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Bridging microbiology and therapy in conditions with damaged skin barrier

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DEPARTMENT OF DERMATOLOGY AND VENEREOLOGY | LUND UNIVERSITY



Bridging microbiology and therapy in conditions with damaged skin barrier

Bridging microbiology and therapy in conditions with damaged skin barrier

Sigrid Lundgren



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DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine at Lund University to be publicly defended on 17 of May at 09.00 in Belfragesalen, BMC D15, Klinikgatan 32, Lund

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Abstract: Atopic dermatitis (AD) is one of the most common inflammatory skin diseases characterized by an impaired skin barrier. This makes it prone to colonization by *Staphylococcus aureus* (*S. aureus*), which can exacerbate the condition. Wounds are also common conditions, associated with considerable challenges and exhibit damaged skin barriers. The process of wound healing is delicate and complex, involving a nuanced interplay between skin microflora and host mechanisms.

The overarching aim of this thesis is to investigate therapeutic strategies and underlying mechanisms that impact conditions associated with impaired skin barriers, including atopic dermatitis and the process of epidermal wound healing.

Paper I explored the antimicrobial and anti-biofilm properties of sodium hypochlorite, while Paper II delved into those of potassium permanganate against *S. aureus* isolates from patients with AD. Paper III is a study protocol for a prospective, randomized, double-blinded, first-in-human, placebo-controlled clinical trial testing the tolerability of a novel wound gel. Paper IV considered the control wounds from this trial, exploring the dynamics of bacteria, inflammation and exudation during the healing of epidermal wounds.

In Paper I, sodium hypochlorite showed antibacterial effects against planktonic *S. aureus* at concentrations of 0.01-0.08%. Reduction of *S. aureus* biofilm formation was seen from a concentration of 0.0219%, and eradication of *S. aureus* biofilm was observed at concentrations ranging from 0.01% to 0.16%.

In Paper II planktonic *S. aureus* was killed at potassium permanganate concentrations of 0.05%. Biofilm formations were inhibited when subjected to a concentration of 0.01%, whereas biofilm eradication was observed at concentrations of 1% or above.

Paper IV observed a rapid increase in bacteria following epidermal wounding, which stabilized by the Day 8. The microbial composition, identified by MALDI-TOF mass spectrometry, consisted mainly of commensal bacteria. Peaks in inflammatory cytokines and neutrophil proteins were seen on Day 5 and did not correlate to the amount of bacteria in the wound.

Key words: Atopic dermatitis, *Staphylococcus aureus*, biofilm, epidermal wound healing

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In memory of my beloved, and ever-encouraging grandmother

Kjerstin Eriksson, 1927 – 2012

“Turn your wounds into wisdom”

Oprah Winfrey

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Abstract

Atopic dermatitis (AD) is one of the most common inflammatory skin diseases characterized by an impaired skin barrier. This makes it prone to colonization by *Staphylococcus aureus* (*S. aureus*), which can exacerbate the disease. Wounds are also common conditions, associated with considerable challenges and exhibit damaged skin barriers. The process of wound healing is delicate and complex, involving a nuanced interplay between skin microflora and host mechanisms.

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Populärvetenskaplig sammanfattning

Atopisk dermatit (AD), som även kallas atopiskt eksem, är en av de vanligaste inflammatoriska hudsjukdomarna och drabbar ungefär vart femte barn och ses hos ca 2-10% av vuxna. AD karaktäriseras av röda utslag, eksem, som ofta uppstår i arm- eller knäveck. Sjukdomen är förenad med nedsatt livskvalitet, klåda och försämrad sömnkvalitet. Vid AD har man en försvagad hudbarriär som inte fungerar som den skall. En rad faktorer bidrar till utvecklingen av AD, inklusive ärftlighet, miljöfaktorer, förändringar i immunsystemet samt obalans bland hudens mikroorganismer, vanligen kallat mikrobiomet.

Staphylococcus aureus (*S. aureus*) är en bakterie som påverkar utvecklingen och svårighetsgraden av AD. Denna bakterier återfinns betydligt oftare i huden hos personer med AD jämfört med friska individer. *S. aureus* har utvecklat flera överlevnadsstrategier, bland annat har den en förmåga att skapa biofilm, en sorts samhälle av bakterier som omges av en självproducerad, skyddande substans. Denna biofilm bidrar till bakteriernas överlevnad genom att öka deras motståndskraft mot såväl det mänskliga immunförsvaret som behandlingar med antibiotika, något som kan resultera i utmaningar vid behandling. Man har set kopplingar mellan förekomst av *S. aureus* biofilm och svårighetsgraden av AD.

Behandling vid AD syftar till att angripa angriper faktorer, däribland att reducera mängden *S. aureus* för att förbättra förmågan att läka eksemen. Med tanke på problematiken med antibiotikaresistens, finns det ett behov av effektiva antibakteriella behandlingar som inte främjar resistensutveckling.

De två substanserna natriumhypoklorit (hushållsklorin) och kaliumpermanganat har använts under många år för att behandla eksem som bedömts vara bakteriellt belastade. Både patienter och hälsovårdspersonal rapporterar ofta om positiva effekter av dessa behandlingar men det finns trots detta relativt lite vetenskapligt underlag som styrker att behandlingarna är effektiva. Ännu mindre vet man om ämnenas effekt mot *S. aureus* biofilm.

En skadad hudbarriär ses även vid sår. Det finns många olika tillstånd som innefattar sårbildning, och dessa är ofta förknippade med försämrad livskvalité och höga kostnader för samhället. Sårsläkning är en komplex process i flera faser där olika celltyper och signalmolekyler är involverade. Hudens mikrobiom tros också spela en roll i denna process. Hur bakterier och kroppsegna faktorer förhåller sig till varandra vid normal sårsläkning är dock sparsamt utrett.

Det övergripande syftet med denna avhandling var att undersöka behandlingar och grundläggande mekanismer vid dessa tillstånd med skadad hudbarriär.

I delarbete I undersökte vi natriumhypoklorits antibakteriella effekter samt dess påverkan på biofilm bildad av *S. aureus* från patienter med AD. Bakterierna odlades upp och utsattes i labbmiljö för natriumhypoklorit i olika koncentrationer. Vi kunde

visa att natriumhypoklorit har en dosberoende antibakteriell effekt mot fria *S. aureus* (som inte är i biofilm). Vidare sågs att natriumhypoklorit från en koncentration av 0.02% har en kraftfullt hämmande effekt mot biofilmsproduktion samt nedbrytning av etablerad biofilm från *S. aureus*. Detta får jämföras med den vedertagna koncentrationen 0.005% som ofta används i kliniken. Mot bakgrund av våra resultat skulle man därför kunna överväga att gå upp i koncentration för effektivare biofilmbekämpning. Dock testades natriumhypoklorit även mot odlade hudceller, och där observerades en negativ påverkan vid dessa, högre koncentrationer. Sammantaget behöver man vara försiktig med att rekommendera en ökade koncentration och fler studier kan komma att krävas för väga riskerna mot de terapeutiska fördelarna.

I delarbete II undersökte vi på liknande sätt om kaliumpermanganat har en antibakteriell eller biofilmshämmande effekt gentemot *S. aureus* från patienter med AD. Studien visade att effekten mot fria bakterier var god i de koncentrationer som används kliniskt idag. Däremot konstaterades att substansen är ineffektivt mot att bryta ned etablerad biofilm. Resultaten stödjer fortsatt användning av kaliumpermanganat för behandling av eksempatienter då det sannolikt minskar bakteriemängden. Dock bör man ha i åtanke att behandling av biofilmer kan kräva tilläggsbehandling.

Delarbete III är ett studieprotokoll för en klinisk prövning där vi för första gången hos människa utvärderade säkerheten och toleransen för en ny sårgel innehållande proteinet TCP-25. I studien deltog 24 friska, frivilliga personer som samtliga fick tillverkat fyra ytliga sår på låren. Därefter behandlades två av sårerna med sårgelen och de andra två fick placebo. Deltagarna följdes under 15 dagar, under vilka vi tog prover för att undersöka eventuellt upptag av TCP-25 i blodet samt tecken på biverkningar. Vidare togs bakteriella prover från sårytan och förbanden samt nogsamt dokumentation av sår läkningen.

Delarbete IV bygger på observationer och data insamlat från de placebobehandlade sårerna i tidigare nämnda studie (delarbete III). Målet med delarbete IV var att undersöka dynamiken mellan bakterier, inflammation och sårvätskning under läkningsprocessen av ytliga, så kallade epidermala sår. Resultaten visade att bakteriemängden tenderade att öka fram till dag 8 för att sedan avta till dag 11. I sårerna påvisades främst harmlösa bakterier, så kallade kommensaler, även om *S. aureus* noterades i några fall. De bakterier som vi såg med hjälp av prover från sårytan förekom även i sår förbanden. Proteinmängden i förbanden användes som ett mått på sårens vätskning. Ingen tydlig korrelation observerades mellan sårvätskans mängd och bakteriehalten. Inte heller såg vi något samband mellan bakteriemängden och inflammationsmarkörer. Detta tyder på att läkningsprocessen i normala ytliga sår till stor del styrs av kroppens egna mekanismer oberoende av bakterieförekomst.

Denna avhandling har bidragit med ökad förståelse för vanligt förekommande behandlingar som syftar till att minska bakteriebördan vid AD. Vidare har vi kartlagt den komplexa processen vid epidermal sårläkning. För att fullt ut utnyttja dessa rön krävs vidare forskning, patientstudier och test av nya behandlingsstrategier.

List of Papers

Paper I

Eriksson S, van der Plas MJA , Mörgelin M , Sonesson A

Antibacterial and antibiofilm effects of sodium hypochlorite against *Staphylococcus aureus* isolates derived from patients with atopic dermatitis. *British Journal of Dermatology*. 2017 Aug;177(2):513-521

Paper II

Lundgren S, Sonesson A.

The Effect of Potassium Permanganate on Staphylococcal Isolates Derived from the Skin of Patients with Atopic Dermatitis. *Acta Derm Venereol*. 2024;104:adv18642

Paper III

Lundgren S, Wallblom K, Fisher J, Erdmann S, Schmidtchen A, Saleh K

Study protocol for a phase 1, randomised, double-blind, placebo-controlled study to investigate the safety, tolerability and pharmacokinetics of ascending topical doses of TCP-25 applied to epidermal suction blister wounds in healthy male and female volunteers. *BMJ Open*. 2023 feb 22;13(2):e064866.

Paper IV

Lundgren S, Petruk G, Wallblom K, Cardoso J, Strömdahl AC, Forsberg F, Luo C, Nilson Bo, Hartman E, Fisher J, Puthia M, Saleh K, Schmidtchen A.

Analysis of bacteria, inflammation, and exudation in epidermal suction blister wounds reveals dynamic changes during wound healing

In manuscript

Related papers not included in the thesis

Wallblom K, **Lundgren S**, Saleh K, Schmidtchen A, Puthia M.

Image-based non-invasive assessment of suction blister wounds for clinical safety and efficacy. *Wound Repair Regen.* 2024

Sonesson A, Przybyszewska K, **Eriksson S**, Morgelin M, Kjellstrom S, Davies J, et al.

Identification of bacterial biofilm and the *Staphylococcus aureus* derived protease, staphopain, on the skin surface of patients with atopic dermatitis. *Sci Rep.* 2017;7(1):8689.

Abbreviations

AD	atopic dermatitis
AMP	antimicrobial peptide
APC	antigen presenting cell
CFU	colony-forming units
CV	crystal violet
DALY	Disability-Adjusted Life Year
DLQI	Dermatology Life Quality Index
EASI	Eczema Area and Severity Index
EPS	extracellular polymeric substances
<i>FLG</i>	filaggrin gene
HBP	heparin-binding protein
HOCl	hypochlorous acid
IgG	immunoglobulin G
IgE	immunoglobulin E
IL	interleukin
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization Time-of-Flight
MBEC	minimum biofilm eradication concentration
MHC	major histocompatibility complex
MIC	minimum inhibitory concentration
MPO	myeloperoxidase
NE	neutrophil elastase
OCl ⁻	hypochlorite ion
PDGF	Platelet-Derived Growth Factor
QS	Quorum sensing
SEA	<i>staphylococcal</i> enterotoxin A
SEB	<i>staphylococcal</i> enterotoxin B
SEM	scanning electron microscopy
SCORAD	SCORing Atopic Dermatits
TEWL	transepidermal water loss
TNF- α	tumor necrosis factor-alpha

Introduction

The human skin

The human skin is one of the largest organs of the body, with a surface area of 1.5-2.0 m². Functioning as the initial barrier between an organism and its environment, the skin plays a crucial role against external threats. It enables sensation, thermoregulation and facilitates metabolic processes. With a diversity in structure and function, the skin is composed of three fundamental layers: epidermis, dermis and subcutis (Figure 1). The epidermis, the outermost layer, consists primarily of keratinocytes. These cells undergo a process of keratinization in which they differentiate, lose their nucleus and migrate from the basal layer to the surface forming the cornified stratum corneum (1). This semipermeable surface is capable of enduring mechanical forces and serves as protection against the penetration of pathogens and chemicals. The lower, nucleated parts of the epidermis also play a significant role in barrier function with cell-cell junctions and lipid production fundamental to preventing water loss (2, 3). Other specialized cells in the epidermis are melanocytes which produce pigment, antigen-presenting Langerhans cells, Merkle cells involved in sensory reception and resident T cells, part of the adaptive immune system (1).

The dermis, situated beneath the epidermis, is a vascularized connective tissue layer providing the skin with structural support, elasticity and nutrition. It is histologically divided into two distinct but connected regions. The superficial part, the papillary dermis is recognized for its fine collagen fibrils and plentiful ground substance. By extending fingerlike projections, known as papillae, towards the epidermis, the surface area for nutrient transport across the two layers is increased. Located below the papillary dermis is the reticular dermis. This is characterized by its dense, irregularly organized collagen fibers. A multitude of nerve endings are also found in the dermis, along with sweat and sebaceous glands, hair follicles, lymphatic structures and blood vessels (1).

The deepest layer of the skin, the subcutis, consists of adipocytes distributed in looser connective tissue. It functions as thermal insulation, energy reserve and protective padding.

An impaired skin barrier can have substantial implications for comfort and health and is seen in a wide range of dermatological conditions such as atopic dermatitis and wounds (4).

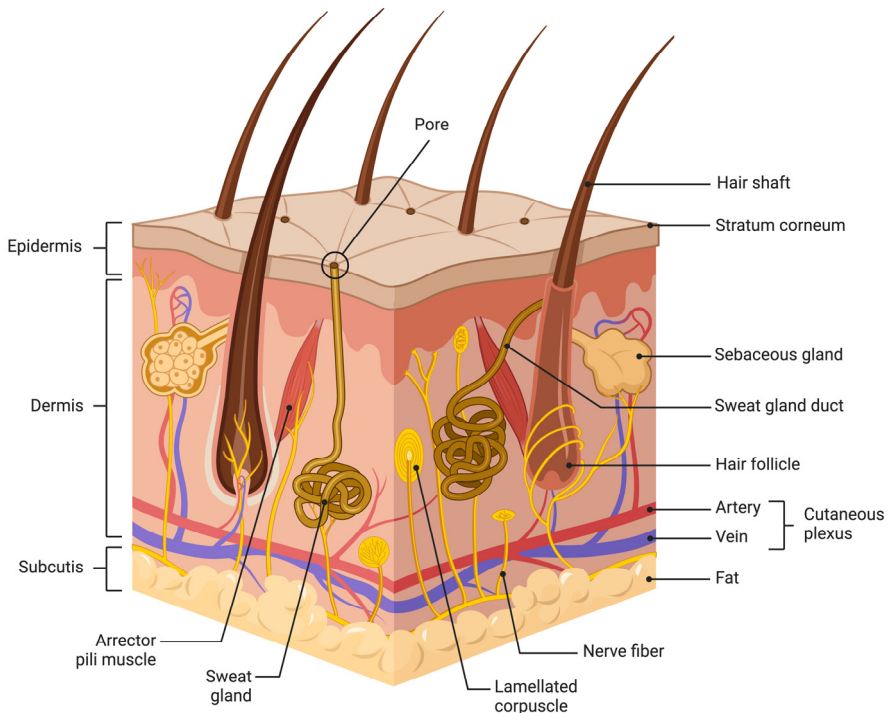


Figure 1 – Anatomy of human skin

The three layers of the skin, epidermis, dermis and subcutis. Created with BioRender.com

The immune system

The immune system is an essential protective system comprising an array of cell types and signal molecules to defend against pathogens. It is divided into the innate and adaptive immune systems. Innate immunity is characterized by its immediate but unspecific response to pathogens (5). Pathogen-associated molecular patterns (PAMPs) are structures on pathogens recognized through pattern recognition receptors (PRRs) expressed by macrophages, dendritic cells, neutrophils and epithelial cells (6). This recognition initiates the innate immune response, stimulating the production of signal molecules and serves to recruit additional immune cells and enhance phagocytosis (7).

The adaptive immune system, also known as the acquired immune system, has a longer activation time but offers a more specific response and develops an immunological memory. Unlike the innate immune system, it generally requires a prior exposure to the pathogen to initiate a response. The key cells of the adaptive immune system are the B- and T-lymphocytes, commonly called B- and T-cells (5).

Antigens are molecules from pathogens and non-pathogenic substances like pollen, food proteins and dust mites. When entering the body, the antigen encounters antigen presenting cells (APCs) like dendritic cells, macrophages and B-cells. The APCs process the antigen into smaller peptides and display them on their surface, bound to major histocompatibility complex (MHC) molecules (7).

T-cells that haven't faced their specific antigen, so called naïve T-cells, circulate the bloodstream and lymphoid organs. When they encounter an APC that presents an antigen that matches its T-cell receptor (TCR), they bind with this to the MHC. This interaction, combined with co-stimulatory molecules (the most well-known being CD28 on T-cells and CD80 and CD86 on APC) leads to an activation, differentiation and proliferation of the T-cell. There are two main subcategories of the naïve T-cells; these are distinguished based on specific cell surface proteins (CD4 and CD8) and differentiate into various subsets. The CD4+ T-cell differentiate into different helper T-cells (Th1, Th2, Th17, regulatory Treg) that can secrete various signal molecules, cytokines that regulate the inflammatory response. CD8+ T-cells differentiate into cytotoxic T-cells responsible for killing infected or abnormal cells (7-9).

The B-cells are essential to the humoral immune response. Upon antigen binding to B-cell receptors (BCRs) on the cell surface, B-cells become activated. Subsequently, the activated B-cell transforms into a plasma cell, generating large quantities of specific antibodies. These antibodies bind to the pathogen, neutralizing it and tagging it for destruction (5, 9).

Skin microbiome

The skin hosts millions of bacteria, fungi, and viruses - commonly called the skin microbiome or microbiota (10, 11). These microorganisms, the commensals, are essential to maintaining of immune homeostasis and inhibiting the growth of pathogens by production of antimicrobial peptides (AMPs) and proteases (12, 13).

Of all commensal communities of the body, the skin harbors the most diverse, comprising over 1,000 distinct bacterial species (14, 15). The microbial populations differ in quantity and composition across various body sites. Using traditional culture methods, the average microbial count ranges from 10^3 to 10^4 colony forming units (CFU)/cm², but in moister regions, like the groins, armpits and knee creases,

microbial counts can exceed 10^6 CFU/cm² (16). A study found that more than 90% of the skin bacteria belongs to four phyla: Actinobacteria (52%), Firmicutes (24%), Proteobacteria (16%), and Bacteroidetes (6%) (15). Further investigations of healthy adult skin have demonstrated that *Staphylococcus* and *Corynebacterium* species are frequently found in humid places, whereas lipophilic *Cutibacterium* species are abundant in sebaceous areas, like the face, chest and back. These bacteria produce lipases that break down sebum lipids into fatty acids, which then serve as a food source. This process also acidifies the skin, helping to maintain a skin pH that combats pathogenic bacteria. Drier areas of the skin, like the forearm and buttocks, tends to have a higher diversity of microbial species and are also less stable over time (15, 17, 18).

S. aureus is a species that regularly appears in the microbiota, sometimes as a commensal but is also associated with skin disorders; especially when the skin barrier is compromised, as in atopic dermatitis or wounds (19). When wounds occur, they provide an entry point for microorganisms to infiltrate underlying tissues (20, 21). Commensal microorganisms' interactions during wound healing are believed to promote the innate system, while pathogens may hinder the process, leading to delayed healing (21-23).

Children exhibit a more diverse microbial composition compared to adults. However, during puberty, there is a compositional shift (24). In adulthood, the microbial makeup remains relatively stable despite continuous exposure to external microorganisms from the surroundings and other individuals (25).

Staphylococcus aureus

Staphylococcus aureus (*S. aureus*) is a Gram-positive bacterium, characterized by its rounded coccoid shape and ability to form clusters resembling grapes when observed under a microscope. The bacteria was first described in the 1880's by the surgeon Alexander Ogston who discovered the bacteria in pus from surgical abscesses (26). Over a century later, *S. aureus* continues to be an adaptable and dangerous pathogen in humans responsible for a wide array of infections in multiple organ systems. *S. aureus* is found as a commensal in the nares in about 20-30% of the population but is known to be a risk factor for subsequent infection (27-29). In case of clinical infection, 80% of *S. aureus* strains detected in the bloodstream are consistent with the isolates found in the nares (29). Treatment of infections caused by *S. aureus* has become more challenging due to the rise of multidrug-resistant strains. It is a successful pathogen due to its many virulence mechanisms allowing it to invade host tissues and evade the immune system (30, 31) (Figure 2).

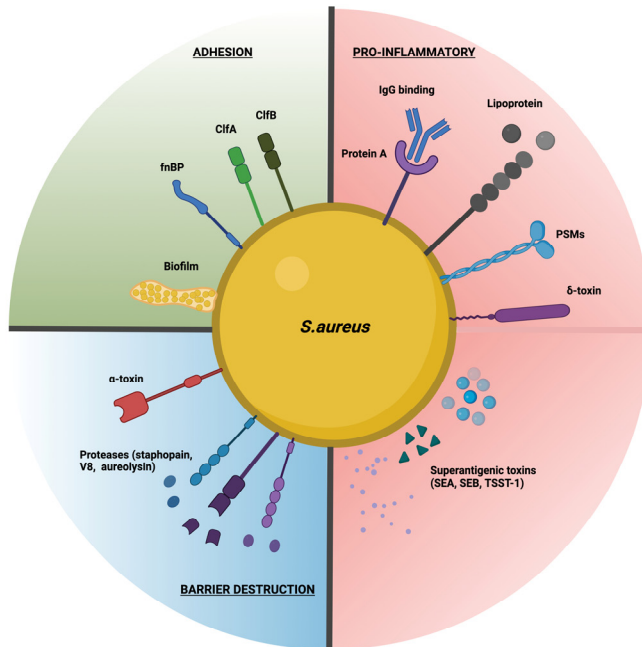


Figure 2 – Virulence factors of *S. aureus*

S. aureus exhibit a wide array of virulence factors that aid in adhering to host tissue, disrupting barriers and trigger inflammatory responses. Modified from Paller et al. 2019 (31) Created with BioRender.com

Virulence mechanisms of *Staphylococcus aureus*

Adhesion

Enhanced adhesion, both to host tissues and medical implants, is promoted by special surface proteins such as clumping factors and fibronectin-binding proteins. Clumping factor A (ClfA) is a fibrinogen-binding protein that facilitates platelet aggregation and clumping of the bacteria; an important process for infiltrating host cells. By binding to fibrinogen, ClfA can also shield the bacteria from opsonization and neutrophil phagocytosis (32, 33). Clumping factor B (ClfB) binds predominantly to cytokeratin 10, a protein in squamous epithelial cells of the skin and nasal epithelium (32). Fibronectin-binding proteins (FnBP) A and B assist in bacterial invasion of host cells by binding to the glycoprotein fibronectin found in blood and connective tissues (34).

Destruction of host cells

Alpha toxin is an important virulence factor that destroys a wide range of host cells by producing pores in their membranes. By disrupting adherence junctions, the toxin

also breaches epithelial and endothelial integrity (35). In addition *S. aureus* also produces extracellular proteases, such as V8, staphopain and aureolysin, all of which contribute to the breakdown of host proteins, assisting in tissue invasion, and modulate host immune response (36).

Evading the immune system

Many strains of *S. aureus* produce a protective polysaccharide capsule, which surrounds the peptidoglycan cell wall that serves as an antiphagocytic barrier. For effective phagocytosis, the immune system marks the bacteria with antibodies and complement components, a process called opsonization (37). Protein A is a surface protein that binds to the Fc portion of immunoglobulin G (IgG) and thereby protects the bacteria from being recognized and destroyed by the immune defence (38). Another way to escape the immune system is to produce leukocidins, which are toxins that destroy leukocytes (39). Another known virulence factor is coagulase, which is an enzyme that converts fibrinogen into fibrin, leading to the formation of blood clots (coagulation) around the bacteria and thus inhibiting phagocytosis (40). *S. aureus* can also produce varying amounts of biofilm. This is an efficient way of protecting the bacteria from phagocytosis (41). Biofilm formation will be discussed more in depth in subsequent sections.

Exfoliative toxins

Some strains of *S. aureus* produce exfoliative toxins, resulting in staphylococcal scaled skin syndrome (SSSS) characterized by extensive blistering and peeling of the skin. The toxins responsible, exfoliative toxin A (ETA) and exfoliative toxin B (ETB), target desmoglein-1, a key component of desmosomes, and lead to disruption of cell-cell adhesion and detachment of keratinocytes (42).

Staphylococcal superantigens and toxins

S. aureus produces superantigenic toxins. These activate a large number of T-cells, non-specifically leading to immense cytokine release and systemic effects. Toxic shock syndrome toxin 1 (TSST-1) is infamous for being the cause of the dreaded toxic shock syndrome associated with tampon use, by creating an environment in which toxin-producing *S. aureus* thrives (43). Other examples of superantigenic toxins are the staphylococcal enterotoxins A and B (SEA, SEB), best known for their role in food poisoning. However, they can also implicate skin health by exacerbating inflammatory responses (44). Another set of toxins comprises the phenol-soluble modulins (PSMs) which are amphipathic peptides divided into two groups: α -type PSMs and β -type PSMs. PSM α stimulates the production of proinflammatory cytokines such as IL-1 α and IL-36 α from keratinocytes. This activates $\gamma\delta$ T cells and trigger Th17 responses, as well as recruiting neutrophils. Moreover, certain strains of *S. aureus* produce a similar toxin called delta-toxin (δ -

toxin), which induces mast cell degranulation and raises levels of IL-4 and IgE (45, 46).

Lipoproteins

These molecules, anchored to the cell membrane of the bacteria, play an important role in facilitating attachment to host cells. *S. aureus* lipoproteins interact with the Toll-like receptor 2 (TLR2) that is expressed on immune cells, such as macrophages and dendritic cells. They trigger signaling pathways that induce the production of proinflammatory cytokines like tumor necrosis factor alpha (TNF- α), interleukin 1 β and 6 (IL-1 β , and IL-6). Inflammation is a mechanism aimed at defending against infections but can also result in skin damage and clinical manifestations of *S. aureus* skin infections (47-49).

Staphylococcal biofilm

The first report describing bacterial biofilms dates back to Antonie van Leeuwenhoek, who famously detailed his observations in a letter dated October 9, 1676. In it, he documented his investigation of what he termed “animalcules” (a historic expression referring to microscopic organisms or tiny creatures) in the dental plaque from his own teeth. He noted that the microorganisms within the plaque had a remarkable resilience to vinegar in contrast to those found outside the plaque, which were killed (50). While many characteristics had been described over time over the years, it was only in 1978 that Costerton et al. introduced the term “biofilm” (51, 52).

Bacterial biofilms consist of aggregates of sessile bacteria which, unlike their planktonic, free-flowing counterparts, are attached to biotic or abiotic surfaces or grouped together, encapsulated in a self-produced matrix. This matrix, a conglomeration of extracellular polymeric substances (EPS) such as proteins, polysaccharides and extracellular DNA (eDNA), affords the bacteria with structural integrity and protection (53).

Within the biofilm, bacteria undergo alterations in their phenotype, adjusting to the environmental conditions of low oxygen and nutrient levels. Consequently, their metabolic activity is decreased and their rate of cell division reduced, leading to a lower susceptibility to cell-wall-active antibiotics like the beta-lactams (54, 55). Biofilm formation is an ancient adaptation and displays a distinctive growth strategy that enables bacteria to survive in hostile conditions and colonize new niches (55, 56).

The capability to form biofilm varies among species and even among isolates of the same species. A biofilm may consist of either of single a species or an array of microbial populations (57).

Biofilm formation

Attachment

Biofilm formation (58) (Figure 3) is a process involving multiple steps, starting with attachment. During this phase, free-floating (planktonic) bacteria encounter a surface, which can be biotic or abiotic. This attachment typically occurs through reversible mechanisms mediated by physical forces, such as van der Waals forces, cellular surface charge, and hydrophobicity. Surface proteins, such as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), are crucial in this stage as they facilitate adhesion. Once adhered, certain bacteria advance to irreversible attachment, forming a strong bond with the surface. The bacteria then initiate the production of EPS, which serve as an anchor to the surface providing structural support to the developing biofilm (59).

Microcolony

Following attachment, bacteria initiate replication and assemble into three-dimensional microcolonies on the surface. These microcolonies are embedded in a self-generated EPS matrix which holds the bacterial cells together, and provides a structural support, and acts as a protective barrier hindering the penetration of antimicrobial agents to the biofilm bacteria. Within the microcolonies, bacterial communication and coordination intensify, enabling collective responses to environmental changes (60).

Maturation

The microcolonies continue to grow, initiating the biofilm maturation process in which the EPS matrix continues to accumulate and shape biofilms or varied appearance. Depending on the local nutrient availability, these range from flat formations to elevated, mushroom-like structures. This stage of maturation is marked by an important diversification within the biofilm's ecosystem, leading to the emergence of numerous of specialized microenvironment (61). These microenvironments coexist depending on the gradients of nutrients, oxygen and pH. This heterogeneity emphasizes the adaptability and resilience of the bacterial collective (62-64).

Interbacterial coordination and communication is required in the biofilm and is managed via a process known as quorum sensing (QS). QS is mediated by the release and detection of small signaling molecules and can synchronize the gene expression across the biofilm and control various behaviors like virulence factors, antibiotic resistance and further EPS production (65).

Bacteria in biofilms are shielded from immune defense mechanisms such as phagocytosis and complement system activation, making them exceedingly difficult to eliminate from the host. As biofilms reach maturity, their resistance to antibiotics

peaks (66). Notably, bacteria within a biofilm can exhibit the capacity to withstand antibiotic concentrations 10 to 1,000 times higher than the dose required to kill the planktonic equivalent (67).

Dispersion

The biofilm cycle ends with detachment, whereby bacteria are released from the mature biofilm and regain their planktonic state enabling them to spread and colonize new surfaces. This strategy contributes to the adaptability and survival of the bacterial species.

The dispersal procedure involves a variety of mechanisms. *Erosion* happens when physical forces, like fluid movement, act on the biofilm resulting in a continued detachment of single bacterial cells or small clusters. By contrast, to this, spreading can also occur through *sloughing*, when larger portions of the biofilm detach due to structural weakness or significant shear forces (68). These processes are often a passive way of spreading the biofilm, but the detachment can also be made in an active way by *seeding*. This involves the regulated release of clumps of bacterial cells. This dispersal mechanism is initiated by the bacteria and involves the production of enzymes, such as dispersin B and deoxyribonuclease. These break down parts of the EPS-matrix and reduce biofilm adhesion (69, 70). Environmental alterations such as changes in nutrient supply, oxygen levels, and flow dynamics can trigger detachment of the biofilm (71, 72).

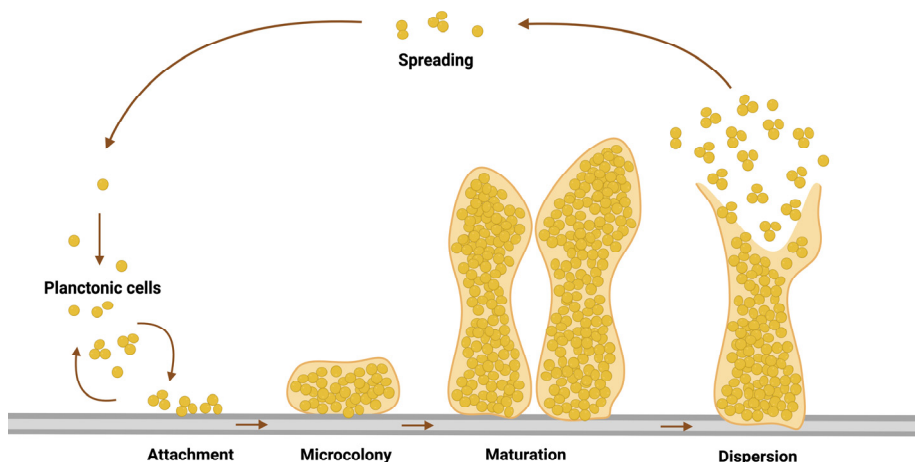


Figure 3 – Biofilm formation cycle

Bacterial biofilm formation begins with planktonic cells attaching to a surface. This is followed by the development of microcolonies and culminates in maturation. Finally, dispersion occurs, enabling bacteria to migrate to new locations. Modified from Peng et al. 2022 (58). Created with BioRender.com

Staphylococcus epidermidis

Staphylococcus epidermidis (*S. epidermidis*) is a coagulase-negative staphylococcus species, meaning that it does not produce coagulase. It is considered a non-pathogenic commensal of the skin. It is frequently found in lesional skin in AD and reports have suggested that *S. epidermidis* can trigger inflammation in AD, potentially by playing a role in exacerbations (73, 74). Isolates that produce biofilm may even further contribute to the pathogenesis of the disease (75).

Atopic dermatitis

Background

Atopic dermatitis (AD) is one of the most common inflammatory skin diseases and has been described in medical history since the Roman Age. In 1923, Arthur Coca and Robert Cooke recognized a relationship between rhinitis and asthma and coined the term *atopy* (76). This term originates from the Greek word *atopia* which means “without place”. Ten years later, in 1933, Fred Wise and Marion Sulzberger introduced the term *atopic dermatitis*, referring to infantile eczematous lesions concentrated on the face and flexural folds in a people with a family history of atopic conditions.

The prevalence and incidence of AD have increased over the past decades, in 2010 it was calculated that some 230 million persons have this disease (77, 78). According to the Global Burden of Disease study, the prevalence among children was 15-20% and up to 10% for adults (79). These numbers place AD as the 15th most common of nonfatal diseases, and it carries the highest disease burden among skin disorders in terms of disability-adjusted life years (DALY) (80). This has further been underscored by reports of high Dermatology Life Quality Index (DLQI) scores. These indicate impaired life quality connected to a limited lifestyle, avoidance of social connections, compromised activities and itching (81).

Pathogenesis of atopic dermatitis

The pathogenesis of AD is caused by a complex interplay between skin barrier dysfunction, genetics, immune dysregulation, environmental factors and microbial imbalance (82) (Figure 4).

Impaired skin barrier function can be indicated by an increased level of transepidermal water loss (TEWL). This is a measurement of the amount of water evaporating from the skin's surface and is associated with AD (83). Further

dysfunctional barrier transformations in AD are altered lipid composition and an increased pH level (84). These changes are associated with diminished function of antimicrobial peptides and adverse shifts in the skin microbiome (85, 86). These are marked by a decrease in microbial community diversity enabling increased colonization by *S. aureus*.

A strong heritability is seen in AD and atopic disorders in the family history is the strongest risk factor in developing the disease (87, 88). Mutation of the filaggrin gene (*FLG*) is connected to a reduction in filaggrin production and is considered an important genetic component in the pathogenesis of AD. Filaggrin organizes keratin fibers together and is crucial in maintaining a healthy stratum corneum. Deficiency leads to an increased permeability of the skin, allowing letting water to escape out and allergens, irritants and pathogens to penetrate in (89).

Inflammation is central to AD and is believed to be initiated a disruption of the epidermal barrier. A breach in the barrier triggers the release of alarmins, endogenous molecules that signal tissue damage. These alarmins, such as interleukin-33 (IL-33) and thymic stromal lymphopoietin (TSLP), activate skin dendritic cells and innate immune cells to interact with Th2 cells. These are predominant in AD, especially in patients of Caucasian origin (90, 91). When the Th2 cells are activated, they release cytokines into the skin, chiefly interleukin 4 (IL-4), interleukin 5 (IL-5), interleukin 13 (IL-13), and interleukin 31 (IL-31) (92). These cytokines stimulate inflammation downstream of Janus kinase (JAK) pathways, activating sensory nerve ends, promoting pruritus and leading to stimulation of B-cells to produce IgE antibodies (90)

Environmental factors such as extreme temperatures, air pollution, increased water hardness and frequent use of household cleaning products have been associated with a worsening of AD (93).

Elevated levels of total immunoglobulin E (IgE) are more frequently observed in patients with AD compared to healthy controls, and this elevation has been associated with poorer disease outcomes. However, the precise role of IgE in the pathogenesis of AD is not completely understood (94).

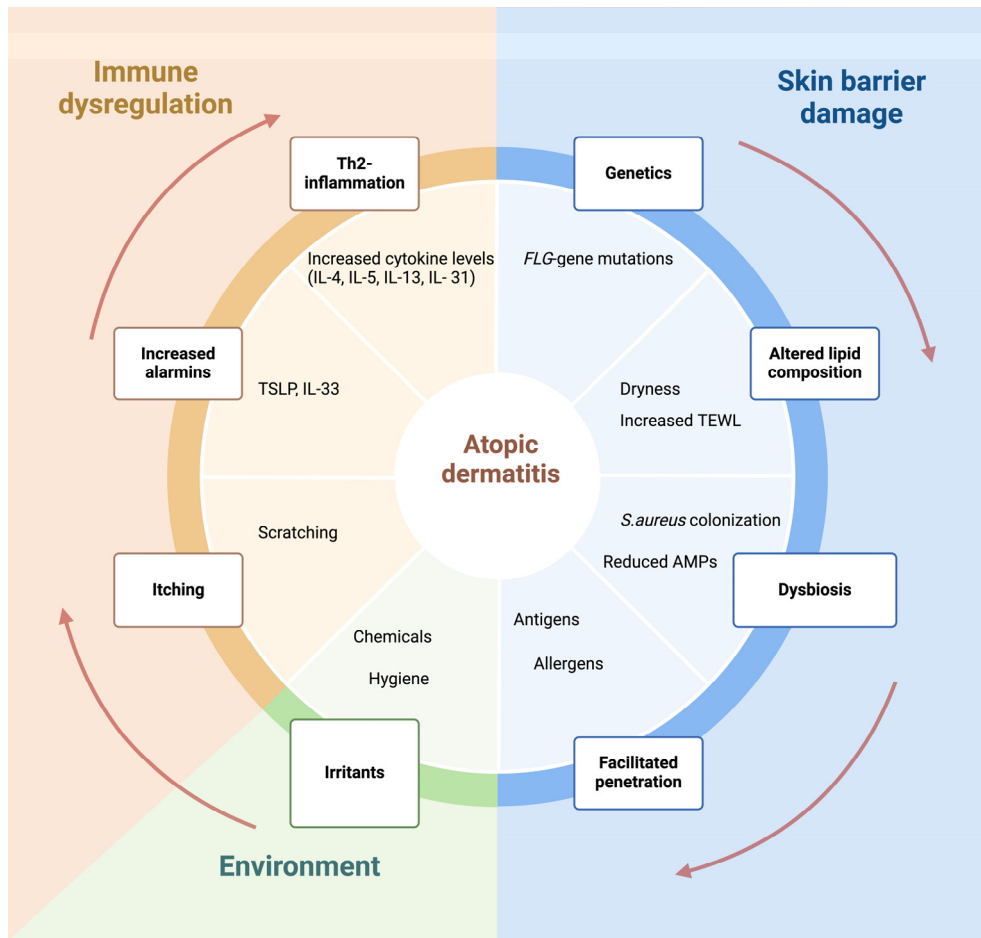


Figure 4 – Pathogenesis of atopic dermatitis

The pathogenesis of atopic dermatitis (AD) is multifactorial and is connected to skin barrier damage, impaired immune mechanisms and environmental influences. Modified from Eyerich et al. 2015 (82). Created with BioRender.com

Clinical presentation of atopic dermatitis

The clinical presentation of AD is heterogenic and varies according to age, pigmentation of the skin and stage in the disease. However, essential characteristics are eczematous lesions with papules or papulovesicles, edema, crusting and scaling, severe pruritus and a chronic or relapsing development (80) (Figure 5). Different diagnostic criteria exist to facilitate accurate diagnosis. In 1980 Hanifin and Rajka published a list of 23 clinical signs and symptoms, which is still commonly used (95, 96). The severity of AD may be further evaluated using scoring tools such as

the Eczema Area and Severity Index (EASI) and the Scoring Atopic Dermatitis (SCORAD) scale. These are used in both research and clinical practice (97, 98).



Figure 5 – Clinical presentation of atopic dermatitis

A typical distribution of eczematous lesions in a patient with atopic dermatitis. Published with permission of the patient. Photo: Department of Dermatology and Venereology, Skåne University Hospital, Lund.

The role of *Staphylococcus aureus* in atopic dermatitis

The prevalence of *S. aureus* is high in patients with AD (99). A meta-analysis, comprising 95 observational studies, indicated a colonization rate of 70% in lesional skin and 39% in nonlesional skin, as compared to 10% colonization in the skin of healthy individuals (93, 100). There is evidence for a correlation between the density of colonization and the severity of the disease (31, 49, 100) (Figure 6).

The normal microbiota is disturbed in AD, and the diversity of microbes on the skin is diminished. AD patients exhibit fewer protective commensal bacteria like *S. epidermidis* and *Staphylococcus hominis* (*S. hominis*), which are known to produce lantibiotics and promote the secretion of AMPs to protect against harmful pathogens (86, 101). Endogenous AMPs such as LL-37 and HBD-2 are reduced in lesional AD skin, in comparison to psoriatic lesions or healthy skin (102).

Asymmetrical or deformed corneocytes are created in AD due to the reduction of filaggrin degradation products in the stratum corneum. *S. aureus* shows an enhanced binding affinity to these deformed corneocytes (103).

S. aureus struggles to thrive in acidic conditions, such as the healthy stratum corneum. However, in AD, reduced sweat secretion and diminished levels of free fatty acids leads to a change in pH with a shift towards alkalinity contributing to a milieu favorable to *S. aureus* (3, 31).

As described earlier, *S. aureus* expresses several virulence factors that contribute to the intensity of symptoms in AD . By enhanced adhesion to the skin surface, facilitation of tissue invasion, and stimulation of Th2 immune response with increased production of IL-4, IL-5 and IL-13 an increased inflammation, erythema, and pruritus is seen (104). *S. aureus* strains capable of forming biofilms have been found to predominate in AD lesions, with a correlation the to the disease severity (105, 106).

Thymic stromal lymphopoietin (TSLP) is a cytokine produced by keratinocytes and is found to be overexpressed in patients with AD. The presence of *S. aureus* and its virulence factors can further trigger the enhanced production of TSLP by the keratinocytes, amplifying the inflammatory response and worsening disease severity (104, 107).

Topical treatments

Management of atopic dermatitis is multifaceted and varied considering the patients specific requirements. The main goal is to address triggering factors and enhance overall condition of the skin. Key therapies such as topical corticosteroids and calcineurin inhibitors plays a crucial role in reducing skin inflammation. When combined with moisturizers, these treatments form the cornerstones for the topical treatment of AD (108, 109). The insight regarding a correlation between *S. aureus* and increased severity of AD has led to considerations that anti-staphylococcal treatments may offer additional benefits in the disease management (110). Various options, including oral and topical antibiotics, as well as alternative antimicrobial substances have been discussed (86, 110-112).

Sodium hypochlorite

Sodium hypochlorite (NaOCl), commonly known as bleach or household bleach, is a substance with historical significance. The French chemist Claude Louis Berthollet was the first to isolate and produce hypochlorite in 1789, initially for use in bleaching of clothes (113). In 1825, a Hungarian obstetrician, Ignaz Semmelweis, realized that handwashing with hypochlorite before delivery reduced the frequency of “post partum fever” (114). During World War I, soldiers’ wounds injuries often became infected, not seldom leading to amputation or death. A chemist named Henry Dakin developed an antiseptic solution containing buffered sodium hypochlorite at a concentration of around 0.5%, which served as a non-irritating antiseptic wound cleanser (115). Since then, it has become a part of medicine in the management of burns, ulcers and dental work, as well as an important household cleaning product worldwide.

When used in the medical profession, a low incidence of toxicity is reported. However, as sodium hypochlorite is extremely accessible in higher concentrations as a household cleaner, there have been numerous reports of adverse effects upon topical exposure, including reversible skin and eye irritation (114, 116). In cases of ingestion it has been shown to cause irritation and first-degree burns of the gastrointestinal tract (117), renal injuries (118), signs of pulmonary toxicity (119) and, in large quantities, even death (120).

Sodium hypochlorite is the sodium salt of hypochlorous acid and is an oxidative agent (121). When dissolved in water, hypochlorous acid (HOCl) and hypochlorite ions (OCl^-) are formed (Figure 7). These two products are assumed to be responsible for the disinfectant properties of the substance (122). The hypochlorite ion carries a charge which hinders it from penetrating the cell walls of bacteria, limiting its antibacterial effects. Conversely, the hypochlorous acid, considered to be a powerful

oxidant, is a small, uncharged molecule similar to water, that passes easily through the cell membrane and exerts antimicrobial effects both inside and outside the bacterial cells by disrupting pathogen's essential enzyme functions (123, 124). This mechanism is also appears in nature, with HOCl as one of the main oxidants formed by neutrophils and is essential part of a successful innate immune response (125).

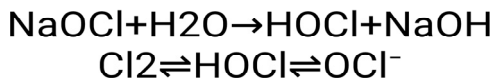


Figure 7 - Chemical reaction of sodium hypochlorite

When sodium hypochlorite (NaOCl) is added to water (H₂O) hypochlorous acid (HOCl) and hypochlorite ions (OCl⁻) are formed.

Over the years, several studies have shown the usage of sodium hypochlorite to improve the disease status of AD (126-128). In a study published in 2023, 15 patients with AD and five people with non-atopic skin were instructed to take a five to ten-minute bleach bath (0.005% NaOCl) twice per week for 12 weeks. Eight of the 15 patients achieved 50% improvement of EASI scores (EASI₅₀) and significant reduction in itching. The patients with AD also showed a reduction in TEWL values in non-lesional skin. However, this treatment showed no significant reduction of *S. aureus* levels (129).

Potassium permanganate

Potassium permanganate (KMnO₄) is a purple crystal salt consisting of potassium and permanganate ions (Figure 8). It is manufactured and used in different forms, such as tablets, crystals and solutions. When resolved in water, the aqueous solution varies in color from light pink to violet, depending on the concentration(130).

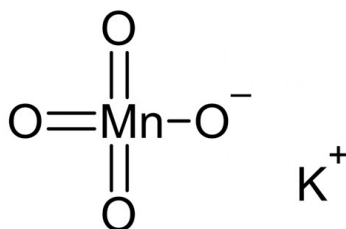


Figure 8 - Potassium permanganate

Potassium permanganate is a salt consisting of potassium and permanganate ions.

Potassium permanganate is a strong oxidative agent with astringent and odor eliminating effects (130). It has been used in medicine since the early 1800s. In dermatology, it is used as a topical treatment for different types of wounds and eczematous conditions (131). Concentrations in treatment range between 0.001% and 0.1%, depending on the condition, the age of the patient, and local traditions (132).

Potassium permanganate can be used either by soaking dressings and leaving them on the skin for local treatment or, for managing larger areas, by diluting it in a bathtub for full body exposure (Figure 9). The standard treatment duration is usually 10 to 20 minutes (131).



Figure 9 – Potassium permanganate bath

Treatment of atopic dermatitis may involve soaking in potassium permanganate for 10-20 minutes. Photo: Sigrid Lundgren, Department of Dermatology and Venereology, Skåne University Hospital, Lund.

Patients should be informed that the treatment will temporarily stain their skin and nails brown, and permanently discolor clothing and basins that comes in contact with the product. Furthermore, caution needs to be taken due to the risk of irritation or burns from incompletely dissolved crystals and tablets. Cases of caustic burns have been documented, including one where a patient inadvertently sat on a tablet during bathing, resulting in a wound on to their buttocks (133). Caustic injuries of the gastrointestinal tract have been observed after ingestion (134), and there are older reports of vaginal burns after illegal usage as an abortifacient (135).

Being a powerful oxidizing agent, potassium permanganate has been showed to play a role in the degradation of toxins in water systems. It triggers reactions that cause the toxins to decompose into simpler and potentially less harmful compounds (136). Potassium permanganate is described and widely used as an antibacterial substance in AD but, apart from one study from 1992, which observed a reduction in *S. aureus* colonization on the skin of AD patients after potassium permanganate treatment (137), there has been minimal investigation into its actual impact on bacteria derived from AD-affected skin.

Wounds and wound healing

Skin wounds exist in many forms. They and have a profound impact on quality of life and healthcare systems, resulting in significant economic costs (138, 139). Reports suggest that nearly one billion individuals globally suffer from acute or chronic wounds (140).

Wound healing is a precisely regulated biological process characterized by a sequence of well-coordinated, overlapping stages, including hemostasis, inflammation, proliferation, and remodeling, all directed towards the restoration of tissue integrity and functionality(141) (Figure 10).

While compensatory mechanisms allow for some degree of skin repair despite imbalances among the involved cells and signalling molecules, disruption in various stages can result in incomplete wound closure, delayed healing, or excessive scarring (142).

Hemostasis

The first stage of wound healing, hemostasis, occurs immediately after the skin is injured and ends after a few hours. This phase strives to terminate bleeding and minimize blood loss, while also initiating the inflammatory response. Exposure of collagen activates thrombocytes, which forms a provisional plug (141, 143). Additionally, clotting factors initiate clotting cascades, leading to the formation of

a fibrin-rich blood clot. This diminish hemorrhaging and serves as a scaffold, facilitating further cell migration (141, 144). Platelets also secrete platelet-derived growth factor (PDGF) drawing macrophages and fibroblasts to the site (145). Leukocytes and platelets further release cytokines and growth factors, activating inflammation (IL-1 α , IL-1 β , IL-6, IL-8 and TNF- α), stimulating collagen synthesis, and initiating angiogenesis (141, 146). IL-8 plays an important role as a potent neutrophil chemoattractant (141).

Inflammation

The inflammatory phase is initiated during hemostasis and is characterized by the recruitment of immune cells to the wound. The first cells to reach the site are the neutrophils, typically arriving within minutes to hours after injury, and reaching their peak about 24 to 48 hours thereafter (147). They are responsible for cleansing the area from debris and pathogens by phagocytosis, and releasing of antimicrobial substances and proteinases like neutrophil elastase (NE) (142). The neutrophils also release inflammatory mediators, like tumor necrosis factor alpha (TNF- α), and interleukins (IL-1 β , IL-6) further amplifying the recruitment of inflammatory cells and initiation of the tissue repair process (141). Although the neutrophils plays an important role, prolonged neutrophil activity can result in tissue damage and impede wound healing (148).

In the second phase of the inflammatory stage, macrophages enters the injury zone and continue the phagocytosis process and removal of apoptotic cells (149, 150). They secrete proinflammatory cytokines like IL-1, IL-6 and TNF- α , plus important growth factors such as transforming growth factor β (TGF- β), vascular endothelial growth factor (VEGF), and PDGF. This stimulates cell proliferation and the creation of an extracellular matrix and brings the wound into the proliferate phase of wound healing (151).

The last cells to infiltrate in this stage are the lymphocytes, important producers of IL-2 which contributes to the fibroblast recruitment. The inflammatory stage normally continues for two weeks with the classic inflammatory signs: dolor (pain); rubor (redness); calor (warmth); and tumor (swelling) (152).

Proliferation

During this stage, there is a shift from inflammation to the active proliferation of cells essential for tissue repair, reepithelializing, the formation of granulation tissue, and restoration of blood vessels via angiogenesis (153).

Reepithelialization of the wound starts within 24 hours after the injury, when keratinocytes from the wound edges and epithelial stem cells from hair follicles

becomes activated and undergo phenotypic and functional changes (154-156). This allows for the epithelial cells to migrate laterally and proliferate (143).

Fibroblasts plays an important role in this stage. They are present in the surrounding tissue and becomes activated by PDGF, which serves as a chemoattractant, guiding them into the wound area. Fibroblasts are involved in the formation of granulation tissue by the production of fibronectin, vitronectin and collagen, thus creating a temporary matrix to support further cell ingrowth (143, 157). This matrix undergoes gradual replacement, forming a more collagen-rich matrix. Once a sufficient amount of collagen has been deposited, fibroblasts halt their production and undergo apoptosis. Dysregulation in this process could result in the formation of abnormal scars (143, 158).

Matrix metalloproteinases (MMPs), produced by a wide range of cells involved in wound healing, are a family of enzymes that plays a crucial role in remodeling of the extracellular matrix by balancing synthesis and degradation (141, 157).

Angiogenesis is important in sustaining the granulation tissue with oxygen and nutrients. This process is initiated by growth factors such as VEGF, PDGF, and fibroblast growth factor (FGF).

Remodeling

The remodeling phase, also known as the maturation phase, represents the final stage in wound healing. This may last from months to years depending on the size of the wound. During remodeling inflammatory cells leave, this results in an acellular, mature wound, marking the shift from granulation tissue to scar tissue. This process involves reduced angiogenesis and replacement of collagen type III with the stronger collagen type I. The process is balanced by MMPs, which degrade excess collagen (159). Myofibroblasts are responsible for contraction of the wound, reducing its size (160). The tensile strength of a fully mature scar is only about 80% of the original tissue (157, 161).

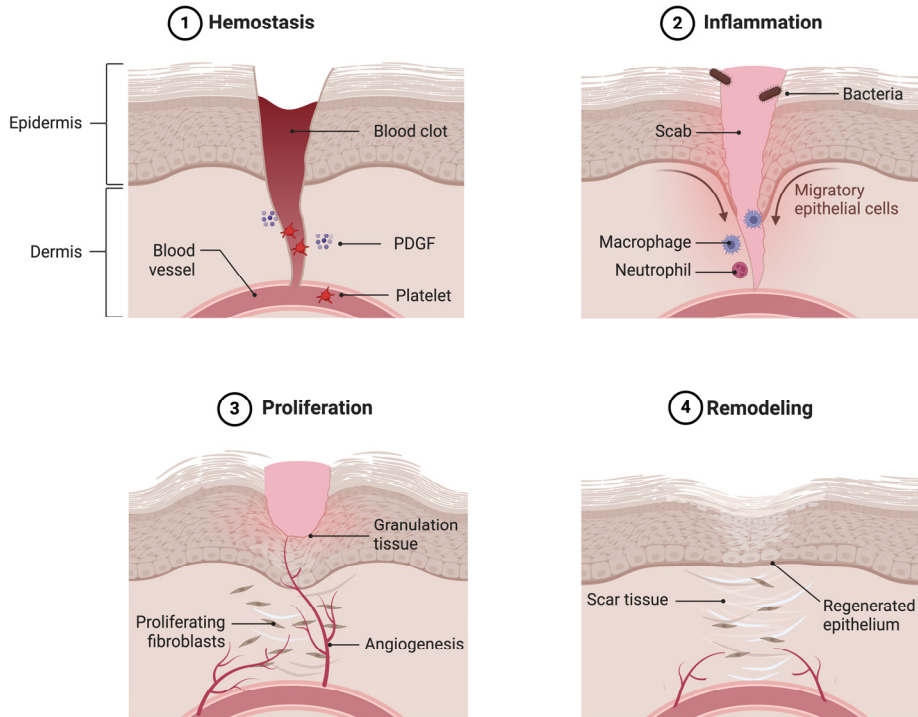


Figure 10 – Wound healing

The four phases of wound healing. Created with BioRender.com

Aims

The overarching objective of this thesis is to explore the therapeutic approaches and fundamental mechanisms affecting conditions that involve compromised skin barriers, including atopic dermatitis and epidermal wound healing.

Paper I

To investigate the antimicrobial and anti-biofilm efficacy of sodium hypochlorite *in vitro* against *Staphylococcus aureus* isolates derived from patients with atopic dermatitis.

To explore potential cytotoxic effects on keratinocytes *in vitro*.

Paper II

To investigate the antimicrobial and anti-biofilm efficacy of potassium permanganate *in vitro* against *Staphylococcus aureus* isolates derived from patients with atopic dermatitis.

Paper III

A study protocol for a prospective, randomized, double-blinded, first-in-human, placebo-controlled clinical trial assessing the safety and tolerability of three dose levels of TCP-25 gel when applied to epidermal suction blister wounds.

The secondary aim was to assess the systemic exposure of TCP-25 subsequent to its topical application on epidermal suction blister wounds.

The exploratory aims encompassed evaluation of wound conditions through photographs and the collection and storage of samples from dressings and wound surfaces for further exploratory research.

Paper IV

To study the dynamics of bacteria, inflammation and exudation during the healing process of epidermal wounds.

Material and methods

Background

This thesis comprises a series of studies exploring the efficacy of antimicrobial agents commonly used in the treatment of atopic dermatitis. It also examines the clinical aspects of epidermal wound healing, focusing on the complex interactions of the microflora in these contexts. The methodologies range widely, spanning from laboratory-based *in vitro* assays to detailed clinical studies. Every method possesses its unique strengths and limitations, emphasizing the necessity for careful consideration to obtain the desired information.

In vitro, Latin for “in glass”, refers to studies conducted outside a living organism or outside a subject’s normal biological milieu. The tests are normally performed in a controlled laboratory environment, enabling manipulation of specific, isolated specific variables. These studies also benefit from a replicability and less complex ethical considerations, since no animals or humans are examined in the actual assays. *In vitro* studies can provide important insights into potential effective treatments. However, whether the same outcome would be seen in a clinical milieu, for example real patients with atopic dermatitis, is uncertain, due to the complex biochemical environment present in living organisms.

Study design

The types of studies in this thesis vary. Paper I and II are laboratory-based experimental studies using *in vitro* and *ex vivo* methods. They are unblinded and follows an experimental design evaluating how bacterial isolates respond to antimicrobial treatments.

Paper III is a study protocol connected to a Phase 1 clinical trial. This is designed to assess the safety and tolerability of a novel, peptide-based (peptide TCP-25) wound gel (Fig 11).

The study is a prospective, randomized, double-blinded, first-in-human, placebo-controlled clinical trial. In the study twenty-four healthy volunteers receive four suction blister wounds each: two treated with the active product and the other two

receiving a placebo gel. Over a span of 15 days, we meticulously monitored the healing process and collected an extensive array of samples. One wound on each thigh was randomly assigned to receive the active drug, while the other received a control gel devoid of the drug substance.

Paper IV is the exploratory part of the clinical study referred to in the study protocol (paper III). As the study did not include wounds treated with the drug, we only included the samples from control wound, analysing a total of 48 control wounds from 24 subjects.

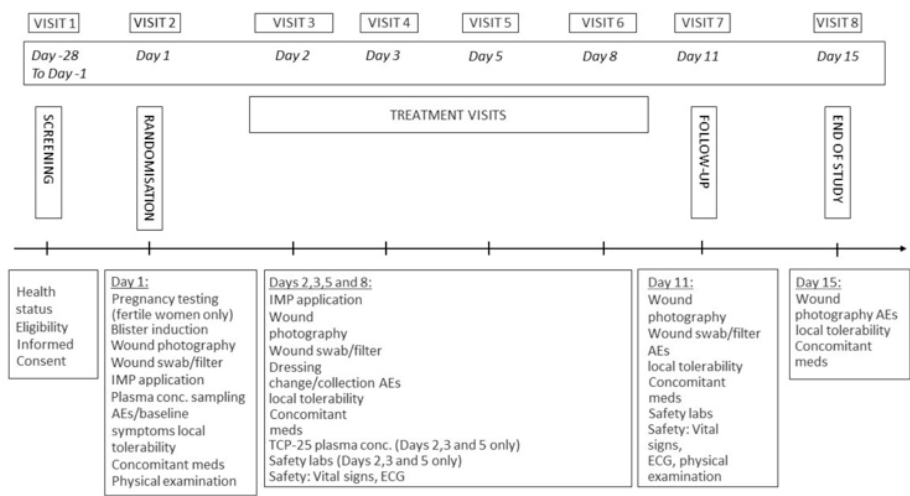


Figure 11 – Study design
 A summary of the prospective, randomized, double-blinded, first-in-human, placebo-controlled clinical trial examining the safety and tolerability of a novel wound gel based on the peptide TCP-25.
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Formation of suction blister wounds

The suction blister technique is a controlled and standardized method used to create epithelial wounds on the skin that are useful for research purposes. After cleansing, a suction device (in form of a negative pressure chamber or a suction pump) is applied to the skin surface with a cup tightly pressed against it. The device then creates a vacuum and the skin is pulled into the cup separating the epidermis from the dermis and creating a suction blister. The fluid in the blister is collected with a syringe, and the roof of the blister is removed with sterile forceps and scissors, exposing the wound beneath (162-164) (Figure 12)

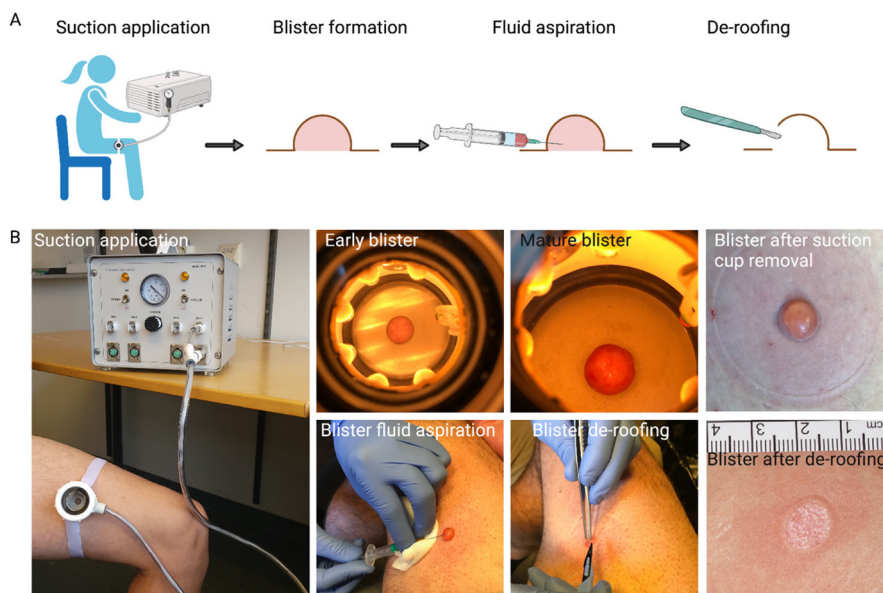


Figure 12 – Suction blister formation

A) Schematic illustration of the set-up. B) Suction blisters produced by separating the epidermis from dermis using vacuum chambers. Standardized epidermal wounds emerge after removal of blister roof. Authorized use granted by © Wound Repair and Regeneration

Methodological considerations

Wound formation for research purposes can be done in various ways, depending on what kind of information is wanted. Our study included healthy individuals with intact skin and its aim was to create epidermal wounds. It was desirable to use a technique that was well tolerated, reproducible and with minimal risk of scarring. In contrast to punch biopsies which normally create deeper wounds associated with a risk of bleeding, infection, discomfort, and scarring the suction blister technique provides a minimally invasive, non-painful way of creating superficial wounds in the skin.

Microbiological methods

Microorganisms

The clinical isolates used in paper I and II were collected from eczematous lesions, usually from the antecubital fossa, in patients with AD after informed consent. The

identification of *S. aureus* and *S. epidermidis* was performed by standard routines at Department of Clinical Microbiology at Skåne University Hospital.

Swab and dressing collection from wounds

Sample collection from epidermal wounds can include swabbing with a pre-wetted swab rotated over the wound to gather bacterial samples.

Wound dressings harbor information regarding the bacterial composition of the wound, as well as protein content linked to the degree of exudate and inflammatory markers. The weight of dressings from the wound is an indirect measurement of the amount of exudate from the wound.

By pressing the dressing together with a buffer in a syringe, it is possible to extract bacteria and proteins could be extracted from the material. Bacterial counts performed from dilutions from swabs and dressing extracts provides a quantification of the bacterial levels on the wound surface and dressing.

Viable count assay

Viable count assay, also known as colony-forming unit (CFU) counting, is a basic assay to evaluate the number of viable bacteria in a sample. The bacteria are incubated with the tested substance, in our case potassium permanganate. The incubation time can be altered to fit the purpose; we chose 10 minutes to mimic the treatment time for patients with atopic dermatitis. The samples are serially diluted to ensure that the number of colonies will be countable after they are plated onto agar plates. After incubation, colonies are counted and the number of CFUs per milliliter in the original sample is calculated by multiplying this number by the dilution factor (165).

Radial diffusion assay

Another classic method used for assessing the antibacterial activity of a substance is the radial diffusion assay (RDA). The principle of RDA is to evaluate the suppression of bacterial growth by diffusion of an antibacterial agent through a semi-solid agar medium. Bacteria of a specific concentration are added to molten agar and then cast on a solid plate in which punch wells are made. The test substance is added to the wells and allowed to diffuse into the plate during incubation at room temperature. An overlay covers and seals the compound in place, offering a nutrient-rich milieu for bacterial growth during incubation. Measurement of the diameter of the clear zones (indicating antimicrobial efficacy) is compared with the control. The wider the zone, the greater the effect (166, 167).

***Ex vivo* assay on skin biopsy**

To examine the antimicrobial effect of sodium hypochlorite on bacteria with varying adherence levels to the skin, we used an *ex vivo* model. In this setup, punch biopsies from AD affected skin, which was colonized with *S. aureus*, were dissected into four segments. These segments were subjected to sodium hypochlorite at three distinct concentrations, with the fourth immersed in filtered tap water to serve as a control. After incubation for an hour, bacterial counts were conducted on the incubation fluid. This was followed by a buffer wash to detach weakly attached bacteria after which another bacterial count was performed. The biopsies were then vortexed and sonicated to assess the effect of sodium hypochlorite on tightly adhered bacteria. This was followed by a final bacterial count.

MALDI-TOF MS

Matrix-Assisted Laser Desorption Ionization – Time Of Flight Mass Spectrometry, (or commonly called MALDI-TOF MS) is an analytical technique used for quick identification of microorganisms, proteins and biomolecules. The sample is placed on a metal plate together with a matrix compound, usually a crystalline organic acid. The sample is then exposed to a laser beam, causing ionization and sublimation. The charged molecules are accelerated by an electrical field into a flight tube traveling through a vacuum, and then separated and sorted based on their mass-to-charge ratio. As the ions reach the detectors, their flight times are recorded and a mass spectrum is generated. This yields a molecular fingerprint that can be matched against protein profiles from a database for identification. Bacterial samples from swabs and dressings grown on blood agar plates allow bacterial colonies to be collected and identified using MALDI-TOF MS (168, 169).

Methodological considerations

Radial diffusion assay (RDA) is a simple and inexpensive way of obtaining a fairly quick, and visual determination of a substance's antimicrobial properties. It is limited by being less sensitive to the sub-lethal effects of the bacteria than other methods such as the epsilometer test, also known as the E-test. An E-test can, to some extent, provide a gradient of concentrations and, more specifically, determine the Minimum Inhibitory Concentration (MIC) of an antibacterial substance (170, 171).

The viable count assay is frequently used to determine the efficacy of antimicrobial agents. Although it is a well-established assay that can be very accurate if performed correctly, it is both time-consuming and labour-intensive, especially if many dilutions are required. Quicker alternatives such as flow cytometry can save time

but requires more expensive equipment and does not differentiate between live and dead cells unless appropriate stains are used (170).

A notable limitation in paper IV is the manual selection of only six colonies via MALDI-TOF MS analysis. This provides insights into the wound bacterial composition but prevent us from gaining a full picture of the microbiome. While 16S rRNA sequencing could have been an alternative method giving a broader analysis. However, our primary focus was not to offer a thorough microbiome analysis, but rather, broader exploration into the dynamics where bacterial analysis is just one facet together with inflammatory biomarkers, protein leakage, novel biomarkers. Furthermore, it is important to notice that 16S-based methods sometimes lack the resolution needed to differentiate between commensal staphylococci (172, 173).

Biofilm assays

One of the main areas of interest in this thesis was examining biofilm inhibitory properties of substances used clinically in treating atopic dermatitis with suspected high bacterial load. Traditional microbiological tests to evaluate the efficacy of antimicrobial agents are usually performed on planktonic, free-floating bacteria. However, other methods are required to gain an understanding of the concentrations needed to affect biofilms produced by the same bacteria other types of methods are needed. The methods we used for these purposes are described briefly below.

Biofilm formation assay

A recognized method of quantifying the biomass of biofilm produced by bacterial isolates is the crystal violet (CV) biofilm assay. The bacteria are cultivated, with or without the tested substance, in a microtiter plate with a suitable surface to which biofilm can adhere. After incubation, non-adherent cells are washed away, and the remaining biofilm in the plate is stained with crystal violet, a plain dye that binds to negatively charged extracellular molecules (such as surface molecules and polysaccharides). The dyed biofilm is solubilized in ethanol and the absorbance is measured spectrophotometrically, providing a quantitative evaluation of the biofilm's biomass (Figure 13). This method is suitable for screening of the biofilm-forming capacity of microbial isolates, and for examining the ability of different substances to inhibit biofilm formation (174).

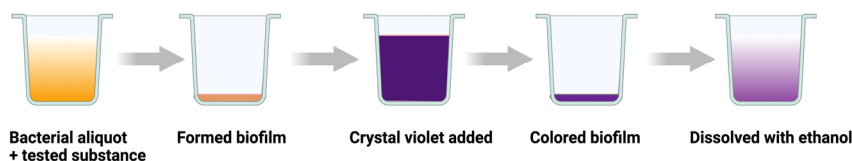


Figure 13 – Biofilm formation assay

Quantification of biofilm formation by coloration with crystal violet. Created with BioRender.com

MIC and MBEC

The Minimum Inhibitory Concentration (MIC) refers to the lowest concentration of an agent required to prevent the visible growth of a bacteria after an incubation period. This parameter is critical in assessing the susceptibility of the bacteria in a planktonic state. The Minimum Biofilm Eradication Concentration (MBEC) represents the lowest concentration of an antimicrobial that can eradicate an established biofilm. The Calgary Biofilm Device (175) provides a sophisticated method of evaluating the sensitivity of bacteria (like *S. aureus*) to antibacterial substances in both planktonic and biofilm states. Bacterial aliquots are placed in the wells of a 96-well microtiter plate, and a lid is added which has pegs protruding down into the wells is added. During incubation, biofilm forms on these pegs. The lid with biofilm covered pegs is then transferred to another 96-well plate with a serial dilution of the tested substance. This is usually referred to as the “challenge plate”. The lid, now with treated biofilms, is then carefully washed and moved to a new plate, called the “recovery plate”, which has wells containing growth medium. The whole plate is sonicated to facilitate the disruption of biofilm from the pegs into the wells. Both the challenge plate and the recovery plate are incubated, and their bacterial growth evaluated. Determination of MIC is made by examining the challenge plate. No visible growth indicates effective inhibition of planktonic bacteria. Similarly, the MBEC value is read from the recovery plate (175, 176).

Confocal Laser Scanning Microscopy

A more detailed examination of treated biofilm can be performed using the high-resolution capabilities of Confocal Laser Scanning Microscopy (CLSM) combined with fluorescent LIVE/DEAD staining. Biofilm, treated or untreated, is stained with two different fluorescent dyes. These are SYTO 9, which binds to DNA and stains both living and dead cells green and propidium iodide (PI), a red dye, which only penetrates damaged cell membranes, and thus only stains dead cells. CLSM scans the layers of biofilm with a laser, activating the fluorescent dyes. This is a powerful method for studying biofilms, visualizing both living and deceased cells, and

gaining a more thorough understanding of the biofilm architecture using three dimensional imaging (177, 178).

Scanning Electron Microscopy

The powerful imaging technique of Scanning Electron Microscopy (SEM) is used to acquire high-resolution images of a variety of surface materials, including biofilms. Samples are prepared, in this thesis, these consist of a punch biopsy derived from eczematous skin plus the biofilm-coated pegs detached from the lid of the CBD. The samples are then prepared by fixation and dehydration, and then covered with conductive material. The sample is placed in a vacuum chamber, an essential milieu if the electron beam is to travel unhindered by any interactions with air molecules. The electron beam is focused and scans the sample surface. It interacts with the atoms of the material, resulting in the emission of secondary electrons, backscattered electrons and X-rays. These signals are detected and used to construct a detailed and highly magnified image of the sample structures (179).

Methodological considerations

The Crystal Violet method to examine biofilm inhibition properties is a straightforward assay that is suitable to simultaneously analyse a large number of samples. This assay is appropriate for quantifying biofilm biomass. However, it is limited by its non-specific way of staining both living and dead cells, which may give an imprecise indication of viable biofilm. An alternative, but more time consuming and expensive, method we used was Confocal Laser Scanning Microscopy (CLSM), with fluorescent dyes to distinguish between living and dead cells. As a strictly *in vitro* model, this does not test the substance and biofilm interactions of the immune system (179, 180).

The Calgary Biofilm Device is a favorable assay as it can perform simultaneous assessment of MIC and MBEC values. Due to the complexity of biofilm formation in a natural environment, it could be contemplated that the CBD may not fully mimic these settings. If more dynamic conditions are needed, a flow cell system could be considered, as this could better simulate the clinical conditions of biofilm formation. Since these studies are mainly deemed applicable to eczematous skin we assumed that the CBD method to be sufficient enough for this purpose (175).

Cell methods

In dermatological research, especially when investigating conditions like atopic dermatitis, the preservation of the skin barrier is a critical factor. In addition to the

important determination the antimicrobial effect of a substance's, cytotoxicity aspects needs to be considered if we are to gain knowledge of what treatment conditions can promote healing with minimal harm to the host cells.

The cells used in these procedures were transformed human adult keratinocytes (also known as HaCaT cells) derived from a spontaneously immortalized, human keratinocyte cell line from the late 1980s. They are non-tumorigenic and retain differentiation capabilities similar to normal keratinocytes making them frequently used in research exploring various characteristics of skin biology (181).

LDH

The Lactate Dehydrogenase (LDH) assay is a commonly used technique to evaluate cell cytotoxicity. LDH is a cytoplasmic enzyme found in all living cells. The enzyme is released into the culture medium when the plasma membrane is damaged. An enzymatic reaction mediated by LDH release transform tetrazolium salt to a formazan compound resulting in a color change of the sample. The amount of LDH released is reflected in the intensity of the color shift. Detection of the absorbance by a microplate reader, allows for precise quantification of the amount of cell damage (182).

3-(4,5-Dimethylthiazolyl)-2,5-diphenyl-tetrazolium bromide assay

This assay, also known as the MTT assay, is a standard colorimetric method for assessing cell viability and proliferation. The method relies on the principle that living, metabolic active cells use mitochondrial dehydrogenase enzymes to convert the yellow tetrazolium salt to purple coloured formazan. Cells grown in a 96-well plate are treated with a chosen substance and then incubated with MTT, thus enabling the described reaction. Once the medium is discarded, dimethyl sulfoxide (DMSO) is applied, to effectively dissolving the formazan compounds. The optical density (OD) of the solubilized formazan is measured using a microplate reader. This indicates the quantity of viable cells in the sample (183).

Methodological considerations

LDH is an ideal assay for evaluation of the cytotoxic impact of a drug, providing a quick, cost-effective and direct quantitative measure of cell damage. A limitation to consider however is the fact that neither of these assays distinguish between different types of cell death, both necrotic and apoptotic result in membrane disruption (182).

Protein analyses

Protein content

Protein content in a sample can be measured by using of the bicinchoninic acid (BCA) assay. This relies on a protein's capacity to reduce copper ions in an alkaline medium, creating a purple-colored complex with BCA. The intensity of the color is proportional to the amount of protein in the sample. Spectrophotometric measurement of the absorbance is compared to a standard curve with a known protein concentration, thus determining the protein content of the sample (184).

ELISA

Enzyme-linked immunosorbent assay (ELISA) is a frequently used technique for qualitative and quantitative detection of proteins in a sample. In this thesis, ELISA was used to quantify neutrophil proteins. The fundamental principle of ELISA involves using a microplate, precoated with a specific antibody that binds to the protein of interest in the sample added to the plate. After washing off excess unbound substances through washing, a substrate solution is added. This triggers an enzymatic reaction yielding a detectable signal, such as a color change or fluorescence. The intensity of the signal is proportional to the quantity of the protein (185).

Meso Scale Discovery technology

Cytokine quantification using Meso Scale Discovery (MSD) technology allows for simultaneous detection of multiple proteins in a sample through a combination of multiarray technology and electrochemiluminescence. Compared to conventional ELISA, the MSD technology offers increased sensitivity, a wider dynamic range, and provides the flexibility to be conducted as a single or multiplex assay, effectively conserving valuable sample material (186).

MSD assays use specific plates containing microwells coated with capture antibodies selective for the target protein are utilized. When the sample is added, the target protein (such as a cytokine) selectively binds to the capture antibodies immobilized on the well surface. Following a washing to remove unbound proteins, detection antibodies are added and labeled with electrochemiluminescent (ECL) tags. These antibodies bind to the captured protein, forming a "sandwich" complex with the target protein nestled between the two antibodies. An electric current is then applied, prompting the ECL tags to radiate light. This is captured by a photodetector. The intensity of the light emission corresponds to the amount of target protein, thus enabling quantification of the sample (186).

Methodological considerations

Both ELISA and MSD technology provides sensitive techniques to examine the protein content in a sample. The major benefit for MSD is the ability to make simultaneous measurement of multiple proteins. However, this method is usually costlier and requires more specialized equipment.

Ethical approval

Papers I and II complied with the Declaration of Helsinki and were approved by the Ethics Examination Board of Lund, permit numbers 144/2010 and 82/2012.

Papers III and IV were conducted in accordance with the Declaration of Helsinki, ICH/GCPE6 (R2), and the European Union Clinical Trials Directive and were approved by the Swedish Medical Products Agency and the Swedish Ethical Review Committee; registration number 2022-00527-01.

Ethical considerations

To make progress in life and medicine, mankind is dependent on the development of new ideas and knowledge. However, this cannot be achieved at any cost and the potential benefits needs to be balanced against possible risks. This concept has ancient origins going back to the Hippocratic Oath circa 400BC, which urged doctors to prioritize beneficence and non-maleficence in their practice, while also laying the groundwork for patient autonomy and justice (187).

The pledge to beneficence implies that the practitioner should work with the patient's best interests at heart. In research, this translates to conducting studies that aim to enhance health and knowledge, while ensuring that the benefits outweigh any potential risks involved.

Connected to this is the principle of non-maleficence, which emphasizes the importance of physicians not causing any harm or injury. In research contexts, this involves minimizing of the risks to participants.

Autonomy refers to respect for patients' individual rights. This is fundamental to medical research by obtaining informed consent to participate in studies and allowing the right to withdraw at any given time.

Justice underscores the importance of equality in medicine, advocating for compassionate treatment of all individuals without discrimination. In medical

research, this involves striving for a uniform selection and treatment of study participants.

As medicine and society have evolved, so have the frameworks of ethical considerations. Notable historical benchmarks are the Nuremberg Code of 1947 (188) and the Declaration of Helsinki in 1964 (189). The latter, with its detailed guidelines for medical research, serves as the ethical cornerstone of the work presented in this thesis.

In papers I-II, bacterial samples were collected from patients with AD who were registered at the Department of Dermatology and Venereology, Skåne University Hospital, Lund. The sampling did not interfere with these patients' medical care and treatment. In paper I punch biopsies, that are a standard procedure at the Dermatological clinic, were performed and examined. All patients involved have given a written informed consent and the studies were approved by the regional Ethics Examination Board of Lund.

Papers III-IV include a study protocol and a clinical study involving healthy volunteers who received superficial epidermal wounds on their thighs. The potential study subjects were provided both verbal and written information regarding the study and the purpose of it. They were given the opportunity to ask questions privately, and it was emphasized that participation was entirely voluntary with the freedom to withdraw at any point without explanation. Subsequently, written consent was obtained from all the participants before any study-related procedures commenced. The study was conducted as part of a first-in-human trial for a topical wound gel and was executed at the Phase 1 Unit, Skåne University Hospital, Lund. All personnel involved with the participants of the study had undergone training and education in Good Clinical Practice (GCP). The choice of suction blister technique was considered to reduce the likelihood of persistent marks and scarring of the skin and thereby minimizing the risk of harm. The study was conducted in strict adherence to ethical standards aligning with the Declaration of Helsinki, ICH/GCPE6 (R2), and the European Union Clinical Trials Directive. Approval for this study has been granted by the Swedish Medical Products Agency and the Swedish ethics committee bearing the registration number 2022-00527-01

Statistical analyses

Paper I

In paper I, the data was reported as means \pm standard deviation (SD). Group differences were assessed using one-way ANOVA with Dunnett's multiple

comparison test. A P-value < 0.05 was considered as significant. The analyses were conducted using GraphPad PRISM version 6.0c statistical software.

Paper II

In paper II, the data was depicted as means along with their \pm SD. To investigate variations among groups, we used the Wilcoxon signed-rank test. A significance level of $P < 0.05$ was chosen to indicate statistical significance. All statistical analyses were conducted using GraphPad PRISM 8.

Paper III

Paper III is a study protocol for a prospective, randomized, double-blinded, first-in-human, placebo-controlled clinical trial testing the tolerability of a novel wound gel. The statistical analysis plan involves presenting continuous data, including mean \pm SD, median, and minimum and maximum values, along with details on evaluable and missing observations. Categorical data will be displayed as counts and percentages. All statistical analyses will be conducted using SAS V.9.4 or a newer version (SAS Institute). The baseline will be expressed as the most recent non-missing observation recorded before the initial administration of the intervention.

Outliers will be integrated into summary tables and listings without separate handling in analyses. Missing data will not be imputed, but if a clinical safety laboratory value falls outside the limit for detection, this limit will be utilized in statistical calculations. Similarly, for pharmacokinetics plasma concentrations, values below the lower limit of quantification will be substituted with the lower limit of quantification if more than 50% of the values for a specific time point exceed this limit.

Paper IV

In paper IV, summary data were graphically represented by plotting the median and interquartile range. Spearman correlation coefficients were computed to assess the correlation between two variables. Despite each participant having two control wounds included in the study, individual wounds were treated as independent entities in all analyses, in total 48 wounds ($n=48$). Non-parametric analyses were chosen due to the non-normal distribution of the data, as determined through visual inspection. All statistical tests conducted were two-tailed. Considering the exploratory design of the study, p-values were left unadjusted for multiple comparisons, serving as hypothesis-generating indicators only. Data were analyzed and visualized using GraphPad Prism 10 software.

Results

Readers are encouraged to read the included papers for more details of the work in this thesis.

Paper I

Sodium hypochlorite demonstrated antimicrobial properties against *S. aureus* isolates derived from the lesional skin of AD patients. In the RDA the results showed that sodium hypochlorite dose-dependently inhibited the growth of planktonic *S. aureus* with progressive widening of clear zones at rising concentrations (Figure 14). When using the CBD method, MIC values for sodium hypochlorite were determined for 11 clinical isolates, ranging from 0.01% to 0.08%. The effects against the clinical isolates were similar to the reference ATCC strain.

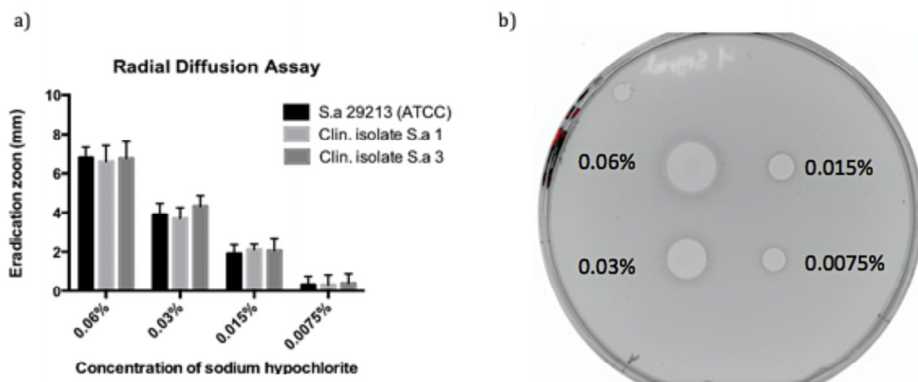


Figure 14 – Radial diffusion assay

The antibacterial efficacy of sodium hypochlorite against *S. aureus* examined with radial diffusion assay (RDA). a) results represent the mean size of inhibitory zones \pm standard deviation from three separate experiments. b) A representative image displays a treated plate with visible clear zones, demonstrating antibacterial effects. Authorized use granted by © British Journal of Dermatology

In this study, SEM analysis revealed the presence of clustered coccoid bacteria surrounded by a matrix of extracellular substances on a punch biopsy derived from eczematous skin (Figure 15).

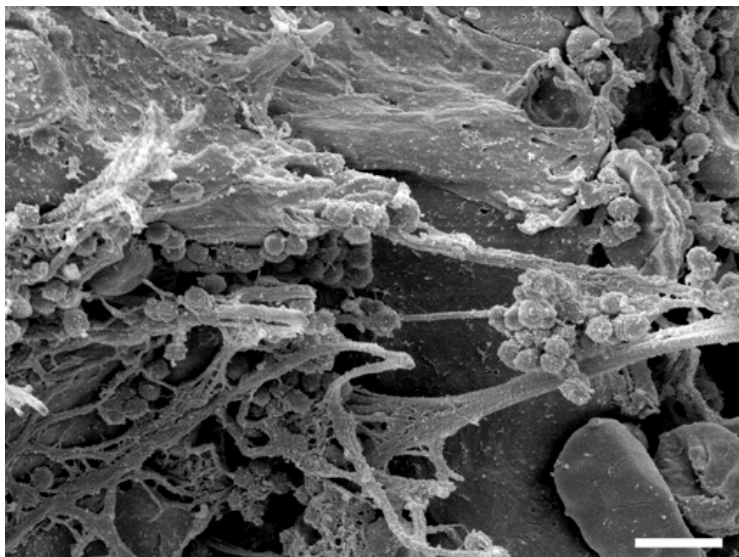


Figure 15 – Scanning electron microscopy

Analysis using a scanning electron microscope (SEM) of a punch biopsy obtained from a patient with atopic dermatitis revealed the presence of clustered coccoid bacteria embedded within extracellular substances. Authorized use granted by © British Journal of Dermatology

Investigates to analyse the biofilm formation with the CV method showed that, when untreated, all the tested strains produced biofilm. When subjected to sodium hypochlorite, the inhibition of biofilm formation was observed. A significant reduction was presented in some strains from a concentration of 0.0219%, whereas a majority of the strains showed a significant inhibition when exposed to a concentration of 0.0875% or higher.

The capacity of sodium hypochlorite needed to eradicate established *S. aureus* biofilm was determined using the CBD method and provided MBEC values ranging from 0.01% to 0.16%.

Further effects of sodium hypochlorite against *S. aureus* biofilm were visualized during the examination using confocal microscopy and LIVE/DEAD staining, where a concentration of 0.04% killed nearly all the bacteria (red fluorescent) (Figure 16).

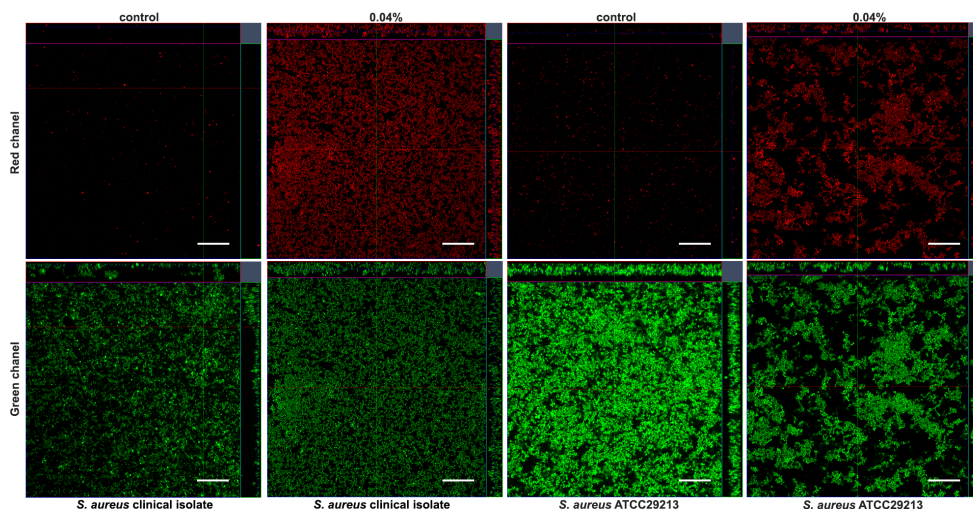


Figure 16 – Confocal laser scanning microscopy

Confocal laser scanning microscopy images show representative views

of control and treated biofilms of a clinical *S. aureus* isolate and the ATCC 29213 strain. These biofilms were stained using the LIVE/DEAD® kit (Molecular Probes, Eugene, OR, U.S.A.). In the top row, dead bacteria are highlighted by propidium iodide staining (red fluorescence), whereas the bottom row depicts both live and dead bacteria stained with green fluorescent SYTO9. The magnification is 963x, and the scale bar represents 20 μ m. Authorized use granted by © British Journal of Dermatology

In the *ex vivo* assay we examined the effect of sodium hypochlorite against bacteria with varying degrees of adherence to the skin in a punch biopsy. We observed that incubation with a 0.02% concentration killed >90% of bacteria released from the biopsy into the incubation solution. However, both weakly and strongly attached bacteria remained abundant. Increasing the concentration to 0.04% killed >90% of the weakly attached bacteria following washing. Complete eradication of weakly attached occurred with 0.16% sodium hypochlorite, but approximately 10% of the strongly attached bacteria (after vortex and sonication) survived this concentration.

Cytotoxic effects analyzed with LDH assay showed a significant increase in damaged cells when treated with a concentration of 0.02%, but non significant changes when exposed to 0.005%, 0.01% and the higher 0.04%.

The viability examined with MTT assay was significantly affected when the cells were exposed to a concentration of 0.01% or higher.

Paper II

The antimicrobial effect of potassium permanganate against planktonic *S. aureus* and *S. epidermidis* from patients with AD and two reference ATCC strains was tested using the viable count assay. After a ten-minute exposure to concentrations ranging from 0.01% to 0.5%, the results presented a partial or complete decrease in CFU for all the tested isolates. When subjected to 0.05%, potassium permanganate was found to completely eliminate *S. aureus*. For the two tested *S. epidermidis* strains, this was seen when a 0.1% concentration was applied (Figure 17).

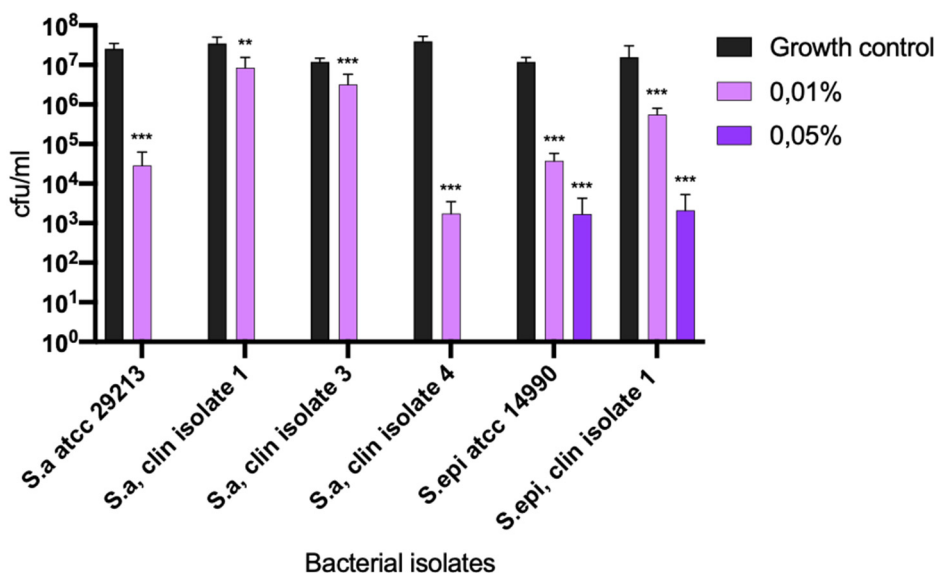


Figure 17 - Antibacterial effects against planktonic *S. aureus* and *S. epidermidis* isolates

Viable colony counts of *S. aureus* and *S. epidermidis* obtained from patients with AD, along with two ATCC strains, after potassium permanganate exposure with a concentration range of 0.01% to 0.5% for 10 minutes. Values marked with an asterisk are significantly (**p ≤ 0.002, ***p ≤ 0.0005) different from the growth controls. Bacteria exposed to potassium permanganate concentrations of 0.1% and 0.5% exhibited a total elimination of colony-forming units, with no detectable growth observed. Authorized use granted by © ACTA DV

The antibacterial properties were further demonstrated in the RDA, where potassium permanganate exhibited a dose-dependent effect against bacterial growth. Determination of MIC values using the CBD method found values ranging from 0.5% to 1% for the *S. aureus* isolates.

The Crystal Violet method was used to examine the capability of potassium permanganate to inhibit biofilm formation. The results found that all the tested

strains (nine clinical isolates of *S. aureus*, one clinical isolate of *S. epidermidis* and two reference ATCC strains) produced biofilm. However, biofilm production was suppressed in the presence of potassium permanganate in a manner that tended to be dose-dependent for the majority of the bacterial isolates tested. Most of the strains displayed a reduction in biofilm formation upon exposure to a minimum concentration of 0.01% potassium permanganate when compared to the untreated growth control.

The ability to affect established staphylococcal biofilm was tested in a modified version of the CBD method, whereby potassium permanganate was serially diluted in concentrations from 1% to 0.03%. For the *S. aureus* strains the MBEC values ranged from 1% to beyond the highest tested concentration of 1%. For the tested *S. epidermidis* strains, the MBEC values were lower, from 0.25% to 1%.

Some of the pegs were further examined using SEM, revealing coccoid bacteria. These were densely packed in the untreated controls, with a reduced amount observed as the concentration rose. When subjected to the highest concentration of 1%, the *S. aureus* ATCC strain presented deformed and broken bacterial structures (Figure 18).

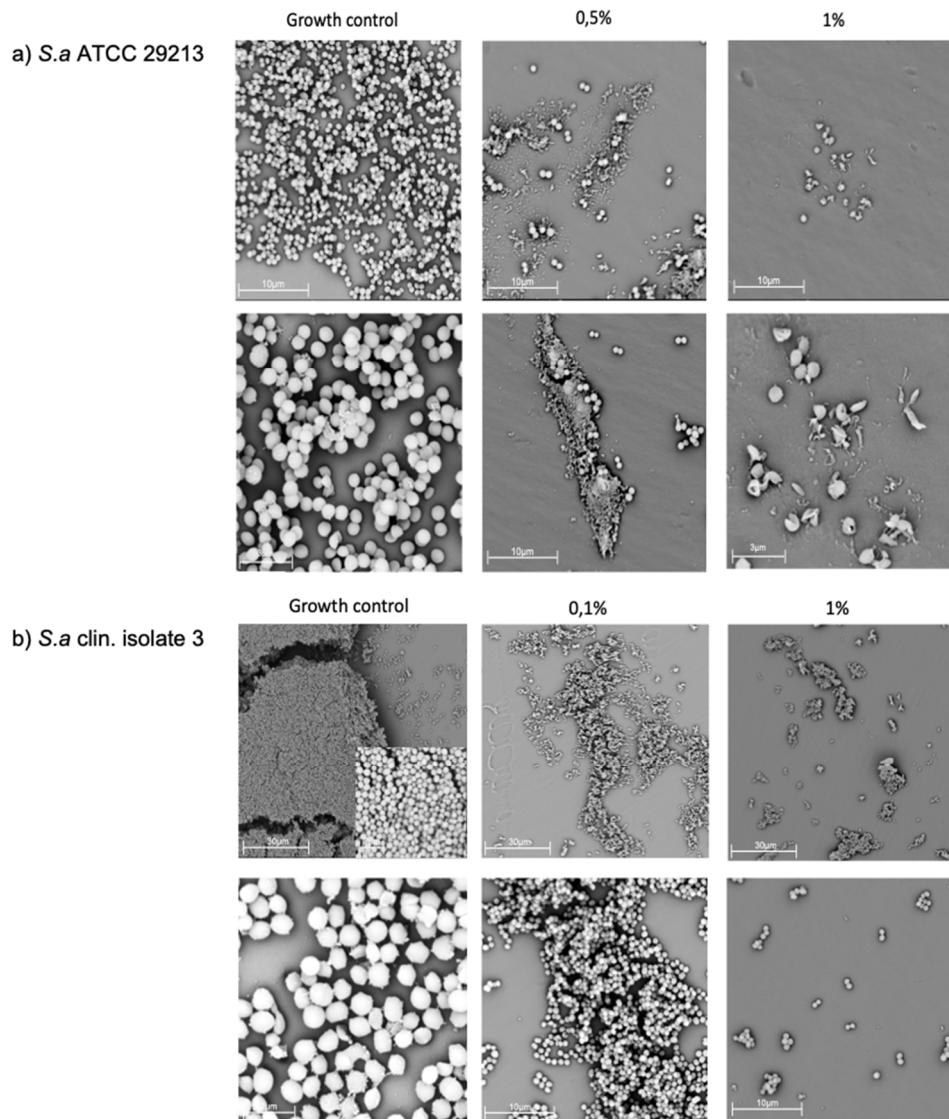


Figure 18 - Scanning electron microscopy

The bacterial distribution within bacterial biofilms on plastic pegs is shown after exposure to varying concentrations of potassium permanganate. Higher concentrations of potassium permanganate result in a reduced bacterial number. (a) Displays *S. aureus* ATCC 29213 treated with different concentrations of potassium permanganate, where the morphology of the bacteria is notably changed at a 1%. (b) Shows *S. aureus* clinical isolate 3 after treatment with potassium permanganate. Authorized use granted by © ACTA DV

Paper III

The clinical study described in this protocol was performed in April to June 2022, when 24 healthy adults were included. Each participant underwent the creation of four epidermal wounds. Two of the wounds received treatment using an active wound gel and two received a placebo gel. Throughout the 15-day observation period following the protocol, meticulous monitoring was conducted, and a biobank of samples was established.

Paper IV

The aim for this study was to create epidermal wounds and investigate the healing process in regard to bacterial amount and composition, plus exudation levels and inflammation.

Directly after wound formation, the bacterial levels were zero, or close to zero in the swab samples. The bacterial amount then increased steadily in the subsequent days, peaking on Day 8 with a median of 1.7×10^6 CFU in the swabs, and 1.6×10^7 CFU in the dressing. The bacteria levels declined on Day 11, to a median of 1.3×10^6 CFU/swab and 1.4×10^6 CFU/dressing. Variability in bacterial levels between individuals were observed, but there was an overall correlation between bacterial counts in swab and dressing (Figure 19).

MALDI-TOF MS was used to investigate the bacterial composition of the wounds on Days 3 and 8 by analyzing six colonies each from swabs and dressing. The findings showed the formation of unique bacterial composition for each individual, generally consisting of commensals but, in some cases, *S. aureus*. The composition was quite stable between the days and between swabs and dressings. Regardless of the bacterial composition, all the wounds exhibited similar healing patterns in terms of appearance and timing.

The amount of protein in the dressings was analyzed to investigate exudation levels over time. A peak was seen on Day 5 and then a decline until Day 11, when the protein content in dressings was close to zero. The correlation of protein levels between the left and right legs was moderate to strong, while the correlation between protein content and bacterial levels was faint to none.

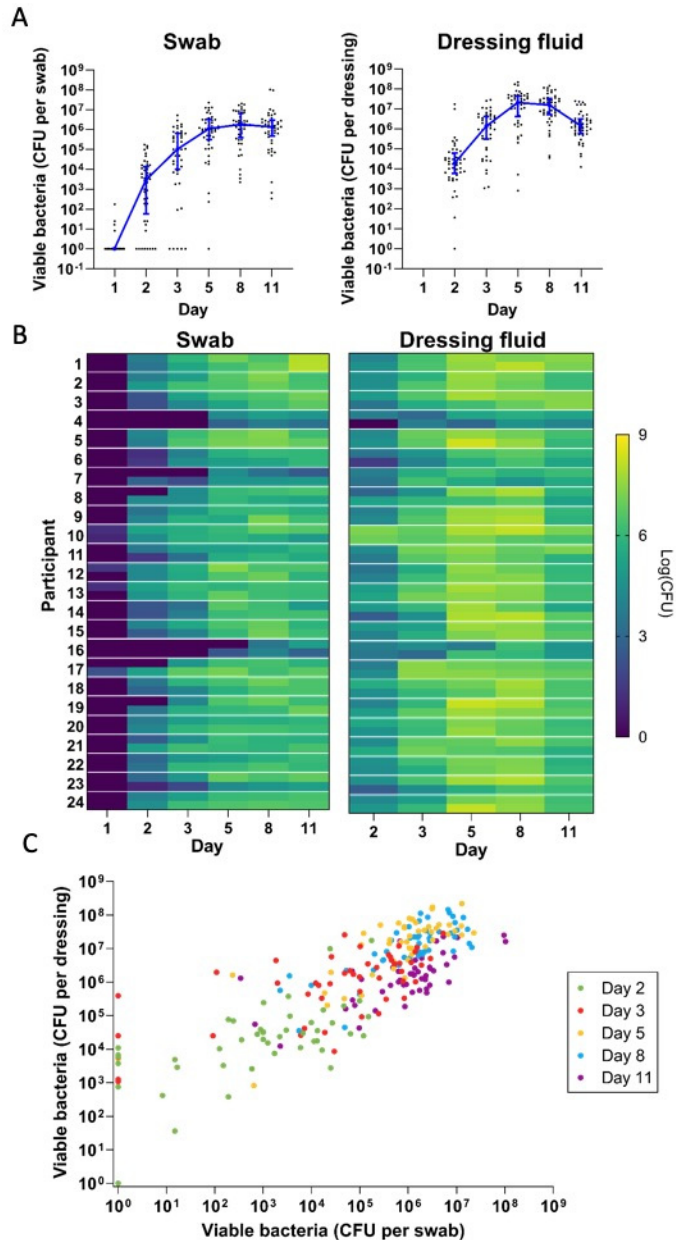


Figure 19 – Bacterial development over time during epidermal wound healing

A) Bacterial counts in swab samples (left) and dressing fluid samples (right) plotted across a timeline. The blue line indicates the median of all wounds for that day ($n=48$) and the whiskers represent the interquartile range. B) A heatmap providing a comparison of bacterial counts from swab (left) and dressing fluid (right) samples corresponding to each wound. Data from the left and right wounds are

presented in separate cells at the top and bottom of each row, respectively. C) The scatterplot showcases the correlation of bacterial counts in swab and dressing fluid samples from each wound.

To monitor the progression of inflammation, the levels of 10 different cytokines (IL-1 β , IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, IFN- γ , TNF- α) from dressing fluids were measured. The findings showed a similar pattern over time for all the cytokines with a slight increase or decrease between Days 2 and 3, peaking on Day 5 and then with levels reduced again at Day 8. Due to minimal exudation on Day 11, no cytokine measurements were conducted on this day (Figure 20).

For a majority of the cytokines, we found weak or no correlation to bacterial amounts. However, stronger correlations were observed for TNF- α on Days 3 and 5 ($R = 0.475$ and 0.488), IL-10 on Day 5 ($R = 0.531$) and IL-1 β on Days 5 and 8 ($R = 0.497$ and 0.480) (Figure 20).

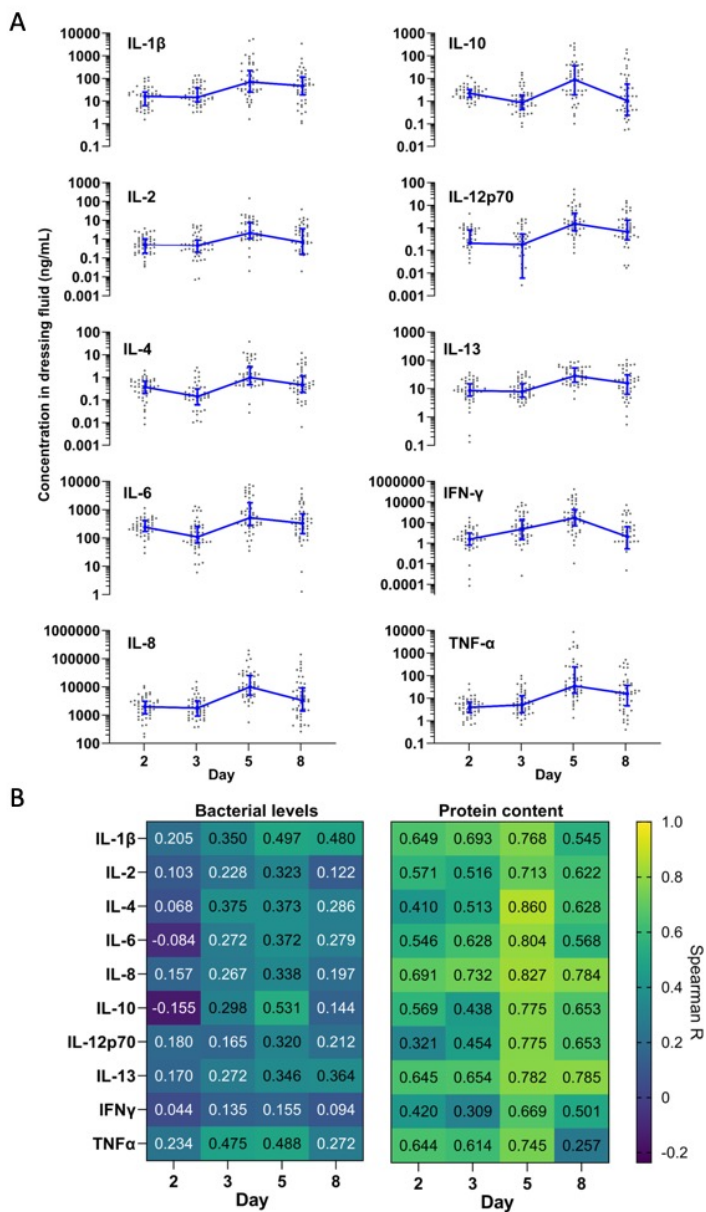


Figure 20 – Dynamics of inflammation

A) The chart presents the concentrations of ten distinct cytokines found in dressing fluids, monitored over time using MesoScale technology. Each point represents the concentration from an individual wound, with the median for all wounds on a given day depicted by the blue line ($n=48$), accompanied by whiskers indicating the interquartile range. B) This section shows Spearman correlation coefficients that link each cytokine's levels to the bacterial amount in swab samples (left) and the protein content of the corresponding wound (right)

Another measurement of inflammation is exudation levels, measured in this study as the protein content in dressings. A moderate to strong correlation between protein content and cytokine levels was detected, peaking on Day 5.

The amount of the neutrophil proteins, myeloperoxidase (MPO), neutrophil elastase (NE) and heparin binding protein (HBP) was measured from the exudation fluid. MPO and NE levels were about the same on Days 2 and 3, then peaking on Day 5 before declining on day 8. HBP showed a gradual escalation until Day 5, and then a fall on Day 8. The correlations between these neutrophil markers and the cytokine IL-8 were found to be moderate to strong, whereas the correlation with bacterial levels was little to none. Furthermore, a moderate to strong correlation was found between neutrophil proteins and protein content, especially on Day 8.

Discussion

Paper I

Atopic dermatitis is one of the most common dermatological conditions and *S. aureus* is known to cause exacerbations of the disease (104). Furthermore, studies indicate that staphylococcal biofilm contributes to the severity and persistence of AD (190). Our SEM examination of a punch biopsy from lesional skin in an AD patient support the presence of biofilm therein.

Reduction of the *S. aureus* bioburden in AD is thought to be beneficial to the healing process (191). The use of topical antibiotics has been considered to decrease *S. aureus* colonization. However, this strategy is not always favorable due to concerns over the rising development of antibiotic resistance (30, 192). Thus, sustainable antimicrobial therapies targeting *S. aureus* (but without the risk of fostering resistance) are consequently of interest.

The use of sodium hypochlorite as an adjunctive treatment for AD has been recognized in clinical practice for many years (100, 108). The standard concentration of the substance in bleach baths is 0.005% (122, 126, 193)

Several clinical studies have been conducted in which patients with AD were treated with bleach baths. The results are mainly positive, with a decrease in disease severity although the effect on the presence of *S. aureus* seemed to vary (126, 127). Both reduction (194) of the bacteria, and no significant change (129) after similar treatments have been reported.

An *in vitro* study tested the susceptibility of sodium hypochlorite against *S. aureus* from varying sources, with the MIC values obtained ranging between 0.0128% and 0.82% (195). This is comparable to the MIC values found in our study, which ranged between 0.02% and 0.08%. The slight variation might be explained by different origins of the bacteria, plus altered methods of use. The RDA results from in our study further supports the antistaphylococcal effect against *S. aureus* derived from AD patients and favor its potential role as a treatment possibility to reduce the bacterial load of AD.

Biofilms pose a significant therapeutic challenge due to their resilience against the host immune system and antibiotics. Therefore, the possible ability of sodium hypochlorite to disrupt these biofilm formations is therefore of particular clinical

interest. Not only would the breakdown of biofilm allow for more efficient bacterial killing, it would also expose the underlying bacterial cells to the host's defense mechanisms. Even though sodium hypochlorite is frequently discussed as an antistaphylococcal agent in the context of AD treatment, there is very little knowledge of how the substance affects *S. aureus* biofilm.

The *ex vivo* model showed that 0.02% of sodium hypochlorite, the concentration closest to the levels used in clinical practice, was ineffective in killing skin-adhered bacteria. However, a concentration of 0.04% not only effectively reduced the loosely attached bacteria but also diminished the tightly attached bacteria by 80-90%.

Our study demonstrated that sodium hypochlorite possesses both inhibitory effects regarding biofilm formation and eradication properties against established biofilm. When the *S. aureus* biofilm was treated with 0.04% of sodium hypochlorite, the confocal microscopy revealed effective killing of the bacteria inside the biofilm. The MBEC values, ranging from 0.01% to 0.16%, were only slightly higher than the MIC values. This indicates that the substance is nearly as effective against *S. aureus* biofilms as it is against planktonic bacteria.

From a clinical perspective, the outcomes of our findings suggest that a higher concentration of sodium hypochlorite than typically used today in bleach baths could offer superior capability for reducing the bacterial burden and targeting against *S. aureus* biofilms. This might enhance the treatment outcomes for patients with infected AD.

However, it remains crucial to consider the balance between the desired effectiveness of a treatment and the possibility of cytotoxic side effects. Multiple studies report excellent skin tolerance of bleach baths at the commonly used concentration 0.005%, with no signs of impairment to the skin barrier compared to water baths (193, 196). A higher concentration may pose a risk of skin irritations and potentially harm the skin barrier. In this *in vitro* study, we found that when keratinocytes were exposed to sodium hypochlorite concentrations of $\geq 0.02\%$, they showed signs of cytotoxic impact. However, these findings are based on *in vitro* data and the translation to clinical practice requires careful consideration, as the *in vivo* environment is much more complex.

Paper II

Potassium permanganate is commonly used to treat dermatological conditions such as wounds and AD. The concentration range used in clinics is usually between 0.001% and 0.1%. The substance is deemed an antibacterial agent, although the evidence for its efficacy against bacteria from AD skin is not well studied. One of

the few investigations made was a study conducted by Stadler et al. in 1992, in which bacteria derived from the skin of AD patients demonstrated a dose-dependent decrease in survival when exposed to clinically relevant concentrations of potassium permanganate for five minutes (137). Beyond this study, only a limited number of investigations have been conducted, and the understanding of the effects of potassium permanganate on *S. aureus* biofilm is practically non-existent. To support the continued use of this well-tolerated, cost-efficient and anecdotally beneficial treatment there is a need for concrete data demonstrating its efficacy

In the viable count assay, mimicking clinical conditions (131) with an incubation time of 10 minutes and the use of clinically relevant concentrations (ranging from 0.01% to 0.5%), *S. aureus* and *S. epidermidis* isolates were significantly reduced or totally eradicated. The antibacterial effect is further underscored by the results from the RDA, in which a dose-dependent inhibition of bacterial growth is evident. These findings suggest that potassium permanganate may be effective in reducing the bacterial burden in AD.

The presence of *staphylococcal* biofilm formation is thought to further contribute to the persistence and severity of lesions in AD (190). Data from our study indicate that a majority of *staphylococcal* strains from AD patients form a reduced amount of biofilm when subjected to potassium permanganate at an *in vitro* concentration of 0.01%. These findings further reinforce the anecdotally reported favorable properties of potassium permanganate as a treatment in infected AD. However, the findings regarding the weak efficacy of potassium permanganate in eradicating established *S. aureus* biofilm needs to be recognized. In our study, the MBEC values were somewhat inconsistent and several of the biofilms formed endured even the highest tested concentration of 1 %. This was further demonstrated by examining pegs from CBD with scanning electron microscope. As the concentrations of potassium permanganate rose, there was a decrease in the amount of bacteria observed, along with alterations in bacterial structures. However, even at the highest concentration of 1%, bacteria were still observed attached to the pegs. At this intensity, the persistence of biofilm despite the application of potassium permanganate implies that it may not be as efficient enough as a standalone antibiofilm treatment in AD. These results carry essential implications for clinical practice. They emphasize clinicians to be aware of the limitations of potassium permanganate and that they should contemplate alternative or complementary therapies when managing biofilm-related infections in AD.

Taken as a whole, our findings revealed that potassium permanganate exhibited antibacterial effects *in vitro* against planktonic *S. aureus* and *S. epidermidis* derived from AD skin, but a weak capacity to erase preformed biofilm. The dose-dependent effects seen in this study triggers a desire to establish an optimal concentration that is both effective against *S. aureus* and tolerable for the patient. If used inappropriately, potassium permanganate can cause skin irritation (133) which instead of helping the patient might cause an exacerbation of the AD. This study

supports the consideration of potassium permanganate as a potential adjunctive therapy in managing AD, but additional research is required to explore the optimal concentration and application methods.

Paper III

The study protocol aimed to assess the safety and tolerability of a novel wound gel containing ascending doses of TCP-25. Alongside this, the exploratory aim was to collect samples from the wounds and dressings for exploratory research. Being a first-in-human clinical trial the safety considerations were paramount. To achieve this, a comprehensive and detailed protocol developed outlining safety measures, including inclusion and exclusion criteria, dosing regimen, monitoring parameters, and procedures for reporting adverse events. Adherence to the protocol was essential to ensure participant safety throughout the trial and to maintain consistency and standardization across study procedures and participants. The clear endpoints helped to keep focus and ensured that the trial generated meaningful data.

Paper IV

Wound healing is one of the most intricate mechanisms in the human body, involving the activation of numerous biological pathways (197). The purpose of this study was to follow, in a controlled manner, the dynamics of normal epidermal wound healing in respect to bacterial colonization, exudation and inflammation.

The study design with manufacturing of standardized wounds on human skin and a thorough follow up at multiple time points is a great strength in this study. This enabled us to monitor a wide range of aspect throughout the normal healing process of epidermal wounds. However, it also has a few limitations, one being the uncertainty of how the occlusive Meplix dressing affect the bacterial growth. Other types of dressings might have given another outcome (198).

Directly after wounding, there was none or close to none bacterial growth, but on the following measurement points there was a noticeable increase until Day 8, followed by a drop in bacterial levels on Day 11. A damp environment promotes bacterial growth and the moist conditions within the wound caused by exudation and occlusive dressing is thought to further enhance bacterial proliferation (199). The decline on day 11 could be explained by the decreased exudation when the wound heals.

Throughout the healing process, the bacterial composition remained largely consistent for most of the wounds, primarily consisting of commensal skin bacteria.

However, there were a few exceptions presenting the possible pathogen *S. aureus*, recognized for its association with wound infections (200). Nevertheless, it is important to note that wounds can heal successfully despite the presence of *S. aureus* (201). In our study, we found no substantial disparities in the healing outcome between wounds containing commensals or *S. aureus*.

The strong correlation between the bacterial amounts in swabs and dressings supports the idea that swabs accurately reflect the bacterial composition present in wound dressing fluid. Furthermore, these findings propose that when the swabbing method is not advised (for example if the patient experience pain) a substitute could be to examine the dressing fluid instead.

Exudation is a vital component of wound healing. It facilitates the removal of debris, bacteria and dead tissue, while simultaneously providing the wound area with vital nutrients and growth factors needed for tissue repair (202).

An interesting finding in our study was the weak to nonexistent correlation between bacterial amounts and protein levels in the wounds. This indicates that the quantity of bacteria does not affect the level of exudate in normal epidermal wound healing.

Inflammation is known to play a crucial role in wound healing. In our study, we analyzed 10 different cytokines to elucidate inflammatory mechanisms during normal epidermal wound healing. We observed a consistent pattern, with the majority of cytokines peaking on Day 5. This correlated well with the protein content and supported the association between inflammation and the production of exudate through capillary leakage (202). However, the cytokine levels showed little to none correlation with bacterial levels, a finding which suggests that bacteria may not be the primary factor in the inflammatory process of normally healing wounds.

Another crucial participant in the wound healing process is the neutrophils involved in pathogen control, and promotion of inflammatory processes, and tissue repair (203). To assess their presence in the wound, we measured neutrophilic protein levels and found that they had a pattern much alike the inflammatory cytokines with MPO and NE exhibiting increased levels on Day 5. However, the HBP was increasing as early as Day 3, possibly due to it having been stored in vesicles with a greater tendency to release than for the other two proteins (204). IL-8 is known to be an important chemoattractant for neutrophils, guiding them to the site of injury (205). Our study supports this interaction with a high correlation between the neutrophil protein levels and IL-8 in the wounds. As with the cytokine levels, we found a weak correlation between neutrophil proteins and bacterial quantities. This allowed us to speculate that host factors rather than bacterial amounts might play more of a dominant role in neutrophil activation during the wound healing process (Figure 21).

Collectively, this study provides valuable insights into the intricate process of wound healing. We have demonstrated that different factors, like bacterial levels,

exudation, inflammatory cytokines and neutrophil activity all contribute to this process without being entirely dependent on each other.

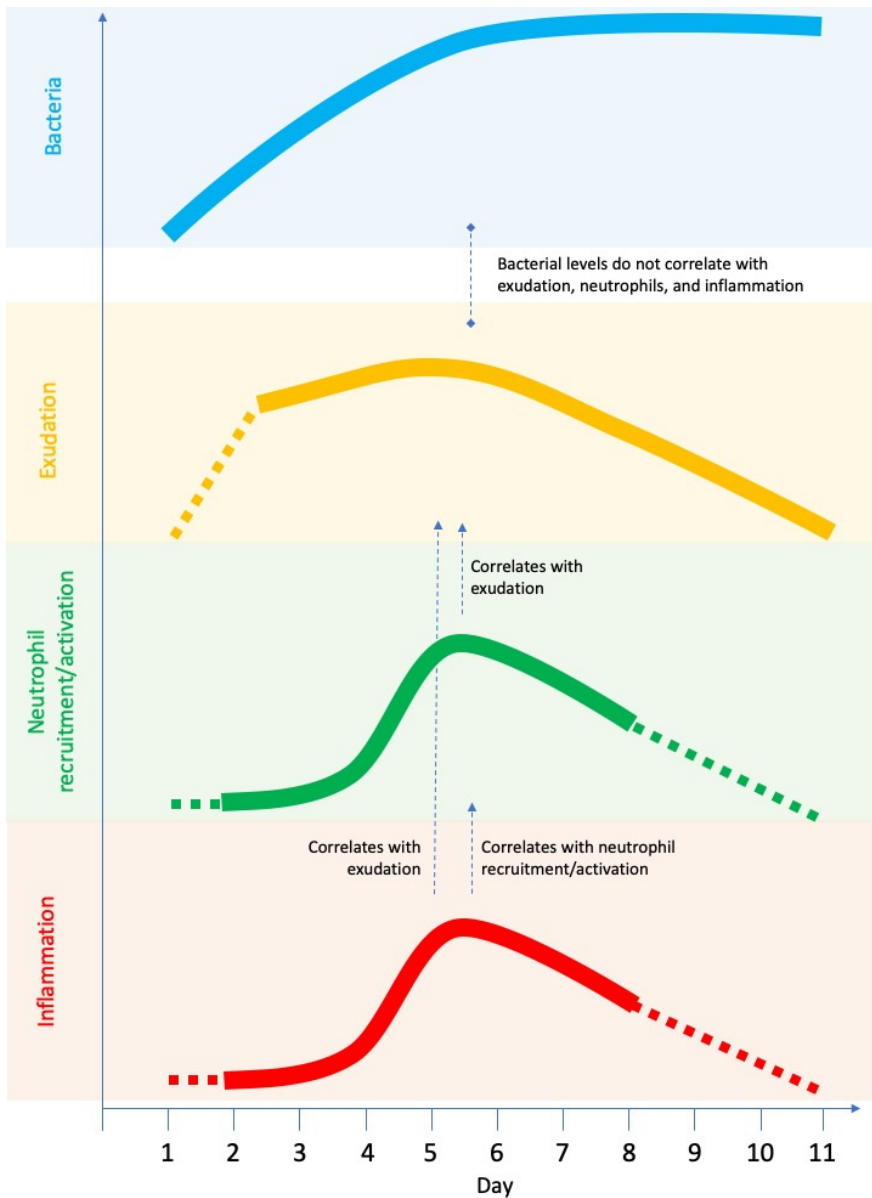


Fig 21 – Dynamic overview of epidermal wound healing

An overview of the patterns of bacterial presence, fluid secretion, neutrophil protein levels, and inflammatory processes in normally healing epidermal wounds over time.

Main conclusions

Paper I

In conclusion, our findings reinforce the usage of sodium hypochlorite in the management of AD to reduce the burden of *S. aureus*. Our *in vitro* findings points to the possibility that higher concentrations than those typically applied could be more beneficial to reduce *S. aureus* biofilm. Although, there must be a careful consideration of safety to avoid any adverse outcomes.

Paper II

The primary takeaway from this study is that potassium permanganate exhibits *in vitro* antibacterial effects against *S. aureus* and *S. epidermidis* sourced from AD skin. Nevertheless, its capacity to eliminate established staphylococcal biofilm is limited. Collectively, potassium permanganate presents a potential as a valuable adjunctive therapy for AD.

Paper III

Further clinical trials are needed to examine to the tolerability and efficacy of the novel TCP-25 containing wound gel.

Paper IV

In this study we found that during normal epidermal wound healing the bacterial numbers increase rapidly after wounding and stabilize by Day 8. Inflammation, exudation and neutrophil activity were not correlated to bacterial counts indicating that the process of healing is primarily driven by the host mechanisms regardless of colonizing bacteria.

Future perspectives

The landscape of treatment for AD is undergoing an expansion of new therapeutic options with the introduction of topical and systemic Janus kinase inhibitors (JAK-inhibitors) (206). These advancements are undoubtedly beneficial additions for management of this common disease. However, I remain convinced that there will remain a need for wide array of treatment alternatives enabling for a patient oriented treatment focusing on tolerability, management and efficacy.

The bacterial components in AD plays a role in the disease severity underscoring the importance of addressing this aspect effectively (49). Moreover, as challenges related to antibiotic resistance escalate there will be an ongoing need for efficient antimicrobial therapies as alternatives to the use of topical or oral antibiotics.

The findings of anti-biofilm effects against *S. aureus* biofilm from clinical isolates of sodium hypochlorite in paper I prompts consideration of raising the current concentrations utilized in clinical practice. However, this adjustment must be approached cautiously to minimize the risk of adverse effects on the skin. Therefore, additional research is encouraged to thoroughly investigate this matter further.

Up to now, potassium permanganate has primarily been used based on clinical experiences. During my residency at a dermatological clinic, I have witnessed firsthand its beneficial effects on numerous patients, with oozing, itching dermatitis, troublesome wounds. Sometimes however, questions have been raised about the evidence supporting its use. The study detailed in Paper II of this thesis has demonstrated its antibacterial effects against *S. aureus* isolated from patients with AD, providing empirical support for its role as an antimicrobial treatment for such conditions.

Patients commonly describe a reduction of itchiness in lesions following potassium permanganate treatment. This improvement may be linked to decreased bacterial levels and subsequent reduction in inflammatory responses. However, other mechanisms may also contribute to this effect. Evidence indicates that potassium permanganate has the ability to break down bacterial toxins in drinking water (136). It is possible that this mechanism could extend to neutralizing bacterial toxins on the skin as well.

With this consideration in mind, we have conducted initial tests indicating that potassium permanganate may impact the protein structures of bacterial toxins, as

well as proinflammatory and pruritic cytokines. This work will be continued as it could offer a potential explanation for the observed itch-relieving effects of the substance in clinical settings. While it may not represent a groundbreaking advancement in medicine, it holds potential interest for everyday dermatologists supporting this frequently used substance. And if this theory will be validated, I am delighted to have contributed to this exploration.

Another emerging field addressing the altered microbiome in AD is skin probiotics. The idea is to promote the growth of beneficial commensal bacteria on the skin, which in turn inhibits the colonization of pathogenic bacteria like *S. aureus*. This can be achieved by introducing beneficial microorganisms or factors that promote their growth, thereby strengthening the microbial barrier and leading to long-term improvement of the skin barrier.

The microflora and its interactions with the host during wound healing is a field scarcely examined and could give important information that could yield valuable insights for developing effective treatments opting for a successful healing. Further analyses of this material will be undertaken to deepen our understanding of normal wound healing mechanisms.

Following the initial part of this clinical trial two additional sections were incorporated where the tolerability of the wound gel were tested on venous leg ulcers and dystrophic epidermolysis bullosa wounds. For these subsequent parts I assumed the role of primary investigator, and I am eager to continue leveraging this experience to make further contributions in the future.

Alongside the work with this thesis, I concurrently served as a resident for 5.5 years at the Department of Dermatology and Venereology at Skåne University Hospital. I find the combination of clinical work and research within the same field to be advantageous and stimulating for new ideas and adds a significance to the research process. During my clinical practice, I have developed a growing interest in the treatment of leg ulcers. With the increased aging population, there will be a demand for efficient treatments that promote successful healing and support active aging. These advancements are something I aspire to be a part of and feel hopeful that it will be possible in the future, thanks to the encouraging environment I'm positioned in with supportive colleagues and research partners.

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