecycline 300mg bid 17 ISO/11 LYM/16 HV	• Sterile swabs • Pre-dose and after 6 weeks • Cheek, back and armpit	16S rRNA V1-V3	• Positive correlation *Propionibacterium* abundance and acne severity grade • Both treatments reduced clinical acne grades • *Propionibacterium* decreased in cheek samples after both treatments • *Propionibacterium* decreased in back samples after lymecycline, but not isotretinoin treatment • Diversity increased after treatment	• Small cohort • No non-lesional patient sampling	Moderate
ATOPIC DERMATITIS						
Sugita et al. 2004[58]	Case control 13 AD/12 HV	• OpSite® transparent adhesive dressings • Lesional skin • HV matched sites	26S and 5S rRNA intergenic spacer region 1	• *M. restricta* colonizes both AD and HV	• Small cohort • No serial sampling • Limited analysis • Patients on active treatment	Low
Dekio et al. 2007[49]	Case control 13 AD/10 HV	• Sterile swabs • Forehead skin	16S rRNA	• In both AD and HV there was a high rate of *Streptococcus* species • In AD *Strenotrophomonas maltophilia* was significantly more common	• Small cohort • No serial sampling • Patients on active treatment	Low
Kaga et al. 2009[50]	Case control 56 AD/32 HV	• OpSite® transparent adhesive dressings • Lesional skin AD • Face HV	26S and 5S rRNA intergenic spacer region 1	• In mild and moderate AD, *M. restricta* was predominant over *M. globose* • In patients with severe AD, proportions of *M. restricta* and *M. globose* were almost identical	• Limited analysis • No serial sampling • Variation in skin sample sites • Patients possibly on active treatment	Low to moderate
Yim et al. 2010[51]	Prospective cohort 60	• Sterile swabs • 5 body sites	26S	• There were no significant differences between positive *Malassezia* culture, *Malassezia* species, and severity of AD	• Limited analysis • Patients on emollient treatment	Low to moderate
Akaza et al. 2010[52]	Case control 67	• Sterile swabs • Lesional and non-lesional skin • Face and trunk	26S	• For the total number of *Malassezia* species, there were no significant differences between lesional and non-lesional areas	• No serial sampling • Patients on active treatment	Low to moderate
Kong et al. 2012[60]	Prospective cohort 12 AD/11 HV	• Sterile swabs • Multiple time points • Baseline, flare, post-flare	16S rRNA V1-V9	• Flare ups were associated with an increased proportion of *Staphylococcus* sequences, particularly *S. aureus*, and correlated with disease severity • Increases in *Streptococcus*, *Propionbacterium*, and *Corynebacterium* species were observed following therapy	• Small cohort • Only moderate to severe patients • Different treatments regimens during flare	Low to moderate
Seite et al. 2014[54]	Prospective cohort Emolliens treatment 46	• Sterile swabs • Lesional and non-lesional skin • Multiple time points	16S rRNA V1-V2	• Affected skin harbored a greater relative abundance of *Staphylococcus*, and in particular *S. epidermis*, compared to healthy skin • Responders had increased microbial diversity and decrease in *Staphylococcus* species	• Large time between first and second sample • Only moderate patients	Low to moderate
Chng et al. 2016[55]	Case control 19 medical history AD/15 HV/5 positive skin prick	• Tape stripping anti-cubital fossa	16S rRNA V3-V6 WMS	• Non-flare, baseline skin microbiome signatures enriched for *Streptococcus* and *Gemella* in AD prone skin versus normal skin • Increased percentage of *S. aureus* carriers noted in AD cohort over control subjects	• Small cohort • No serial sampling • No lesional samples	Low
Gonzalez et al. 2016[56]	Randomized, placebo-controlled, single-blinded Topical steroid or Topical steroid + dilute bleach bath 21 AD/14 HV	• Sterile swabs • Lesional and non-lesional skin • Multiple time points	16S rRNA V4	• Affected skin harbored a greater relative abundance of *S. aureus* • Microbial diversity at all lesional sites inversely correlated with overall EASI Index score • Taxonomic normalization occurred on lesional following treatments • Bacterial communities on lesional skin resemble non-lesional skin but remain distinct from healthy control skin	• Small study	Moderate
Seite et al. 2017[57]	Double-blind, Randomized, comparative Emollient A or emollient B 53	• Sterile swabs • Lesional and non-lesional skin • Multiple time points	16S rRNA V1-V2	• Significant increased levels of *Xanthomonas* genus in patients treated with emollient A • Levels of *Staphylococcus* genus increased between Day 1 and Day 28 in patients treated with emollient B •	• Only moderate patients • No wash-out other treatments	Moderate
Kim et al. 2017[59]	Prospective cohort Wet dressings Topical steroids Antihistamines Antibiotics 27 AD/6 HV	• Saline soaked gauzes	16S rRNA V1-V3	• Proportion of *Staphylococcus* significantly decreased after treatment • Diversity (Shannon Index) significantly increased after treatment	• Small study • Patients on wide variety of treatments • No non-lesional skin analysis	Low to moderate

TABLE 2 **Evaluation of the microbiome as clinical biomarker for each dermatological disease included in the review based on the criteria of a useful biomarker as defined by de Visser et al.[4]** Scoring system indicated as follows: +; studies in general report a positive outcome, 0; no studies available, -; studies in general report a negative outcome.

Indication	Manuscripts (No.)	Evidence level overall	Consistency	Therapeutic response	Dose-response relation	Relationship with disease	Recommendation for trial implementation
PV	10	Low	-	0	0	0	Negative, more evidence needed
HS	2	Low	+	0	0	+	Negative, more evidence needed
UC	10	Low	+	0	0	+	Negative, more evidence needed
SD	4	Low	-	0	0	+	Negative, more evidence needed
AV	5	Moderate	+	+	0	+	Positive
AD	11	Moderate	+	+	0	+	Positive

FIGURE 1 Flowchart of the study.



CHAPTER VI

INTER- AND INTRA-PATIENT VARIABILITY OVER TIME OF LESIONAL SKIN MICROBIOTA IN PATIENTS WITH ATOPIC DERMATITIS

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Short communication adapted from the following full article

ABSTRACT

Atopic dermatitis (AD) is a common chronic, inflammatory skin disorder associated with *Staphylococcus aureus* colonization and reduced microbiota diversity. The current standard for evaluating the effect of treatment on the skin microbiota is by comparing its composition before and after treatment. The aim of the current evaluation was to determine whether limited sampling is sufficient to capture the full extent of variability in the skin microbiota. To analyze inter- and intra-patient variability of the skin microbiota of 20 patients with mild to moderate AD over a period of 42 days, the coefficient of variation (CoV) was calculated for microbial diversity, relative abundance of *Staphylococcus spp.* and *S. aureus* concentration. The inter-patient variability of microbial diversity was high for lesional skin compared to non-lesional skin (CoVs of 35.5-45.9% vs 16.3-28.0%). For the other test results, high CoVs, in the range 45.3-94.1%, were found for lesional skin. Interestingly, a wide range of intra-patient variability was observed for lesional skin compared to non-lesional skin (CoVs of 7.1-173% vs 3.5-29.3%). Based on these data, 3 groups with significantly different microbiological phenotypes were defined. In conclusion, lesional skin microbiota is associated with a large inter- and intra-patient variability. A high sample frequency, e.g. once weekly, yields excellent time-dependent insight into the changes of the variable skin microbiota, which can be used to determine the treatment effect on the lesional skin microbiota in clinical trials.

INTRODUCTION

Atopic dermatitis (AD) is a chronic, inflammatory skin disorder characterized by periodic flares of dry, red itchy skin lesions. AD is a very common skin disorder in developed countries with a prevalence of approximately 20% in children and 3% in adults.[1] The pathophysiology of AD is complex and still only partially understood. Current evidence strongly points to a disruption of the skin barrier and subsequent immune dysregulation as the primary pathological drivers in AD.[2] The microbiome of the skin is important in maintaining immune homeostasis and preventing the skin from being colonized by pathogens, such as *Staphylococcus aureus*. Approximately 90% of the patients suffering from AD are colonized by *S. aureus* and the relative abundance of *S. aureus* increases during an AD flare due to a reduction in the relative abundance of colonizers of the skin.[3,4] *S. aureus* can produce several molecules with potential to cause inflammation and to promote further immune dysregulation.[2] Moreover, the increase in relative abundance of *S. aureus* and the reduction of the microbial diversity of the skin seem to be linked to the severity of the disease, promoting the skin microbiota as a potential biomarker in AD.[5] Nonetheless, the potential usefulness of this as an AD biomarker has yet to be defined.

Treatments of AD involve emollients and topical anti-inflammatory corticosteroids. There are limitations to the use of steroids, because of possible skin atrophy and systemic side-effects as well as limited patient tolerance after long-term usage. Currently, more specific treatments are being developed.[6,7] The effects of new treatments are increasingly evaluated using subjective clinical AD scores and the microbiota composition of lesional skin before and after treatment.[8–11] The design of the majority of these studies includes the collection of a single sample before and after treatment.

However, healthy skin of each human has a specific microbial 'fingerprint', which depends on the physical and chemical features of the skin as well as on host and environmental factors, including colonization at birth, antibiotic exposure, hygiene, lifestyle, and geographic location.[12,13] The level of variation depends on the topographical diversity of the skin as well as on individual factors.[14–16] Lesional skin may also be characterized by large inter- and intra-patient variability of the skin microbiota, implying the need for frequent sampling when the effect of a treatment on the lesional skin microbiota is being investigated. However, data of longitudinal studies analyzing the inter- and intra-patient variability of lesional skin microbiota is lacking.

The aim of the current evaluation was to analyze inter- and intra-patient variability of the skin microbiota of patients with AD over time to determine whether limited sampling is sufficient to capture the full extent of variability in the skin microbiota.

MATERIALS AND METHODS

SOURCE OF SAMPLES AND ASSOCIATED DATA ❋ Microbiological test results of skin swabs, along with selected clinical data from the placebo group of 2 randomized, double-blind, placebo-controlled mono-centre phase 2 clinical trials conducted at the Centre for Human Drug Research (Leiden, The Netherlands) between June 2015 and December 2017, were used in this evaluation. Both clinical trials were approved by the independent Medical Ethics Committee ('Evaluation of Ethics in Biomedical Research', Assen, The Netherlands) and were designed to assess the pharmacodynamics of omiganan in patients with mild to moderate AD. The Declaration of Helsinki was the

guiding principle for trial execution. Written informed consent was obtained from all patients.

Data from 250 samples obtained in the initial clinical trial (ClinicalTrials.gov: NCT03091426) were used to determine the variability of the skin microbiota. In this trial, the placebo group (n = 20) consisted of 11 (55%) females and 9 (45%) males with a mean ± standard deviation age of 24 ± 5 years and clinical AD score (objective-scoring atopic dermatitis: oSCORAD) of 21.1 ± 5.6. Briefly, each patient administered the vehicle gel (hydroxyethyl cellulose, sodium benzoate, glycerin, purified water) without the active compound twice daily for 28 consecutive days on all AD lesions. At the start of this treatment period (Day 0), the severity of the lesional skin was assessed clinically. Two skin swabs were collected for bacterial culture and molecular methods using an ESwab and a sterile cotton swab (Puritan, Guilford, ME, USA), respectively. Swabs were dipped in a NaCl-Tween solution, before rubbing the tip of the swab firmly over 4 cm^2 of the target lesion for 5 times. Hereafter the swab material was placed in a vial containing 1 mL NaCl-Tween solution. The skin swabs were obtained from a predefined part of an AD lesion (preferably the antecubital fossa) and from a predefined part of non-lesional skin (preferably the contralateral site). Both clinical assessment and sample collection were repeated each week during a period of 42 days. During the treatment period, patients were allowed to use bland emollients (unguentum leniens) as maintenance therapy. The patients were not allowed to wash the selected sites 6 h prior to the clinical assessment and sample collection and had to avoid prolonged exposure of their involved skin to sunlight during the complete study period. Incomplete datasets or data of samples obtained after concomitant use of corticosteroids were excluded from the analysis.

Data from 76 skin swabs obtained in a separate clinical trial with a comparable study population (ClinicalTrials.gov: NCT02456480) were used for verification purposes. In this trial, the placebo group (n = 12) consisted of 8 (67%) females and 4 (33%) males with an age of 25 ± 11 years and clinical AD score of 19.0 ± 7.4. This clinical trial differed in study design as: (I) the vehicle gel without the active compound was administered once daily on only the predefined AD lesion on the antecubital fossa; (II) only lesional skin was sampled each week; (III) clinical assessment of lesional skin was not measured at day 35 and 42; and (IV) bacterial culture was not performed.

CLINICAL ASSESSMENT OF LESIONAL SKIN ❋ The severity of the lesional skin was assessed clinically based on the oSCORAD system, calculated as: A/5+7B/2.[17] 'A' in the calculation was defined as the extent of AD, which was assessed as a percentage of each defined body area and reported as the sum of all areas, with a maximum score of 100%. 'B' in the calculation was defined as the severity of 6 specific symptoms of AD (erythema, excoriation, swelling, oozing/crusting, lichenification and dryness), which were scored 0-3 and reported as the sum of all symptoms, with a maximum score of 18. A total score of 0-7.9 was categorized as clear skin, 8.0-23.9 as mild AD, 24.0-37.9 as moderate AD, and 38.0-83.0 as severe AD.

BACTERIAL CULTURE ❋ Skin swabs were inoculated on blood agar (Becton, Dickinson and Co., Franklin Lakes, NJ, USA) and incubated at 35 °C in a 5% CO_2 incubator for 24 h. Species identification was performed by MALDI-TOF (Bruker Corp., Billerica, MA, USA) and colony-forming units (CFU) were calculated for *S. aureus* after dilution if necessary.

DNA EXTRACTION ❋ Each skin swab was diluted by addition of 50 µL 10x phosphate-buffered saline (PBS) to 450 µL swab in NaCl-Tween solution. DNA was extracted and eluted in a final volume of 100 µL with the Magna Pure 96 instrument using the Magna Pure 96 DNA and viral NA Large Volume Kit and the Pathogen universal 500 protocol (Roche Diagnostics, Meylan, France).

MICROBIOTA ANALYSIS ❋ Microbiota analysis was performed as described elsewhere.[18] Briefly, a fragment of approximately 464 bp of the V3–V4 regions of the 16S ribosomal RNA (rRNA) gene was amplified and sequenced with the MiSeq desktop sequencer (Illumina, San Diego, CA, USA). Sequencing data was processed using the QIIME pipeline and a pre-clustered version of the Augustus 2013 GreenGenes database. High-quality sequences (> 100 bp in length; quality score > 20) were clustered into operational taxonomic units (OTUs) using an open reference-based approach that implements reference-based clustering following by *de novo* clustering at a 97% similarity level. No low abundance filtering was used. For the bar charts, a limited number of genera were selected, representing the microbiota composition of each sample. Only genera with a relative abundance ≥ 1% of the total reads were included. The remaining genera formed the other genera category.

QUANTITATIVE REAL-TIME PCRS ❋ *S. aureus* was detected by quantitative real-time PCRs (qPCRs) aimed at the *nuc* gene, using primers and a probe described elsewhere.[19] The total bacterial DNA load (16S rRNA gene) was established using a primer set (Fw 5'-CGAAAGCGTGGGGAGCAAA-3', RV1 5'-CCGTACTCCCCAGGCGG-3'

and RV2 5'-GTCGTACTCCCCAGGCGG-3') based on Bogaert *et al.*[20] and 20X EVA green (Biotium, Inc., Fremont, CA, USA). Both qPCRs were carried out in a total volume of 10 µL, containing 5 µL (2x) LC480 Probes Master mix (Roche) and 2 µL of extracted DNA. Amplification reactions were performed using a LightCycler 480 II Instrument (Roche) under the following conditions: 5 min at 95 °C followed by 45 cycles of 95 °C for 10 s, 60 °C for 50 s and 72 °C for 1 s (*nuc* gene) or 5 min at 95 °C followed by 45 cycles of 95 °C for 10 s, 60 °C for 15 sec and 72 °C for 1 s (16S rRNA gene). For quantification, a 10-fold dilution series of a plasmid was included in each run and the second derivative analysis method was used for data analysis.

The total load of human DNA (RNaseP gene) was determined using primers and a probe described elsewhere.[21] Each qPCR was carried out in a total volume of 25 µL, containing 12.5 µL (2x) IQ Supermix (Bio-Rad Laboratories Inc., Hercules, CA, USA) and 5 µL of extracted DNA. Amplification reactions were performed using a CFX96 instrument (Bio-Rad Laboratories Inc.) under the following conditions: 3 min at 95 °C followed by 45 cycles of 95 °C for 15 s and 60 °C for 50 s. For quantification, a 10-fold dilution series of MOLT cell line DNA was included in each run. For data analysis, the threshold was set on 850 relative fluorescence units.

STATISTICAL ANALYSIS ❋ The statistical software package SPSS was used for statistical analysis. To characterize the microbiota of lesional and non-lesional skin over time, paired sample t-tests and unstructured linear mixed models were performed on the first set of samples. The paired-samples t-test was used to compare microbial diversity (Shannon diversity index) and the relative abundance of *Staphylococcus* spp. of lesional and non-lesional skin at baseline. Unstructured linear mixed model with time as repeated factor was used to compare clinical AD score (oSCORAD), microbial diversity, relative abundance of *Staphylococcus* spp. and *S. aureus* concentration (culture and qPCR) of lesional and non-lesional skin at baseline with data obtained 7, 14, 21, 28, 35 and 42 days later.

To analyze the inter- and intra-patient variability of the lesional and non-lesional skin microbiota, the coefficient of variation (CoV) was calculated for the microbial diversity, relative abundance of *Staphylococcus* spp. and *S. aureus* concentration by dividing the standard deviation by the mean. This was performed for the first and second set of samples. For inter-patient variability, the CoV was calculated per time-point and for intra-patient variability per patient over time. A CoV ≤ 25% has been considered as an acceptable level of variation for analytical methods (22, 23 Clinical data for patient groups were compared using the 1-way analysis of variance (ANOVA) and chi-square tests.

RESULTS

COMPARISON OF LESIONAL AND NON-LESIONAL SKIN MICROBIOTA ❋ To characterize the microbiota of lesional and non-lesional skin over time, microbiota composition was first compared at baseline. A significant lower microbial diversity of 3.8 ± 1.7 was observed for lesional skin compared to 5.1 ± 1.0 for non-lesional skin (p = 0.011; *Figure 1A*). The lower microbial diversity of the lesional skin was due to the presence of a lower number of OTUs and the relatively high abundance of the genus *Staphylococcus* (*Figure 1B*). Subsequent detection and quantification of *S. aureus* showed a correlation between the relative abundance of the genus *Staphylococcus* and the concentration of *S. aureus* (*Figure 1C, D*). This confirms that the relative abundance of *S. aureus* was higher on lesional skin compared to non-lesional skin as expected.

During the following 42 days, the mean clinical AD score of lesional skin was significantly lower (p ≤ 0.036) compared with the baseline scores (*Figure 2a*). During these days, the mean clinical AD score ranged between 16.1 ± 5.6 and 19.3 ± 4.9, still corresponding with mild to moderate AD. In comparison with the clinical AD score, there was no significant difference in the mean microbial diversity, mean relative abundance of *Staphylococcus* spp. and the mean *S. aureus* concentration determined by qPCR over time (*Figure 2b-E*). The mean microbiota composition of non-lesional skin remained relatively stable over time.

INTER-PATIENT VARIABILITY AT EACH TIME-POINT ❋ To quantify the extent of inter-patient variability of the skin microbiota, the CoV was calculated at each time-point for all test results. For lesional skin, high CoVs were observed, in the range 35.5-45.9% for microbial diversity, 46.9-65.2% for relative abundance of *Staphylococcus* spp., and 45.3-94.1% for *S. aureus* concentration. For microbial diversity of non-lesional skin, low CoVs, in the range 16.3-28.0%, were found. These data strongly indicate that there was considerable variation in lesional skin microbiota between patients.

INTRA-PATIENT VARIABILITY OVER TIME ❋ To analyze the skin microbiota variability within an individual patient over time, the CoV for microbial diversity, relative abundance of *Staphylococcus* spp. and *S. aureus* concentration was calculated per patient. For all test results of lesional skin, CoVs ranging between 7.1% and 173% were observed. For microbial diversity of non-lesional skin, low CoVs, ranging between 3.5% and 29.3%, were found. These data indicate that there was a wide range of intra-patient variability for lesional skin.

Defining microbiological phenotypes ❋ The patient population could be divided into 3 groups with different microbiological phenotypes, as shown by 3 representative patients in *Figure 3*. The lesional skin microbiota of group I (n = 7; *orange*) and II (n = 8; *blue*) were dominated by *Staphylococcus* spp., resulting in a different profile compared to their non-lesional skin microbiota. These groups differed in variability, as the lesional skin microbiota of group II was relatively unstable (Supplementary Figure S1). The lesional skin microbiota of group III (n = 5; *red*) was not dominated by *Staphylococcus* spp. Its composition and variability were similar to their non-lesional skin microbiota. This group had a significantly higher microbial diversity ($p < 0.001$), lower relative abundance of *Staphylococcus* spp. ($p < 0.001$), lower *S. aureus* concentration ($p < 0.001$) and lower clinical AD score ($p = 0.032$) compared with group I and II. There was no significant difference between the 3 groups in age, sex, Fitzpatrick skin type, season of participation, target area for sample collection or total bacterial load.

Confirmation of large inter- and intra-patient variability for lesional skin microbiota ❋ The large inter- and intra-patient variability for lesional skin microbiota was confirmed by data of the second sample set obtained from an independent but comparable study population (Supplementary Tables S1, S2). For lesional skin, the CoV for microbial diversity, the relative abundance of *Staphylococcus spp.* and the *S. aureus* concentration at each time-point was between 27.0% and 68.8%. The variability of all test results within an individual patient in time ranged between a CoV of 1.3% and 161.3%. This second sample set also confirmed the existence of 3 different microbiological phenotypes (Supplementary *Table S3*, *Figure S2*).

Discussion

To our knowledge, this is the first longitudinal analysis of inter- and intra-patient variability of skin microbiota of patients with mild to moderate AD. While the sampling method was strictly standardized, large inter- and intra-patient variability for lesional skin microbiota were found. The large inter-patient variability originated from variable *S. aureus* abundance and environmental factors that vary significantly among humans.[12,13] The wide range of intra-patient variability indicated that the skin microbiota of some individuals varied more than others. Based on these data, three patient groups with different microbiological phenotypes were defined. The microbiological phenotype for group I and II can be described as high *Staphylococcal* bioburden, low microbial diversity and either microbiologically stable, or unstable, respectively. The observation that the variability within each of these two groups is consistent within subjects across longitudinal samples, as well as concordant in multiple microbiological assessments, suggests that this difference is not caused by variable sample quality. This difference might be caused by the same unidentified individual (genetic) factors that determine the degree of variability of healthy skin microbiota.[15,16] However, influences of uncontrolled factors (e.g. number of showers, washing with soap, direct UV-exposure) on the stability of the microbiota cannot be excluded. Group III was characterized by a significantly different lesional microbiota compared to group I and II. It could be described as low *Staphylococcal* bioburden and high microbial diversity. The relative lack of dysbiosis was associated with lower clinical AD score.

The data presented in this evaluation suggest that without intervention the individual microbiota composition of the lesional skin can change considerably over a period of 42 days, in particular in patients with a microbiological phenotype of group II. Because the variability over time can be high, single samples collected before and after treatment may not be sufficient to determine the effect of the treatment on an individual's lesional skin microbiota. High sample frequency and statistical analyses methods, which utilize repeated measures across more than one end-of-study time-point, may reduce the effect of the variability in the analyses of clinical trials. The ability to objectively classify subjects to the microbiological phenotypes could be useful in the analyses and interpretation of microbiota data in future clinical trials with larger sample sizes.

The limitation of the presented evaluation is that the sample sets are from patients involved in a clinical trial administering a vehicle gel on AD lesions. Although the vehicle gel did not contain the active compound, this could have had an influence on the lesional skin microbiota as it contains the preservative sodium benzoate. However, this was considered to be minimal because (I) the concentration was far below the minimal inhibitory concentration for *S. aureus* and (II) the diversity increased under treatment (data not shown). In this evaluation, administration of the vehicle gel had no significant effect on the microbial diversity or relative abundance of *Staphylococcus spp.* However, a significant difference in clinical AS score was observed after administration of the bland emollient or vehicle gel. Since only one sample for each subject was available prior to initiation of treatment in the clinical trial, we were unable to undertake analyses to evaluate any method to define pre-treatment microbiological stability which could serve as a covariate in statistical analyses or from which to stratify randomization. Another limitation is the small patient group and the omission of including patients of younger age. A larger and more diverse population is required to study the

microbiological phenotype classifications and generalize more broadly. Lastly, the limited variation in anatomic target areas and disease states at baseline as inter- and intra-patient variability of lesional skin microbiota might be different for patients e.g. with severe AD located at their dorsal neck.

In conclusion, this evaluation shows that lesional skin microbiota of patient with mild to moderate AD is characterized by large inter- and intra-patient variability, reflecting a highly individual profile. Based on these data, lesional skin microbiota remains a potential target engagement biomarker in AD. A high sample frequency, e.g. once weekly, yields excellent time-dependent insight into the changes of the variable skin microbiota, which can be used to determine the treatment effect on the lesional skin microbiota in clinical trials.

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FIGURE 1 Baseline characteristics of lesional (orange) and non-lesional (blue) skin microbiota. (A) microbial diversity, (B) microbiota composition, (C, D) *Staphylococcus aureus* concentration based on culture or quantitative real-time PCR (qPCR) in relation to the relative abundance of *Staphylococcus spp.* determined by microbiota analysis. For microbiota diversity, means ± standard deviations of operational taxonomic units are indicated in the bars. The *S. aureus* concentration based on qPCR is normalized by calculating the NUC gene copies per 1000 16S rRNA gene copies. All p-values are based on a paired-sampled t-test.

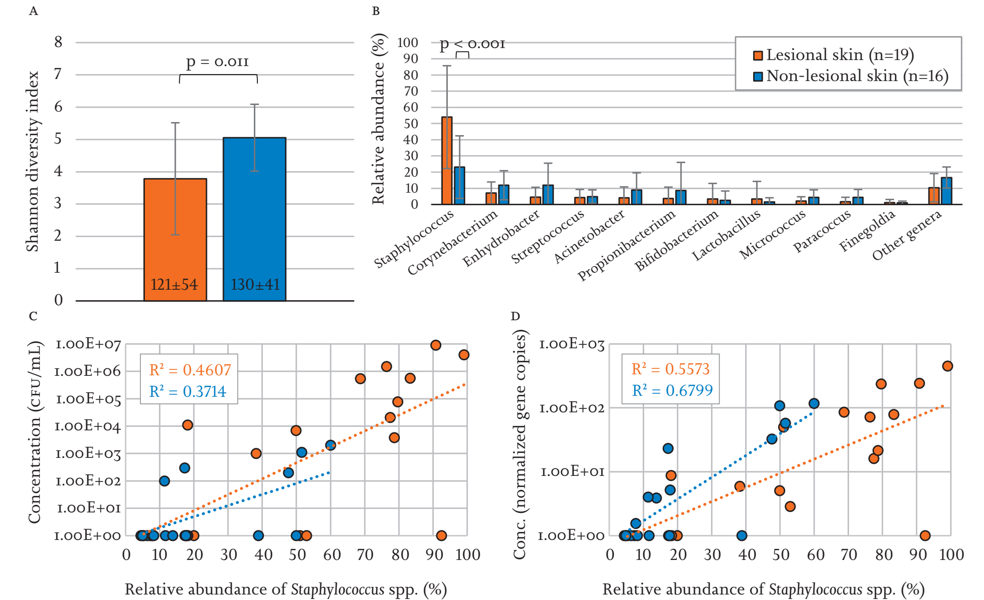


FIGURE 2 Analysis of lesional (orange) and non-lesional (blue) skin microbiota during a period of 42 days. (A) clinical AD score, (B) microbial diversity, (C) relative abundance of *Staphylococcus spp.*, and (D, E) *Staphylococcus aureus* concentration based on culture or qPCR. The *S. aureus* concentration based on qPCR is normalized by calculating the nuc gene copies per 1000 16S rRNA gene copies. Mean values are indicated by crosses and outliers by dots. Number of samples are indicated below the bars. All p-values are based on an unstructured linear mixed model.

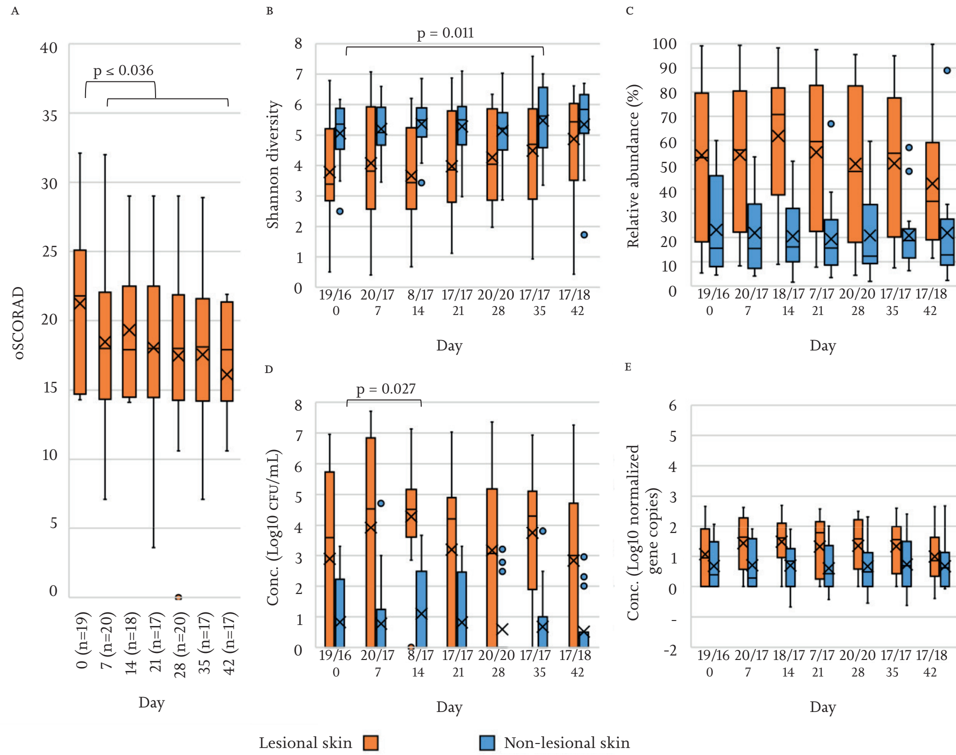
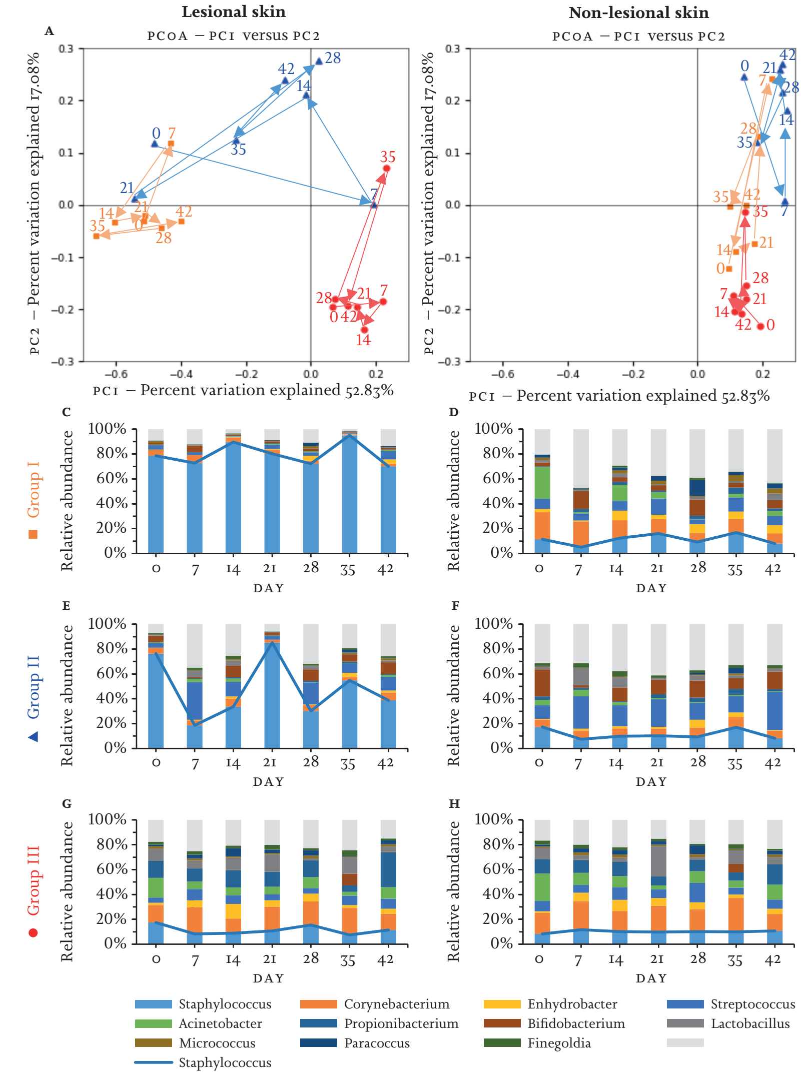


FIGURE 3 Lesional and non-lesional skin microbiota of 3 selected patients representing 3 groups of patients with different microbiological phenotypes shown in (A, B) principal coordinates analysis (PCOA) plots and (C-H) bar charts. In the PCOA plots, the arrows combined with the day numbers show how the microbiota composition changed over time.



SECTION III

CLINICAL APPLICATION IN ATOPIC DERMATITIS

CHAPTER VII

PHARMACODYNAMIC EFFECTS OF TOPICAL OMIGANAN IN PATIENTS WITH MILD TO MODERATE ATOPIC DERMATITIS IN A RANDOMIZED PLACEBO-CONTROLLED PHASE II TRIAL

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ABSTRACT

Omiganan is an indolicidin analogue with antimicrobial properties that could be beneficial for patients with atopic dermatitis. This randomized, double-blind, placebo-controlled phase II trial explored efficacy, pharmacodynamics and safety of topical omiganan once daily in 36 patients with mild to moderate AD. Patients were randomized to apply topical omiganan 1%, omiganan 2.5% or vehicle gel to one target lesion once daily for 28 consecutive days. Small but significant improvements of the local oSCORAD index and morning itch were observed in the omiganan 2.5% group compared to the vehicle gel group (-18.5%; 95%CI=-32.9,-1.0; p=0.04 and -8.2; 95%CI=-16.3,-0.2; p=0.05 respectively). A shift from lesional to non-lesional skin microbiota was observed in both omiganan treatment groups, in contrast to the vehicle group. In conclusion, treatment with topical omiganan improved dysbiosis in patients with mild to moderate atopic dermatitis and small but statistically significant improvement of clinical scores were detected. Our findings warrant further exploration in future clinical trials.

INTRODUCTION

Atopic dermatitis (AD) is a common skin disorder with a prevalence up to 3% in adults and up to 20% in children of the Western world.[1] Patients with AD have a xerotic erythematous skin with oozing and crusting that typically occurs on the flexor sites of the body. Severe pruritus is the main and most bothersome symptom for most patients, and can lead to reduced quality of life and reduced quality of sleep.[2] Current topical therapies for AD include bland emollients in combination with anti-inflammatory agents such as corticosteroids and calcineurin inhibitors. Side effects can be serious including HPA-axis suppression with extensive topical corticosteroid therapy. Recently dupilumab, the first monoclonal anti-IL4 antibody treatment for patients with moderate to severe AD was registered, and it is likely that may others will follow. However, for the patients with mild disease, novel therapeutic agents with a favorable side effect profile are wanted.

The pathophysiology of atopic AD is multifactorial and only partially understood. One of the factors involved in the pathogenesis is colonization and infection of the skin with *Staphylococcus aureus* that can produce and secrete toxins and act as super antigens leading to inflammation.(3) Colonization rates in a study of Park and colleagues show that up to 90% of the AD patients is colonized with this pathogen, compared to only 5-30% of the healthy subjects.(4) A deficiency in antimicrobial peptides (AMPs), which are important in the host defense system of the skin, accounts for the susceptibility to this bacterium in AD patients.[5] A dysregulated type 2 T helper (TH2) response in the skin leading to the production of pro-inflammatory cytokines such as IL-4 and IL-13 is most likely responsible for this AMP deficiency.[6,7] Therefore, topical AMPs are new potential therapeutic options for patients with AD.

Omiganan is a novel, synthetic, cationic peptide and an analogue of indolicidin, a short AMP from the cathelicidin family. It has demonstrated antimicrobial activity against a wide variety of gram positive and negative bacteria and fungi.[8-10] Cationic peptides are also suggested to have immunomodulatory roles, in both pro- and anti-inflammatory pathways depending on the context.[11,12] The combination of anti-bacterial and anti-inflammatory properties make omiganan a promising compound for the topical treatment of AD. While omiganan has been investigated before in several other indications, e.g. acne vulgaris and rosacea, this is the first trial to elucidate its effects in patients with AD.

In this study we aimed to explore the clinical efficacy, patient-reported outcomes, pharmacodynamics, safety and tolerability of topical omiganan on one target lesion in patients with mild to moderate AD.

MATERIALS AND METHODS

STUDY DESIGN, RANDOMIZATION AND TREATMENTS ❊ We conducted a randomized, double-blind, placebo-controlled mono-center phase 2 study to explore the clinical efficacy, patient-reported outcomes (PROs), pharmacodynamics (PD), safety and tolerability of omiganan in 36 patients with mild to moderate AD. The Declaration of Helsinki was the guiding principle for trial execution, and the study was approved by the independent Medical Ethics Committee 'Medisch Ethische Toetsingscommissie van de Stichting Beoordeling Ethiek Biomedisch Onderzoek' (Assen, the Netherlands) prior to any trial procedure. All patients provided written informed consent before participation. The study was conducted from June 2015 to November 2015 at the Centre for Human Drug Research, Leiden, The Netherlands. Eligible patients were randomized to apply either topical omiganan 1%, omiganan 2.5% or vehicle gel, which served as placebo with an identical appearance, once daily (QD) for 28 consecutive days to one target lesion. This was done 1:1:1 in blocks of three according to a randomization list with codes generated by the unblinded statistician. Next to the active ingredient omiganan pentahydrochloride, the excipients of the compound are glycerin, hydroxyethyl cellulose, benzoic acid, sodium benzoate and water. For safety reasons, only the pre-defined target

lesion (one antecubital fossa) was treated in this first in patient study with drug/placebo but not with emollient. Study drug application was recorded with use of a validated mobile phone e-diary application. Subjects, study personnel and investigators were blinded for the allocated treatment throughout the study. Emollients (unguentum leniens) were handed out to use QD, but not on the target lesion. Patient visits were scheduled at day -14 (run-in period), 0, 1, 3, 7, 14, 21, 28 (end of treatment, EOT), 35 and 42 (end of study, EOS). During the run-in period patients applied emollients to the skin (not on the target lesion) and triamcinolone 0.1% ointment to eczema lesions except for the target lesion, if needed. All study details are provided in the protocol and assessment schedule, Supplemental Material S1 and S2.

PATIENTS ❋ Patients were included if: 1) AD was mild to moderate and (intermittently) present for more than one year, 2) aged between 18 and 65 years, and 3) willing to give written informed consent. Inclusion criteria were an oSCORAD index of 8-40, and an affected body surface area (BSA) of 5-15%. For PD assessments throughout the study, all patients should have at least one target lesion (one antecubital fossa) affected by AD, with a BSA of ≥0.5% and a pruritus numeric rating scale (NRS) score of at least 30 on a 0-100 scale. There was a wash-out period for any type of AD medication; for cyclosporine, mycophenolate mofetil and other systemic immunosuppressive drugs 4 weeks, phototherapy 3 weeks, biologics 5 half-lives of the drug, topical calcineurin-inhibitors 10 days, topical corticosteroids 2 weeks, and any other topical medication (prescription or over the counter) 2 weeks. Patients with other clinically significant (skin) conditions in the treatment area were excluded. Health status of included patients was verified by a detailed medical history, physical examination, vital signs, 12-lead ECG and laboratory test (including hepatic and renal panels, complete blood count, chemistry panel, virology and urinalysis). Patients were evaluated the four most prevalent fillagrin mutations in Europe at screening (2282DEL4, R501X, S3247X AND R2447X).

CLINICAL EFFICACY, PHARMACODYNAMIC ASSESSMENTS AND PATIENT REPORTED OUTCOMES ❋ One target lesion was assigned for treatment and PD assessments in the trial. Another non-treated eczema lesion served as a control lesion for part of the PD measurements (off-target side). Clinical efficacy was assessed by the local oSCORAD index (% of BSA, erythema, edema/papulation, oozing/crusting, excoriation, lichenification and dryness) of the target lesion. Standardized pictures were taken by a 3D stereo-camera system (LifeVizTM

QuantifiCare, Valbonne, France) for the assessment of the target lesion size and roughness analysis. The skin barrier status of lesional and non-lesional skin was assessed by trans-epidermal water loss assessment (TEWL, AquaFlux AF200 system, Biox, London, UK). This was done under standardized environmental conditions (temperature 22°C±2°C; relative humidity <60%) and patients were acclimatized under relaxed conditions for at least 15 minutes prior to measurements. All measurements were performed at each study visit. PROs consisted of NRS itch (0-100) and the 5-D itch scale.[13,14] The itch scores were divided in morning and evening itch in order to link the application time with efficacy outcomes.

SWAB COLLECTION FOR MICROBIOLOGY ❋ Patients were instructed not to wash or apply the study drug and keep the target lesion dry for at least 24 hours prior to the study visit. Skin swab samples were collected with sterile swabs that were dipped in a NaCl Tween solution (Puritan Sterile Polyester Tipped Applicators REF 25-806-1PD), before rubbing the tip of the swab firmly over 4cm^2 of the target lesion for 5 times. Hereafter the swab material was placed in a micro tube (REF 72.694.105, Sarstedt, Numbrecht, Germany) containing 0.9% NaCl and 0.1% Tween 20. Analysis was performed as described by van den Munckhof *et al.*[15]

MICROBIOLOGY ANALYSIS – *S. AUREUS* QUANTIFICATION ❋ A single-plex quantitative PCR (qPCR) adapted from literature.[16] targeting the nuc gene was applied to quantify the *S. aureus* bacterial load. Quantification of bacterial load was done by comparing the results of the samples with the results of a standard curve with known concentrations. This standard curve was tested in parallel to the samples in each experiment. Samples that were below the limit of quantfication (LOQ) were used in the analysis as ½ LLOQ.

MICROBIOME ANALYSIS ❋ The DNA extraction was performed automatically with the Magna pure 96 instrument using the Magna Pure 96 DNA and Viral NA Large Volume Kit and the Pathogen universal 500 (Roche Diagnostics, Basel, Switzerland). An input volume of 500µL sample and an elution volume of 100µl was used. After DNA extraction, the variable regions V3 and V4 of the 16S rRNA gene were amplified using the primers described by Klindworth, *et al.*[17]: Bakt_341F (5'CCTACGGGNGGCWGCAG -3') and Bakt_805R (5'- GACTACHVGGGTATCTAATCC -3') with Illumina overhang adaptor sequences added. The generated amplicons of around 460 base pairs were analyzed on a capillary system using a standard protocol, to confirm successful amplification of a PCR fragment of the expected

size. As a next step, PCR products were cleaned up by Ampure XP beads (Beckman Coulter) to remove primer-dimers and small a-specific PCR products and the purified PCR products were quantified using the Quant-iT PicoGreen dsDNA kit (Life Technologies), followed by serial dilution steps to reach the correct amount of input DNA. Index primers (Nextera XT Index kit) were added by limited cycle PCR to the diluted PCR products. Prior to pooling, samples were normalized by using beads with maximum binding capacity (Nextera XT sample preparation kit).

The sequencing was performed on the Illumina MiSeq platform by using the MiSeq v2 sequencing kit with 500 cycles (Illumina). De-multiplexed FASTQ files were generated as output and the sequences of the FASTQ files were analyzed using the Metagenomics workflow of the MiSeq Reporter software of Illumina, resulting in a taxonomy percentage summary of the sequenced bacterial sample. To calculate the relative abundance of microorganisms at genus level the unclassified DNA was excluded, hence the sum of the percentages of the DNA of all microorganisms found was set to 100 %. Furthermore, the microorganisms with an abundance of less than 10% were excluded from the analysis.

BIOPSY BIOMARKERS ❋ Skin punch biopsies (4mm) were collected from lesional skin at day 0 (pre-dose) and day 28 and from non-lesional skin at day 0. Biopsies were placed in RNA later medium directly after harvest of the biopsy and stored and -80ºC. The biopsies were analyzed at DDL Laboratory, Rijswijk, The Netherlands. RNA extraction and real-time quantitative PCR analysis relative to the housekeeping gene GAPDH. Because of limited material a selection of markers in the protocol was made. The final set of markers was chosen based on disease involvement (IL-31, eotaxin, IFN-γ) and expected investigational drug effects (IFN-α, IFN-γ, IL-6).[18]

SAFETY AND TOLERABILITY ❋ Safety and tolerability endpoints were assessed by the frequency of treatment-emergent adverse events (TEAEs), serious adverse events (SAEs), discontinuations due to AEs or deaths occurred, laboratory values (hematology, chemistry, coagulation, and urinalysis), vital signs, electrocardiographic parameters, and physical examination.

TREATMENT COMPLIANCE AND EXPOSURE ❋ Compliance of study drug application was recorded using a mobile e-diary application which entailed a notification and photo capture function enabling the date and time documentation of each gel application.

STATISTICAL METHODS ❋ All calculations were performed using SAS for windows V9.4 (SAS Institute, Inc., Cary, NC, USA). No formal power calculation was performed given the exploratory character of this first in patient study. Clinical efficacy and pharmacodynamic endpoints were analyzed with a mixed model analysis of variance using treatment, time and treatment by time as fixed factors and subject as random factor. Analyses were conducted in the clinical evaluable (CE) population. This population consisted of all subjects that applied the study medication for at least 21 days and completed the EOT visit. The results per variable were generated with estimates of the difference of the different contrasts and a back transformed estimate of the difference in percentage for log transformed parameters, 95% confidence intervals (in percentage for log-transformed parameters) and Least Square Means (geometric means for log transformed parameters), and the p-value of the contrasts. The analyses of the mRNA expressions in the biopsies incorporated normalization for housekeeping gene GAPDH. Moreover, it incorporated the values from non-lesional skin to correct for the high variability. For the organization and visualization of the microbiome data, Python 3.5.2 was used.

RESULTS

PATIENT CHARACTERISTICS ❋ Fifty-nine patients were screened of whom 36 were enrolled in the study. All enrolled patients completed the study (Figure 1). Baseline characteristics were comparable between the treatment groups (Table I). The overall mean BSA of the target lesion was 1.4% (±0.9).

CLINICAL EFFICACY ❋ A reduction of the target lesion oSCORAD index was observed in both active treatment groups compared to vehicle gel, mainly due to a reduction in % of BSA. This reduction was statistically significant for omiganan 2.5% (-18.5%; 95%CI=-32.9,-1.0), but not for omiganan 1% (-13.4%; 95%CI=-28.4,4.6) (see *Figure* 2A), which might indicate a dose dependency. The reduction in the oSCORAD index in the omiganan 2.5% group was accompanied with a trend in reduction of % of BSA of the target lesion (-0.31; 95%CI=-0.64,0.03) (see Figure2B).

PATIENT-REPORTED OUTCOMES ❋ A significant decrease on the 0-100 NRS itch scale was observed during the morning in the omiganan 2.5% group compared to the vehicle gel (-8.2; 95%CI=-16.3,-0.2), but not in the omiganan 1% group (-1.1; 95%CI=-9.5,7.4). Itch during the evening slightly decreased in

both active treatment groups. However, these decreases were not statistically significant (see *Figure* 2C,D).

EXPLORATORY PHARMACODYNAMICS ❋ Skin barrier function as measured by TEWL improved in all treatment groups (-4.5, -8.8 and -12 in the vehicle gel group, omiganan 1% and omiganan 2.5% group respectively) (*Figure* 3A). The 3D photo analysis revealed no significant changes in the roughness of the skin surface (data not shown). Biomarkers demonstrated a high degree of variability in general. At baseline a significant difference between lesional and non-lesional mRNA expression in skin relative to GAPDH was observed for the markers eotaxin, IFN-γ and IL-31 (see Figure3B, C and D). There was no difference between lesional and non-lesional skin for the markers IFN-α and IL-6. No significant reductions of biomarkers were observed in any of the treatment groups. An example of the relative mRNA expression of eotaxin before and after treatment is shown in *Figure* 3E.

MICROBIOLOGY AND MICROBIOME ❋ No significant reductions in total bacterial load were observed in both active treatment groups, i.e. omiganan 1% and omiganan 2.5%, compared to the vehicle gel group (data not shown). For the bacterial load data of *S. aureus* data by qPCR, a high proportion of samples showed results below the limit of quantification. There were no differences between the treatment groups of the quantified samples. Microbiome data demonstrated a high degree of variability between patients in the presence and abundances of the genera. However, in general, the presence of the *Staphylococcus* genus dominated lesional skin compared to non-lesional skin (*Figure* 4). Individual data can be found in Supplemental material S3. After both active treatments *Staphylococcus* abundance decreased significantly from 64% to 37% for omiganan 1% (p=0.05) and from 70% to 42% (p=0.01) for omiganan 2.5% compared to vehicle (*Figure* 4 and *Figure* 5A), while the abundance in the vehicle group remained stable. With the decrease of *Staphylococcus* abundance, the summary of the microbiome diversity (Shannon index) significantly increased up to a total change of 0.11 for omiganan 1% (p=0.03) and 0.08 for omiganan 2.5% (p=0.03 compared to vehicle). At baseline, a moderate correlation was found between the target lesion oSCORAD index and *Staphylococcus* abundance (r=0.409) and between the target lesion oSCORAD index and diversity index (r=0.333). However, the decrease in the target lesion oSCORAD index as seen in both treatment groups did not correlate with the reduction in *Staphylococcus* abundance and increase in diversity (r=0.182 and r=-0.096, respectively) (*Figure* 6).

SAFETY AND TOLERABILITY ❋ One or more TEAEs were experienced by 18 of 36 patients (50%). All TEAEs were of mild (n=24) or moderate severity (n=3), The most frequent TEAEs were headache, upper respiratory tract infections and influenza like illness. They were all self-limiting and were considered unlikely related (n=28) to treatment. No local AEs were reported. No discontinuations due to adverse events occurred. Application did not result in any clinically significant changes in safety laboratory parameters or vital signs. No SAEs, discontinuations due to AEs or deaths occurred.

TREATMENT COMPLIANCE AND EXPOSURE ❋ Treatment compliance was high and comparable in all treatment groups and ranged from 89-100%.[19] The mean usage of study drug per day ranged from 1.2 to 1.3 mg per cm^2 in all treatment groups.

DISCUSSION

This is the first randomized, double-blind, controlled clinical trial exploring the clinical efficacy, pharmacodynamics and safety of omiganan in patients with AD. Treatment with omiganan 2.5% resulted in a clinically small but statistically significant reduction in the target lesion oSCORAD index and morning itch after 28 days of treatment compared to treatment with a vehicle gel. This reduction was mainly caused by a reduction in BSA. As *proof-of-pharmacology*, a shift from lesional to a non-lesional microbiome profile in terms of a reduction of *Staphylococcus* genus and increase in diversity was seen in both active treatment groups compared to the vehicle gel group.

The shift in microbiome profile observed, was predominantly driven by a reduction in *Staphylococcus* genus. This can be explained by the previously reported activity of omiganan against this genus.[8] The abundance of *Staphylococcus* is known to increase in skin affected by AD. Our study did show a moderate correlation between the clinical score (oSCORAD index) and the disturbed microbiome profile at baseline, which is in concordance with the correlations of around 0.50 found by others.[20-22] The correlation of improvement of the oSCORAD index and the degree of dysbiosis would suggest an important role of the microbiome in the pathogenesis of AD. However, a decrease in abundance of *Staphylococcus*, or an increase of microbial diversity, did not correlate with oSCORAD index improvement in our study. In some patients, this relationship was even reversed. In contrast, in previous studies with other treatments, such as topical corticosteroids and emollients, a correlation of clinical improvement and *Staphylococcus* reduction and/or increase

in diversity index was reported.[20,22] A delay in clinical improvement after a recovery of the microbiome or variable individual responsiveness of the microbiome in the small treatment group in our study, may be underlying mechanisms prohibiting a clear insight in the relation between the microbiome and clinical efficacy. It is also known that some subjects by nature have a higher abundance of *Staphylococcus* up to 30% and/or a greater microbial diversity on the skin than others while this does not appear to contribute to disease activity.[23,24] An alternative explanation could be that recovering the microbiome in mild to moderate AD patients does not lead to an improvement of clinical symptoms, but the effect is the other way around, in which reduction of inflammation leads to normalization of the microbiome. This notion is supported by the fact that there is no evidence for a beneficial effect of antimicrobial interventions in non-infected AD, and the fact that the microbiome can recover with topical corticosteroids alone, which evidently lack antimicrobial properties.[25] More studies are needed to provide full insight in the relationship between the microbiome and inflammation in AD, as the outcomes of our study do not give a clear insight.

The clinical effects on the target lesion oSCORAD and affected BSA might be explained by the immunomodulatory effects of omiganan. Although no data of omiganan on this is available yet, comprehensive data of immunomodulatory potential is available on another member of the cathelicidin family, i.e. LL-37, the only endogenous human cathelicidin. LL-37 has anti-inflammatory properties including the inhibition of AIM2 inflammasome formation and suppression of IFN-γ, TNF-α, IL-4 and IL-12.[11,26,27] IFN-γ expression remained unchanged after treatment (data not shown), the other pro-inflammatory markers were not included in this study. Moreover, LL-37 is involved in skin barrier homeostasis and presumably suppresses itch.[28] On the contrary, LL-37 is also involved in several pro-inflammatory pathways when excessively present, e.g. in the downregulation of IL-10 and mast cell release of inflammatory mediators.[29,30] In summary, there is evidence from AMP family members that the clinical effects of omiganan can rely on the immunomodulatory properties rather than the antimicrobial properties but more *in vitro* and *in vivo* studies with omiganan are needed to draw conclusions.

MICROBIOME AS BIOMARKER ❋ In a previous literature review we described the potential of skin microbiome associated outcomes as biomarker in early phase clinical AD trials.[31] Although the exact relation between the skin microbiome and AD pathophysiology remains debatable based on this review the implementation looked promising with respect to antimicrobial therapies.[31] In a longitudinal observation of AD patients no major dissimilarity and robust microbiome profiles over time of lesional and non-lesional skin were observed.[15] With the current study we observed that the clear improvements in the microbiome only weakly correlated with clinical response. Therefore the microbiome may be considered as disease biomarker in AD to a lesser extent. For this part, since only a single AD lesion was treated and since a high inter-individual microbiome variability was observed, a larger study with total body application is needed to explore the full potential. However, the microbiome in this study provided *proof-of-pharmacology* of topical omiganan in patients with mild to moderate atopic dermatitis by serving as drug mechanistic biomarker when assessing target engagement.

ITCH REDUCTION NOT CLINICALLY MEANINGFUL ❋ Although there was a small statistically significant reduction in the morning itch with omiganan 2.5% treatment compared to vehicle, the minimal clinically important difference (MCID), which is the smallest patient-reported outcome change that is considered clinically meaningful,[32] was not achieved. The mean reduction in NRS morning itch in the 2.5% treatment group was 8.2 (on a scale of 0-100), while the MCID for itch is determined as at least 20 to 30-point reduction.[33] The evening itch did not decrease significantly. When looking at the time of dose administration, most patients applied the gel in the morning (60%), which might explain why the effects on itch in the evening are less apparent. The itch reduction did not correlate with a reduction of IL-31 mRNA expression, a biomarker for itch, in the skin biopsies at EOT which supports the debate about the questionable clinical relevance of the observed reduction.[34]

LIMITATIONS ❋ Only one target lesion was treated with the study drug for safety reasons, therefore potential efficacy when applied on all lesions remains unknown. When treating and trying to recover only one lesion in terms of inflammation and *Staphylococcus* reduction, auto contamination of other lesions may occur, which might be important assuming *Staphylococcus* plays a major role in the pathogenesis. However, our results indicated a clear shift in microbiome profile with a reduction in *Staphylococcus* genus. It remains unclear whether this includes *S. aureus* or other *Staphylococcus* species since analysis on species level was not feasible with our NGS determination method. Unfortunately, the qPCR analysis was not able to perform quantitation in many samples which precluded statistical analysis. Cultures to compare with our NGS method were not performed in this study. Another limitation concerns the scoring of itch of the target lesion only. I may be hard for the patients to discern the itch from

the target lesion from the itch related to other AD lesions and this might have influenced the scoring. Furthermore, study groups were relatively small to make definite conclusions about efficacy, and the clinical relevance of the decrease in target lesion oSCORAD and morning itch is therefore debatable.

In conclusion, in patients with mild to moderate atopic dermatitis, the topical administration of omiganan QD to a limited treatment area for up to 28 days is safe and well tolerated. Pharmacological activity in terms of a significant reduction in the oSCORAD of the predefined target lesion and the patient-reported itch was observed with the highest dose of 2.5%. However, since these reductions were small, clinical relevance of both is debatable. The microbiome showed a significant shift from lesional to non-lesional skin profile with both active treatments, which did only show low correlations with the clinical improvement. Future studies with optimization of the treatment regimen, i.e. dose (exploration of more concentrations), frequency (BID instead of QD), and other sub-indications (such as infected AD) are necessary to explore the true potential of omiganan in patients with AD.

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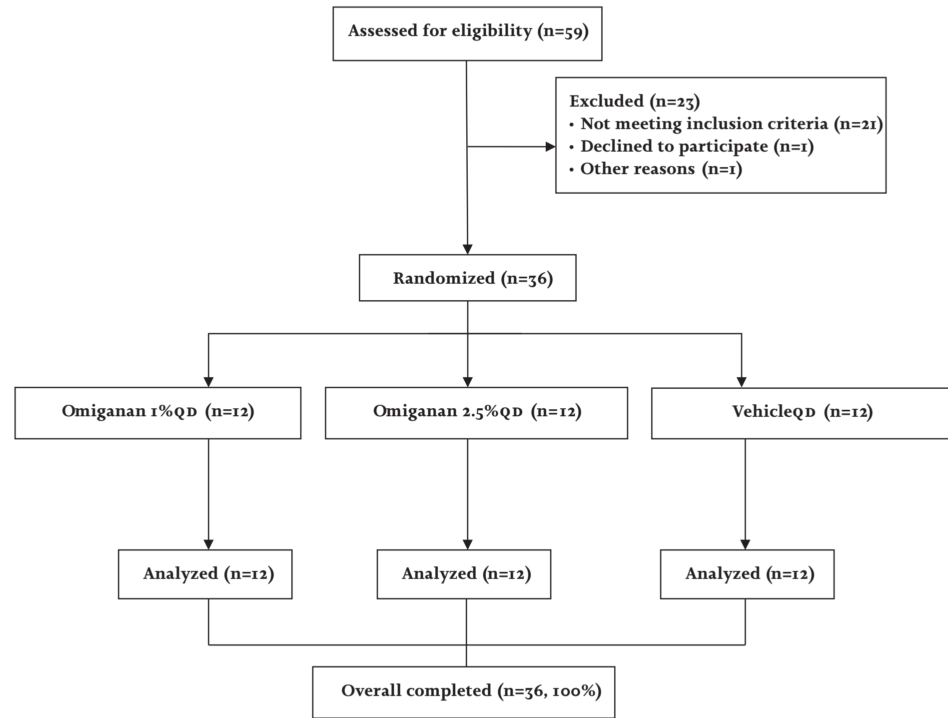
TABLE 1 **Baseline characteristics of the study.**

	Omiganan 1% N = 12	Omiganan 2.5% N = 12	Vehicle gel N = 12	Total N = 36
Sex, n (%)				
Female	9 (75%)	10 (83%)	8 (67%)	27 (75%)
Male	3 (25%)	2 (17%)	4 (33%)	9 (25%)
Age, years (SD)	25.0 (5.2)	25.1 (7.1)	24.7 (10.9)	24.9 (7.8)
Fitzpatrick Skin Type, n (%)				
I	0 (0%)	0 (0%)	0 (0%)	0 (0%)
II	4 (33%)	5 (42%)	5 (42%)	15 (42%)
III	4 (33%)	5 (42%)	3 (25%)	12 (33%)
IV	1 (8%)	1 (8%)	2 (17%)	4 (11%)
V	2 (17%)	0 (0%)	1 (8%)	3 (8%)
VI	1 (8%)	0 (0%)	1 (8%)	2 (6%)
Years since diagnosis – mean (SD)	20.5 (9.4)	19.2 (10.9)	21.1 (12.2)	20.3 (10.6)
Exacerbations per year – mean (SD)	7.3 (6.9)	9.1 (6.4)	11.2 (9.8)	9.2 (7.8)
Subjects with Filaggrin mutation – n (%)	0 (0%)	3 (25%)	4 (33%)	7 (19%)
% BSA - target lesion – mean (SD)	1.5 (0.9)	1.0 (0.5)	1.6 (1.1)	1.4 (0.9)
oSCORAD index - target lesion – mean (SD)	17.6 (7.5)	16.3 (4.5)	18.1 (8.4)	17.3 (6.8)
% BSA - all lesions – mean (SD)	9.1 (5.7)	7.0 (7.2)	8.9 (3.4)	8.3 (5.6)
oSCORAD index - total body – mean (SD)	18.4 (8.4)	18.9 (6.4)	17.8 (5.4)	18.4 (6.7)
Previous treatment with corticosteroids (USA classification)– n (%)				
Class IV corticosteroid	6 (50%)	8 (67%)	6 (50%)	20 (56%)
Class III corticosteroid	6 (50%)	7 (58%)	7 (58%)	20 (56%)
Class II corticosteroid	5 (42%)	7 (58%)	6 (50%)	17 (47%)
Class I corticosteroid	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Calcineurin inhibitor	2 (17%)	7 (58%)	2 (17%)	11 (31%)

SD = standard deviation; BSA = body surface area; oSCORAD = objective SCORAD index

FIGURE 1 Flowchart of the study.



QD = *qualified dose (once daily).*

FIGURE 2 **Change from baseline in body surface area, OSCORAD index and morning and evening itch in the omiganan 1% and 2.5% treatment groups compared to vehicle gel.** Change from baseline graphs: delta least squares means (LSM) over time of clinical assessments BSA (Panel A.) and OSCORAD (Panel B.) of target lesion. In the lower panels the patient-reported outcomes are depicted, i.e. itch morning (C.) and itch evening (D.).

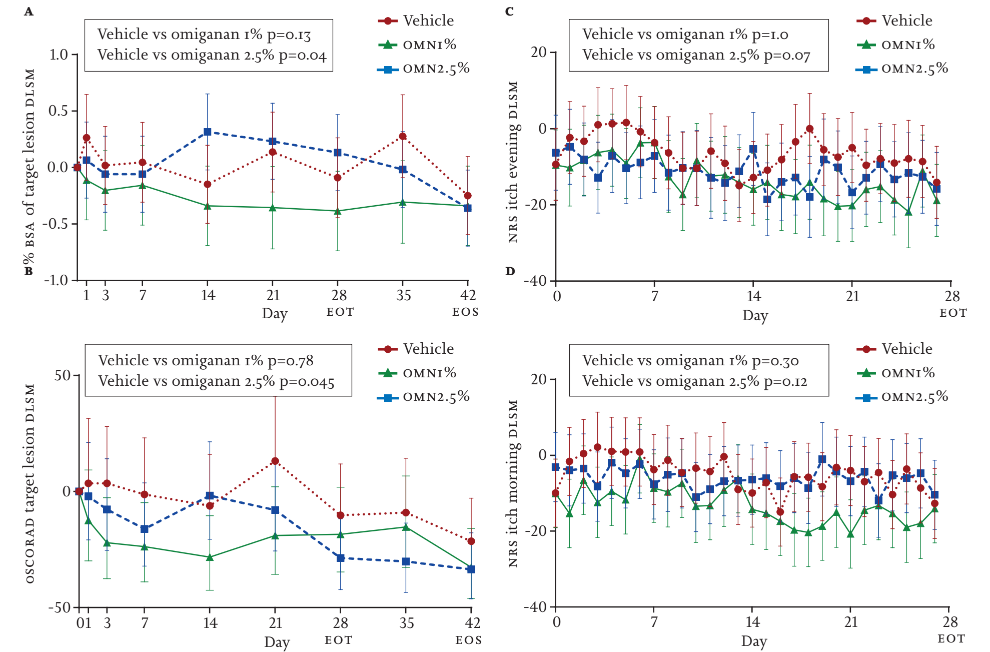


FIGURE 3 Pharmacodynamic effects of topical omiganan in the omiganan 1%, omiganan 2.5% and vehicle gel group. In panel A the trans-epidermal water loss (TEWL) over time is depicted showing improvement in all treatment groups including vehicle. In panels B, C and D relative mRNA expressions in skin punch biopsy of markers IL-31 (B), eotaxin (C) and IFN-γ (D) in lesional versus non-lesional skin in mild to moderate atopic dermatitis patients at baseline, medians with interquartile ranges are presented. Statistical significance is indicated as follows: *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$, based on a paired t-test on log transformed data. E) relative mRNA expression of eotaxin per treatment group before treatment lesional skin (day 0), after end of treatment lesional skin (day 28) and non-lesional (NL). No treatment effect occurred on this marker.

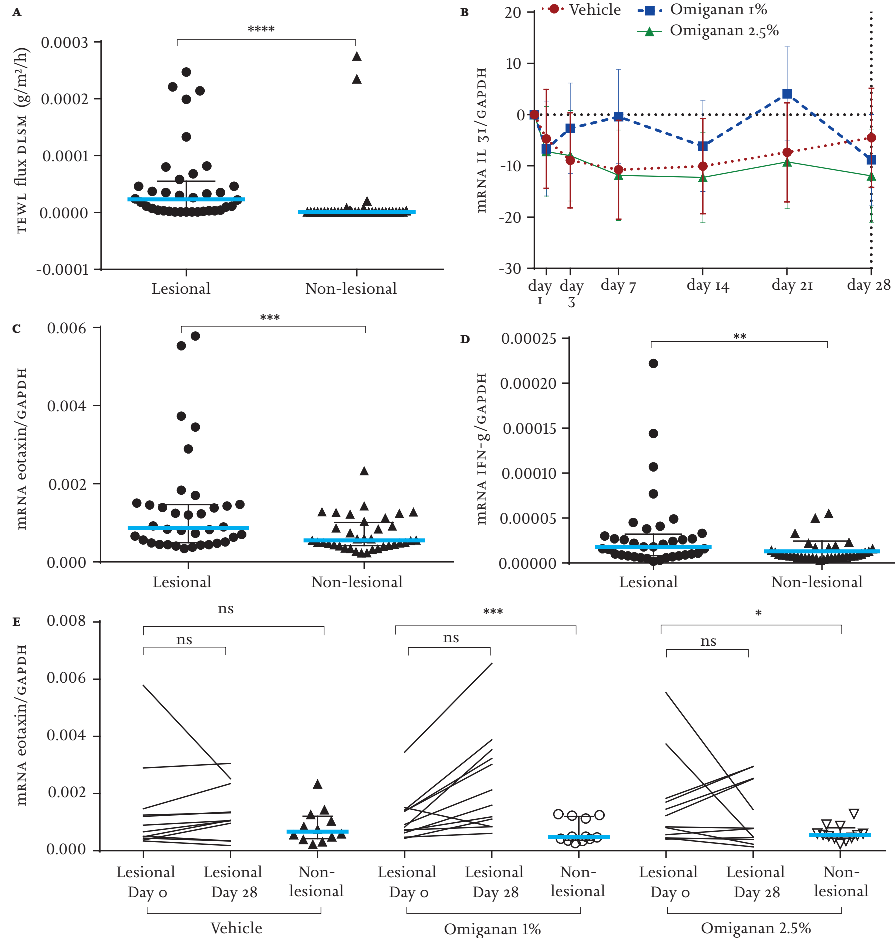


FIGURE 4 Course of cutaneous microbiome after omiganan treatment over time. Relative *Staphylococcus abundance* over time in the omiganan 1% group (panel A), omiganan 2.5% group (panel B.) and vehicle group (panel C) are depicted. A reduction is seen in both active treatment groups while this is not present in the vehicle group. Non-lesional boxes are presented as control on the right side. Above the box plots the values of the median are indicated in blue.

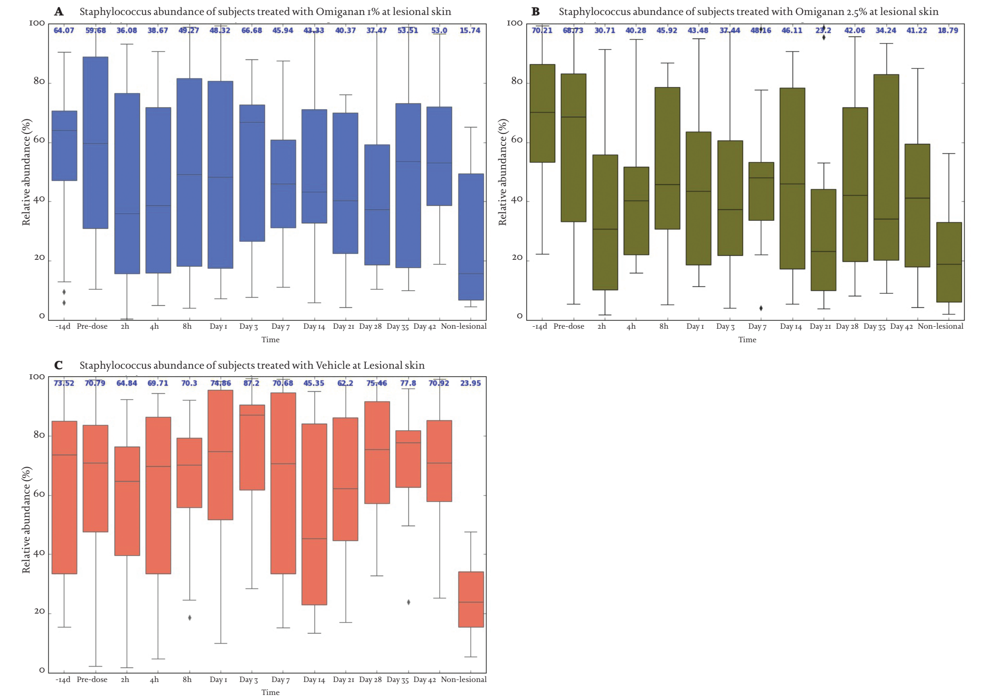


FIGURE 5 **Omiganan improves dysbiosis of target lesion.** *Staphylococcus abundance* (A.) and diversity index (B.) from baseline to day 28 (end of treatment) per treatment group with p-values of the differences as calculated with a mixed model of data over time.

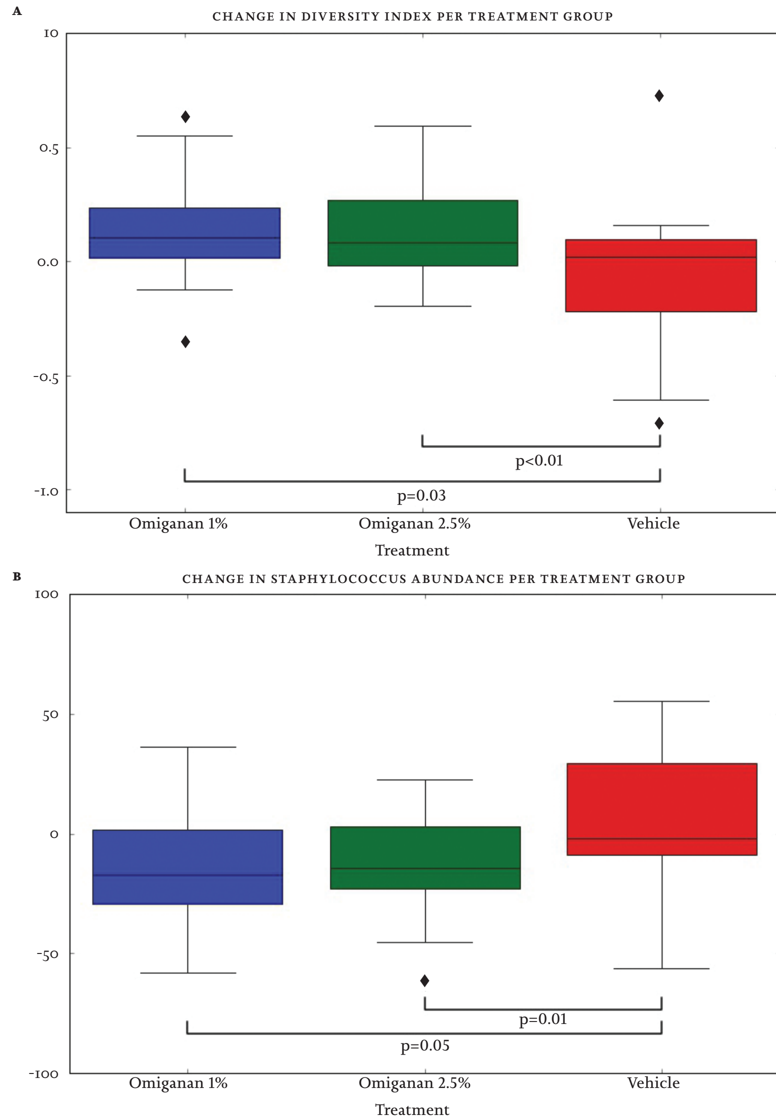
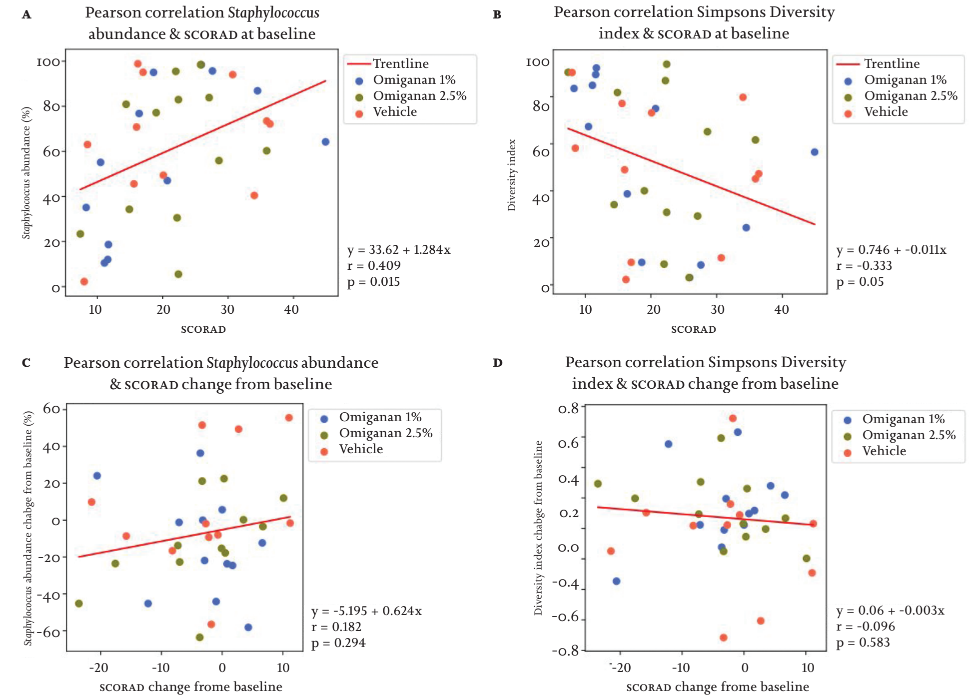
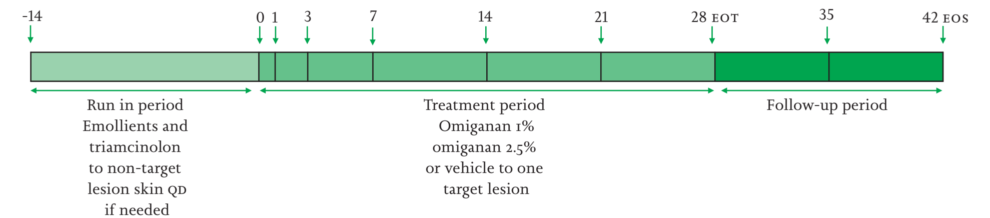


FIGURE 6 **Correlation analysis of target lesion OSCORAD index and *Staphylococcus abundance*.** The correlation between the local target lesion OSCORAD and *Staphylococcus abundance* in the microbiome is shown in panel A, and for the target lesion OSCORAD and diversity index in B. The delta correlations are presented in panels C and D.



SUPPLEMENTAL FIGURE S1 **Assessment schedule of the study.**



CHAPTER VIII

TOPICAL ANTI-MICROBIAL PEPTIDE OMIGANAN RECOVERS CUTANEOUS DYSBIOSIS BUT DOES NOT IMPROVE CLINICAL SYMPTOMS IN PATIENTS WITH MILD-TO-MODERATE ATOPIC DERMATITIS IN A PHASE 2 RANDOMIZED CONTROLLED TRIAL

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ABSTRACT

BACKGROUND Dysbiosis and colonization with *Staphylococcus aureus* is considered to play an important role in the pathogenesis of atopic dermatitis (AD). Recovering this dysbiosis may improve AD symptoms. Omiganan is a synthetic indolicidin analogue antimicrobial peptide with activity against *S. aureus* and could be a viable new treatment option for AD.

OBJECTIVE To explore the tolerability, clinical efficacy and pharmacodynamics of omiganan in mild-to-moderate AD.

METHODS Eighty patients were randomized to omiganan 1%, 1.75%, 2.5% or vehicle twice daily for 28 days on all lesions. Weekly visits included clinical scores, and microbiological and pharmacodynamic assessments of one 'target lesion'.

RESULTS In all omiganan treatment groups dysbiosis was recovered by reducing *Staphylococcus* abundance and increasing diversity. A reduction of cultured *S. aureus* was observed in all omiganan treatment groups, with a significant reduction for omiganan 2.5% compared to vehicle (-93.5%, 95%CI=-99.2%/-28.5% p=0.02). No significant clinical improvement was observed.

CONCLUSION Topical administration of omiganan twice daily for up to 28 days in patients with mild-to-moderate AD led to a recovery of dysbiosis, but without clinical improvement. Therefore, a mono-treatment that selectively targets the microbiome does not appear to be a successful treatment strategy in mild-to-moderate AD.

INTRODUCTION

The pathophysiology of atopic dermatitis (AD) is complex and incompletely understood. Genetic susceptibility, environmental factors, epidermal barrier abnormalities, immunological disturbances and dysbiosis of skin microbiota may explain the heterogeneous character of AD and it remains hard to discern which of these are primary or secondary events, or both.[1]

Staphylococcus aureus is an important player regarding dysbiosis in AD.[2,3] Colonization with this pathogen and a lower overall microbial diversity is apparent in approximately 70% of the lesional skin of AD patients.[4] A deficiency in antimicrobial peptides (AMPs) related to a TH2-response may partially account for the susceptibility to *S. aureus* colonization.[5,6] After adhesion, *S. aureus* may directly cause or increase ongoing inflammation by binding of its superantigens to MHCII molecules which induces excessive production of T-cell cytokines.[7–9] In addition, its superantigens may serve as conventional allergens and will generate a specific IGE-response.[9]

Based on the hypothesis that dysbiosis plays an important role in the pathogenesis of mild-to-moderate AD, the microbiome and more specifically *S. aureus* might be an important target for novel therapies.[6,9,10,11] A potential novel topical treatment from this perspective is omiganan, a synthetic indolicidin analogue. This short AMP from the cathelicidin family has shown *in vitro* and *in vivo* antimicrobial activity against *S. aureus* without the development of bacterial resistance.[12–14] We previously investigated the efficacy and tolerability of omiganan 1% topical gel, 2.5% topical gel or vehicle gel that was applied once daily to one antecubital fossa (target lesion) affected by AD.[15] We observed a statistically significant shift from lesional to non-lesional microbiome profile with both omiganan 1 and 2.5% gel, while there was no change in the vehicle group. Moreover, we found a statistically significant improvement of the target lesion according to local scoring Atopic Dermatitis (oSCORAD) and patient-reported itch scores. Although clinical effects were small, these results suggested a dose-response relationship and therefore we hypothesized that an increased dosing frequency of omiganan twice daily to all AD lesions would lead to a better clinical response.

The objective of this study was to investigate the tolerability, safety, clinical efficacy, microbiological and pharmacodynamic effects of omiganan 1%, 1.75% and 2.5% applied to all lesions twice daily in patients with mild-to-moderate AD.

MATERIALS AND METHODS

The Declaration of Helsinki was the guiding principle for trial execution. The independent Medical Ethics Committee 'Medisch Ethische Toetsingscommissie van de Stichting Beoordeling Ethiek Biomedisch Onderzoek' (Assen, the Netherlands) approved the study prior to any study activity. All patients provided written informed consent before participation. The study was conducted from March 2017 to December 2017 at the Centre for Human Drug Research, Leiden, The Netherlands.

STUDY CHARACTERISTICS, PATIENTS AND TREATMENTS ❋ This was a randomized, double-blind, placebo-controlled mono-center phase 2 study in 80 patients with mild-to-moderate AD. Patients with confirmed mild-to-moderate

AD (EASI score between 1.1 and 21.0 and BSA 2-20%) present for at least 1 year before study participation were included. There was a wash-out period for any AD medication. Patients with any other clinically significant condition were excluded. Health status was verified by taking a detailed medical history, a complete physical examination, vital signs, 12-lead ECG and laboratory tests (including hepatic and renal panels, complete blood count, chemistry panel, virology and urinalysis). Eligible patients were randomized 1:1:1:1 to omiganan 1%, 1.75%, 2.5% or vehicle gel by an independent statistician. Vehicle gel served as placebo with identical appearance to omiganan gel. The study drug was applied to all eczema lesions BID for 28 consecutive days. Bland emollients (Unguentum leniens) were supplied to use as maintenance therapy. Triamcinolon 0.1% was provided as rescue medication when discussed with the study physician. The full study protocol can be found in Supplemental Material S1.

MICROBIOME AND MICROBIOLOGY ❊ For microbiome analysis and *S. aureus* qPCR quantification, skin swab samples of lesional and non-lesional skin were collected. Procedures and conditions are described in detail in Supplemental Material S1 (7.5; p.44-46). The analysis was performed as described by van den Munckhof *et al.*[16] For *S. aureus* cultures sterile swabs (Puritan Sterile Polyester Tipped Applicators REF 25-806-1PD) were taken of lesional and non-lesional skin and transported to the microbiology department of the Alrijne Hospital, The Netherlands. Cultures were performed on blood agar plates.

EFFICACY AND PATIENT-REPORTED OUTCOMES ❊ Efficacy was evaluated by the Eczema Area and Severity Index scores (EASI), oSCORAD score and Investigator Global Assessment (IGA). Patient-Reported Outcomes (PROs) consisted of weekly Patient-Oriented Eczema Measure (POEM) and Dermatology Life Quality Index (DLQI) questionnaires and daily numeric rating scale (NRS) itch scores.

TARGET LESION PHARMACODYNAMICS ❊ One AD lesion, preferably of the antecubital fossa, was assigned as 'target lesion', opposed to a part of healthy skin that served as negative control (non-target lesion). Erythema and roughness were assessed by 3D photo analysis (Antera 3D, Miravex, Ireland). Skin barrier status of lesional and non-lesional skin was assessed by trans-epidermal water loss assessment (TEWL, AquaFlux AF200 system, Biox, London, UK). Skin surface biomarkers IL-10, IFN-γ , IL-13, IL-6, eotaxin3 and IL-31 were assessed with Transdermal Analysis Patches (TAP, FibroTx, Estonia) and qualitatively and quantitatively analysed by spot-ELISA. At day 0 (pre-dose) and day 28 (end-of-treatment, EOT), 3 mm skin punch biopsies were collected from lesional and non-lesional skin. RNA extraction and real-time quantitative PCR analysis was performed by the Erasmus Medical Centre, Rotterdam, The Netherlands for the following biomarkers: IFN-α, IFN-γ, IL-31, IL-6, IL-13 and eotaxin3. Details of the procedures and conditions are described in the Supplemental Material S1 (7.5; p.44-46).

TREATMENT COMPLIANCE, SAFETY AND TOLERABILITY ❊ Treatment compliance was recorded with a mobile e-diary app including a notification and photo capture function to enable documentation. Safety and tolerability were evaluated by adverse events monitoring, physical examination, vital signs, 12-lead ECGs and laboratory tests.

STATISTICAL ANALYSIS ❊ All calculations were performed using SAS for windows V9.4 (SAS Institute, Inc., Cary, NC, USA). No formal power calculation was performed given the exploratory character of this study. Efficacy/pharmacodynamic endpoints were analyzed with a mixed model analysis of variance using treatment, time and treatment by time as fixed factors and subject as random factor. Analyses were conducted in all patients that applied the study medication for at least 21 days and completed the EOT visit. Estimates of the difference, 95% confidence intervals, Least Square Means and p-values were generated. Analyses of mRNA expressions in the biopsies incorporated normalization for housekeeping gene GAPDH and incorporated the values from non-lesional skin to correct for the high variability. For the organization and visualization of microbiome data, Python 3.5.2 was used. Supplemental Material S2 includes the methodology for the microbiome analysis.

RESULTS

PATIENT DISPOSITION AND BASELINE CHARACTERISTICS ❊ Eighty patients were enrolled in the study, of whom 72 (90%) completed the study. For the participant flow, see Supplemental material S3. For the baseline characteristics of patients in all treatment groups, see *Table 1*.

MICROBIOME ❊ Microbiome composition of lesional skin was dominated by *Staphylococcus* and exhibited a low diversity index in general. After omiganan treatment, abundance of *Staphylococcus* was reduced in all active treatment

groups (Supplemental material S4). This reduction was statistically significant in the omiganan 1% and 2.5% treatment vs. vehicle group (-15.1, 95%CI=-28.6/-1.7, p=0.03 and -17.2, 95%CI=-30.4/-4.1, p=0.01, respectively). After treatment discontinuation, *Staphylococcus* abundances in the 1.75% and 2.5% treatment groups returned to pre-dose levels and remained low in the omiganan 1% treatment group. The diversity index increased in all omiganan treatment groups. This was statistically significant for omiganan 1% vs. vehicle (0.15, 95%CI=0.03/0.26, p=0.01) and omiganan 2.5% vs. vehicle (0.15, 95%CI=0.04/0.27, p=0.01). A summary of the change in *Staphylococcus* abundance and diversity data is depicted in Figure 1. See Supplemental material S2 for the full microbiome report with individual plots.

MICROBIOLOGY ✻ A reduction in *S. aureus* in the cultures was observed in all active treatment groups, which was most pronounced and statistically significant in the omiganan 2.5% group compared to vehicle at end-of-treatment (EOT) (*Table* 2). In the omiganan 1% and 1.75% treatment groups, a substantial, but not statistically significant numerical mean reduction was observed. After cessation of treatment, *S. aureus* culture values increased up to the end of the study. For the qPCR S. aureus data, a non-significant decrease was observed in all omiganan treatment groups.

Pearson correlation coefficients of the relation between *S. aureus* in culture and the relative abundance of the genus *Staphylococcus* by 16S rRNA gene profiling using next-generation sequencing (NGS) for vehicle were 0.46, 0.33, 0.10 and 0.38 for omiganan 1%, 1.75% and 2.5% respectively. For the relation between *S. aureus* qPCR and the relative abundance of the genus *Staphylococcus* by NGS, correlation coefficients were 0.60, 0.68, 0.55 and 0.78 for vehicle, omiganan 1%, 1.75% and 2.5% respectively (*Figure* 2 and 3).

EFFICACY AND PATIENT-REPORTED OUTCOMES ✻ Clinical efficacy showed no significant improvements in the active treatment groups compared to vehicle. The omiganan 1.75% group exhibited a small but significant clinical worsening, see *Table* 2. POEM scores showed a similar pattern in line with the clinical efficacy scores. Daily NRS itch scores were highly variable and no statistically significant changes occurred in any of the treatment groups.

BIOMARKERS ✻ Based on 3D photography of the target lesion, erythema and roughness slightly worsened (*Table* 2). Transepidermal water loss significantly differed between lesional and non-lesional skin at baseline (32.27, 95%CI=28.31/36.24, p<0.0001) but no significant changes were observed after omiganan and vehicle treatment (*Table* 2 and Supplemental material S5). Biopsy biomarker data were highly variable in general. At baseline, a significant difference between lesional and non-lesional skin was found for IL-13 only (0.99, 95%CI=0.83/1.14, p<0.0001). Non-significant differences were observed between lesional and non-lesional skin for eotaxin and IL-31 (0.1, 95%CI=-0.01/0.21, p=0.08 and -0.08, 95%CI=-0.18/0.01, p=0.09, respectively), *Figure* 3. Although there were no significant differences between lesional and non-lesional skin at baseline for IL-6, a significant reduction was observed in the omiganan 1% and 2.5% group. In the vehicle and omiganan 1.75% group no significant changes were observed. For all other biopsy biomarkers, no significant changes occurred. TAP revealed no differences in any of the biomarkers between lesional and non-lesional skin at baseline, nor after any of the treatments. Serum TARC increased significantly with 26.4% in the omiganan 1.75% group, parallel to the small increase in clinical severity scores. The other serum biomarkers showed no notable changes.

TREATMENT COMPLIANCE AND SAFETY ✻ Overall treatment compliance ranged from 90-98% across the treatment groups. Average usage of study medication per day ranged from 1.1 to 2.0 mg per cm^2. No serious adverse events (SAEs), or deaths occurred during the study. Eight patients discontinued because of a significant increase in atopic dermatitis symptoms, not related to the study drug. Most frequently occurring AEs were application site AEs (n=13), headache (n=14) and influenza-like illnesses (n=8). All TEAEs were of mild or moderate severity and self-limiting. There were no clinically significant changes in any of the safety laboratory parameters, vital signs and ECG recordings.

DISCUSSION

In this study we observed that treatment of AD skin with the topical AMP omiganan led to a recovery of lesional skin dysbiosis across all active treatment groups. The statistically significant reduction of *S. aureus* in culture, of *Staphylococcus* genus in the microbiota and an increase in diversity index of the microbiota profile, did not translate into clinical efficacy or significant improvement of molecular biomarkers.

We observed a similar recovery of dysbiosis in our previous study evaluating a single target lesion using oSCORAD. [15] The results of the current study however indicate that BID administration to all AD lesions does not lead to a significant

improvement of clinical symptoms. Firstly, this could be explained by the absence of a linear dose-response pharmacology of omiganan. Secondly, the effects on the target lesion oSCORAD of the previous QD study might have been a coincidence, and with a greater variability of the measurement in this study no effects could be observed. Lastly, cationic peptides including omiganan are suggested to have pro-inflammatory effects which might interfere with the anti-inflammatory properties.[17,18] However, for omiganan the pro-inflammatory capacities are only triggered by a viral co-stimulus which is not relevant in AD patients.[19]

Whether microbial skin dysbiosis is a primary or secondary factor in the pathogenesis of AD remains uncertain. Kobayashi and colleagues (2015) highlighted the microbiota-host immunity axis as a potential target for future AD treatment in their study in which Adam17(fl/fl)Sox9-(Cre) mice developed eczematous dermatitis with dysbiosis similar to the dysbiosis observed in AD. Mice were treated with targeted antibiotics against *S. aureus*, *C. mastitidis* and *C. bovis* species, which recovered dysbiosis and eliminated skin inflammation clinically and in the (biopsy) biomarkers.[20] Our study focused on this axis with the strong antimicrobial capacity of omiganan against the species involved in the dysbiosis of AD.[12,14] Our findings of reversed dysbiosis, without improvement of clinical scores seem to suggest that dysbiosis is more likely to be a secondary effect in the pathogenesis of AD, by susceptibility for *S. aureus* colonization through a deficient barrier related to TH2 skewed immune dysregulation. This hypothesis is supported by the observed dysbiosis recovery after corticosteroids and coal tar treatments, which do not have any specific antimicrobial properties.[21,22] Apparently, by suppressing inflammation, the epidermal barrier will heal and the susceptibility to *S. aureus* colonization will decrease leading to a normalization of the microbiota profile.

Baseline data of the current study did show numerical differences between lesional and non-lesional skin but no statistically significant differences for the proximal *S. aureus*-associated downstream biomarkers, while *S. aureus* is known to activate the TLR-2 pathway and to produce Staphylococcal enterotoxin B which can induce the production of a variety of cytokines including IL-6, IFN-γ, IL-31 and eotaxin.[7,8] However, with active treatment we did observe a statistically significant decrease of IL-6, but in general, the *S. aureus* reduction did not yield a clear signal of the downstream markers.

The relative short duration and the mono-therapy approach are potential limitations of this study. However, a recently published study showed no corticosteroid-sparing effect with an antimicrobial endolysin specifically targeting *S. aureus*.[23] Another limitation could be that our patient population could have been mixed in terms of *S. aureus* colonization, as this was based on one single culture, and may have benefitted less from omiganan treatment than a population with abundantly colonized or infected AD lesions.

Based on our results, the future development of omiganan may be focused on diseases where *S. aureus* plays a more central role, e.g., in superinfected AD a non-antibiotic drug like omiganan may reduce the need for oral antibiotics that are increasingly associated with bacterial resistance. A clear clinical benefit was demonstrated in a case series of three patients with *S. aureus*-related dermatoses that were treated with a topical bacteriophage derived endolysin.[24] Omiganan might also have clinical utility in the eradication of multi-drug resistent *S. aureus* strains in long-term carriers, where it might replace the long-term use of oral antibiotics.

In conclusion, this study showed that topical administration of omiganan twice daily for up to 28 days in patients with mild-to-moderate AD led to a recovery of dysbiosis, but without clinical improvement. Our findings do not support dysbiosis as a viable monotherapy drug target in mild-to-moderate atopic dermatitis, and indirectly suggest that *S. aureus may* play a less prominent role in the pathogenesis of mild-to-moderate AD.

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TABLE 1 Baseline characteristics.

	All patients	Omiganan 1%	Omiganan 1.75%	Omiganan 2.5%	Vehicle
	N=80	**N=20**	**N=20**	**N=20**	**N=20**
Patients dosed	80 (100%)	20 (100%)	20 (100%)	20 (100%)	20 (100%)
Patients completed	72 (90%)	19 (95%)	16 (80%)	19 (95%)	18 (90%)
Age (years)					
Mean (SD)	24.4 (6.5)	24.5 (8.4)	25.9 (8.2)	23.6 (3.5)	23.5 (4.5)
Median	23.5	22.5	23.0	24.0	22.0
Min,Max	18, 49	18, 49	18, 48	18, 31	18, 33
Sex					
Female (%)	44 (55.0%)	11 (55.0%)	11 (55.0%)	11 (55.0%)	11 (55.0%)
Male (%)	36 (45.0%)	9 (45.0%)	9 (45.0%)	9 (45.0%)	9 (45.0%)
OSCORAD					
mean (SD)	19.9 (4.6)	19.3 (4.1)	20.7 (4.3)	20.8 (5.4)	18.8 (4.4)
Min-max	10.9, 29.8	10.9, 25.4	14.4, 29.8	10.9, 28.8	10.9, 28.5
EASI					
mean (SD)	3.8 (2.0)	3.8 (1.6)	3.8 (2.2)	4.1 (2.3)	3.5 (2.1)
Min-max	1.1, 9.8	1.4, 5.8	1.4, 9.8	1.2, 9.3	1.1, 8.1
IGA					
mean (SD)	16.9 (4.4)	18.0 (3.9)	15.9 (5.6)	16.9 (4.3)	16.8 (3.5)
Min-max	6, 26	10, 25	6, 26	6, 24	10, 22
Serum TARC					
Mean (SD)		146.3 (122.9)	190.1 (87.3)	140.1 (125.2)	140.4 (85.4)
Fitzpatrick Skin Type					
Fitzpatrick 1	7 (8.8%)	2 (10.0%)	1 (5.0%)	3 (15.0%)	1 (5.0%)
Fitzpatrick 2:	39 (48.8%)	8 (40.0%)	12 (60.0%)	7 (35.0%)	12 (60.0%)
Fitzpatrick 3:	20 (25.0%)	7 (35.0%)	4 (20.0%)	6 (30.0%)	3 (15.0%)
Fitzpatrick 4:	9 (11.3%)	2 (10.0%)	2 (10.0%)	3 (15.0%)	2 (10.0%)
Fitzpatrick 5:	4 (5.0%)	0 (0%)	1 (5.0%)	1 (5.0%)	2 (10.0%)
Fitzpatrick 6:	1 (1.3%)	1 (5.0%)	0 (0%)	0 (0%)	0 (0%)
Filaggrin mutation present*	12/73 (16%)	4/17 (24%)	3/19 (16%)	4/18 (22%)	1/19 (5%)
S. aureus colonization lesional skin	50 (63%)	11 (55%)	16 (80%)	11 (55%)	12 (60%)
Mean colony forming units/ml**	933503 (5650793)	908232 (5637456)	744972 (4560153)	856325 (5371280)	933503 (5650793)

** for patients that consented for polymorphism analysis / ** of S. aureus positive patients*

TABLE 2 Analysis results – intention-to-treat analysis.

Outcome	Omiganan 1% vs vehicle			Omiganan 1.75% vs vehicle			Omiganan 2.5% vs vehicle		
	Estimated difference	95% CI	p-value	Mean	95% CI	p-value	Mean	95% CI	p-value
OSCORAD	2.0	0.52/4.51	0.12	3.83	1.3/6.36	0.003	2.52	0.0/5.04	0.05
EASI	0.2	-0.8/1.19	0.69	1.36	0.35/2.36	0.008	0.57	-0.41/1.55	0.25
IGA	0.66	-2.07/3.39	0.63	4.31	1.59/7.02	0.002	2.68	-0.01/5.38	0.05
NRS itch first half of the day	-0.4	-10.3/9.4	0.93	3.4	-6.5/13.3	0.49	4.7	-5.2/14.6	0.35
NRS itch second half of the day	1.2	-8.2/10.5	0.80	4.9	-4.5/14.3	0.30	3.7	-5.6/13.1	0.43
Erythema target lesion by 3D photography	5.5	-5.93/16.92	0.34	17.84	6.44/29.24	0.003	1.02	0.04/2.0	0.04
Roughness target lesion by 3D photography	0.19	-0.78/1.16	0.7	1.35	0.37/2.33	0.008	1.02	0.04/1.99	0.04
TEWL flux (g/m²/h)	-0.49	-8.25/7.27	0.90	5.57	-2.27/13.41	0.16	4.0	-3.91/11.91	0.32
IFNα biopsy (mRNA relative to ABL)	-15.9%	-80.4%/261.3%	0.81	-35.9%	-85.1%/175.3%	0.54	46.6%	-65.4%/521.1%	0.60
IFNγ biopsy (mRNA relative to ABL)	-51.8%	-87.4%/83.8%	0.28	-33.2%	-82.2%/150.6%	0.54	-30.6%	-81.2%/156.1%	0.58
IL-6 biopsy (mRNA relative to ABL)	-80.5%	-94.8%/-26.9%	0.02	-30.6%	-81.5%/160.3%	0.58	-71.3%	-92.2%/5.9%	0.06
IL-31 biopsy (mRNA relative to ABL)	-16.1%	-76.9%/204.1%	0.79	25.4%	-66.3%/366.1%	0.73	-48.2%	-85.7%/87.9%	0.31
Eotaxin biopsy (mRNA relative to ABL)	-8.4%	-62.4%/122.9%	0.84	24.8%	-48.7%/203.8%	0.62	-4.7%	-60.4%/129.4%	0.91
IFNγ TAP (ng/ml)	64%	0%/169.2%	0.05	33.1%	-18.6%/117.5%	0.25	25.1%	-22.6%/102.3%	0.36
IL-10 TAP (ng/ml)	15.3%	-25.7%/79.0%	0.52	-9.5%	-42%/41.2%	0.66	-11.5%	-43.2%/37.8%	0.58
IL-13 TAP (ng/ml)	20%	-45.5%/164.3%	0.65	7.7%	-51.4%/138.8%	0.85	20%	-45.7%/165.3%	0.65
IL-31 TAP (ng/ml)	75%	-30%/337.4%	0.23	24.3%	-50.6%/212.4%	0.64	18.4%	-51.6%/189.4%	0.71
IL-6 TAP (ng/ml)	-7.6%	-58.8%/107.1%	0.85	-20.6%	-65.3%/81.7%	0.58	-17.7%	-62.6%/81.2%	0.62
Eotaxin TAP (ng/ml)	-17.1%	-52.7%/45.4%	0.51	-24.1%	-56.7%/33.1%	0.33	8.6%	-36.8%/86.8%	0.76
IFNα serum (pg/ml)	-4.8%	-15.1%/6.8%	0.40	-3.7%	-14.2%/8.0%	0.51	-4.7%	-15%/5.9%	0.41
IFNγ serum (pg/ml)	-7.1%	-38%/39.2%	0.72	13.8%	-24.4%/71.2%	0.53	21.9%	-19%/83.6%	0.34
TARC serum (pg/ml)	-7.4%	-27.3%/18.1%	0.53	26.4%	-1.7%/62.7%	0.07	2.7%	-19.5%/30.9%	0.83
IL-31 (pg/ml)	-9.3%	-28.3%/14.8%	0.41	-12%	-31.2%/12.5%	0.30	-13.8%	-32.5%/10.0%	0.23
Eotaxin (pg/ml)	5.5%	-2.6%/14.3%	0.19	-4.1%	-11.7%/4%	0.31	-1.2%	-8.8%/7.1%	0.77
S. aureus in culture (cfu/ml)	-84.3%	-98.4%/51.6%	0.11	-83.6%	-98.3%/61.9%	0.12	-92.5%	-99.2%/-28.5%	0.02
qPCR *S. aureus* (copies/uL)	-70.5%	-96%- 119.7%	0.23	-53.8%	-94.2% - 265.4%	0.46	-63.2%	-95.2% - 184.9%	0.34

FIGURE 1 Mild-to-moderate atopic dermatitis. Summary of the change in diversity index of the target lesion, boxplots and medians (blue text).

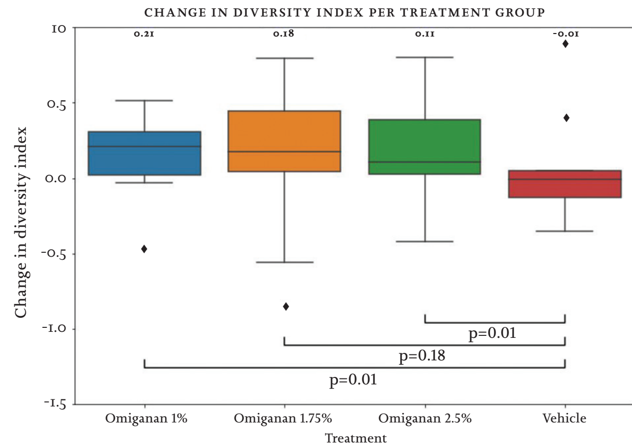


FIGURE 2 Mild-to-moderate atopic dermatitis. Presence of *S. aureus* based on culture or qPCR in relation to the relative abundance of *Staphylococcus* determined by next-generation sequencing (NGS) at baseline of the target lesion of each patient. Red: vehicle, blue: omiganan 1%, orange: omiganan 1.75%, green: omiganan 2.5%.

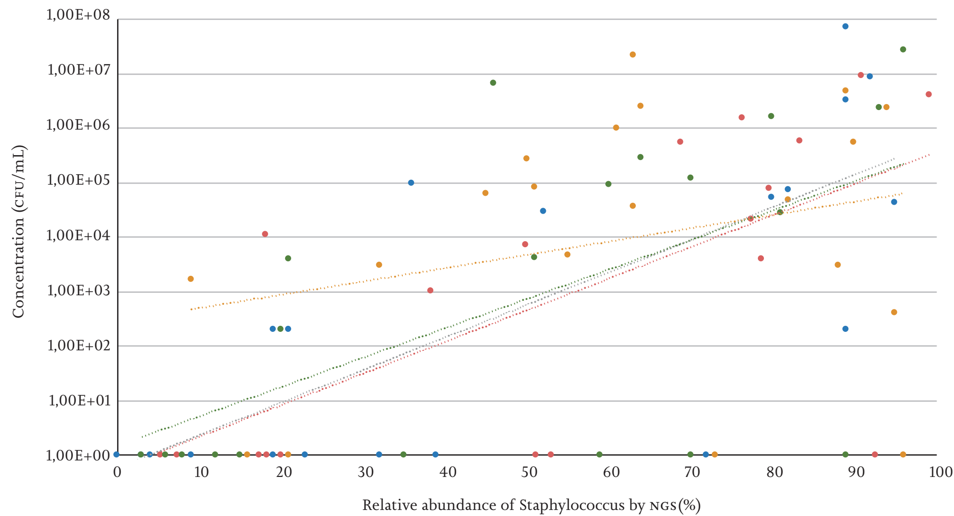
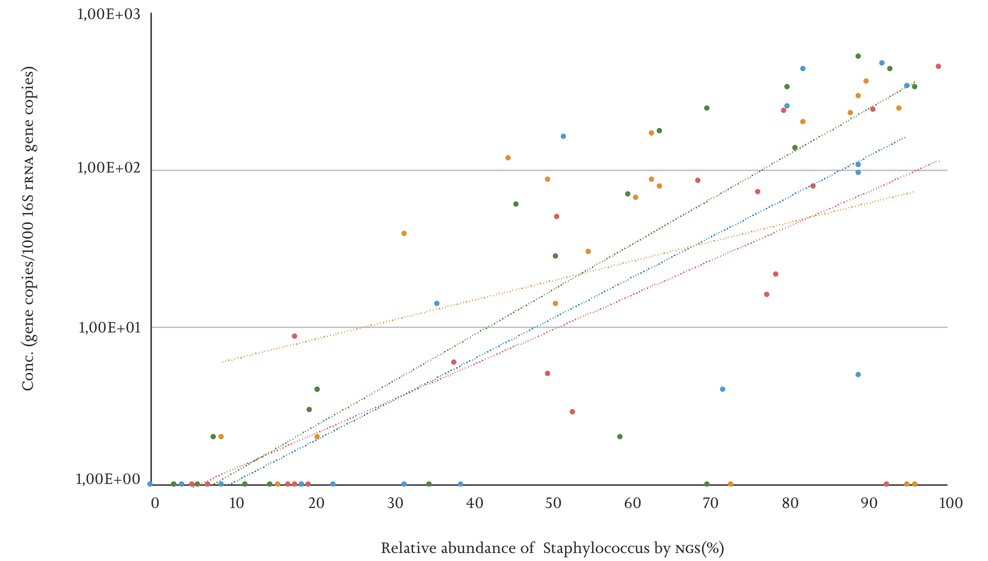
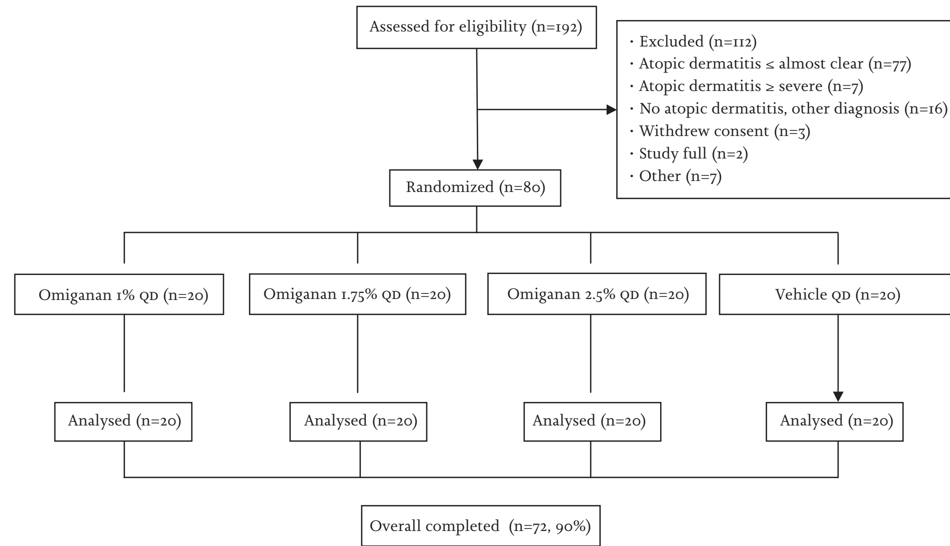


FIGURE 3 Mild-to-moderate atopic dermatitis. Presence of *S. aureus* based on qPCR in relation to the relative abundance of *Staphylococcus* determined by next-generation sequencing (NGS) at baseline of the target lesion of each patient. Red: vehicle, blue: omiganan 1%, orange: omiganan 1.75%, green: omiganan 2.5%

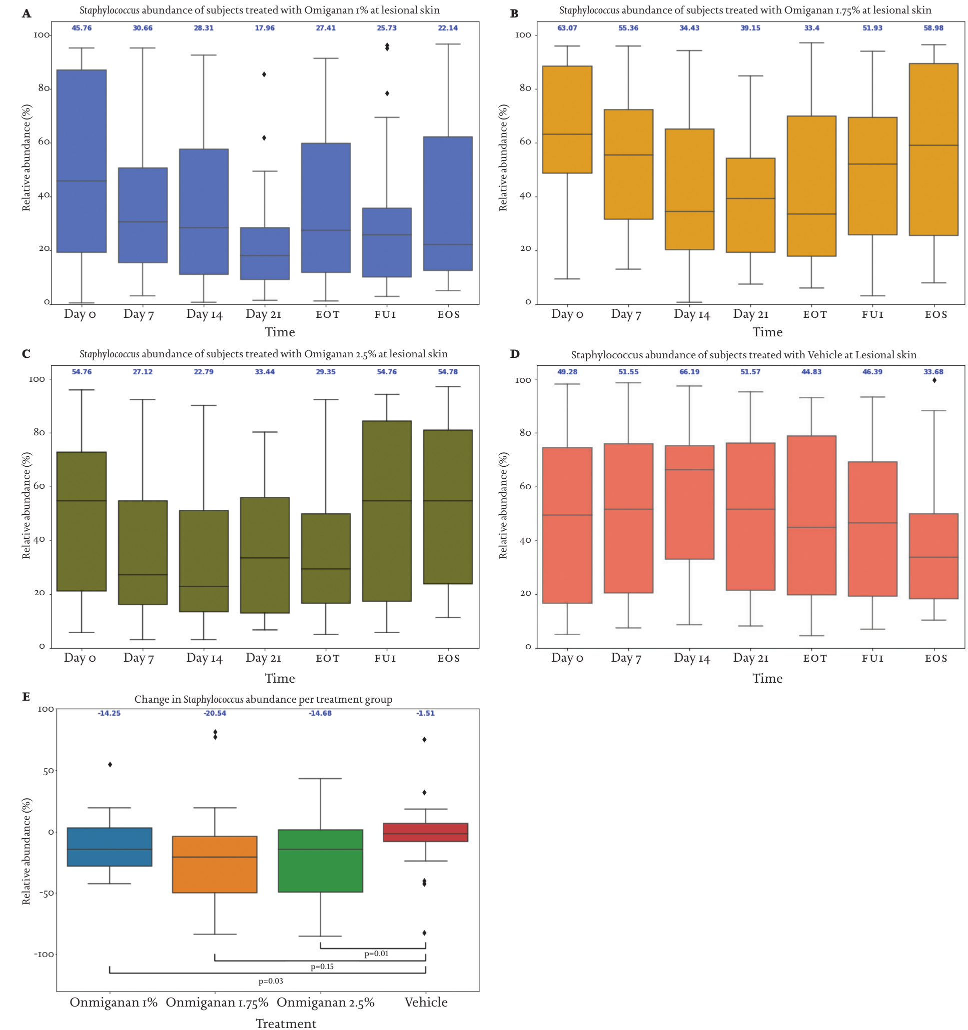


SUPPLEMENTAL MATERIAL S3 Flowchart of the study.

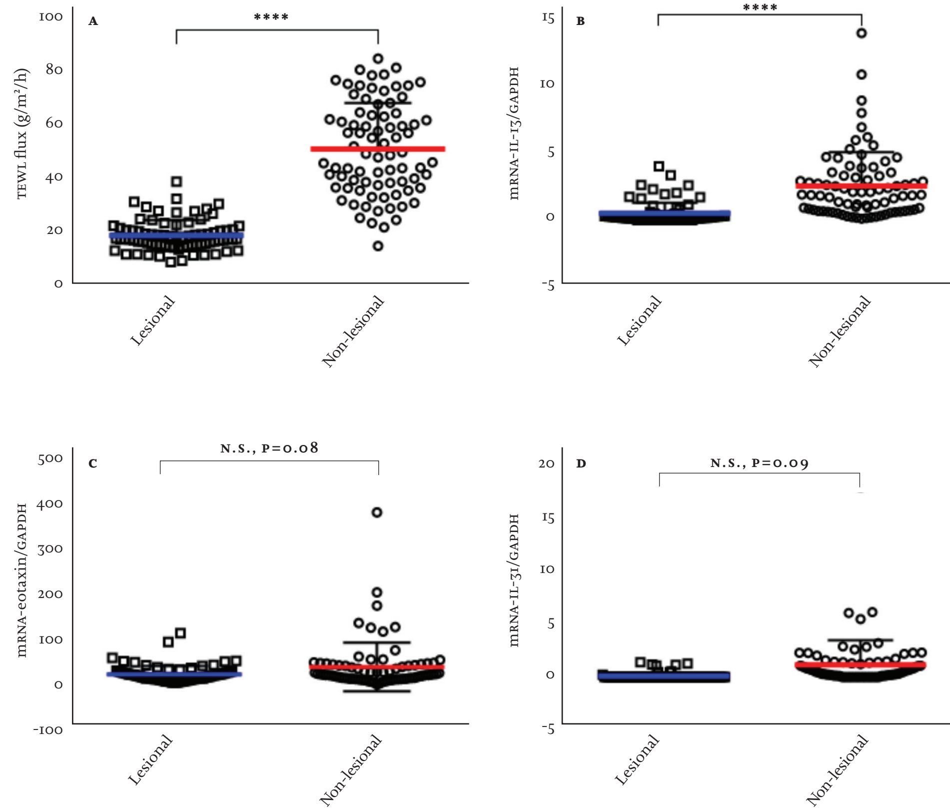


SUPPLEMENTAL MATERIAL S4 *Staphylococcus* abundance over time.

A) *Staphylococcus* abundance over time of the target lesion in the omiganan 1% treatment group, B) *Staphylococcus* abundance of the target lesion over time in the omiganan 1.75% treatment group, C) *Staphylococcus* abundance of the target lesion over time in the omiganan 2.5% treatment group, D) *Staphylococcus* abundance of the target lesion over time in the vehicle treatment group, E) Summary of the change in *Staphylococcus* abundance of the target lesion, boxplots and median (blue text), and F).



SUPPLEMENTAL MATERIAL S5 **Biomarkers.** A) Transepidermal water loss lesional vs. non-lesional skin, B) mRNA expression of IL-13 relative to GAPDH lesional vs. non-lesional skin, C) mRNA expression of eotaxin relative to GAPDH lesional vs. non-lesional skin, D) mRNA expression of IL-31 relative to GAPDH lesional vs. non-lesional skin.



CHAPTER IX

SUMMARY AND PERSPECTIVES

There is a high need for novel treatments for patients with chronic immune-mediated inflammatory skin diseases. Success rates from phase I to market registration in auto-immune/inflammation treatments are as low as 6.3%, which is primarily related to the lack of clinical information (unknown active dose, unclear regimen, uncertain pharmacological activity) on the drug in the early phases of clinical development.[1] Since the number of therapeutic candidates in clinical development programs is rapidly increasing, new strategies to optimize the development process are needed. However, the literature on rational drug development in dermatology is scarce. Though there is movement into the direction of a question-based drug (QBD) development approach,[2] still a lot of early clinical development plans are rather traditional. Recently, we proposed a systematic approach derived using QBD for early phase clinical pharmacology studies in dermatology with five important cornerstones, i.e.

1. investigate dermal and systemic pharmacokinetic properties
2. elucidate pharmacodynamics properties including dose-response
3. use sensitive and objective endpoints
4. profile disease and drug effects by a complementary, multimodal approach
5. foster multi-disciplinary collaborations for trial conduct.[3]

In line with this dermatological drug development blueprint, the scope of this thesis was to develop new tools for this rational drug development approach in inflammatory skin disease, in particular atopic dermatitis. Subsequently, we investigated a novel drug candidate for atopic dermatitis using this approach.

This thesis is divided into three parts:

SECTION 1

Describes the development and application of a skin inflammation challenge model in healthy volunteers.

SECTION 2

Reports on the development and selection of novel biomarkers as tools for *proof-of-pharmacology* trials in dermatology.

SECTION 3

A novel therapy for atopic dermatitis (AD) is explored utilizing the tools described in foregoing sections.

This chapter sums up the results and highlights the perspectives of rational drug development in inflammatory skin disease.

RATIONAL DRUG DEVELOPMENT IN CHRONIC INFLAMMATORY SKIN DISEASE

SECTION 1

SKIN INFLAMMATION MODELS AS TOOL FOR EARLY PHASE PROOF-OF-PHARMACOLOGY Investigating the pharmacodynamics of a novel compound next to safety, tolerability and pharmacokinetic properties is mandatory as recently re-emphasized by the EMA first in human guideline.[4] To obtain this information in healthy volunteers different approaches are taken, e.g. investigating target engagement by receptor occupancy studies or downstream signaling elucidation or assessing functional biomarkers of the pathway. However, difficulties arise when investigating anti-inflammatory drugs in healthy volunteers who obviously do not suffer from an inflammatory skin disease. In this case, pharmacological challenge models that mimic the pathophysiological characteristics of skin inflammation can be applied. These models can be used to obtain early insight into target engagement and dose-response relationships. In dermatology, many drugs that are currently in the development pipeline have an immunomodulatory mechanism of action. Preclinical animal models are routinely used to elucidate the pharmacodynamic effects in vivo, e.g. imiquimod and lipopolysaccharide models in mice.[5] While these models give some indication, the predictiveness for activity in the target indication remains low. Therefore, translational models that mimic skin inflammation are required to help answering important questions for the future development of a new drug. For example, questions regarding drug profiling, dose or drug regimen selection and possibly refining the right target indication for the compound. The answers to these questions would directly influence the next steps of the development process. For this purpose, an imiquimod (TLR7 stimulation) induced skin inflammation challenge model was developed in this thesis in **section 1.**

In **chapter 2** two models to induce a local temporary and reversible inflammatory response by activating the innate immune system of the skin were investigated, i.e.

1. imiquimod application under occlusion,
2. tape stripping of the skin prior to imiquimod application under occlusion.

The inflammatory response of the skin in this study was quantified with a toolbox of novel measurements and biomarkers to characterize skin inflammation from different perspectives, i.e. clinically (erythema grading of the skin by physician), imaging (erythema of the skin by 2D photo analysis), biophysical (perfusion of the skin by Laser Speckle Contrast Imaging), molecular (mRNA

expression of CXCL10, IFN-β, IFN-γ, TNF-α, IL-1β, IL-6, HBD-2, MX1, MXA and ICAM-1 in skin punch biopsies), cellular (immunohistochemical staining of CD11C, CD14, CD1A, CD4, CD8 and HLA-DR in skin punch biopsies) and patient reported (itch and pain). In general, both models induced a temporary local inflammatory response as expected. However, a stronger inflammatory skin response with a more rapid onset was obtained with the second model; tape stripping of the skin prior to 2 days of imiquimod application under occlusion. Tape stripping in combination with imiquimod treatment under occlusion showed larger responses in several domains including erythema and perfusion ($p<0.0001$), mRNA expression of the inflammatory markers ($p<0.01$) and inflammatory cell influx compared to vehicle and imiquimod alone treatment. After 2 days, the imiquimod inflammatory response reached a plateau phase in which the erythema blood flow and biopsy biomarkers remained stable compared to 3 days of application. Subject reported that itch and pain at the treatment sites were acceptable. Furthermore, the induced inflammation was fully reversible.

To validate and test the applicability of the developed imiquimod model, an interaction study of the imiquimod model with omiganan, a topically applied antimicrobial peptide (AMP) with immunomodulatory properties was performed in **chapter 3**. Previous *in vitro* work with omiganan showed that omiganan enhances anti-viral IFN responses induced by imiquimod, which suggests future application of omiganan as co-treatment with imiquimod for viral skin disease in humans e.g. in anogenital warts and high grade squamous intraepithelial lesion (HSIL).[6] A translation from *in vitro* to *in vivo* in healthy volunteers of this concept with a dose regimen selection was preferred instead of going directly into a patient population. With our local skin inflammation challenge model with imiquimod we were able to perform this translational step. In this study we investigated different doses and different dosing regimens of combined omiganan and imiquimod treatment. Skin responses were measured from different perspectives as described in **chapter 2**. Two days of imiquimod application under occlusion with tape stripping prior to application induced the same local inflammatory response as observed previously **(chapter 2)**, confirming the repeatability of the developed model. Co-treatment with omiganan enhanced this inflammatory response to imiquimod, with increases in perfusion ($p<0.01$) and erythema ($p=0.02$). Inflammatory responses in the biopsy biomarkers following TLR7 stimulation were also enhanced by omiganan (increases in IL-6, IL-10, MXA, and IFNɣ), and more immune cell infiltration was observed (in particular CD4+, CD8+ and CD14+ cells). These results are in line with the earlier mechanistic *in vitro* results and therewith this study supports a future imiquimod/omiganan combination therapy study e.g. in patients with anogenital warts or actinic keratosis.

To summarize **section 1**, the imiquimod skin inflammation challenge model in healthy volunteers was found to be a suitable pharmacological challenge model for future early phase *proof-of-pharmacology* as well as immunomodulatory profiling or interaction trials. More specifically for development programs with novel compounds targeting the innate immune system and drug development programs where TLR7 is involved. While previously models with imiquimod were developed in psoriasis patients or with a lengthy treatment-period of 28 days in healthy volunteers without TS to mimic psoriasis lesions, our study showed a rapid and reproducible way of inducing short-term inflammatory skin lesions for the purpose of drug profiling and interactions studies.[7,8] **Chapter 3** is an illustrative example of an interaction study with the developed imiquimod model to translate previous *in vitro* work to *in vivo* data.[6] This study showed a thorough review of the drug effects in healthy volunteers which helped with stratifying the next steps in development of the compound, which was finding the right target population for the drug in this case. In conclusion this chapter shows integrating a skin inflammation challenge model in a drug development program can lead to a more rational way of early clinical development. In future development programs in inflammatory skin disease, the integration of skin inflammation challenge models should be considered.

SECTION 2

DEVELOP AND INTEGRATE OBJECTIVE CLINICAL ENDPOINTS

Clinical scores still play a key role in all phases of clinical trials for inflammatory skin disease. These scores provide a general standardized routine-based estimation of disease severity for clinical practice and as demanded by the regulatory agencies. However, they also have multiple disadvantages such as limited objectivity since the physician that performs the assessment might introduce a response quantification bias and the lack of sensitivity needed to quantify smaller effects of novel drug candidates, which are important parameters in early clinical phase research. With the many new innovative techniques that are currently available, the integration of surrogate endpoints that supports unbiased objective evaluation of a drug in clinical trials in dermatology is proposed. To facilitate this approach more objective endpoints are needed. In **section 2** we report on the development and selection of novel biomarkers as tools for *proof-of-pharmacology* with a multimodal approach.
Chapter 4 entails the development and application of an e-diary phone application with reminder function to monitor treatment adherence and patient reported outcomes in a clinical trial. It is known that the adherence to topical drugs in clinical practice can be as low as 25%[9–11] Many drugs in dermatological clinical trials

are topical, and will be commonly applied at home. To make conclusions on safety and efficacy it is essential to obtain valid data on treatment adherence. To monitor this, often paper diaries are used which have a high recall bias, a low-to-moderate adherence rate and a limited reliability.[12] These diaries are also used to obtain data on patient reported outcomes, e.g. pain and itch. The median adherence (actual administrations divided by the expected administrations) range of the e-diary was 98% (range 97-99%), which is excellent. In addition, patient reported outcomes were added to the application to monitor patient reported itch and pain, which also had excellent adherence rates (89% (range 87-95%) and 94% (range 87-96%) respectively). Previously published results indicate that 67%-95% of the patients using topical treatments under dose their medication because of low adherence. Integration of the e-diary in a clinical trial could prevent this.[13,14] Moreover, adherence means the drug is applied to the lesions and therewith reaches the site of action. Although this does not necessarily mean the drug penetrates the skin, this can be supported with *in vitro* and *in vivo* models. Also the e-diary could facilitate a 'trial at home' approach for clinical trials, in which remote monitoring of a patient will lower the burden for participants.[15]

Another potential biomarker that is currently of interest for many skin diseases is the skin microbiota (i.e. the specific microorganisms that are present on the skin). Healthy skin of each human has a specific microbial 'fingerprint', which depends on the physical and chemical features of the skin as well as on host and environmental factors, including colonization at birth, antibiotic exposure, hygiene, lifestyle, and geographic location.[16,17] This fingerprint can also be influenced by skin disease. To explore whether skin microbiota analysis would be interesting for our multimodal approach a systematic literature review was performed on the potential of the skin microbiota as biomarker in six dermatological conditions with an inflammatory component, i.e. atopic dermatitis, acne vulgaris, psoriasis vulgaris, hidradenitis suppurativa, seborrheic dermatitis/ pityriasis capitis and ulcus cruris. Presented in **chapter 5,** the search yielded 841 papers of which 11 were included in the review. In general, the review showed that there was a high variability in study design and sampling methods between studies, which made comparisons of specific findings difficult. A standard approach for skin microbiota study design, collection, storage, processing and analysis should be followed in future studies.[18] However, for two indications, i.e. atopic dermatitis and acne vulgaris there is preliminary evidence from multiple studies that supports implementation of the skin microbiota as disease or drug-specific biomarker in early phase clinical trials as there seems to be a clear relationship between changes in the microbiota and disease severity, mostly for

atopic dermatitis (higher abundance of *Staphylococcus* combined with a lower general diversity).[19-21] But, despite this clear relationship, the review indicated that the skin microbiota within an individual patient can highly vary over time.[22-25] Therefore, data of longitudinal studies characterizing and analyzing the inter-patient and intra-patient variability of lesional skin microbiota is needed for final conclusions on implementing the skin microbiota as biomarker in clinical trials in atopic dermatitis.

In **chapter 6** it was therefore aimed to characterize the microbiota of lesional and non-lesional skin of atopic dermatitis patients. In particular, the inter-patient and intra-patient variability of the skin microbiota of atopic dermatitis patients over time was analyzed. This to determine whether limited sampling before and after treatment is sufficient to determine the effect of an atopic dermatitis treatment on the lesional skin microbiota or that multiple time point sampling is needed when implementing skin microbiota analysis in a clinical trial in atopic dermatitis. The inter-patient variability of microbial diversity was high for lesional skin compared to non-lesional skin (coefficient of variation of 35.5-45.9% vs 16.3-28.0%). For lesional skin, the degree of inter-patient variability of the relative abundance of *Staphylococcus* spp. and the concentration of *Staphylococcus aureus (S. aureus)* was also high with coefficients of variation ≥46.9%. The large inter-patient and intra-patient variability for lesional skin microbiota was confirmed by microbiota data obtained from an additional set of swabs from atopic dermatitis patients. Therefore a high sample frequency, e.g. once weekly, is needed to time-dependent insight into the changes of the highly variable skin microbiota in clinical trials.

Taken together, in **section 2** we showed the evaluation of novel biomarkers (e-diary and skin microbiota) for clinical trials in dermatology. The e-diary provides more valid data on treatment adherence (and indirectly 'does the drug reach the site of action' as well as clinical efficacy) and can facilitate a so-called 'trial at home' approach and should therefore be considered as important tool for a clinical development program. For AD, the skin microbiota as biomarker in a clinical trial should be further explored, but attention should be given to a standardized sampling method and high sampling frequency.

SECTION 3

MULTIMODAL PROFILING TO THOROUGHLY STUDY DRUG EFFECTS

With the number of novel measuring techniques rapidly expanding, the opportunity arises to thoroughly study drug effects in different domains (multimodal profiling) for the individual patient instead of focusing on one (mostly clinical)

endpoint. The different domains in this multimodal approach include clinical outcomes, imaging, biophysical, cellular and molecular biomarkers complemented by patient reported outcomes. By integrating the results of this multimodal approach, a so-called 'systems dermatology' approach is constructed, in which the effects of a drug can be described in a highly detailed and mechanistic manner. In **section 3** this multimodal profiling approach was applied in two phase II studies with a novel topical compound for the treatment of AD.

In **chapter 7** the pharmacodynamics, efficacy and safety of omiganan gel in two concentrations, i.e. 1% and 2.5%, on a target lesion of 36 atopic dermatitis patients was explored. Atopic dermatitis is a multifactorial disease and the pathophysiology is not completely understood. Genetic susceptibility, environmental factors, epidermal barrier abnormalities, immunological disturbances and dysbiosis of the skin microbiota all play a role in the disease and the variability of these mechanisms may explain the heterogeneous character of atopic dermatitis. It remains hard to discern which of these mechanisms are primary events (causing atopic dermatitis), secondary events (resulting from atopic dermatitis), or both.[26] *S. aureus* is an important player regarding dysbiosis in atopic dermatitis.[19,27,28] *S. aureus* can produce and secrete toxins and act as super antigens leading to inflammation.[29–31] A deficiency in AMPs, which are important molecules in the host defence system of the skin, plays among other factors an important role in the susceptibility to *S. aureus* colonization in patients with atopic dermatitis.[32,33] As presented in **chapter 3**, omiganan is a novel, synthetic, AMP and might therefore be beneficial in atopic dermatitis. Because this was the first clinical study investigating this indication, only a single target lesion was treated with omiganan gel (1% or 2.5%) or vehicle once daily. The target lesion was extensively measured using all modalities, including the microbiota profile (**chapter 6)**. As clinical measurement the objective SCORing Atopic Dermatitis (oSCORAD) of the target lesion was performed. Moreover 2&3D photos were taken to quantify erythema and roughness, the skin barrier function was evaluated using trans epidermal water loss measurements, skin biopsies were taken to analyze mRNA expression, swabs were taken for microbiota and qPCR *S. aureus* analysis and patients reported on treatment compliance and itch by an e-diary. Significant improvements of the target lesion oSCORAD and morning itch were observed in the omiganan 2.5% group compared to the vehicle gel group (p=0.04 and p=0.05, respectively). In addition, a shift from lesional skin microbiota to non-lesional microbiota was observed, which was mainly related to a reduction of the *Staphylococcus* genus and an increase in microbial diversity in both omiganan treatment groups when compared with vehicle. In conclusion, using multimodal assessments of a target lesion we were able to obtain *proof of pharmacology* for omiganan already in the first human study. However, the improvement of the microbiota did not correlate with the target lesion oSCORAD improvements, while this correlation was observed previously with topical corticosteroid and emollient treatment.[24,34] An explanation could be that dysbiosis is a secondary and not a primary factor in the pathogenesis of AD. Because of the multiple limitations of the study (small group, target lesion treatment, non-optimized dose) it was decided to perform a larger phase II study with dose optimization to reach a more robust conclusion on the potential of omiganan in atopic dermatitis, which is presented in **chapter 8**.

Chapter 8 entailed the evaluation of the pharmacodynamics, efficacy and safety of omiganan gel in three concentrations (1%, 1.75% and 2.5%) twice daily to all lesions in 80 patients with atopic dermatitis. In this study we mostly used the same outcome measures as in **chapter 7**, with the addition of *S. aureus* culturing next to the microbiota and qPCR analysis and total body AD scores (Eczema Area and Severity Index (EASI), oSCORAD and Investigator Global Assessment (IGA)). Similar to the study in **chapter 7**, all three concentrations of omiganan recovered the dysbiosis of lesional skin by inducing a shift from a lesional to a non-lesional microbiota profile. This was mainly related to a reduction in *S. aureus* (observed in the microbiota analysis and cultures). However, the clinical scores and patient reported outcomes remained stable or even deteriorated during the course of this study. These findings support the hypothesis that the dysbiosis is more likely to be a secondary effect in the pathogenesis of atopic dermatitis, by susceptibility for *S. aureus* colonization through a deficient barrier caused by skin immune dysregulation (primary effect). This hypothesis is supported by the fact that treatment with corticosteroids and coal tar can normalize dysbiosis without having any direct antimicrobial properties.[34,35] By suppressing the inflammatory reaction, the epidermal barrier will heal and the susceptibility to *S. aureus* colonization will decrease leading to normalization of the microbiota profile. Because of the absent clinical effects of omiganan in AD, future development of omiganan may focus on diseases where *S. aureus* plays a proven central role, e.g. superinfected atopic dermatitis or the treatment of long term multiresistent S. aureus carriers. A clear clinical benefit was demonstrated by Totté *et al.* in a case series of three patients with related dermatoses that were treated with a topical bacteriophage derived endolysin.[36] In addition, bacterial resistance of omiganan has not been described before *in vitro* and *in vivo*.[37–39] It might therefore be that omiganan can substitute for topical antibiotics in which resistance is a major problem.

Overall, in **section 3** a thorough evaluation of the pharmacodynamic effects of omiganan on atopic dermatitis was performed using the multimodal approach.

By integrating the multimodal profiling approach, *proof-of-pharmacology* of omiganan in terms of effects on the skin microbiota was shown, but no clinically relevant effects on the AD lesions were observed. This led to important new insights. Firstly, it gave insight into the perspectives of omiganan, and secondly it may change our view on the role of dysbiosis in the pathogenesis of atopic dermatitis. In conclusion, by integrating data from the different domains, response or nonresponse of a drug can be elucidated and explained in a highly detailed and mechanistic manner, which can directly affect the next steps in the clinical development of the drug.

PERSPECTIVES

THE FUTURE OF THE IMIQUIMOD CHALLENGE MODEL AND SKIN INFLAMMATION MODELS IN GENERAL ❊ Integrating a skin inflammation challenge model with imiquimod (**chapter 2** and **chapter 3**), aided in the profiling of a novel drug candidate and influenced its next steps in the development directly. Currently, several drugs are under development targeting TLR7/TLR8, mostly focusing on anti-tumor characteristics with more than 30 leads to be explored within the next years.[40,41] This will create many opportunities for the imiquimod model to be integrated in the new strategies of clinical development programs. Besides the imiquimod skin inflammation challenge model, also other models are available targeting other pathways of the skin immune system. *Table 1* gives an overview of the currently available models. For some pharmacological models (i.e. BCG and KLH), studying systemic inflammation next to the local inflammation is even possible. However, most of these models could and should be optimized in future studies with the multimodal profiling approach (characterizing the induced skin inflammation clinically, by imaging, biophysical, molecular, cellular and with patient reported outcomes) to be ready for implementation as translational model in future clinical development programs. With the increasing number of leads for drugs with an immunomodulatory mode of action this would be an enormous step forward to a more rational way of early clinical development.

OTHER CLINICAL ENDPOINTS TO BE EXPLORED FOR AD ❊ Although some novel biomarkers and methodologies have been presented in this thesis, more objective endpoints are needed to enhance the monitoring of drug and disease effects. For AD one can think of computer guided technologies that allow automated EASI and Body Surface Area (BSA) measurements after total body imaging and digital image analysis. This was previously explored for the Psoriasis Area and Severity Index (PASI) that is used as clinical score in psoriasis patients and showed excellent results.[77] However, in AD this would be a challenge due to the poorly demarcated borders of the lesions compared to psoriasis. Artificial intelligence techniques might provide more accurate assessments and are currently being developed.[78] Digital scoring of the EASI by trained persons from digital images has been investigated before and showed excellent correlations.[79] This could be a solution to objectify the EASI score when scored by independent trained persons in a clinical trial instead of the research physician. Another area of interest in AD are serum biomarkers. For example, the chemokine thymus and activated regulated chemokine (TARC) was found to correlate very well with AD clinical severity and a combination of serum biomarkers as objective measurement for disease severity has already been proposed.[80,81] Nevertheless, still more insight can be gained in this field. A preferred future approach to explore additional biomarkers for AD would be an observational study in which patients of all disease severity groups (mild, moderate and severe) are characterized on the different modalities with an extensive set of potential and known biomarkers as presented in Figure 1.

THE CLINICAL PERSPECTIVE: THE PIPELINE OF NOVEL DRUGS FOR AD ❊ The insight in the pathophysiology of AD is rapidly evolving, and the identification of specific inflammatory pathways involved in AD has led to the development of an increase in novel topical as well as systemic compounds.[82] As AD is a heterogeneous disease a variety of compounds is currently being investigated or have been recently approved, an overview is provided in *Table 2*. The main trend for novel treatments are systemic targeted drugs (monoclonal antibodies) antagonizing a specific cytokine involved in pathogenesis of AD, mainly focusing on the TH2 axis, e.g. dupilimab that targets both IL-4 and IL-13 by blocking their common receptor (IL-4Rα/IL-13Rα1). Dupilumab is the first approved monoclonal antibody for AD and is highly efficacious with a favorable safety profile.[83,84] As presented in *Table 2*, it is likely that many other drugs such as tralokinumab (targeting IL-13 by binding the receptor complex IL-4Rα/ IL-13Rα1 and IL-13Rα2) will follow.[85] As mentioned before, AD is a very heterogeneous disease and therefore also targeted therapies blocking cytokines of the TH17 axis are being explored, such as secukinumab (anti IL-17) which is currently registered for psoriasis and psoriatic arthritis. Another area of interest is the development of Janus Kinase (JAK) inhibitors, in both topical and systemic formulations, which act by blocking the phosphorylation of cytokine receptors and therewith

blocking signal transduction and broadly reducing inflammation.[86] To lower the risk that the drug will fail in a pivotal trial, the multimodal patient profiling approach (Figure 1) can be integrated to increase the probability that response or nonresponse of the drug will be detected early in the clinical development process, therewith saving valuable time and resources.

OVERALL CONCLUSIONS

In this thesis, a new approach for rational drug development in inflammatory skin disease was described by focusing on three important cornerstones:
1. developing skin inflammation models for early phase *proof-of-pharmacology*
2. integrating objective clinical endpoints
3. profiling disease in a multimodal fashion to more thoroughly evaluate drug effects

The results of this thesis show that integration of this approach in a clinical development program can directly influence the next steps in clinical development of the drug candidate. While each new drug needs a tailored approach, these general aspects need to be considered when designing dermatological drug development programs. This will lead to a more rational, efficient and less expensive way of dermatological drug development.

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TABLE 1 Overview of human skin inflammation challenge models.

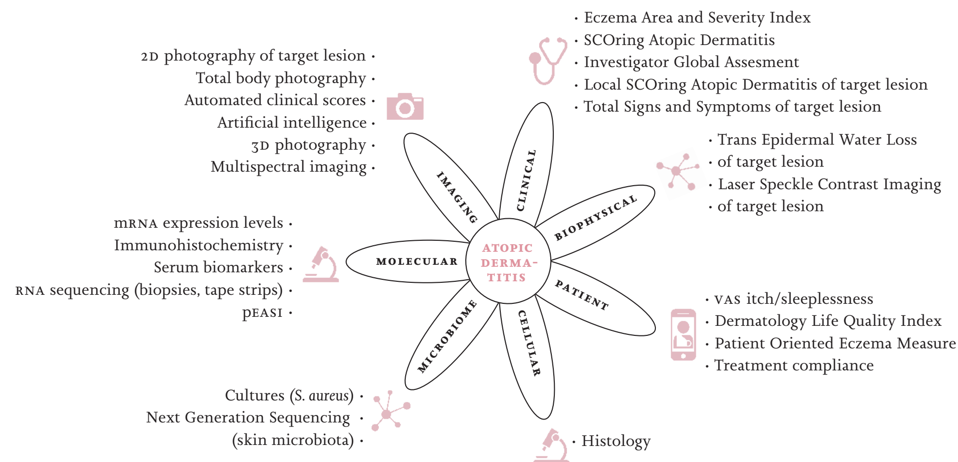
Skin inflammation challenge	Application	Mode of action	Condition induced	Immune response	Reference
INFLAMMATION					
BCG	Intradermal	TLR4, 9 agonist	Local inflammation, systemic immune response	Adaptive	42-44
Imiquimod	Local under occlusion	TLR7 agonist	Local inflammation, mimicking psoriasis	Innate+ Adaptive	7, 45-48
LPS Challenge +AL(OH)3	Intradermal	TLR4 agonist	Inflammatory response	Innate+ Adaptive	49, 50
Cantharidin	Paper disc with cantharidin	Neutrophils	Local inflammation	Innate	49, 51
Injected UV Killed E.Coli	Intradermal	Neutrophils	Erythema, heat, swelling and pain	Innate	49, 52
KLH	Intradermal, Intramuscular	Neo-antigen	Local inflammation, systemic immune response	Adaptive	53-57
ITCH					
Capsaicine	Intradermal, intra muscular, topical	TRPV 1 receptor	Itch	Innate	58-60
Histamine	Intradermal, intramuscular	H1,2,3,4 receptor CMIA fibers	Itch	Innate	61-68
Cowhage	Cutaneous	Par-2	Itch Burning	Unknown	69, 70
UV EXPOSURE					
UV-B irradiation	Local Thermode	PI3K/AKT/ MTOR-upregulation	Pain, pigmentation, erythema, inflammation	Innate Adaptive	58, 71-76

BCG: Bacillus Calmette- Guérin /LPS: Lipopolysaccharide/Injected UV Killed E.Coli: Injected ultraviolet killed Escherichia Coli/ KLH: Keyhole Limpet Hemocyanin

TABLE 2 What's new in atopic dermatitis? Drugs recently approved or currently investigated in AD.

Mechanism of action	Compounds	References
TOPICALS		
Phosphodiesterase (PDE) 4 antagonist	Crisaborole	87
Janus Kinase (JAK) inhibitor	Tofacitinib, JTE-032, ruxolitinib	88, 89
SYK/JAK inhibitor	Cerdulatinib	
Microbiome transplantations	-	90
SYSTEMICS		
IL-4/IL13 antagonist	Dupilumab	83, 84
IL-13 antagonist	Tralokinumab, Lebrikizumab	85, 91, 92
IL-31 antagonist	Nemolizumab, BMS-981164	93-95
Thymic stromal lymphopoietin (TSLP) antagonist	Tezepelumab, MK-8226	96
OX40 antagonist	GBR 830, KHK4083	97
IL-33 antagonist	Etokimab	
IL-22 antagonist	Fezakinumab	98
IL-23 antagonist	Ustekinumab	99
IL-17 antagonist	Secukinumab, MOR106	
JAK inhibitor	Baricitinib, upadacitinib, abrocitinib	100-103
Histamine 4 receptor antagonist	ZPL-3893787	104

FIGURE 1 Multimodal patient profiling of atopic dermatitis.



CHAPTER X

SAMENVATTING EN PERSPECTIEVEN

Er is een grote behoefte aan nieuwe geneesmiddelen voor chronische immuungemedieerde inflammatoire huidziekten. De slagingspercentages van ontwikkelingstrajecten voor nieuwe behandelingen zijn laag. Slechts 6.3% van de kandidaat geneesmiddelen haalt uiteindelijke registratie. Dit komt voornamelijk door de ezigheid van klinische informatie over het geneesmiddel in de vroege fase van ontwikkeling, het is bijvoorbeeld onbekend wat de actieve dosis is, onduidelijk wat het regime moet zijn en onzeker wat de farmacologische activiteit van het middel is. Gezien de sterke toename van het aantal potentiële nieuwe geneesmiddelen zijn nieuwe strategieën nodig om het proces van ontwikkeling te optimaliseren. Dit wordt 'rationele ontwikkeling van geneesmiddelen' genoemd. Er is echter weinig literatuur beschikbaar over de rationele ontwikkeling van geneesmiddelen binnen de dermatologie. De zogeheten 'Question-Based Drug (QBD) development' benadering is een nieuwe benadering voor geneesmiddelenontwikkeling. De meeste programma's volgen echter nog steeds het traditionele pad van ontwikkeling. Recentelijk hebben wij een voorstel gedaan voor QBD voor vroege fase geneesmiddelenstudies binnen de dermatologie. Dit omvat de volgende vijf hoekstenen:

1. het onderzoeken van farmacokinetische ('wat doet het lichaam met het geneesmiddel') eigenschappen van het geneesmiddel in de huid en systemisch
2. het onderzoeken van de farmacodynamische eigenschappen ('wat doet het geneesmiddel met het lichaam') inclusief dosis-respons van het geneesmiddel
3. het gebruik van gevoelige en objectieve eindpunten in de onderzoeken
4. het profileren van de ziekte en de reactie van de ziekte op het geneesmiddel door middel van een multimodale aanpak
5. multidisciplinaire samenwerkingen voor uitvoer van het onderzoek.

De strekking van dit proefschrift is op deze hoekstenen gebaseerd, en omvat de ontwikkeling van nieuwe methoden ter ondersteuning van deze aanpak binnen de geneesmiddelenontwikkeling voor inflammatoire huidziekten, met name atopisch eczeem. Daarnaast wordt in dit proefschrift een nieuw geneesmiddel onderzocht voor de behandeling van atopisch eczeem met behulp van de nieuwe rationele aanpak. Dit proefschrift is onderverdeeld in drie delen:

- sectie 1 beschrijft de ontwikkeling en toepassing van een huidinflammatiemodel in gezonde vrijwilligers,
- sectie 2 rapporteert de bevindingen van onderzoeken naar nieuwe biomarkers voor geneesmiddelonderzoeken binnen de dermatologie,
- in sectie 3 wordt een nieuwe behandeling voor atopisch eczeem onderzocht met behulp van de methoden die beschreven zijn in de voorgaande secties.

Dit hoofdstuk is een samenvatting van de resultaten van alle secties, en belicht de perspectieven van rationele geneesmiddelenontwikkeling op het gebied van inflammatoire huidziekten.

RATIONELE GENEESMIDDELONTWIKKELING VOOR CHRONISCHE IMMUUNGEMEDIEERDE INFLAMMATOIRE HUIDZIEKTEN

SECTIE I

HUIDINFLAMMATIEMODELLEN ALS 'TOOL' VOOR BEWIJS VAN FARMACOLOGISCHE ACTIVITEIT IN GEZONDE VRIJWILLIGERS ✻ Recentelijk is in de EMA richtlijn voor geneesmiddelenonderzoek met mensen beschreven dat het onderzoeken van de farmacodynamiek van een geneesmiddel verplicht is naast het onderzoeken van de veiligheid, verdraagbaarheid en farmacokinetische eigenschappen. Het onderzoeken van de farmacodynamiek van anti-inflammatoire geneesmiddelen in gezonde vrijwilligers die niet lijden aan een inflammatoire huidziekte is echter lastig. In dit geval kan een farmacologisch 'challenge' model gebruikt worden dat, in ieder geval de belangrijkste, oorzakelijke kenmerken van de inflammatoire huidziekte nabootst. Zo'n model kan inzicht geven in de wisselwerking van een geneesmiddel met het beoogde doel-eiwit ('target engagement') en dosis-respons relaties. Binnen de dermatologie zijn er veel geneesmiddelen in ontwikkeling met een immunomodulatoir werkingsmechanisme. Preklinische diermodellen worden vaak gebruikt om de farmacodynamische eigenschappen *in vivo* (in het leven) te onderzoeken, bijvoorbeeld in muismodellen met imiquimod en lipopolysacharide. Deze modellen geven een idee van het effect, de voorspellende waarde voor activiteit in de daadwerkelijke doelpopulatie in de mens blijft echter laag. Daarom zijn modellen in de mens die huidinflammatie nabootsen noodzakelijk om belangrijke vragen te kunnen beantwoorden omtrent de toekomstige ontwikkeling van een geneesmiddel. Bijvoorbeeld vragen aangaande geneesmiddelprofilering, dosis of doseerschema en het vinden van een goede doelpopulatie voor het geneesmiddel. De antwoorden op deze vragen beïnvloeden direct de volgende stappen in het ontwikkelingstraject van het geneesmiddel. Voor dit doel is in sectie 1 van dit proefschrift een imiquimod-geïnduceerd huidinflammatiemodel ontwikkeld.

In **hoofdstuk 2** werden twee modellen onderzocht die een lokale inflammatoire respons van de huid opwekken door het activeren van het aangeboren immuunsysteem, namelijk:

- imiquimod aanbrengen onder occlusie (afdekking van de huid)
- tapestrippen (het verwijderen van een deel van de bovenste laag van de huid met speciaal plakband) van de huid voor aanbrengen van imiquimod onder occlusie.

De inflammatoire respons van de huid in deze modellen werd gemeten met een 'toolbox' vol nieuwe meetapparatuur en biomarkers om de inflammatoire respons vanuit verschillende perspectieven in kaart te brengen, namelijk: klinisch (scoren van erytheem door arts), beeldvorming (erytheem (roodheid) van de huid d.m.v. 2D foto-analyse), biofysisch (perfusie (doorbloeding) van de huid d.m.v. Laser Speckle Contrast Imaging), moleculair (gen-expressie van verschillende biomarkers in huidbiopten), cellulair (immunohistochemische kleuringen van huidbiopten) en patiënt gerapporteerde uitkomsten (jeuk en pijn). Beide modellen wekten een lokale, tijdelijke inflammatoire respons zoals wij hadden verwacht. In het tweede model waarbij tapestripping van de huid werd toegepast voor de eerste maal aanbrengen van imiquimod onder occlusie werd een sterkere inflammatoire response gezien die sneller op gang kwam. Dit tweede model zorgde ook voor een grotere respons op verschillende domeinen waaronder erytheem en perfusie ($p<0.0001$), gen expressie van de inflammatoire markers ($p<0.01$) en inflammatoire cel instroom in de huid vergeleken met de placebogroep en de groep waarbij imiquimod werd aangebracht zonder tapestrippen van de huid. Na 2 dagen bereikte de imiquimod-respons een fase waarbij het erytheem, perfusie en de biopt-biomarkers stabiel bleven ten opzichte van 3 dagen behandeling. Proefpersonen rapporteerden dat jeuk en pijn acceptabel bleven op de behandellocaties. De opgewekte inflammatoire respons was volledig omkeerbaar na het stoppen van de behandeling.

Om de toepasbaarheid van het ontwikkelde imiquimodmodel te testen werd een interactiestudie uitgevoerd van het imiquimodmodel met omiganan, een antimicrobieel peptide (AMP) in gel vorm voor lokale aanbrenging met immunomodulaire eigenschappen. Dit is terug te lezen in **hoofdstuk 3**. Eerder uitgevoerd *in vitro* (in reageerbuis) werk met omiganan toonde aan dat omiganan de antivirale respons opgewekt door imiquimod versterkt. Dit suggereert de toekomstige toepassing van omiganan met imiquimod voor virale huidziekten in mensen, bijvoorbeeld voor anogenitale wratten. Een vertaling van dit concept van *in vitro* naar *in vivo* in gezonde menselijke vrijwilligers met een selectie van de juiste dosis was in dit geval gewenst in plaatst van directe uitvoer van een studie in patiënten. Met het ontwikkelde imiquimodmodel was het mogelijk deze vertalende stap uit te voeren. In deze studie onderzochten we verschillende doseringen en verschillende doseerschema's van de gecombineerde behandeling met omiganan en imiquimod. De huidrespons werden gemeten vanuit verschillende perspectieven zoals beschreven in **hoofdstuk 2**. Twee dagen imiquimod onder occlusie met tapestripping van de huid voorafgaand aan het eerste smeermoment wekte een identieke respons op zoals eerder geobserveerd werd in **hoofdstuk 2**, wat de herhaalbaarheid van het ontwikkelde model bevestigt. Extra behandeling met omiganan versterkte deze inflammatoire respons van imiquimod, met een toename van perfusie en erytheem. Ook de inflammatoire respons in de huidbiopten werd versterkt door omiganan (toename van antivirale biomarkers), en meer instroom van immuuncellen in de huid werd geobserveerd. Deze resultaten zijn in lijn met de eerder uitgevoerde mechanistische *in vitro* studies, en deze studie ondersteunt daarom een studie waarbij de combinatie van imiquimod/omiganan onderzocht wordt in patiënten.

Samenvattend bleek het imiquimod huidinflammatiemodel in gezonde vrijwilligers een geschikt farmacologisch 'challenge' model voor toekomstige vroege fase geneesmiddel studies om farmacodynamische activiteit aan te tonen, en daarnaast voor immunomodulatoire profileringsstudies en interactiestudies. In **hoofdstuk 3** wordt een voorbeeld beschreven van een interactiestudie met het ontwikkelde imiquimod model, waar eerder *in vitro* werk vertaald werd naar *in vivo* uitkomsten. In deze studie werden de effecten van een nieuw geneesmiddel zeer uitgebreid onderzocht wat hielp bij de volgende stap van het ontwikkeltraject voor het geneesmiddel: het vinden van de juiste doelpopulatie voor het geneesmiddel. Concluderend laten de beschreven gegevens zien dat het toevoegen van een huidinflammatiemodel in een geneesmiddel ontwikkeltraject kan leiden tot een rationelere manier van vroege fase geneesmiddelonderzoek. In toekomstige ontwikkeltrajecten van geneesmiddelen voor inflammatoire huidziekten moeten de toevoeging van een huidinflammatie 'challenge' model overwogen worden.

SECTIE II

ONTWIKKELING EN TOEVOEGING VAN OBJECTIEVE KLINISCHE EINDPUNTEN ❋ Klinische scores spelen nog steeds een zeer grote rol in alle fasen van geneesmiddelenonderzoek bij inflammatoire huidziekten. Deze scores uitgevoerd door de arts geven een algemene gestandaardiseerde schatting van ziekte-ernst en zijn verplicht gesteld door de regelgevende organisaties. Echter hebben klinische scores ook verschillende nadelen, zoals een beperkte

objectiviteit door de afhankelijkheid van de arts die het onderzoek uitvoert, en de beperkte gevoeligheid om kleine effecten van potentiële geneesmiddelen op te sporen, wat juist heel belangrijk is in vroege fase geneesmiddelonderzoek. Tegenwoordig zijn er veel nieuwe innovatieve technieken beschikbaar, wat een objectievere beoordeling van een geneesmiddel mogelijk maakt in klinische onderzoeken binnen de dermatologie. In **sectie 2** wordt via een multimodale aanpak de ontwikkeling en selectie van nieuwe biomarkers onderzocht, die bewijs van farmacologische activiteit (‘*proof-of-pharmacology*’) kunnen verschaffen.

Hoofdstuk 4 beschrijft de ontwikkeling en toepassing van een elektronisch dagboek (e-dagboek) als telefoonapp met herinneringsfunctie om therapietrouw en patiënt-gerapporteerde uitkomsten te vervolgen in een klinische studie. Het is bekend dat de therapietrouw bij plaatselijk aangebrachte geneesmiddelen in de praktijk zeer laag is, met 25% als dieptepunt. In klinische studies binnen de dermatologie zijn de meeste geneesmiddelen in deze vorm, en zullen vaak thuis aangebracht moeten worden op de huid. Om uitspraken te kunnen doen over de veiligheid en effectiviteit van een geneesmiddel is het essentieel om betrouwbare data te verkrijgen over therapietrouwheid. Om dit op te volgen worden vaak papieren vragenlijsten gebruikt, welke een lage betrouwbaarheid hebben. Deze vragenlijsten worden ook gebruikt voor het verzamelen van patiënt-gerapporteerde uitkomsten, bijvoorbeeld over pijn en jeuk. De therapietrouw (aantal daadwerkelijke toedieningen gedeeld door het aantal verwachtte toedieningen) voor het e-dagboek was excellent, namelijk 98%. Daarnaast werden ook patient-gerapporteerde uitkomsten toegevoegd aan het e-dagboek om jeuk en pijn te monitoren. Dit had ook een excellente therapietrouw (89% voor jeuk en 94% voor pijn). Eerder gepubliceerde resultaten laten zien dat 67-95% van de patienten die een geneesmiddel moet aanbrengen op de huid een te lage dosering gebruikt vanwege slechte therapietrouw. Het gebruik van een e-dagboek in een klinische studie kan dit voorkomen. Daarnaast betekent therapietrouw dat het geneesmiddel is aangebracht op de huidafwijking en dus het gebied bereikt is waar het terecht moet komen. Het e-dagboek ondersteunt ook de ‘trial@home’ benadering van het Centre of Human Drug Research, bij waarbij proefpersonen op afstand kunnen worden vervolgd en zij minder naar het onderzoekscentrum hoeven te komen. Dit leidt tot een lagere belasting voor de proefpersoon.

Een andere potentiële nieuwe biomarker voor meerdere huidziekten is de huidmicrobiota (de specifieke micro-organismen die aanwezig zijn op de huid). De gezonde huid van ieder mens heeft een unieke microbiële ‘vingerafdruk’, welke afhankelijk is van verschillende kenmerken van de huid evenals van de gastheer en omgevingsfactoren zoals kolonisatie bij de geboorte, antibioticagebruik, hygiëne, leefstijl en locatie. Deze ‘vingerafdruk’ kan ook beïnvloed worden door een huidziekte. Om uit te zoeken of analyse van de huidmicrobiota interessant zou zijn als biomarker in onze multimodale aanpak werd een systematische literatuur review uitgevoerd, waarin we de potentie van de huidmicrobiota als biomarker onderzochten in zes verschillende huidziekten met een inflammatoire component: atopisch eczeem, acne vulgaris, psoriasis vulgaris, hidradenitis suppurativa, seborroisch eczeem/hoofdroos en ulcus cruris. Zoals gepresenteerd in **hoofdstuk 5** leverde de zoekstrategie 841 artikelen op, waarvan er 11 artikelen werden geïncludeerd in deze review. In het algemeen was er een hoge variabiliteit in onderzoeksopzet en manier van materiaalafname tussen de studies, wat een vergelijking lastig maakte. Een standaard onderzoeksopzet (met standaard manier voor materiaalafname, verwerking en analyse) voor huidmicrobiota studies is gewenst voor toekomstige studies. Voor twee indicaties, namelijk atopisch eczeem en acne vulgaris, zijn er meerdere studies gevonden die de toevoeging van de huidmicrobiota als ziekte- of geneesmiddel-specifieke biomarker ondersteunen, gezien er een duidelijke relatie tussen verandering in de huid microbiota en ziekte-activiteit lijkt te zijn. Hierover is het meest bekend bij atopisch eczeem. Het is bekend dat lesionale eczeem huid een grotere hoeveelheid *Staphylococcus* in combinatie met een lagere algemene microbiële diversiteit heeft. Maar ondanks deze relatie is het ook bekend dat de huidmicrobiota enorm kan variëren in de tijd bij één individuele patiënt. Daarom is het nodig om studies uit te voeren om de variabiliteit van lesionale huidmicrobiota tussen patiënten (inter-patiënt) en binnen één patiënt (intra-patiënt) te karakteriseren en analyseren. Zo zullen er conclusies getrokken kunnen worden over de toepassing van de huidmicrobiota als biomarker in klinische studies voor atopisch eczeem.

In **hoofdstuk 6** was het doel om de huidmicrobiota van lesionale en niet-lesionale huid van patiënten met atopisch eczeem te karakteriseren. Voornamelijk de inter-patient en intra-patiënt variabiliteit van de huid microbiota bij patiënten met atopisch eczeem in de tijd werden geanalyseerd. Dit om te bepalen of materiaalafname voor en na behandeling voldoende is om een effect van een behandeling op atopisch eczeem te detecteren, of dat uitgebreidere materiaalafname nodig is om de huidmicrobiota als biomarker te gebruiken in een klinische studie bij patiënten met atopisch eczeem. De inter-patient variabiliteit van de microbiële diversiteit was hoog voor de lesionale huid vergeleken met de niet-lesionale huid (variatie-coëfficiënt van 35.5-45.9% vs. 16.3-28%). De mate van inter-patient variabiliteit van de relatieve hoeveelheid *Staphylococcus* soorten en *Staphylococcus aureus* (*S. aureus*) op lesionale huid was ook hoog met variatiecoëfficiënten van ≥46.9%. De grote inter-patient en intra-patiënt variabiliteit voor lesionale huidmicrobiota

werd bevestigd door huidmicrobiota data van een extra set materiaal van patiënten met atopisch eczeem. Daarom is een hoge frequentie van materiaalafname, bijvoorbeeld een keer per week, nodig om inzicht te krijgen in de zeer variabele huidmicrobiota in klinische studies.

Samenvattend laat **sectie 2** de evaluatie zien van nieuwe biomarkers (e-dagboek en huidmicrobiota) voor klinische studies binnen de dermatologie. Het e-dagboek verstrekt meer betrouwbare data over therapietrouw (en indirect over of het geneesmiddel de doelbestemming bereikt) en kan een 'trial@home' benadering faciliteren. Daarom is het belangrijk te overwegen het e-dagboek op te nemen als tool in een ontwikkeltraject. De geschiktheid van de microbiota van de huid als biomarker in een klinische trial voor atopisch eczeem moet verder worden uitgezocht, waarbij extra wordt gelet op gestandaardiseerde materiaalafname en veelvuldige afnames van materiaal.

SECTIE III

MULTIMODALE PROFILERING OM EEN GENEESMIDDELEFFECT UITVOERIG TE BESTUDEREN ❋ Door het toenemende aantal beschikbare meetinstrumenten bestaat de mogelijkheid om een geneesmiddeleffect uitvoerig te bestuderen op verschillende domeinen (multimodale profilering) in plaats van te focussen op één (meestal klinisch) eindpunt. Deze verschillende domeinen omvatten klinische uitkomsten, beeldvorming, biofysische uitkomsten, cellulaire en moleculaire biomarkers met hierbij ook door de patiënt gerapporteerde uitkomsten. Het samenvoegen van de resultaten van alle domeinen kan een zeer gedetailleerd en mechanistisch profiel vormen van de effecten van een geneesmiddel. In **sectie 3** was deze multimodale aanpak geïntegreerd in twee fase 2 studies over een potentieel nieuwe behandeling voor patiënten met atopisch eczeem.

In **hoofdstuk 7** werd de farmacodynamiek, effectiviteit en veiligheid van omiganan in twee verschillende concentraties (1% en 2.5%) op één eczeemplek onderzocht bij 36 patiënten met atopisch eczeem. Atopisch eczeem is een complexe huidziekte en de oorzaak is nog niet compleet bekend. Genetische aanleg, omgevingsfactoren, epidermale barrière-afwijkingen, verstoringen van het afweersysteem en dysbiose van de huidmicrobiota spelen allen een rol bij het ontstaan van de ziekte. Het blijft lastig te onderscheiden welke van deze mechanismen primair (veroorzakers van atopisch eczeem), secundair (gevolgen van atopisch eczeem), of beiden zijn. *S. aureus* is een belangrijke bacterie bij dysbiose in atopisch eczeem. *S aureus* kan toxines produceren en uitscheiden en als super-antigeen functioneren, wat leidt tot inflammatie van de huid. Een tekort in AMPs, wat belangrijke moleculen zijn in het immuunsysteem van de huid, speelt onder andere een rol bij de gevoeligheid voor *S. aureus* overgroei bij patiënten met atopisch eczeem. Omiganan is een nieuw synthetisch AMP wat mogelijk gebruikt kan worden voor de behandeling van atopisch eczeem. In **hoofdstuk 3** is een klinische studie met omiganan beschreven waarbij één eczeemplek (onderzoeksplek) eenmaal daags behandeld werd met omiganan gel (1% of 2.5%) of placebo. Deze onderzoeksplek werd zeer uitgebreid geëvalueerd op verschillende domeinen inclusief de huidmicrobiota zoals beschreven in **hoofdstuk 6**. Klinisch werd de onderzoeksplek gescoord met behulp van de 'objective Scoring Atopic Dermatitis' (OSCORAD). Daarnaast werden 2D en 3D foto's genomen van de onderzoeksplek om erytheem en de structuur van de huid te kwantificeren. De huidbarrière-functie werd gemeten met behulp van metingen van transepidermaal waterverlies, en er werden biopten genomen voor de bepaling van gen expressie van bepaalde markers. Ook de huidmicrobiota werd in kaart gebracht door middel van afname van huid uitstrijkjes ('swabs') voor microbiota en *S. aureus* analyse. Patiënten vulden een e-dagboek in voor therapietrouw, jeuk– en slaapscores. In de omiganan 2.5% groep werden significante verbeteringen gevonden op de OSCORAD van de onderzoeksplek ($p=0.04$) en ochtendjeuk ($p=0.05$) ten opzichte van de placebo groep. Daarnaast vond er een verschuiving plaats van lesionaal microbiotaprofiel naar niet-lesionaal microbiotaprofiel op de onderzoeksplek, door een afname van *Staphylococcus* en toename van microbiële diversiteit in de omiganan-groepen ten opzicht van placebo. Concluderend konden wij met de multimodale metingen op één onderzoeksplek bewijs van farmacodynamische activiteit van omiganan verkrijgen in een kleine studie waarbij het middel voor het eerste toegediend werd aan mensen. Verrassend genoeg correleerde de verbetering van de OSCORAD van de onderzoeksplek niet met de verbetering in de huidmicrobiota, terwijl dit in eerdere studies wel gezien werd bij behandeling met lokale corticosteroïden en emolliens. Een verklaring hiervoor zou kunnen zijn dat dysbiose een secundaire factor is in de ontstaanswijze van atopisch eczeem, en geen primaire factor. Gezien de beperkingen van deze studie (kleine groep, alleen behandeling van één onderzoeksplek, niet geoptimaliseerde dosis) werd besloten een grotere fase 2 studie uit te voeren met optimalisatie van de dosis om sterkere uitspraken te kunnen doen over de effectiviteit van omiganan bij atopisch eczeem. Dit is gepresenteerd in **hoofdstuk 8.**

In **hoofdstuk 8** werd de farmacodynamiek, effectiviteit en veiligheid van omiganan gel tweemaal daags in drie concentraties (1%, 1.75% en 2.5%) op alle eczeemplekken geëvalueerd in 80 patiënten met atopisch eczeem. In deze studie werden grotendeels dezelfde uitkomstmaten gebruikt als in **hoofdstuk 7**, met als toevoeging het kweken van *S. aureus* naast de huidmicrobiota en *S. aureus*

analyse. Daarnaast werden enkele eczeemscores op basis van een evaluatie van het hele lichaam toegevoegd ('Eczema Area and Severity Index' (EASI), OSCORAD en 'Investigator Global Assessment (IGA)). Alle drie omiganan-concentraties herstelden de dysbiose van lesionale huid door een verschuiving van lesionaal naar niet-lesionaal huidmicrobiota-profiel. Dit werd voornamelijk veroorzaakt door een afname van *S. aureus* in zowel de kweken als op basis van de microbiota-analyse. De klinische eczeemscores en patiënt-gerapporteerde uitkomsten bleven stabiel of verslechterden tijdens de behandelperiode. Deze bevindingen ondersteunen de hypothese dat dysbiose naar alle waarschijnlijkheid een secundair effect is in de ontstaanswijze van atopisch eczeem. De huid wordt gevoeliger wordt voor *S. aureus* kolonisatie door een defecte huidbarrière, welke veroorzaakt wordt door een verstoring van het immuunsysteem van de huid (primair effect). Deze hypothese wordt ondersteund door het feit dat behandeling met lokale corticosteroïden en teerbehandeling dysbiose in atopisch eczeem kan normaliseren zonder dat deze producten antimicrobiële eigenschappen hebben. Door het onderdrukken van de inflammatoire reactie van de huid kan de huidbarrière helen, wat leidt tot een lagere gevoeligheid voor *S. aureus* kolonisatie en dus voor normalisatie van de huidmicrobiota. Gezien het afwezige klinische effect van omiganan bij atopisch eczeem zullen toekomstige studies focussen op indicaties waarbij *S. aureus* een bewezen centrale rol speelt, bijvoorbeeld bij geïnfecteerd atopisch eczeem of de behandeling van dragers van multiresistente *S. aureus*. Totté et al. lieten eerder de werkzaamheid van een topisch endolysine zien in een 'case series' studie van drie patiënten met aan *S. aureus* gerelateerde huidafwijkingen. Bacteriële resistentie van omiganan is nog niet eerder beschreven *in vitro* en *in vivo*. Daarom zou omiganan mogelijk ook een vervanger kunnen zijn voor lokale antibiotica waarbij resistentie een groot probleem is.

Samenvattend werd in **sectie 3** een uitgebreide evaluatie van de farmacodynamische effecten van omiganan op atopisch eczeem beschreven door toepassing van de multimodale aanpak. Door middel van een multimodale profilering van omiganan werd bewijs van farmacodynamische activiteit geleverd op de huidmicrobiota, terwijl er geen klinisch relevante effecten werden gezien op het atopisch eczeem. Dit heeft tot belangrijke inzichten geleid. Allereerst geeft dit inzicht in de potentie van omiganan. Daarnaast kunnen deze uitkomsten mogelijk onze kijk op de rol van dysbiose in de ontstaanswijze van atopisch eczeem veranderen. Concluderend kan door de multimodale aanpak de respons of non-respons van een geneesmiddel worden geëvalueerd op een zeer gedetailleerde en mechanistische manier, wat vervolgens direct de volgende stappen in het ontwikkeltraject kan beïnvloeden.

PERSPECTIEVEN

DE TOEKOMST VAN HET IMIQUIMOD CHALLENGEMODEL EN HUIDINFLAMMATIEMODELLEN IN ZIJN ALGEMEENHEID ❊ Toevoeging van een huidinflammatiemodel met imiquimod (**hoofdstuk 2** en **hoofdstuk 3**) hielp bij het profileren van een nieuw potentieel geneesmiddel en had directe invloed op de volgende stappen in het ontwikkeltraject. Momenteel zijn er verschillende geneesmiddelen in ontwikkeling die hetzelfde als aangrijpingspunt hebben als imiquimod. Er zijn meer dan 30 kandidaat geneesmiddelen die in de aankomende jaren zullen worden geëxploreerd. Dit creëert mogelijkheden voor het imiquimod model om onderdeel te worden van nieuwe strategieën in geneesmiddelontwikkelingstrajecten. Naast het imiquimod huidinflammatiemodel zijn er ook andere modellen beschikbaar die andere paden van het immuunsysteem van de huid als aangrijpingspunt hebben. Tabel 1 geeft een overzicht van deze modellen. Voor sommige farmacologische modellen (BCG en KLH) kan ook een systemische inflammatoire respons bestudeerd worden. De meeste van deze modellen moeten nog geoptimaliseerd worden in toekomstige studies met de multimodale profileringsaanpak (het karakteriseren van de geïnduceerde inflammatie vanuit verschillende perspectieven: klinisch, met beeldvorming, biofysisch, moleculair, cellulair en patiënt-gerapporteerde uitkomsten). Gezien het hoge aantal potentiële kandidaat geneesmiddelen met een immunomodulatoir werkingsmechanisme zouden zulke modellen een enorme stap vooruit zijn op het gebied van een rationelere manier van vroege fase geneesmiddelonderzoek.

ANDERE KLINISCHE EINDPUNTEN BIJ ATOPISCH ECZEEM ❊ Hoewel enkele nieuwe biomarkers en methodologieën zijn gepresenteerd in dit proefschrift, zijn er nog steeds meer objectieve eindpunten nodig om geneesmiddel en ziekte-effecten in (vroege fase) klinische studies te detecteren. Voor atopisch eczeem kan gedacht worden aan geautomatiseerde berekeningen van de EASI score en de beoordeling van door atopisch eczeem aangedaan lichaamsoppervlak ('body surface area'(BSA)) op basis van beeldvorming en digitale foto-analyse. Dit is eerder onderzocht voor de 'Psoriasis Area and Severity Index (PASI) die gebruikt wordt het scoren van ziekte-ernst bij patiënten met psoriasis. Deze geautomatiseerde berekening liet excellente resultaten zien. Echter is dit lastiger in atopisch eczeem vanwege de onscherpe begrenzing van de lesies in vergelijking met psoriasis, waarbij de lesies duidelijk begrensd zijn. Kunstmatige intelligentie zou kunnen bijdragen aan meer betrouwbare metingen, deze technieken zijn in ontwikkeling. Digitale scoring van de EASI op basis van foto's door

getrainde personen is ook eerder onderzocht en liet excellente correlaties zien. Een ander gebied van interesse in atopisch eczeem zijn de serum biomarkers. De biomarker 'chemokine thymus and activated regulated chemokine' (TARC) blijkt zeer goed te correleren met de ernst van atopisch eczeem. Een combinatie van serum biomarkers om ziekte-ernst in te schatten is al voorgesteld. Desalniettemin kan er nog veel kennis worden opgedaan in dit veld, bij voorkeur in een toekomstige observationele studie waarbij patiënten met atopisch eczeem met verschillende ziekte-ernst (mild, matig en ernstig) gekarakteriseerd worden op basis van de verschillende modaliteiten, met een zeer uitgebreide set van potentiële en bekende biomarkers zoals gepresenteerd in Figuur 1.

HET KLINISCHE PERSPECTIEF: DE PIJPLIJN VAN NIEUWE GENEESMIDDELEN VOOR ATOPISCH ECZEEM ❋ Het toegenomen inzicht in de oorzaak van atopisch eczeem en de betrokken inflammatoire paden heeft geleid tot een toegenomen ontwikkeling van lokale en systemische kandidaat geneesmiddelen. Gezien het ziektebeeld van atopisch eczeem erg verschillend kan zijn tussen patiënten (heterogeen) is er een grote variatie aan recent goedgekeurde geneesmiddelen en geneesmiddelen die zich nog in een ontwikkelingstraject bevinden. Een overzicht hiervan is te vinden in Tabel 2. Er is een waarneembare trend van systemische geneesmiddelen (monoklonale antilichamen) die specifieke cytokines blokkeren die betrokken zijn bij de ontstaanswijze van atopisch eczeem. Deze focussen meestal op de TH2-as van het immuunsysteem, bijvoorbeeld dupilumab dat aangrijpt op zowel IL-4 en IL-13 door het blokkeren van de gezamenlijke receptor (IL-4Rα/IL-13Rα1). Dupilumab is het eerste goedgekeurde monoklonale antilichaam voor atopisch eczeem en is zeer effectief met een gunstig bijwerkingenprofiel. In de toekomst zullen vele anderen volgen, bijvoorbeeld tralokinumab (grijpt aan op IL-13 door binding aan het receptorcomplex IL-4Rα/ IL-13Rα1 en IL-13Rα2). Vanwege het heterogene beeld van atopisch eczeem worden er momenteel ook andere monoklonale antilichamen onderzocht die aangrijpen op cytokines van de TH17-as van het immuunsysteem, bijvoorbeeld secukinumab (anti-IL17) welke geregistreerd is voor de behandeling van psoriasis en artritis psoriatica. Ook interessant zijn de Janus Kinase (JAK) remmers, zowel in lokale als systemische formulering, die inflammatie in zijn algemeenheid reduceren. Het toevoegen van de multimodale patiëntprofileringsaanpak (zie Figuur 1) in vroege fase studies vergroot de kans dat de respons of non-respons van het geneesmiddel in een vroeg stadium wordt gedetecteerd. Dit zal het risico dat een potentieel geneesmiddel zal falen in klinische studies verkleinen en dit zal waardevolle tijd en middelen besparen.

CONCLUSIES

In dit proefschrift werd een nieuwe aanpak voor rationele geneesmiddelontwikkeling voor inflammatoire huidziekten beschreven met focus op drie belangrijke hoekstenen:

1. de ontwikkeling van huidinflammatiemodellen voor vroege fase geneesmiddelenstudies, waarbij bewijs van farmacodynamische activiteit centraal staat,
2. de toevoeging van objectieve klinische eindpunten in een klinische studie,
3. het profileren van een ziekte op een multimodale manier om geneesmiddeleffecten zeer grondig te kunnen onderzoeken.

De resultaten van dit proefschrift laten zien dat de toevoeging van deze aanpak in een ontwikkeltraject van een geneesmiddel direct de volgende stappen in het traject kunnen beïnvloeden. Natuurlijk heeft elk geneesmiddel een op maat gemaakte aanpak nodig, echter dienen de bovengenoemde drie hoekstenen overwogen te worden bij het ontwerpen van het ontwikkeltraject. Dit zal uiteindelijk leiden tot een meer rationele, efficiënte en duurzamere manier van geneesmiddelontwikkeling.

TABEL 1 Overzicht van beschikbare humane huidinflammatie modellen.

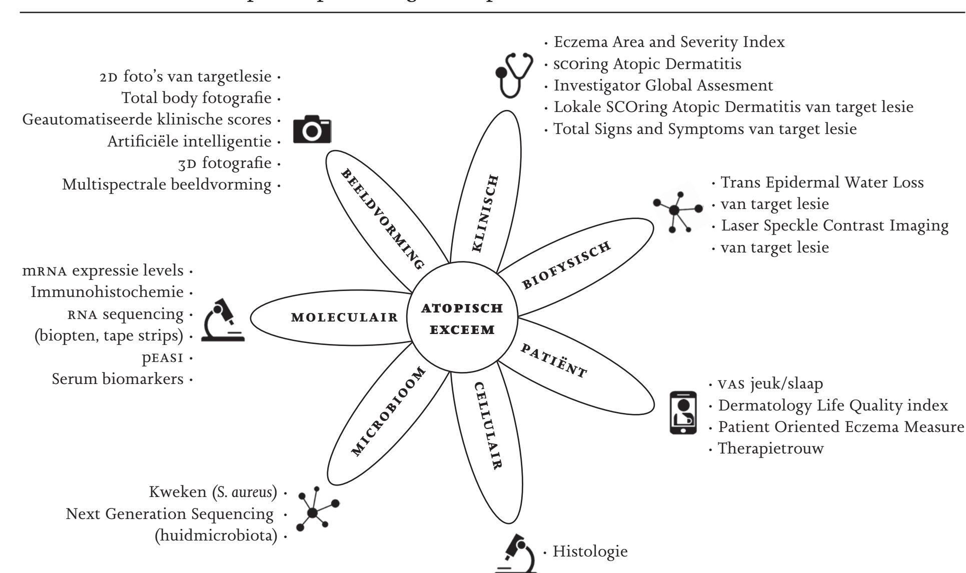
Model	Toepassing	Werkings-mechanisme	Geinduceerde conditie	Immuun respons
INFLAMMATIE				
BCG	Intradermaal	TLR4, 9 agonist	Lokale inflammatie + systemische respons	Adaptief
Imiquimod	Lokaal onder occlusie	TLR7 agonist	Lokale inflammatie, nabootsen psoriasis	Aspecifiek + adaptief
LPS +AL(OH)3	Intradermaal	TLR4 agonist	Inflammatoire respons	Aspecifiek + adaptief
Cantharidin	Papieren schijf met cantharidine	Neutrofielen	Lokale inflammatie	Aspecifiek
UV gedood E.Coli	Intradermaal	Neutrofielen	Erytheem, hitte, zwelling en pijn	Aspecifiek
KLH	Intradermaal, Intramusculair	Neo-antigeen	Lokale inflammatie + systemische respons	Adaptief
JEUK				
Capsaicine	Intradermaal, intra musculair, topisch	TRPV 1 receptor	Jeuk	Aspecifiek
Histamine	Intradermaal, intramusculair	H1,2,3,4 receptor CMIA vezels	Jeuk	Aspecifiek
Cowhage	Cutaan	Par-2	Jeuk, brandend gevoel	Onbekend
UV BLOOTSTELLING				
UV-B verbranding	Locale Thermode	PI3K/AKT/MTOR-opregulatie	Pijn, pigmentatie, erytheem en inflammatie	Aspecifiek + adaptief

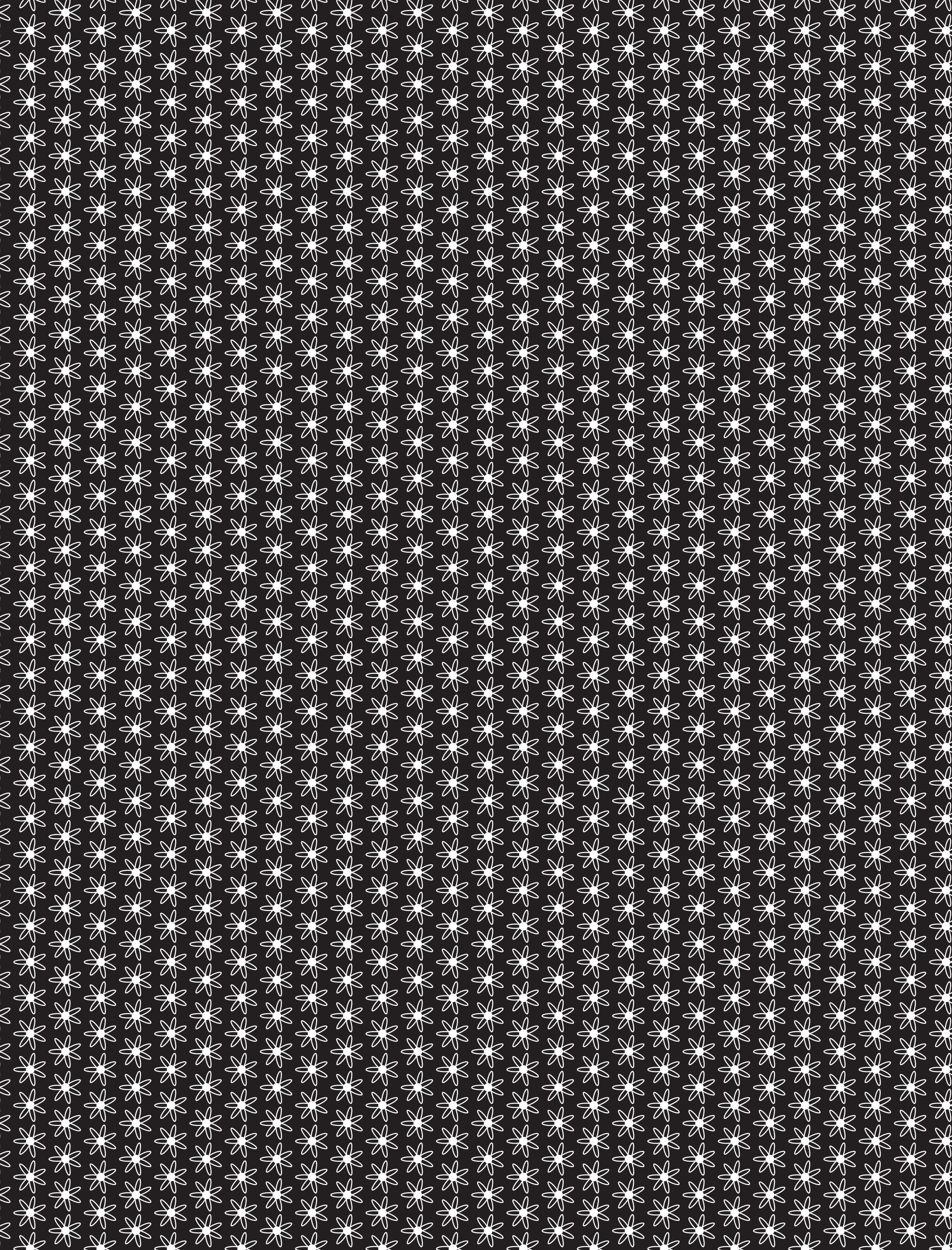
BCG: Bacillus Calmette-Guérin / LPS: Lipopolysaccharide / Geïnjecteerd UV gedood E.Coli: Ultraviolet gedood Escherichia Coli
KLH: Keyhole Limpet Hemocyanin

TABEL 2 Een overzicht van geneesmiddelen in ontwikkeling of recentelijk goedgekeurd voor atopisch eczeem.

Werkingsmechanisme	Geneesmiddel
TOPISCH (LOKAAL)	
Phosphodiesterase (PDE) 4 antagonist	Crisaborole
Janus Kinase (JAK) remmer	Tofacitinib, JTE-032, ruxolitinib
SYK/JAK remmer	Cerdulatinib
Microbioom transplantaties	-
SYSTEMISCH	
IL-4/IL13 antagonist	Dupilumab
IL-13 antagonist	Tralokinumab, Lebrikizumab
IL-31 antagonist	Nemolizumab, BMS-981164
Thymic stromal lymphopoietin (TSLP) antagonist	Tezepelumab, MK-8226
OX40 antagonist	GBR 830, KHK4083
IL-33 antagonist	Etokimab
IL-22 antagonist	Fezakinumab
IL-23 antagonist	Ustekinumab
IL-17 antagonist	Secukinumab, MOR106
JAK remmer	Baricitinib, upadacitinib, abrocitinib
Histamine 4 receptor antagonist	ZPL-3893787

FIGUUR 1 Multimodale patiënt profilering van atopisch eczeem.





CURRICULUM VITAE

Tessa Niemeyer-van der Kolk was born on the 1st of September 1990 in Heemskerk, the Netherlands. She graduated from secondary school in 2008 at the Martinus College in Grootebroek. After graduating she started Medical school at the University of Amsterdam and obtained her MD in 2015 (cum laude). Because of her interest in Dermatology she started her professional carreer as a physician at the sexually transmitted infection (STI) outpatient clinic of the GGD Amsterdam. In January 2016 she started working as research physician and PhD student at the Centre for Human Drug Research (CHDR) under supervision of prof. Robert Rissmann, dr. Martijn van Doorn and prof. Koos Burggraaf, on the topic of early phase clinical drug development in dermatology. She completed her training in clinical pharmacology in 2020. Since January 2021 she works as experienced clinical scientist at CHDR. Tessa Niemeyer-van der Kolk lives in Amsterdam with her spouse Albert and their two children Lilian (2018) and Noah (2020).

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