Human skin equivalents to study the prevention and treatment of wound infections

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CHAPTER 1

General introduction

1. Burn wound infections

1.1 Burns

Burns constitute a major health problem worldwide; fire-related burns alone account for over 300,000 deaths per year globally [1]. In addition, many more people suffer from lifelong disabilities, immobilization and disfigurements due to the complications of such burns. In the United States approximately 100,000 hospitalizations and about 5,000 deaths can be contributed to burns and their complications, such as burn-induced inflammation with fever, tachycardia and leukocytosis as well as wound infections and sepsis [2, 3]. In the Netherlands, 420 individuals per 100,000 are medically treated for burn injuries, and 8/100,000 need hospitalization of which 2-7% die annually [4] . Patients with serious burn injuries require immediate specialized care in order to minimize morbidity and mortality [5]. Due to improvements in medical care for burn patients in the past three decades mortality has decreased by approximately 50% [6]. Still, 75% of burn-related deaths are related to sepsis due to burn wound infections and/or inhalation injury [7]. Furthermore, infectious complications are often associated with difficulties in wound management resulting in delayed wound healing and enhanced scarring [2]. Treatment of (burn) wound infections with antibiotics is complex and often unsuccessful due to the emergence of antibiotic resistant strains. Treatment is further complicated by the ability of bacteria to form biofilms, i.e., highly specialized bacterial communities that are encased in a self-produced matrix in which they are protected from the actions of various types of antibiotics because of, for instance, alteration of their metabolic state.

1.2 Burn wound infections

The (burn) wound bed is a protein-rich environment consisting of avascular necrotic tissue that provides a favorable niche for microbial colonization and proliferation. Therefore, (chronic) wounds often become colonized with a wide variety of pathogens, which can interfere with wound healing [8]. A colonized wound can become a focus of wound invasion and infection when the immune response fails or wound management is not effective [9]. Wounds comprising more than 10^5 organisms per gram of tissue are usually considered to be infected [10].

A wide variety of microorganisms can colonize the burn wound bed. Infection of burn wounds often starts with colonization by Gram-positive bacteria such as *Staphylococcus aureus*, in a later stage Gram-negatives are introduced as well. Most common bacterial species colonizing burn patients are *S. aureus*

(13.2-75% of patients), coagulase-negative Staphylococci (11.6-63.0%), *Pseudomonas aeruginosa* (2-25%), *Klebsiella pneumoniae* (0-15.2%), Streptococcus pyogenes (20%), *Escherichia coli* (1.2-13.6%) and various coliform bacilli (5%) [11, 12]. Anaerobic organisms, yeast and fungi (Candida albicans and Aspergillus fumigatis) can also cause infection but are rarely encountered [13, 14] . Differences in wound colonization were related to burn extent and location, hospitalization and cross-infection due to poor hygiene, age and normal skin flora [14]. Moreover, both studies showed that long hospitalization periods were associated with the occurrence of antibiotic resistant *S. aureus* in the wound bed and an increased frequency of *P. aeruginosa* infection, underscoring the need for strict hospital hygiene [11, 12].

2. Treatment of burn wound infections

2.1 Current treatment strategies

Currently used treatments for burn wound decolonization or prevention of colonization are the application of topical antibiotics like mupirocin [15] and neosporin [16]. However, these antibiotics are ineffective when resistant bacteria colonize the wound [17, 18]. Both *S. aureus* and *P. aeruginosa* have been associated with increasing antibiotic resistance and with biofilm-related wound infections that are difficult to eradicate by first-line antibiotics such as beta-lactams [19]. Topical disinfectants, often used in wound dressings, such as chloride hexidine [20], silver sulfadiazide [21] and iodine preparations [16], may be more effective in clearing (biofilm-associated) pathogens from the wound bed but are also associated with negative effects on wound healing and can inflict pain.

2.2 Antibiotic resistance

Almost as soon as antibiotics were introduced in the early fifties, the first antibiotic resistant bacteria were isolated. In recent years, due to extensive and often incorrect use of antibiotics, there has been a huge increase in drug resistance. Even to the extent that it becomes less and less uncommon to isolate extensive drug resistant (XDR) and even pan-drug resistant (PDR) strains. As a consequence, infections are harder and may even become impossible to control, and the risk of spreading of infections due to such pathogens is increased, and patient's illness and hospital stays are prolonged, with added economic and social costs [22, 23].

Due to improper and widespread usage in both human and animals [23] and environmental presence of antibiotics in sub-inhibitory concentrations [24], bacteria

are constantly exposed to antibiotics. Therefore, the chances of selecting for and spreading drug resistance are increased. Recently, the World Health Organization published a report on antimicrobial resistance, in which they urge the medical industry to review their research and development policies and address the great need for new antibiotics and alternatives to antibiotics [25]. There are a couple of strategies to combat the problem. First, current antibiotic usage should be guided by antibiotic stewardship to help restrict unjust use and target more efficient use of antibiotics where indicated. This means, reduction of over-prescription, advising of patients to finish their prescription and preparation of clear guidelines for good antibiotic usage. Secondly, existing drug compound libraries should be explored for active compounds with a narrow spectrum activity. Finally, the number of new antimicrobial therapies reaching the market should be increased. As between 2003 and 2012 only 7 new antimicrobial agents were approved by the US Food and Drug Administration for usage in patient care, the development of alternative treatments should also be stimulated [26].

2.3 Alternative treatments

The search for alternatives to antibiotics to treat biofilm-associated wound infections has been given a boost in recent years. Kiedrowski et al. recently reviewed novel strategies for eradication of biofilms, including not only antibiotics, but also biofilm degrading enzymes, targeting bacterial quorum sensing and the use of lipo- and glyco-peptides [27]. Moreover, different experimental therapies to clear bacterial biofilms [28] have been reported, ranging from treatment with bacteriophages [29], maggot secretions [30], nanoparticles delivering drugs in biofilms [31] to the usage of honey-based gels [32]. Moreover, experimental treatments using live bacteria are being developed. For instance, genetically modified effector *E. coli* that "seek" pathogens by detection of quorum sensing molecules and subsequently produce DNAse I and the antimicrobial peptide microsin S have been described [33]. Lu *et al.* created bacteria and bacteriophages that carry RNA-guided nucleases that target specific DNA from pathogens. These DNA sequences can be, for example, directed against antibiotic resistance genes [34]. Another strategy is building on the defenses already evolved by nature. Almost all mammals, plants and even bacteria and fungi express defense peptides that are also called antimicrobial peptides (AMPs) [35]. Antimicrobial peptides generally have a broad spectrum of antimicrobial activity and can have anti-biofilm activity.

3. Biofilms and resistance to antimicrobials

3.1 Discovery of biofilms

In 1683, Antoni van Leeuwenhoek made an observation in dental plaques using his own microscopes, and wrote 'The number of these animalcules in the scurf of a man's teeth are so many that I believe they exceed the number of men in a kingdom.' Moreover, he observed using microscopy, that the 'animalcules' that dispersed from the plaques appeared more susceptible to be washed away by wine-vinegar than the bacteria within the plaques [36]. This was a first observation that biofilm-associated, i.e. plaque bacteria are more difficult to eradicate than their planktonic, free-living counterparts. In 1933, Henrici described the relevance of investigating bacteria in their natural habitats, since not all bacteria could be cultured *in vitro* or did not appear identical phenotypically on agar or gelatine as in nature. Moreover, he described biofilms as films of bacteria formed on glass slides in his aquarium. He wrote 'It is quite evident that for the most part the water bacteria are not free floating organisms but grow upon submerged surfaces; they are therefore the *benthos* rather than the *plancton*' [37]. Years later, ZoBell described the various steps in the process of biofilm formation by bacteria and larger organisms in seawater, and named it 'fouling' [38]. Based on Zobells research, Heukelekian and Heller hypothesized that nutrients reach the necessary concentration for bacterial survival and growth at the surface of a glass slide earlier than in a suspension. This nutrient availability allows the bacteria to grown on, or in the proximity of these surfaces. They wrote: 'Surfaces enable bacteria to develop in substrates otherwise too dilute for growth. Development takes place either as bacterial slime or colonial growth attached to surfaces.' [39].

The first time microbial communities were named 'biofilms' in scientific publications was by Mack and colleagues in the mid-seventies [40]. Currently, biofilms are defined as self-secreted extracellular matrix (ECM) that encloses bacterial populations adherent to each other and/or to surfaces or interfaces [41]. The ECM is composed of exopolysaccharides, proteins, glycoproteins, glycolipids and DNA. Nowadays it is believed that, just as van Leeuwenhoek described, biofilms are the predominant phenotype in natural environments, including the human body [42].

3.2 Biofilm formation

Biofilm formation takes place in several stages [43, 44] (figure 1). First, there is initial attachment of planktonic bacteria to a surface. Attachment depends on type of surface and nutrient availability. In the second step, bacteria bind irreversibly and

begin to multiply while emitting chemical signals for communication between the bacterial cells, a process called quorum sensing. A monolayer is formed and once the concentration of quorum sensing molecules exceeds a certain threshold level, the genetic mechanisms controlling ECM production are activated. The ECM supports microcolonies of (bacterial) cells, allows cell–cell communication, forms water channels, retains and concentrates nutrients, and can support gene transfer through conjugation, transformation, and transduction [45].

Next, the monolayer expands to a microcolony by formation of aggregates, resulting in a reduction of motility Finally, the biofilm matures, and reaches its ultimate size, which is generally larger than 100 µm. During the final stage, some bacteria disperse from the biofilm and colonize a new surface to establish a new biofilm [46, 47].

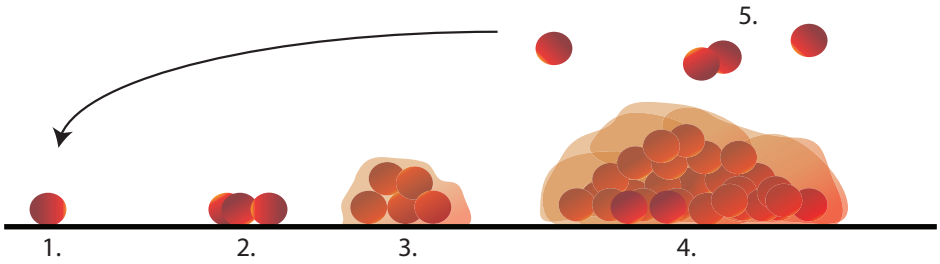

Figure 1. The different stages of biofilm formation. 1. Initial attachment of a single bacterial cell to a biological or artificial surface. 2. Proliferation and formation of a monolayer. 3. Start of ECM production once a certain concentration of bacteria has been reached. 4. Formation of the mature biofilm. 5. Dissemination of bacterial cells from the biofilm, which can attach to a new surface. Asterisk indicates ECM. EMC=extracellular matrix.

3.3 Phenotypic resistance to antimicrobial agents due to biofilm formation

It has been demonstrated that *S. aureus* residing within biofilms can persist in the presence of concentrations of antibiotics and antimicrobial agents that are 100-1,000 times higher than those active against planktonic cells [8, 48-50]. There are several mechanisms proposed to explain such phenotypic resistance to antibiotics in bacterial biofilms [51, 52]. The first is slow or incomplete penetration of the antibiotic into the biofilm. Polymeric substances like those that make up the ECM of a biofilm are known to slow down the diffusion of antibiotics, especially the larger molecules, and also solutes in general diffuse at slower rates within biofilms than they do in water. Also, the center of a biofilm can be depleted of nutrients limiting cell growth, or have a high waste accumulation in which antibiotic action

is antagonized [44]. However, others have reported that antibiotics may just as easily penetrate biofilms [53, 54] as diffuse in water, but this penetration does not translate into activity and elimination of the biofilm associated bacteria. Secondly, alterations in the microenvironment of the biofilm may lead to depletion of oxygen, an altered pH and accumulation of waste products. It has been shown that some antibiotics the ability to enter the bacterial cells is absent in low pH and anaerobic circumstances [55]. Finally, at least some of the cells in a biofilm experience nutrient limitation and therefore exist in a slow-growing or starved state, with an altered metabolism, sometimes designated as persister cells [56]. Thus, in the absence of cell wall production, for instance, cells are not susceptible to the action of beta-lactam antimicrobial drugs. Heterogeneity in the physiological state of bacteria within biofilms constitutes an important survival strategy as at least some of the cells will survive incubations with antibiotics targeted at metabolically active bacteria. Development of persister cells is thought to be a biologically programmed response to growth on a surface, as shown by the observation that resistance to antibiotics can already be seen in newly formed biofilms that have not yet formed a physically large barrier against antimicrobial agents (reviewed in [57, 58]).

Currently, the ability of bacteria to adhere to and form a biofilm on artificial, abiotic surfaces, such as the surface of a microtiter plate or flow-cell surface [59, 60] is exploited to investigate the effects of a variety of potential anti-biofilm agents, including (synthetic) AMPs [61-65], antibiotics [66] and medicinal maggot secretions [67]. However, these biofilm assays do not fully represent the detailed characteristics of biofilm-associated infections of a medical device *in vivo*. It should be kept in mind that soon after its insertion into a patient the surface of the medical device will be covered with host-derived proteins, indicating that specific interactions between these proteins and the bacteria are probably of more importance than adherence to the abiotic surface [68]. In addition, *in vitro* models involving biotic surfaces are required for studying bacterial biofilm formation.

4. Antimicrobial peptides in human skin

4.1 Human skin

The skin is composed of two anatomical layers, the dermis and epidermis. The epidermis is nonvascular and consists of several layers of melanocytes and keratinocytes and forms the first line of defense against the outside environment (figure 2). The barrier properties of this layer prevent fluid and temperature loss and

provide a physical barrier against invasive pathogens. The epidermis is constantly renewed by proliferating keratinocytes that move upwards to form several differentiated layers: the basal layer; the spinous layer; the granular layer and; once they are completely cornified, the *stratum corneum* (SC). Through this process, the epidermis can also replace cells lost after burning, mechanical and other injuries [2].

4.2 Antimicrobial peptides of the human skin

Besides acting as a physical barrier, the skin acts as a chemical barrier against pathogens as well, partly formed by antimicrobial peptides (AMPs), also known as host defense peptides, present in the skin layers [69]. AMPs are part of a natural defense mechanism against pathogens and are produced by almost all complex organisms [35, 70]. Production of AMPs can be both constitutive and inducible, e.g. produced after wounding, infection, UV-light or irritation. The importance of AMPs in the barrier function of the skin is illustrated by some diseases that are characterized by either high AMP production, e.g. psoriasis, or low AMP production, e.g. atopic dermatitis (AD). Skin of AD patients is more prone to bacterial colonization, with 70-90% of AD patients being colonized by *S. aureus*, compared to about 20% of the normal population [71, 72]. Moreover, AD patients also often become colonized by biofilms of staphylococci [73]. Surprisingly, also in psoriasis patients an increased nasal colonization with *S. aureus* is reported [74].

Initially, it was believed that the role of AMPs in skin immunity was confined to killing invading microorganisms, but in recent years the importance of AMPs as immune modulatory agents has been shown [75-78]. The main AMPs present in the skin are LL-37, psoriasin and the human beta-defensins (hBD-1-4) [79]. Figure 2 summarizes the different functions of AMPs in human skin. LL-37 is a cationic peptide cleaved from its parent protein human cathelicidin protein-18 (HCAP-18) after activation. In healthy individuals, the antimicrobial action of LL-37 is present at the physiological concentration of approximately 2-5 µg/ml. During inflammation the local concentration of LL-37 can rise to more than 30 µg/ml [80]. LL-37 has been shown to have anti-bacterial and anti-biofilm activity against amongst others *S. aureus* [81], *P. aeruginosa* [65], *K. pneumoniae* (78) and *E. coli* [64, 82]. The bactericidal activity of LL-37 involves the disruption of the bacterial membrane following interaction with negatively charged bacterial molecules and insertion into the membrane (80). Although LL-37 is a potent antimicrobial agent under the right conditions *in vitro*, its antimicrobial activity is strongly antagonized at physiologic salt concentrations [65, 70]. Therefore, nowadays it is believed that AMPs like LL-37 display an array of functional ac-

tivities, including antibacterial effects, immune modulatory and wound healing properties. For example, LL-37 can aid in wound healing in skin and lung tissue by inducing keratinocyte migration via activation of the endothelial growth factor [83-85] Furthermore, LL-37 has the ability to suppress apoptosis in keratinocytes and thereby accelerates wound healing [86]. LL-37 also has a chemotactic function [87], recruiting neutrophils to the site of infection [88]. Finally, LL-37 can neutralize toxins [89] (Figure 2).

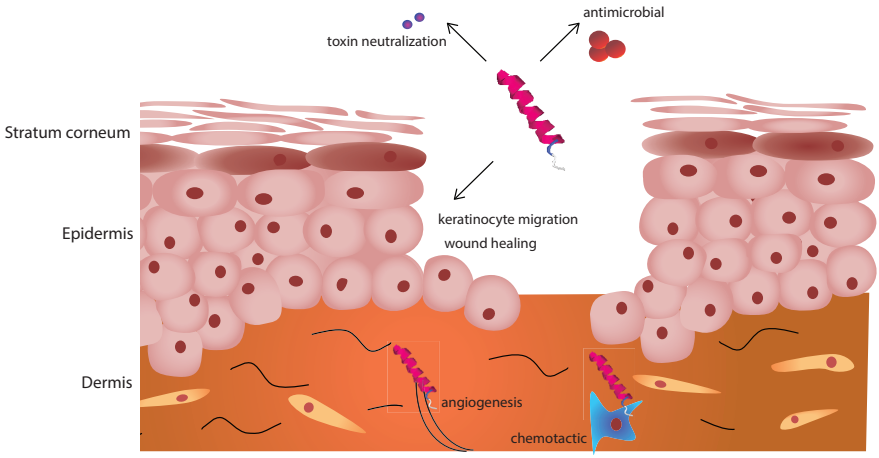

Figure 2. Different functions of antimicrobial peptides in the human skin. These functions include antimicrobial activity, toxin neutralization, induction of angiogenesis, wound healing stimulation, and chemotactic activity [78, 90].

There are four hBDs described in human skin, hBD-1, -2, -3 and -4. HBDs are mainly expressed in the terminally differentiated layers of the skin, and their expression can be induced by the bacterial membrane components lipopolysaccharides (LPS) and peptidoglycan [91]. HBD-2 is mainly present in inflamed skin lesions and is induced by TNF-α, IL-1β, LPS and bacteria. In differentiated keratinocytes, levels of hBD-2 dramatically increase [92]. Expression of hBD-3 is up-regulated in keratinocytes upon stimulation by TNF-α, IL-1β, IFN-γ, and bacteria [93]. HBD-4 is up-regulated by infection with bacteria in epithelial cells. HBD-2, -3, and 4, but not hBD-1, can induce pro-inflammatory cytokine and chemokine production [70, 91, 92, 94].

Finally, the involvement of AMP expression in burn patients is illustrated by elevated expression of hCAP-18/LL-37, hBD2 and hBD3 at the surface of burn wounds, irrespective of wound infection [95].

5. Antimicrobial peptides as therapeutics

The increasing numbers of multidrug resistant (MDR) and even PDR pathogens and their inherent ability to form biofilms stresses the urgent need to develop novel antimicrobial drugs. Synthetic AMPs are considered as possible treatment alternatives due to their multiple activities including broad antimicrobial, anti-biofilm and immunomodulatory properties [96]. Nell *et al* demonstrated that a synthetic derivative of LL-37, called OP-145 or P60.4Ac, showed improved antimicrobial activity, a similar LPS and lipoteichoic acid (LTA) neutralizing activity but lower T cell stimulating, epithelial cell activating and chemotactic activity as compared to LL-37 [62]. Moreover, this 24 amino acid peptide showed no signs of toxicity in preclinical studies and was used as treatment of chronic otitis media in a clinical phase 2 trial [97]. Other peptides derived from human LL-37 have also been used in experimental set-ups, and were demonstrated to have anti-bacterial properties [63, 98].

Thanatin, an AMP with potent antibiotic activity against (extended-spectrum-beta-lactamase-producing) *E. coli*, and R-thanatin, a shorter derivate of thanatin, displays in vitro antimicrobial activity against coagulase-negative staphylococci (*S. epidermidis*, *S. haemolyticus*, and *S. hominis*) [99]. Another promising AMP is Pexiganan, derived from the frog AMP magainin that was described to have antimicrobial activity [61], which had an effect on microbiological eradication rate and clinical improvement rate in the treatment of diabetic foot ulcers [100, 101]. Several other AMPs including the protegrin-1 derivative Iseganan (Intrabiotics Pharmaceuticals Inc.), the indolicin-derived AMP Omiganan (Microbiologix Biotech) and the lactoferrin-derived hLF1-11 (AM Pharma) have been tested in phase 2/3 clinical trials. Based on these results there may be a place for AMPs as topical treatment for infections or decolonization.

6. Models for (burn) wound infection and treatment

6.1 In vivo models

Until recently most research concerning thermal injury, wound healing and wound related infections has been performed *in vivo* using animal models such as rabbits [102], rats [103] and mice [104]. Other animal models for wound healing and scar formation include (red duroc) pigs [105-107]. These experiments have provided much information about the pathophysiology of cutaneous infections. However, there are significant differences between human and animal skin. To name just one, rodent skin is thinner and scar formation is different. The

morphology and wound healing of pig skin is more similar to that of humans. However, the usage of these animals is expensive and raises ethical issues [108].

6.2 In vitro skin models

An alternative to the use of animals are *in vitro* human skin equivalents (HSEs). Twenty-five years of tissue engineering research has led to HSEs that have many properties of native human skin [109-112]. HSEs are used for testing of chemical additives used in human skin products [113-115] and for skin replacement therapy of severe burn wound patients [116]. In research, HSEs are used to investigate for example melanoma invasion [117], squamous cell carcinoma [118], psoriasis [119] and AD [120].

There are different ways of generating HSEs (figure 3). Generally, HSEs are generated from primary keratinocytes and fibroblasts isolated from human surplus skin. To create a HSE, keratinocytes are seeded upon a collagen matrix populated with fibroblasts or on a de-epidermized dermis. Keratinocytes can also be seeded directly upon an inert filter forming epidermal models (EMs) (also known as Leiden epidermal skin model). After culturing to a confluent layer the models are placed air-exposed, and the cells are allowed to differentiate [114, 121, 122]. Apart from these HSEs, ex-vivo skin obtained from a donor can also be used.

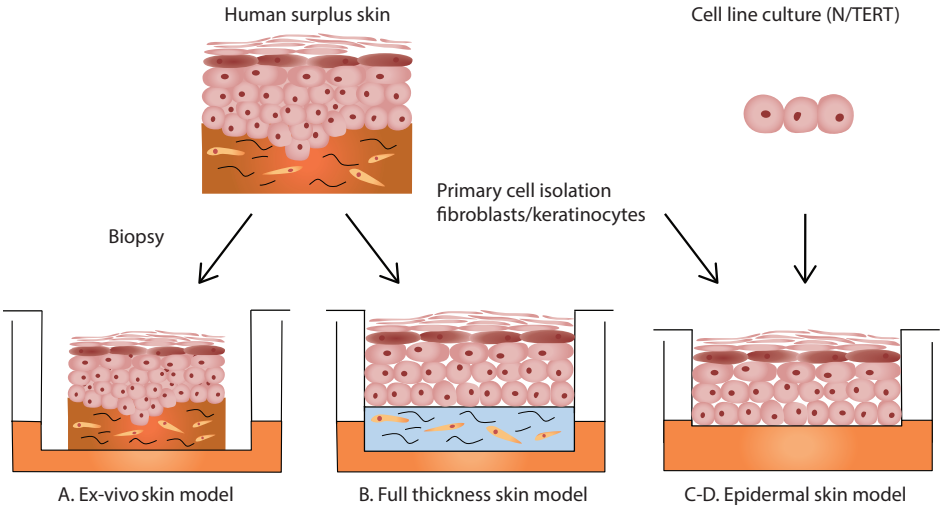

Figure 3. Development of the full thickness skin model and the Leiden epidermal skin models. A, ex-vivo skin model; B, full thickness model with dermal substitute and epidermis. C, epidermal model with either primary keratinocytes. D, epidermal model using immortalized keratinocytes (N/TERT cell lines).

6.3 Skin models to study (burn) wound infection and treatment

In vitro skin models are used for the investigation of wound closure, skin colonization and wound infection (summarized in Table 1). To study (burn) wound healing different models were used. Full thickness HSEs were utilized to make a comparison between (hot and cold) burn injury, demonstrating that after cold injury re-epithelialization is faster, but the expression of pro-inflammatory cytokines is similar [123]. In another study *ex vivo* skin models were used to study how wound healing is influenced by silk fibroin fibers [124]. Moreover, using *ex vivo* skin it was found that fetal skin heals faster than adult skin and scarred skin after burn wounding [125].

To study the colonization of commensal and pathogenic bacteria different HSE models were used. Epidermal skin equivalents have been used for the comparison of adherence of *S. aureus* and *S. epidermides* to a bipolar substrate (Epiderm) and to stainless steel [126]. It was observed that *S. aureus* more easily adheres to Epiderm than *S. epidermides*. To study the effect of colonization on full thickness HSEs, gene analysis was performed, which demonstrated up-regulation of AMPs and pro-inflammatory cytokines after *S. aureus* colonization but not after colonization with *S. epidermis* [127]. A different study describes a HSE infection model either using healthy HSEs or wounded HSEs. The authors observed that *P. aeruginosa* has a better capability to invade the dermis than *S. aureus* which remains in the epidermal layer [128]. An *in vitro* model for biofilm formation was developed by using a collagen matrix that was colonized with *P. aeruginosa* or *S. aureus* [129]. Graft-skin constructs were used to create a wound surface and these wounds were incubated with small aliquots of bacteria, and biofilm formation could be observed [130].

Finally, HSEs are also used to test experimental treatments to clear biofilms. Boekema *et al* used an *ex-vivo* skin model to study the effect of a honey based gel (L-Mesitran Soft) and the commonly used silver sulfazidine on the colonization and wound healing of human skin equivalents. They observed no complete eradication of *P. aeruginosa* after treatment with Mesitran, however wound healing with this substance was significant better than with silver sulfazidine [32].

Table 1. Usage of different *in vitro* skin models in the study of (burn) wounds, wound healing and colonization with skin pathogens.

In vitro model	Subject	Ref
Epidermal model	Adherence of *S. aureus* and *S. epidermides*	[126]
Full thickness model	Colonization of models, gene expression profiles	[127]
	Difference between cold and hot burn wounds	[123]
	Bacterial penetration of the epidermis before and after wounding	[128]
Collagen matrix	Biofilm formation	[129]
Graft-skin constructs	Biofilm formation	[130]
Ex-vivo skin model	Wound healing	[124, 125]
	Treatment of colonized skin models	[32]

7. Thesis aim and outline

Although models for infection and colonization of HSEs exist, few studies have addressed experimental treatment of these infections. The aims of the research described in this thesis were twofold: firstly, to develop an *in vitro* wound infection model of thermal injury, using HSEs prepared from primary keratinocytes and fibroblasts (**Figure 3**) and; secondly, to use this model to investigate new approaches including (synthetic) antimicrobial peptides derived from the human cathelicidin LL-37 that may be developed further as potential treatment for wound infections.

Chapter 2 describes the generation of a burn wound infection model using the full-thickness human skin equivalent. We created a wound in these models with liquid nitrogen prior to allow bacterial colonization. Next, we investigated the immune responses induced by quantifying both the cytokines IL-8 and IL-6 and the AMPs hBD-2 and hBD-3. We continued our experimental treatment approach for methicillin resistant *S. aureus* (MRSA) infections on these infection models by testing the efficacy of synthetic AMPs derived from LL-37. We observed that P60.4Ac, and a novel next generation peptide P10, were able to eradicate MRSA from HSEs. In addition, in **Chapter 3**, we demonstrated the safety of these AMPs in the Leiden epidermal skin model. Moreover, in **Chapter 4**, we showed that the synthetic AMPs, P10, P276 and P145, could also eradicate XDR and PDR MRSA, *P. aeruginosa* and K. pneumoniae. In **Chapter 5** we incorporated P60.4Ac into different formulations, a gel (hypromellose), an oil in water cream (cetomacrogol) and a water in oil cream (Softisan-649), for better topical administration

of the synthetic AMP. We demonstrated strong antimicrobial activity of P60.4Ac in hypromellose.

Next, we assessed the interaction of *S. aureus* with skin models that have an altered barrier function. For this purpose, a model for AD was used. To establish this model, we used N/TERT cells with fillagrin (FLG) knockdown and IL-31 supplementation. Both FLG and IL-31 are associated with AD. We observed that AD models were more prone to *S. aureus* colonization; moreover, these models had decreased AMP expression (**Chapter 6**).

Finally, in **Chapter 7** we used a novel Luminex-based assay to identify bacterial components that play a role in the formation of biofilms on epidermal skin models compared to those formed on polystyrene surfaces. We identified several immunomodulators and toxins, including alpha-toxin, to be specifically expressed in biofilms formed on Leiden epidermal equivalents, and not on polystyrene. These components are potential targets for alternative treatment approaches.

The results obtained in these studies are summarized and discussed in **Chapter 8**. Finally, a Dutch summary is provided in **Chapter 9**.

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