



Universiteit
Leiden
The Netherlands

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

Haisma, E.M.

Citation

Haisma, E. M. (2018, March 28). *Human skin equivalents to study the prevention and treatment of wound infections*. Retrieved from <https://hdl.handle.net/1887/61135>

Version: Not Applicable (or Unknown)

License: [Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden](#)

Downloaded from: <https://hdl.handle.net/1887/61135>

Note: To cite this publication please use the final published version (if applicable).

Cover Page



Universiteit Leiden



The handle <http://hdl.handle.net/1887/61135> holds various files of this Leiden University dissertation

Author: Haisma, Ilse

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

Date: 2018-03-28

CHAPTER 8

Summary and future perspectives

1. Challenges in treating burn wound infections

Infection of burn wounds remains the leading cause of death in burn patients [1, 2]. Treatment of such infections with conventional antibiotics is often unsuccessful, which is due to multiple reasons. Firstly, the presence of multidrug-resistant (MDR), extensively drug-resistant (XDR) and pandrug-resistant (PDR) bacteria (definitions in [3]) in the wound bed render conventional antibiotics ineffective. This problem has increased in recent years due to extensive and improper antibiotic usage that selects for antibiotic resistant microorganisms [4]. Secondly, formation of bacterial biofilms, an extracellular matrix mostly composed of exopolysaccharides, extracellular DNA and proteins, may prohibit entry of antibiotics into the biofilm, leading to failure to reach an adequate concentration around the pathogen or the compounds in the biofilm may antagonize or alter the activity of the antibiotic. Moreover, bacteria in such biofilms can be in a latent, metabolically dormant state, which prohibits antibiotic activity, [5-7]. Finally, systemic antibiotic treatment in burn wound patients may fail since an optimal antibiotic concentration may not be reached in the wound bed due to the presence of debris, clots and necrotic tissue in the wound bed.

Taken all this together, there is a clear need for new antimicrobial agents with modes of action different than that of current antibiotics. Different approaches may be considered (figure 1), e.g. biofilm degradation by maggot secretions [8, 9], inhibition of quorum sensing that is essential for biofilm formation or can make bacteria more susceptible for antibiotics [10, 11], use of bacteriophages [12] or bacteriolysins derived from them [13-16]. Furthermore, the host immune defence can be boosted by, for example, boosting of the endogenous production of antimicrobial peptides (AMPs) by Vitamin D₃ or butyrate ([17], EM Haisma unpublished data)). Identification of new (peptide) antibiotics, e.g. the new antibiotic teixobacin from unculturable bacteria [18] or the peptide antibiotic lugdunin from the commensal bacterium *Staphylococcus lugdunensis* [19, 20] holds another promising approach. In this thesis, assess the potential of synthetic AMPs as topical antimicrobials.

To advance the development of novel antimicrobial agents and identify new drug targets, it is essential to use an appropriate model system. In this thesis, we describe the development and use of different *in vitro* skin models and a mucosal membrane model. There are a couple of advantages to the use of skin models. Firstly, they pose similar properties as normal human skin, including the *stratum corneum* (SC) formation [21] and cell differentiation [22]. The SC of skin models have been demonstrated to resemble that of human skin to a great extent [21]. This is in contrast to studies in animals, especially murine models, where the skin structure,

i.e. epidermal thickness and hair follicle count, is considerably different from that of the human skin [23]. In addition, the immune system of mice and human differ, for example in the expression patterns of defensins as reviewed in [24]. The advantage over *ex vivo* skin is that skin models can be kept in culture longer than *ex vivo* skin biopsies, which stay viable for only a couple of days and experiments can be repeated with cells from the same donor. Finally, with the use of skin models we are able to modify gene expression and thereby mimic disease characteristics (**chapter 6**). Disadvantages of HSE in comparison to the use of animal models is the lack of immune cells such as macrophages and neutrophils; and the absence of some anatomical features such as sweat glands and hair follicles. Currently, investigators are working on skin models that incorporate an immune compartment, for example T-cells [25] or Langerhans-cells [26]. In this thesis, we used different *in vitro* models to study the effect of colonization and infection of healthy skin, (thermally) wounded skin (**chapter 2**), mucosal membranes (**chapter 3**) and skin with disease characteristics of AD (**chapter 6**). In addition, we studied the difference in bacterial colonization and biofilm formation on biotic (*in vitro* skin models) versus a-biotic surfaces (polystyrene) (**chapter 7**). Finally, we used the models to study the antimicrobial activity of (novel) topical synthetic AMPs (**chapter 3,4,5**).

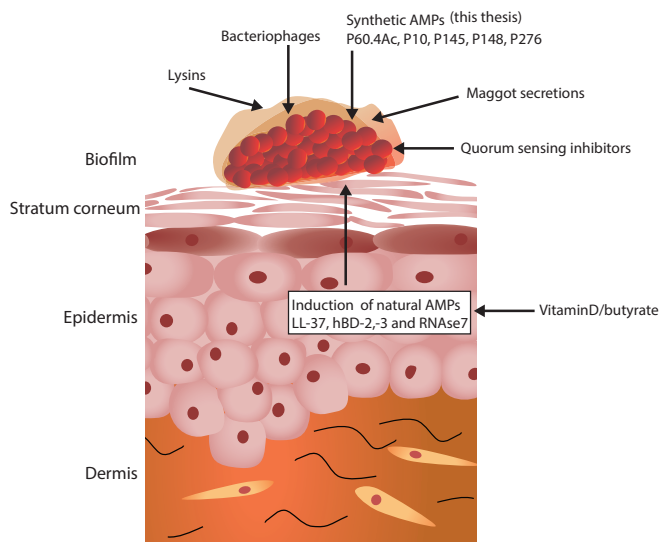


Figure 1: Strategies for the eradication of *S. aureus* and degradation of biofilms. Biofilm degradation by maggot secretions [8, 9], quorum sensing inhibitors [10, 11], bacterial products such as novel antibiotics teixobactin [18], AMP antibiotic lugdunin [19, 20], bacteriolysins [13, 14, 16], bacteriophages [12] or antimicrobial peptides P60.4Ac, P10, P145, P148, P276 (this thesis) or boosting of the endogenous AMP production of for example LL-37, hBD-2, HBD-3 or RNase7 by butyrate and/or Vitamin D₃ (unpublished data).

2. Development of *in vitro* models to study colonization and infection

To study colonization and infection on human skin or mucosae by bacteria we used three different models: (1) the human full thickness skin equivalent (HSE), composed of both a dermal and epidermal component; (2) the (Leiden) epidermal skin model (EM or LEM), composed of differentiated keratinocytes. In addition, we used a (3) mucosal membrane model using human bronchial epithelial models (BEM), composed of differentiated bronchial epithelial cells (explaining figure in introduction).

2.1 Skin models as models for wound healing

Skin models can be used to characterize aspects of the wound-healing process differently than the *in vitro* 'scratch' wound models using monolayer cultures. Monolayers are very limited for such studies, for example due to lack of cell differentiation and cross talk between the keratinocytes and fibroblasts [27]. Different studies have demonstrated similarities between *in vitro* wound-healing models and *in vivo* models, including proliferation, migration, expression of growth factors (e.g. Transforming growth factor beta 1 (TGF- β 1), platelet-derived growth factor- β and keratinocyte growth factor [28, 29]) and cytokine secretion (e.g. Interleukin (IL)-1 α , IL-6, tumor necrosis factor- α [27, 30, 31]), reviewed in [32, 33].

Since skin models consist only from a pre-defined cell population, they can be used as tools for the understanding of individual cellular processes that are involved in tissue repair. This brings also the downside of HSEs as research tools, since HSEs lack the complexity associated with wound-healing mechanisms in the *in vivo* situation. To study molecular mechanisms of tissue regeneration in a complex system, many researchers are now using rat and mouse wound models [34] regardless of the limitations as described above.

To overcome the clear differences between human and animal skin, researchers have used human *ex vivo* skin, HSEs or EMs grafted onto immunocompromised (athymic nude) mice as models. Using this approach, different models can be generated; e.g. to study hypertrophic scarring [35] or wound-healing in epidermolysis bullosa patients [36]. Also EMs composed of human embryonic stem cells have a structure consistent with that of mature human skin 12 weeks after grafting. This type of models could be used for long term research into wound healing, inflammation and skin irritation for example [37].

2.2 HSEs for thermal wounding and colonization

In this thesis we used HSEs to study colonization of (thermally) wounded skin by the Gram-positive bacterium *Staphylococcus aureus* (**chapter 2**). We observed that the course of *S. aureus* adherence and multiplication on thermally wounded and intact HSEs did not differ. Inoculation by MRSA induced the expression of various pro-inflammatory cytokines and chemokines (IL-6 and IL-8) and antimicrobial peptides (human beta-defensin (hBD)-2, hBD-3 and RNase7) in HSEs. However, wounding of the HSEs did not further enhance the induction of these cytokines/AMPs as compared to colonization alone (**chapter 2**). This is in agreement with the findings of others who investigated responses of keratinocyte monolayers exposed to inflammatory stimuli, heat-killed *S. aureus* and skin commensals [38-40] and in HSE after exposure to viable *S. aureus* [41, 42]. Moreover, it is reported that hBD-2 and -3 expression can be induced by superficial barrier disruption [43] and that these are upregulated in the wounds of burn patients [44]. It has been demonstrated that hBDs can be induced by (heat-inactivated) bacteria, including *P. aeruginosa* and *S. aureus*, and toxins like LPS and cytokines like TNF- α [45, 46]. Finally, it has been shown that RNase7 can be induced by heat-killed *S. aureus* in primary keratinocytes [47] and human skin explants [48], and that RNase7 has a role in killing skin colonizing *S. aureus* [48].

HBDs and RNase7 are proven to have antimicrobial activity against (MDR) *S. aureus*, but also Gram-negative bacteria and *Candida*. HBD-3 has a 90% lethal dose (LD₉₀), the concentration that kills 90% of colony forming units, of 2.5-4 $\mu\text{g/ml}$ [46], RNase7 a LD₉₀ of 3-6 $\mu\text{g/ml}$ [48] and hBD-2 keeps the number of CFU static at 100 $\mu\text{g/ml}$ [49]. We analyzed if supernatants produced by HSE were able to kill *S. aureus/P. aeruginosa*. However, we did not observe any killing activity (unpublished data). This may be due to low concentrations of the AMPs present in the supernatant.

2.2 Use of EMs to study colonization and biofilm formation

To investigate differences in biofilm formation on biotic and abiotic surfaces we used EMs. We studied the formation of biofilms of five different *S. aureus* strains on the SC compared to that on an abiotic surface, polystyrene. We screened for the presence of 52 proteins by *S. aureus* in biofilms, and found that six proteins in *S. aureus*, including alpha-toxin were differentially expressed between biofilm on abiotic and biotic surfaces. The detection of several toxins, gamma-hemolysin-B (HlgB), leukotoxins (LUK) -D and -E and alpha toxin in biofilms of multiple strains specifically on EMs suggests that the surface to which the bacteria adhere influences the expression of these bacterial proteins (**chapter 7**). This is confirmed

by others, who have also demonstrated a role for alpha-toxin in the promotion of biofilm development on mucosal membranes [50].

This implicates that currently used biofilm models, such as those using polystyrene plates, might not adequately reflect biofilm formation on a more complex surface such as EMs and, ultimately, the human skin. Others have started research on growth of bacteria on SC, by using human callus as a substrate for the growth of bacteria [51]. By creating an assay using bioluminescent bacteria in a 96-well plate, they created a high throughput screening assay that can be used for the screening of antimicrobial compounds. The results of this study could be used to specifically target biofilm components that are expressed on biotic surfaces like skin and mucosae, or to prevent biofilm development, e.g., the inhibition of quorum sensing to disturb formation of biofilms [10].

2.3 Use of EM to study effect of the atopic dermatitis characteristics on colonization

One of the advantages of using cell models is the ability to knock-down genes to investigate the effect of specific genes on barrier function, colonization or immune responses. Seventy to ninety percent of the AD patients are colonized with *S. aureus* and harbour biofilms [52], which is far more than the general population (where around 20% of people carry *S. aureus*). Although the exact causes of AD are still uncertain, filaggrin (FLG) mutations and overexpression of IL-31 [53] are associated with the occurrence of AD. To study the effect of *S. aureus* colonization on AD skin, we introduced FLG knockdown into our EMs, and/or supplemented the models with IL-31. We demonstrated that knockdown of FLG and/or supplementation with IL-31 leads to higher *S. aureus* colonization, which is in line with the association between AD and *S. aureus* colonization in patients. Moreover, both knockdown of FLG and IL-31 supplementation lead to a *S. aureus*-induced up-regulation of IL-8 mRNA. IL-31 prevented the up-regulation of *S. aureus*-induced AMP expression (hBD-2,-3 and RNase7), whereas FLG knockdown did not, indicating a role for AMPs in the suppression of *S. Aureus* outgrowth. Furthermore, *S. aureus* colonization induced changes in mRNA expression of enzymes involved in cornification of the epidermis. For example in the expression of fatty acid elongase 4 (ELOVL4). ELOVL4 is involved in free fatty acid synthesis, and has been shown to be important for the skin barrier function. Absence of ELOVL4 resulted in impaired barrier function in mice [54]. We found that ELOVL4 expression was up-regulated in response to *S. aureus* colonization, irrespective of FLG-KD, but this was prevented by IL-31 (**chapter 6**). This together may demonstrate why for example extensive washing with soap,

which removes the upper layer of the SC (free fatty acids, AMPs, etc), is also associated with AD and eczema.

In a study where *S. aureus* colonization between AD patients and healthy volunteers were compared, genes like *lukD*, *lukE*, *splB*, *ssl8*, and *sasG* were more frequently found in *S. aureus* isolated from AD patients than from healthy volunteers [55]. All these genes are associated with *S. aureus* virulence. The correlation between AD and bacterial colonization can be further investigated using this model, for example by examining the presence of staphylococcal superantigens, or other virulence genes on AD skin.

3. AMPs as treatment for topical infections

3.1 AMP as alternatives for antibiotics

In 2016, 2235 antibacterial AMPs have been identified according to the antimicrobial peptide database (APD) (<http://aps.unmc.edu/AP/main.php>). There are more than 100 peptide-based drugs for various diseases on the market and almost 500-600 candidates in the preclinical trials, many of which are synthetic AMPs [56]. The first AMP used as a therapeutic agent was Gramicidin, a peptide isolated from a soil bacterium *Bacillus brevis* in 1939 [57]. Gramicidin is almost exclusively active against Gram-positive bacteria. Gramicidin D and Gramicidin S, the cyclic form, are approved by the FDA as topical agents. Due to haemolytic activity at low concentrations they cannot be used as systemic agents. Secondly, polymyxins B and E (Colistin), both polypeptide antibiotics that were discovered in the 1940s, are used as topical and intravenous formulations against mainly Gram-negative bacteria. Although they were mostly abandoned in the 1980s due to reported nephrotoxicity and neurotoxicity [58, 59], in the past few years there has been more attention as a last resort antibiotic because of the emergence of MDR and XDR strains [60]. Colistin has been effective in treating infections, such as pneumonia or bacteraemia, caused by for example *Pseudomonas* or *Acinetobacter* species [61]. Moreover, in combination therapy it can be used to treat biofilms in cystic fibrosis (CF) patients [62]. Finally, Polymyxin B has been used to treat urinary tract infections and meningitis caused by *P. aeruginosa* and *Haemophilus influenza* [59, 60]. There are AMPs, such as omiganan, pexiganan and iseganan that successfully have completed phase III trials, but were denied approval by FDA for clinical use because of lack of advantage over the existing medications [63].

Table1: AMPs in clinical trials or used in the clinic.

AMP	Description	Indication	Development Phase	Clinical trail	Ref
Gramicidin	Gramicidin D and Gramicidin S, the cyclic form	Topical treatment of eye infections	Clinical use		[60]
Colistin	Cationic peptides polymyxin B and E	Clinical use of diverse indications	Clinical use		[64]
Polymyxin B	Polypeptide antibiotic	Last option antibiotic for MDR strains	Clinical use		[58]
Pexiganan	Synthetic analogue of magainin 2 derived from frog skin	Treatment of diabetic foot ulcers with 0.8% cream Locilex	PIII – not approved by FDA	NCT00563433 NCT00563394	[65-67]
Omiganan (Migenix MX-226)	Synthetic cationic peptide derived from indolicidin	Bactericidal, antifungal, catheter associated infections and rosacea	PIII	NCT01784133	[68-70]
		1% gel has been used as a topical antimicrobial	PIIIb	NCT00608959	
hLF1-11	Cationic peptide composed of amino-terminal amino acids 1–11 of human lactoferricin	Allogeneic bone marrow stem cell transplantation–associated infections	PII (terminated)	NCT00509938	[71]
LL-37	Human cathelicidin	Venous leg ulcers	FIM	Eudract-2012-002100-41	[72]
OP-145 (P60.4Ac)	Synthetic 24-mer peptide derived from LL-37 for binding to lipopolysaccharides or lipoteichoic acid	Otitis media	PII		[73]
PMX-30063/Brilacidin	Arylamide oligomer mimetic of a defensin, structure not disclosed	Acute bacterial skin infection caused by <i>S. aureus</i>	PIIb	NCT020388 NCT02324335	[74]
					[75]

Table1: AMPs in clinical trials or used in the clinic. (continued)

AMP	Description	Indication	Development Phase	Clinical trail	Ref
Iseganan (IB-367)	Synthetic 17-mer peptide derived from protegrin 1	Oral mucositis as a mouth rinse against polymicrobial infections in patients treated with chemotherapy	PIII (failed)	NCT00022373	[76-78]
rBP121	Bactericidal/permeability-increasing protein (BPI) derivative	Burn Injury	PIIa	NCT00462904	[79]
LTX-109	Lytic broad spectrum peptide, protected for protease cleavage	Topical, nasal decolonization of methicillin-resistant and -sensitive <i>S. aureus</i> in humans	PI/IIa	NCT01158235	[80]
CZEN-002	derived from alpha-Melanocyte-Stimulating Hormone	Vulvovaginal candidiasis (vaginal)	PII		[81, 82]
XMP-629	BPI derivate	Acne (topical gel formulation)	PII (not continued)		[83]

Development phase (P), first in man (FIM). adapted and updated from [84].

3.2 The use of skin models for the testing of anti-infective drugs

Currently, the *in vivo* active concentrations of a novel antimicrobial compounds is often first associated with the minimal concentration that inhibits growth of a micro-organism *in vitro*, the MIC. The growth of the bacteria in such an assay depends among others on the type of culture medium (often a special bacterial growth agar, Muller-Hinton), which often differs considerably from the conditions in the patient [85]. Moreover, the presence of biofilm-associated infections is not taken into account in this assay. In addition, *in vitro* models involving biotic surfaces are required for studying bacterial biofilm formation. Therefore use of *in vitro* skin models (either HSEs or EMs) for the assessment of novel local topical therapies will have benefits over other *in vitro* systems, like medium or agar. It takes into account the local environment of the pathogen (instead of a standard culture medium or agar) and biofilm formation (table 2). Apart from the local environment of the pathogen, the pharmacokinetics of the drug should also be taken into account. The read-out for pharmacokinetic data generally use the blood (serum/plasma). However, for topical infections it is the local drug concentration in the skin that is relevant for the eradication of the pathogen. The use of skin models to screen for locally applied drugs may result in a more accurate determination of a dosing regimen to treat these type of infections clinically [86].

3.3 Use of skin models to test AMPs

In this thesis, we demonstrated the antimicrobial and anti-biofilm activity of 2nd and 3rd generation synthetic derivatives of the human AMP LL-37. LL-37 has been shown to have antimicrobial, immunomodulatory, anti-biofilm activities, acts as a growth factor during wound healing and has chemotactic ability (44,67-70) (figure 1). In former studies, P60.4Ac, a synthetic derivative of LL-37 has been described to poses higher antimicrobial activity towards Gram-negative bacteria than LL-37, while keeping immunomodulatory functions like toxin (LPS, LTA) neutralization [88]. Also, P60.4Ac has been shown to have additive value in the treatment of patients with chronic otitis media, showing increased efficacy compared to conventional antibiotics [74]. In this thesis, we demonstrated the antimicrobial activity of several new generations of AMPs derived from LL-37 against (MRD/PDR) *S. aureus*, *Klebsiella pneumonia* and *Pseudomonas aeruginosa*. We showed that P10 was the most potent in eradicating MRSA, not only in an *in vitro* killing assay, but also from HSEs. Moreover, we demonstrated that P10 could eradicate established biofilms from EMs (**chapter 3**). Newer generation derivatives from LL-37, P145, P148 and P276 proved to be effective against colonization of EMs with XDR and PDR strains, including a *P. aeruginosa* strain (**chapter 4**). Moreover, we demonstrated that these peptides were also active

against *Mycobacterium tuberculosis* (data not included in this thesis). These results demonstrate the potential of these LL-37-derived peptides as novel treatments against topical infections.

Table 2: Advantages and disadvantages of the use of HSEs as screening tool for novel antimicrobial agents [86, 87].

	Skin models	In vitro MIC assay in 96-well format
Route of administration	Screen for local topical application	Used as model for systemic and local
Representation of bacterial state	Closely mimics the pathogens habitat, e.g. by letting the bacteria for a biofilm	Does not represent the habitat of the pathogen, screens while the bacteria is in suspension/ on agar plate. Medium for MIC tests is broth, which does not exist in any animal or human body.
Through-put	EMs most high throughput	High through-put
Dose-response	Mimics local topical concentrations of the anti-infective drug	Concentrations simulated are either the blood-drug concentrations or the drug-tissue concentrations. However, since these models do not simulate the host immune defense system (they only simulate the antibiotic concentration as a function of time) the end point is the eradication of bacteria.
Disease model	Can use disease models, e.g. for Atopic dermatitis or a wound model	-
Test combination therapies	Yes	Yes
Toxicity	Toxicity, irritation, sensitization and local immunity can also be tested	Give no information about toxicity for human cells
Costs	Expensive	Cheap

3.4.1 Formulation development for peptide antimicrobials

As described above, **chapter 4** demonstrates the activity of the synthetic antibacterial peptides P60.4Ac, P10, P145, P148 and P276 on HSEs. Moreover, these peptides may have additional effects like the parent peptide LL-37, such as

anti-inflammatory activities, pro-angiogenesis, chemotactic and enhancement of wound healing [89]. This makes these synthetic antimicrobial peptides promising candidates, either alone or in the combination with other antimicrobials [90], for the treatment of wound infections by (MDR) bacteria. To further assess the usage of these peptides as topical antimicrobials, we incorporated synthetic peptide P60.4Ac into different formulations and creams. In total two cream-based formulations and one gel was used. The antimicrobial activity of P60.4Ac in hypromellose-4000 gel proved to be the most potent. However, high peptide concentrations of this formulation (>0.5% wt/wt) also showed some toxic effects on the EMs, as mitochondrial activity was reduced and lactate dehydrogenase leakage was observed (**chapter 5**). Thus, it is important to improve safety of these peptides or search for novel peptides with reduced toxicity towards human cells.

3.4 Overcoming the limitations of AMPs

Compared to the number of papers and research efforts put into the therapeutic application of AMPs over the last decades, the number of successful clinical trials remains limited (table 1). There are a couple of reasons for this. First, the antimicrobial activity of natural AMPs under physiological conditions are limited. Many AMPs are sensitive to high salt concentrations [91], have short half lives *in vivo* (e.g., due to protease sensitivity) and limited bioavailability (binding to plasma proteins [71, 92]). While we find good antimicrobial effect of P60.4, P10 and P148 on the HSEs against a variety of (drug resistant) pathogens, we also find a reduced activity in plasma of P60.4 [93] and P10 (**chapter 4**). The 3rd generation peptides P145, P148 and P159, however, remained active in plasma (data not shown). Secondly, although AMPs have been described or claimed as molecules against which bacteria rarely develop resistance, some studies have described the development of resistance towards AMPs via several mechanisms, including modification of the bacterial surface, external trapping of AMPs, efflux pumps, proteolytic degradation, bacterial gene regulation and bacterial regulation of host AMP production [94-96] (table 3).

Table 3: Advantages and disadvantages of AMPs as (topical) drugs [56, 71, 97].

Advantages	Disadvantages
Broad spectrum activity	Costs of development / manufacturing
Rapid killing	Systemic and local toxicity
Suitable for topical application	Activity inhibition by salt, serum and pH
Anti-biofilm activity	Susceptible to proteolysis
Synergy with pharmaceuticals: antibiotics	Pharmacokinetic and pharmacodynamics issues, like a short half-life in vivo
	Sensitization to peptide
Biological other functions (wound healing, chemotaxis)	Natural resistance
	Biological other functions (chemotaxis, angiogenesis, wound healing)

Some of the limitations can be overcome by specific alterations to the peptides. This includes the addition of chemical groups like poly-ethylene glycol (PEG), which increases the stability of peptides *in vivo*; the use of retro-inverso peptides (stereochemical preparation of peptides) to prevent breakdown by enzymes [98]; different delivery systems like via liposomal delivery [99]. Wheat germ agglutinin (WGA)-modified liposomes encapsulating clarithromycin have been used to evaluate *in vitro* and *in vivo* efficacy against MRSA. The researchers found better bio-distribution of clarithromycin when formulated in WGA, and better *in vitro* and *in vivo* antibacterial efficacy against MRSA [99]. It has also been demonstrated that LL-37 can more efficiently protect against viral HPV infection in human epidermal model composed of immortalized keratinocytes when formulated into liposomes than when unformulated [100]. Moreover, multiple AMPs can be combined to get a synergistic effect (e.g., Gramicidin-S and Polymixin B against *P. aeruginosa*) [101]. Also, the combination of a conventional antibiotic and a peptide can be effective against biofilms, for example in the lungs of CF patients [62]. Finally, also the activity or expression of natural AMPs can be enhanced, as has been shown for butyrate [102]. Shigellosis can lead to a decreased LL-37 expression in the rectal epithelium. Butyrate treatment of Shigella-infected rabbits resulted in reduced clinical illness, reduced severity of inflammation in the colon, and reduced bacterial load in the stool, which was associated with a significant up-regulation of CAP-18 (the rabbit equivalent of LL-37) in the mucosal surface epithelium [17].

4. Conclusion

”For the first time, researchers have found a person in the United States carrying bacteria resistant to antibiotics of last resort, an alarming development that the top U.S. public health official says could mean “the end of the road” for antibiotics according to a recent article in the Washington post [103]. This paper emphasises that in the coming years the search for new treatment options to cope with infections caused by resistant bacteria is becoming more and more important.

HSEs can play an important role in the discovery and development of new drugs, as different aspects of these agents can be investigated, including antimicrobial effects, toxicity, effect on wound healing and initiation of host defense mechanisms. Currently, four reconstructed human EMs (Episkin, EpiDerm, SkinEthic, LabCyte EPI-MODEL) have been adopted by the Organization for Economic Cooperation and Development to provide alternative *in vitro* methods for classifying of irritants [104-107]. In the future, the use of HSEs could potentially be extended to that of a first line screening tool for new topical anti-infective agents. A start for this has been made by the use of the callus model [51] and the use of different models to assess the potency of AMPs as antimicrobials (**chapter 3,4,5**). Moreover, in this thesis we used one single pathogen to colonize the models, but also the interaction between different bacterial strains may be investigated, e.g. by the inclusion of commensal bacteria, or test for influence of the skin microbiome on disease phenotype, for example of AD skin [108]).

Although, the results of this thesis demonstrate the potential of the synthetic antibacterial peptides based on the sequence of LL-37, like P60.4Ac, P10, P145, P148 and P276, as novel anti-infective agents, the route from “bench to bedside” is a long one. As described in table 1, many AMPs that proved to be promising in the lab failed in clinical trials. Either due to effectiveness or added benefit from existing therapies. Moreover, these peptides have other roles in the human body like anti-inflammatory activities and enhancement of wound healing. Also the effectivity is depended on local the local environment, like salt concentrations. Therefore the question remains if the systemic use of AMPs will ever be possible. The in this thesis described local use of AMPs seems more obvious, this also in respect to their local expression pattern, e.g. in the skin. However, also in the development of synthetic peptides for local application a lot of knowledge needs to be gained.

References

1. Lionelli GT, Pickus EJ, Beckum OK, Decoursey RL, Korentager RA. A three decade analysis of factors affecting burn mortality in the elderly. *Burns*. 2005;31(8):958-63. doi: S0305-4179(05)00183-X [pii];10.1016/j.burns.2005.06.006 [doi].
2. Bloemsmas GC, Dokter J, Boxma H, Oen IM. Mortality and causes of death in a burn centre. *Burns*. 2008;34(8):1103-7. doi: S0305-4179(08)00071-5 [pii];10.1016/j.burns.2008.02.010 [doi].
3. Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *ClinMicrobiolInfect*. 2012;18(3):268-81. doi: 10.1111/j.1469-0691.2011.03570.x [doi];S1198-743X(14)61632-3 [pii].
4. World Health Organization. *Burns*. 2014.
5. Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin-Scott HM. Microbial biofilms. *AnnuRevMicrobiol*. 1995;49:711-45. doi: 10.1146/annurev.mi.49.100195.003431 [doi].
6. Hoiby N, Bjarnsholt T, Givskov M, Molin S, Ciofu O. Antibiotic resistance of bacterial biofilms. *IntJAntimicrobAgents*. 2010;35(4):322-32. doi: S0924-8579(10)00009-9 [pii];10.1016/j.ijantimicrob.2009.12.011 [doi].
7. Van Acker H, Van Dijck P, Coenye T. Molecular mechanisms of antimicrobial tolerance and resistance in bacterial and fungal biofilms. *Trends Microbiol*. 2014;22(6):326-33. doi: S0966-842X(14)00024-9 [pii];10.1016/j.tim.2014.02.001 [doi].
8. Jiang KC, Sun XJ, Wang W, Liu L, Cai Y, Chen YC, et al. Excretions/secretions from bacteria-pretreated maggot are more effective against *Pseudomonas aeruginosa* biofilms. *PLoSOne*. 2012;7(11):e49815. doi: 10.1371/journal.pone.0049815 [doi];PONE-D-12-18527 [pii].
9. van der Plas MJ, Dambrot C, Dogterom-Ballering HC, Kruithof S, van Dissel JT, Nibbering PH. Combinations of maggot excretions/secretions and antibiotics are effective against *Staphylococcus aureus* biofilms and the bacteria derived therefrom. *JAntimicrobChemother*. 2010;65(5):917-23. doi: dkq042 [pii];10.1093/jac/dkq042 [doi].
10. Brackman G, Coenye T. Inhibition of Quorum Sensing in *Staphylococcus* spp. *CurrPharmDes*. 2015;21(16):2101-8. doi: CPD-EPUB-65771 [pii].
11. Brackman G, Breyne K, De RR, Vermote A, Van NF, Meyer E, et al. The Quorum Sensing Inhibitor Hamamelitannin Increases Antibiotic Susceptibility of *Staphylococcus aureus* Biofilms by Affecting Peptidoglycan Biosynthesis and eDNA Release. *SciRep*. 2016;6:20321. doi: srep20321 [pii];10.1038/srep20321 [doi].
12. Donlan RM. Preventing biofilms of clinically relevant organisms using bacteriophage. *Trends Microbiol*. 2009;17(2):66-72. doi: S0966-842X(09)00004-3 [pii];10.1016/j.tim.2008.11.002 [doi].
13. Daniel A, Euler C, Collin M, Chahales P, Gorelick KJ, Fischetti VA. Synergism between a novel chimeric lysin and oxacillin protects against infection by methicillin-resistant *Staphylococcus aureus*. *AntimicrobAgents Chemother*. 2010;54(4):1603-12. doi: AAC.01625-09 [pii];10.1128/AAC.01625-09 [doi].
14. Fischetti VA. Bacteriophage lysins as effective antibacterials. *CurrOpinMicrobiol*. 2008;11(5):393-400. doi: S1369-5274(08)00129-X [pii];10.1016/j.mib.2008.09.012 [doi].

15. Pastagia M, Euler C, Chahales P, Fuentes-Duculan J, Krueger JG, Fischetti VA. A novel chimeric lysin shows superiority to mupirocin for skin decolonization of methicillin-resistant and -sensitive *Staphylococcus aureus* strains. *Antimicrob Agents Chemother*. 2011;55(2):738-44. doi: AAC.00890-10 [pii];10.1128/AAC.00890-10 [doi].
16. Nelson D, Loomis L, Fischetti VA. Prevention and elimination of upper respiratory colonization of mice by group A streptococci by using a bacteriophage lytic enzyme. *Proc Natl Acad Sci USA*. 2001;98(7):4107-12. doi: 10.1073/pnas.061038398 [doi];061038398 [pii].
17. Raqib R, Sarker P, Bergman P, Ara G, Lindh M, Sack DA, et al. Improved outcome in shigellosis associated with butyrate induction of an endogenous peptide antibiotic. *Proc Natl Acad Sci USA*. 2006;103(24):9178-83. doi: 0602888103 [pii];10.1073/pnas.0602888103 [doi].
18. Ling LL, Schneider T, Peoples AJ, Spoering AL, Engels I, Conlon BP, et al. A new antibiotic kills pathogens without detectable resistance. *Nature*. 2015;517(7535):455-9. Epub 2015/01/07. doi: 10.1038/nature14098. PubMed PMID: 25561178.
19. Zipperer A, Konnerth MC, Laux C, Berscheid A, Janek D, Weidenmaier C, et al. Human commensals producing a novel antibiotic impair pathogen colonization. *Nature*. 2016;535(7613):511-6. doi: nature18634 [pii];10.1038/nature18634 [doi].
20. Lewis K, Strandwitz P. Microbiology: Antibiotics right under our nose. *Nature*. 2016;535(7613):501-2. doi: 535501a [pii];10.1038/535501a [doi].
21. Ponec M, Gibbs S, Pilgram G, Boelsma E, Koerten H, Bouwstra J, et al. Barrier function in reconstructed epidermis and its resemblance to native human skin. *Skin Pharmacol Appl Skin Physiol*. 2001;14 Suppl 1:63-71. doi: 56392 [pii];56392 [doi].
22. El Ghalbzouri A, Jonkman MF, Dijkman R, Ponec M. Basement membrane reconstruction in human skin equivalents is regulated by fibroblasts and/or exogenously activated keratinocytes. *J Invest Dermatol*. 2005;124(1):79-86. doi: JID23549 [pii];10.1111/j.0022-202X.2004.23549.x [doi].
23. Ahrens K, Schunck M, Podda GF, Meingassner J, Stuetz A, Schroder JM, et al. Mechanical and metabolic injury to the skin barrier leads to increased expression of murine beta-defensin-1, -3, and -14. *J Invest Dermatol*. 2011;131(2):443-52. doi: jid2010289 [pii];10.1038/jid.2010.289 [doi].
24. Mestas J, Hughes CC. Of mice and not men: differences between mouse and human immunology. *Journal of immunology (Baltimore, Md : 1950)*. 2004;172(5):2731-8. Epub 2004/02/24. PubMed PMID: 14978070.
25. van den Bogaard EH, Tjabringa GS, Joosten I, Vonk-Bergers M, van RE, Tijssen HJ, et al. Crosstalk between keratinocytes and T cells in a 3D microenvironment: a model to study inflammatory skin diseases. *J Invest Dermatol*. 2014;134(3):719-27. doi: S0022-202X(15)36647-1 [pii];10.1038/jid.2013.417 [doi].
26. Kosten IJ, Spiekstra SW, de Gruijl TD, Gibbs S. MUTZ-3 derived Langerhans cells in human skin equivalents show differential migration and phenotypic plasticity after allergen or irritant exposure. *Toxicol Appl Pharmacol*. 2015;287(1):35-42. doi: S0041-008X(15)00197-0 [pii];10.1016/j.taap.2015.05.017 [doi].
27. Safferling K, Sutterlin T, Westphal K, Ernst C, Breuhahn K, James M, et al. Wound healing revised: a novel reepithelialization mechanism revealed by in vitro and in silico models. *The Journal of cell biology*.

- 2013;203(4):691-709. Epub 2014/01/05. doi: 10.1083/jcb.201212020. PubMed PMID: 24385489; PubMed Central PMCID: PMC3840932.
28. Menon SN, Flegg JA, McCue SW, Schugart RC, Dawson RA, McElwain DLS. Modelling the interaction of keratinocytes and fibroblasts during normal and abnormal wound healing processes. *Proceedings of the Royal Society B: Biological Sciences*. 2012;279(1741):3329-38. doi: 10.1098/rspb.2012.0319. PubMed PMID: 22628464; PubMed Central PMCID: PMC3385718.
29. Wang Z, Wang Y, Farhangfar F, Zimmer M, Zhang Y. Enhanced Keratinocyte Proliferation and Migration in Co-culture with Fibroblasts. *PLoS One*. 2012;7(7). doi: 10.1371/journal.pone.0040951. PubMed PMID: 22911722; PubMed Central PMCID: PMC3401236.
30. Falanga V, Isaacs C, Paquette D, Downing G, Kouttab N, Butmarc J, et al. Wounding of bioengineered skin: cellular and molecular aspects after injury. *The Journal of investigative dermatology*. 2002;119(3):653-60. Epub 2002/09/17. doi: 10.1046/j.1523-1747.2002.01865.x. PubMed PMID: 12230509.
31. Garlick JA, Taichman LB. Fate of human keratinocytes during reepithelialization in an organotypic culture model. *Lab Invest*. 1994;70(6):916-24. Epub 1994/06/01. PubMed PMID: 8015295.
32. Egles C, Garlick JA, Shamis Y. Three-dimensional human tissue models of wounded skin. *Methods in molecular biology (Clifton, NJ)*. 2010;585:345-59. Epub 2009/11/13. doi: 10.1007/978-1-60761-380-0_24. PubMed PMID: 19908015; PubMed Central PMCID: PMC3076317.
33. Ali N, Hosseini M, Vainio S, Taieb A, Cario-Andre M, Rezvani HR. Skin equivalents: skin from reconstructions as models to study skin development and diseases. *The British journal of dermatology*. 2015;173(2):391-403. Epub 2015/05/06. doi: 10.1111/bjd.13886. PubMed PMID: 25939812.
34. Sullivan TP, Eaglstein WH, Davis SC, Mertz P. The pig as a model for human wound healing. *Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society*. 2001;9(2):66-76. Epub 2001/05/15. PubMed PMID: 11350644.
35. Alrobaiea SM, Ding J, Ma Z, Tredget EE. A Novel Nude Mouse Model of Hypertrophic Scarring Using Scratched Full Thickness Human Skin Grafts. *Advances in wound care*. 2016;5(7):299-313. Epub 2016/07/02. doi: 10.1089/wound.2015.0670. PubMed PMID: 27366591; PubMed Central PMCID: PMC4900225.
36. Wang X, Ghasri P, Amir M, Hwang B, Hou Y, Khalili M, et al. Topical application of recombinant type VII collagen incorporates into the dermal-epidermal junction and promotes wound closure. *Molecular therapy : the journal of the American Society of Gene Therapy*. 2013;21(7):1335-44. Epub 2013/05/15. doi: 10.1038/mt.2013.87. PubMed PMID: 23670575; PubMed Central PMCID: PMC3704128.
37. Guenou H, Nissan X, Larcher F, Feteira J, Lemaître G, Saidani M, et al. Human embryonic stem-cell derivatives for full reconstruction of the pluristratified epidermis: a preclinical study. *Lancet*. 2009;374(9703):1745-53. Epub 2009/11/26. doi: 10.1016/s0140-6736(09)61496-3. PubMed PMID: 19932355.
38. Sasaki T, Kano R, Sato H, Nakamura Y, Watanabe S, Hasegawa A. Effects of staphylococci on cytokine production from human keratinocytes. *BrJ Dermatol*. 2003;148(1):46-50. doi: 5017 [pii].

39. Sorensen OE, Thapa DR, Rosenthal A, Liu L, Roberts AA, Ganz T. Differential regulation of beta-defensin expression in human skin by microbial stimuli. *JImmunol.* 2005;174(8):4870-9. doi: 174/8/4870 [pii].
40. Wanke I, Steffen H, Christ C, Krismer B, Gotz F, Peschel A, et al. Skin commensals amplify the innate immune response to pathogens by activation of distinct signaling pathways. *JInvest Dermatol.* 2011;131(2):382-90. doi: jid2010328 [pii];10.1038/jid.2010.328 [doi].
41. Holland DB, Bojar RA, Farrar MD, Holland KT. Differential innate immune responses of a living skin equivalent model colonized by *Staphylococcus epidermidis* or *Staphylococcus aureus*. *FEMS Microbiol-Lett.* 2009;290(2):149-55. doi: FML1402 [pii];10.1111/j.1574-6968.2008.01402.x [doi].
42. Ommori R, Ouji N, Mizuno F, Kita E, Ikada Y, Asada H. Selective induction of antimicrobial peptides from keratinocytes by staphylococcal bacteria. *MicrobPathog.* 2012. doi: S0882-4010(12)00197-0 [pii];10.1016/j.micpath.2012.11.005 [doi].
43. Harder J, Dressel S, Wittersheim M, Cordes J, Meyer-Hoffert U, Mrowietz U, et al. Enhanced expression and secretion of antimicrobial peptides in atopic dermatitis and after superficial skin injury. *JInvest Dermatol.* 2010;130(5):1355-64. doi: jid2009432 [pii];10.1038/jid.2009.432 [doi].
44. Poindexter BJ, Bhat S, Buja LM, Bick RJ, Milner SM. Localization of antimicrobial peptides in normal and burned skin. *Burns.* 2006;32(4):402-7. doi: S0305-4179(06)00031-3 [pii];10.1016/j.burns.2006.01.021 [doi].
45. Chadebecq P, Goidin D, Jacquet C, Viac J, Schmitt D, Staquet M-J. Use of human reconstructed epidermis to analyze the regulation of β -defensin hBD-1, hBD-2, and hBD-3 expression in response to LPS. *Cell Biology and Toxicology.* 2003;19(5):313-24. doi: 10.1023/B:CBTO.0000004975.36521.c8.
46. Harder J, Bartels J, Christophers E, Schroder JM. Isolation and characterization of human beta-defensin-3, a novel human inducible peptide antibiotic. *The Journal of biological chemistry.* 2001;276(8):5707-13. Epub 2000/11/22. doi: 10.1074/jbc.M008557200. PubMed PMID: 11085990.
47. Harder J, Schroder JM. RNase 7, a novel innate immune defense antimicrobial protein of healthy human skin. *The Journal of biological chemistry.* 2002;277(48):46779-84. Epub 2002/09/24. doi: 10.1074/jbc.M207587200. PubMed PMID: 12244054.
48. Simanski M, Dressel S, Glaser R, Harder J. RNase 7 protects healthy skin from *Staphylococcus aureus* colonization. *The Journal of investigative dermatology.* 2010;130(12):2836-8. Epub 2010/07/30. doi: 10.1038/jid.2010.217. PubMed PMID: 20668470.
49. Schneider JJ, Unholzer A, Schaller M, Schäfer-Korting M, Korting HC. Human defensins. *Journal of Molecular Medicine.* 2005;83(8):587-95. doi: 10.1007/s00109-005-0657-1.
50. Anderson MJ, Lin YC, Gillman AN, Parks PJ, Schlievert PM, Peterson ML. Alpha-toxin promotes *Staphylococcus aureus* mucosal biofilm formation. *Front Cell InfectMicrobiol.* 2012;2:64. doi: 10.3389/fcimb.2012.00064 [doi].
51. van der Krieken DA, Ederveen TH, van Hijum SA, Jansen PA, Melchers WJ, Scheepers PT, et al. An In vitro Model for Bacterial Growth on Human Stratum Corneum. *Acta DermVenereol.* 2016. doi: 10.2340/00015555-2401 [doi].

52. Roll A, Cozzio A, Fischer B, Schmid-Grendelmeier P. Microbial colonization and atopic dermatitis. *Curr OpinAllergy ClinImmunol*. 2004;4(5):373-8. doi: 00130832-200410000-00008 [pii].
53. Neis MM, Peters B, Dreuw A, Wenzel J, Bieber T, Mauch C, et al. Enhanced expression levels of IL-31 correlate with IL-4 and IL-13 in atopic and allergic contact dermatitis. *JAllergy ClinImmunol*. 2006;118(4):930-7. doi: S0091-6749(06)01517-X [pii];10.1016/j.jaci.2006.07.015 [doi].
54. Li W, Sandhoff R, Kono M, Zerfas P, Hoffmann V, Ding BC, et al. Depletion of ceramides with very long chain fatty acids causes defective skin permeability barrier function, and neonatal lethality in ELOVL4 deficient mice. *International journal of biological sciences*. 2007;3(2):120-8. Epub 2007/02/22. PubMed PMID: 17311087; PubMed Central PMCID: PMCPMC1796950.
55. Rojo A, Aguinaga A, Monecke S, Yuste JR, Gastaminza G, Espana A. Staphylococcus aureus genomic pattern and atopic dermatitis: may factors other than superantigens be involved? *EurJClinMicrobiolInfectDis*. 2014;33(4):651-8. doi: 10.1007/s10096-013-2000-z [doi].
56. Craik DJ, Fairlie DP, Liras S, Price D. The future of peptide-based drugs. *ChemBiolDrug Des*. 2013;81(1):136-47. doi: 10.1111/cbdd.12055 [doi].
57. Dubos RJ. STUDIES ON A BACTERICIDAL AGENT EXTRACTED FROM A SOIL BACILLUS : I. PREPARATION OF THE AGENT. ITS ACTIVITY IN VITRO. *JExpMed*. 1939;70(1):1-10.
58. Kelesidis T, Falagas ME. The safety of polymyxin antibiotics. *Expert OpinDrug Saf*. 2015;14(11):1687-701. doi: 10.1517/14740338.2015.1088520 [doi].
59. Falagas ME, Kasiakou SK. Colistin: the revival of polymyxins for the management of multidrug-resistant gram-negative bacterial infections. *ClinInfectDis*. 2005;40(9):1333-41. doi: CID35354 [pii];10.1086/429323 [doi].
60. Nation RL, Velkov T, Li J. Colistin and polymyxin B: peas in a pod, or chalk and cheese? *ClinInfectDis*. 2014;59(1):88-94. doi: ciu213 [pii];10.1093/cid/ciu213 [doi].
61. Tre-Hardy M, Vanderbist F, Traore H, Devleeschouwer MJ. In vitro activity of antibiotic combinations against *Pseudomonas aeruginosa* biofilm and planktonic cultures. *IntJAntimicrobAgents*. 2008;31(4):329-36. doi: S0924-8579(08)00013-7 [pii];10.1016/j.ijantimicag.2007.12.005 [doi].
62. Herrmann G, Yang L, Wu H, Song Z, Wang H, Hoiby N, et al. Colistin-tobramycin combinations are superior to monotherapy concerning the killing of biofilm *Pseudomonas aeruginosa*. *JInfectDis*. 2010;202(10):1585-92. doi: 10.1086/656788 [doi].
63. Yeung AT, Gellatly SL, Hancock RE. Multifunctional cationic host defence peptides and their clinical applications. *Cell MolLife Sci*. 2011;68(13):2161-76. doi: 10.1007/s00018-011-0710-x [doi].
64. Biswas S, Brunel JM, Dubus JC, Reynaud-Gaubert M, Rolain JM. Colistin: an update on the antibiotic of the 21st century. *ExpertRevAntiInfectTher*. 2012;10(8):917-34. doi: 10.1586/eri.12.78 [doi].
65. Ge Y, MacDonald DL, Holroyd KJ, Thornsberry C, Wexler H, Zasloff M. In vitro antibacterial properties of pexiganan, an analog of magainin. *AntimicrobAgents Chemother*. 1999;43(4):782-8.
66. Lamb HM, Wiseman LR. Pexiganan acetate. *Drugs*. 1998;56(6):1047-52.

67. Lipsky BA, Holroyd KJ, Zasloff M. Topical versus systemic antimicrobial therapy for treating mildly infected diabetic foot ulcers: a randomized, controlled, double-blinded, multicenter trial of pexiganan cream. *Clin Infect Dis*. 2008;47(12):1537-45. doi: 10.1086/593185 [doi].
68. Fritsche TR, Rhomberg PR, Sader HS, Jones RN. Antimicrobial activity of omiganan pentahydrochloride against contemporary fungal pathogens responsible for catheter-associated infections. *Antimicrob Agents Chemother*. 2008;52(3):1187-9. doi: AAC.01475-07 [pii];10.1128/AAC.01475-07 [doi].
69. Rubinchik E, Dugourd D, Algara T, Pasetka C, Friedland HD. Antimicrobial and antifungal activities of a novel cationic antimicrobial peptide, omiganan, in experimental skin colonisation models. *Int J Antimicrob Agents*. 2009;34(5):457-61. doi: S0924-8579(09)00220-9 [pii];10.1016/j.ijantimicag.2009.05.003 [doi].
70. Vemuri RC, Gundamaraju R, Sekaran SD, Manikam R. Major pathophysiological correlations of rosacea: a complete clinical appraisal. *Int J Med Sci*. 2015;12(5):387-96. doi: 10.7150/ijms.10608 [doi];ijmsv12p0387 [pii].
71. Brouwer CP, Rahman M, Welling MM. Discovery and development of a synthetic peptide derived from lactoferrin for clinical use. *Peptides*. 2011;32(9):1953-63. doi: S0196-9781(11)00305-6 [pii];10.1016/j.peptides.2011.07.017 [doi].
72. Velden WJ, van Iersel TM, Blijlevens NM, Donnelly JP. Safety and tolerability of the antimicrobial peptide human lactoferrin 1-11 (hLF1-11). *BMC Med*. 2009;7:44. doi: 1741-7015-7-44 [pii];10.1186/1741-7015-7-44 [doi].
73. Gronberg A, Mahlapuu M, Stahle M, Whately-Smith C, Rollman O. Treatment with LL-37 is safe and effective in enhancing healing of hard-to-heal venous leg ulcers: a randomized, placebo-controlled clinical trial. *Wound Repair Regen*. 2014;22(5):613-21. doi: 10.1111/wrr.12211 [doi].
74. Peek FAW, Nell MJ, Brand R, Jansen-Werkhoven TM, van Hoogdalem EJ, Frijns JHM. Double-Blind Placebo-Controlled Study of the Novel Peptide Drug P60.4AC in Chronic Middle Ear Infection. *ICAAC-abstract*. 2009;ICAAC(L1-337).
75. Scott RW, DeGrado WF, Tew GN. De novo designed synthetic mimics of antimicrobial peptides. *Curr Opin Biotechnol*. 2008;19(6):620-7. doi: S0958-1669(08)00143-2 [pii];10.1016/j.copbio.2008.10.013 [doi].
76. Trotti A, Garden A, Warde P, Symonds P, Langer C, Redman R, et al. A multinational, randomized phase III trial of iseganan HCl oral solution for reducing the severity of oral mucositis in patients receiving radiotherapy for head-and-neck malignancy. *Int J Radiat Oncol Biol Phys*. 2004;58(3):674-81. doi: 10.1016/S0360-3016(03)01627-4 [doi];S0360301603016274 [pii].
77. Mosca DA, Hurst MA, So W, Viajar BS, Fujii CA, Falla TJ. IB-367, a protegrin peptide with in vitro and in vivo activities against the microflora associated with oral mucositis. *Antimicrobial agents and chemotherapy*. 2000;44(7):1803-8. Epub 2000/06/20. PubMed PMID: 10858334; PubMed Central PMCID: PMCPMC89965.
78. Trotti A, Garden A, Warde P, Symonds P, Langer C, Redman R, et al. A multinational, randomized phase III trial of iseganan HCl oral solution for reducing the severity of oral mucositis in patients receiving radiotherapy for head-and-neck malignancy. *International journal of radiation oncology, biology, phys-*

- ics. 2004;58(3):674-81. Epub 2004/02/18. doi: 10.1016/s0360-3016(03)01627-4. PubMed PMID: 14967419.
79. Domingues MM, Santos NC, Castanho MA. Antimicrobial peptide rBPI21: a translational overview from bench to clinical studies. *CurrProtein PeptSci*. 2012;13(7):611-9. doi: CPPS-EPUB-20121030-2 [pii].
80. Nilsson AC, Janson H, Wold H, Fugelli A, Andersson K, Hakangard C, et al. LTX-109 is a novel agent for nasal decolonization of methicillin-resistant and -sensitive *Staphylococcus aureus*. *AntimicrobAgents Chemother*. 2015;59(1):145-51. doi: AAC.03513-14 [pii];10.1128/AAC.03513-14 [doi].
81. Cutuli M, Cristiani S, Lipton JM, Catania A. Antimicrobial effects of alpha-MSH peptides. *Journal of leukocyte biology*. 2000;67(2):233-9. Epub 2000/02/12. PubMed PMID: 10670585.
82. Csato M, Kenderessy AS, Dobozy A. Enhancement of *Candida albicans* killing activity of separated human epidermal cells by alpha-melanocyte stimulating hormone. *The British journal of dermatology*. 1989;121(1):145-7. Epub 1989/07/01. PubMed PMID: 2547421.
83. Lambert LH, Vanhove GFA. Use of xmp-629 for the treatment of acne. Google Patents; 2005.
84. Fjell CD, Hiss JA, Hancock RE, Schneider G. Designing antimicrobial peptides: form follows function. *NatRevDrug Discov*. 2012;11(1):37-51. doi: nrd3591 [pii];10.1038/nrd3591 [doi].
85. Mattie H. Antibiotic efficacy in vivo predicted by in vitro activity. *International journal of antimicrobial agents*. 2000;14(2):91-8. Epub 2000/03/18. PubMed PMID: 10720797.
86. Nightingale CH. Future in vitro and animal studies: development of pharmacokinetic and pharmacodynamic efficacy predictors for tissue-based antibiotics. *Pharmacotherapy*. 2005;25(12 Pt 2):146s-9s. Epub 2005/11/25. doi: 10.1592/phco.2005.25.12part2.146S. PubMed PMID: 16305285.
87. Mueller M, de la Pena A, Derendorf H. Issues in pharmacokinetics and pharmacodynamics of anti-infective agents: kill curves versus MIC. *Antimicrobial agents and chemotherapy*. 2004;48(2):369-77. Epub 2004/01/27. PubMed PMID: 14742182; PubMed Central PMCID: PMCPMC321563.
88. Nell MJ, Tjabringa GS, Wafelman AR, Verrijck R, Hiemstra PS, Drijfhout JW, et al. Development of novel LL-37 derived antimicrobial peptides with LPS and LTA neutralizing and antimicrobial activities for therapeutic application. *Peptides*. 2006;27(4):649-60. doi: S0196-9781(05)00467-5 [pii];10.1016/j.peptides.2005.09.016 [doi].
89. Hancock RE, Haney EF, Gill EE. The immunology of host defence peptides: beyond antimicrobial activity. *Nature reviews Immunology*. 2016;16(5):321-34. Epub 2016/04/19. doi: 10.1038/nri.2016.29. PubMed PMID: 27087664.
90. Mataraci E, Dosler S. In vitro activities of antibiotics and antimicrobial cationic peptides alone and in combination against methicillin-resistant *Staphylococcus aureus* biofilms. *AntimicrobAgents Chemother*. 2012;56(12):6366-71. doi: AAC.01180-12 [pii];10.1128/AAC.01180-12 [doi].
91. Veldhuizen EJ, Schneider VA, Agustiandari H, van DA, Tjeerdsma-van Bokhoven JL, Bikker FJ, et al. Antimicrobial and immunomodulatory activities of PR-39 derived peptides. *PLoSOne*. 2014;9(4):e95939. doi: 10.1371/journal.pone.0095939 [doi];PONE-D-13-44237 [pii].

92. Svenson J, Brandsdal BO, Stensen W, Svendsen JS. Albumin binding of short cationic antimicrobial micropeptides and its influence on the in vitro bactericidal effect. *JMedChem*. 2007;50(14):3334-9. doi: 10.1021/jm0703542 [doi].
93. de Breij A, Riool M, Kwakman PH, de Boer L, Cordfunke RA, Drijfhout JW, et al. Prevention of *Staphylococcus aureus* biomaterial-associated infections using a polymer-lipid coating containing the antimicrobial peptide OP-145. *Journal of controlled release : official journal of the Controlled Release Society*. 2016;222:1-8. Epub 2015/12/15. doi: 10.1016/j.jconrel.2015.12.003. PubMed PMID: 26658071.
94. Nizet V. Antimicrobial peptide resistance mechanisms of human bacterial pathogens. *CurrIssues Mol-Biol*. 2006;8(1):11-26.
95. Moffatt JH, Harper M, Harrison P, Hale JD, Vinogradov E, Seemann T, et al. Colistin resistance in *Acinetobacter baumannii* is mediated by complete loss of lipopolysaccharide production. *AntimicrobAgents Chemother*. 2010;54(12):4971-7. doi: AAC.00834-10 [pii];10.1128/AAC.00834-10 [doi].
96. Yeaman MR, Yount NY. Mechanisms of antimicrobial peptide action and resistance. *PharmacolRev*. 2003;55(1):27-55. doi: 10.1124/pr.55.1.2 [doi].
97. Stempel N, Strehmel J, Overhage J. Potential application of antimicrobial peptides in the treatment of bacterial biofilm infections. *CurrPharmDes*. 2015;21(1):67-84. doi: CPD-EPUB-62175 [pii].
98. Chorev M, Goodman M. Recent developments in retro peptides and proteins--an ongoing topochemical exploration. *Trends in biotechnology*. 1995;13(10):438-45. Epub 1995/10/01. doi: 10.1016/s0167-7799(00)88999-4. PubMed PMID: 7546569.
99. Meng Y, Hou X, Lei J, Chen M, Cong S, Zhang Y, et al. Multi-functional Liposomes Enhancing Target and Antibacterial Immunity for Antimicrobial and Anti-Biofilm Against Methicillin-Resistant *Staphylococcus aureus*. *PharmRes*. 2016;33(3):763-75. doi: 10.1007/s11095-015-1825-9 [doi];10.1007/s11095-015-1825-9 [pii].
100. Ron-Doitch S, Sawodny B, Kuhbacher A, David MM, Samanta A, Phopase J, et al. Reduced cytotoxicity and enhanced bioactivity of cationic antimicrobial peptides liposomes in cell cultures and 3D epidermis model against HSV. *Journal of controlled release : official journal of the Controlled Release Society*. 2016;229:163-71. Epub 2016/03/26. doi: 10.1016/j.jconrel.2016.03.025. PubMed PMID: 27012977.
101. Berditsch M, Jager T, Stempel N, Schwartz T, Overhage J, Ulrich AS. Synergistic effect of membrane-active peptides polymyxin B and gramicidin S on multidrug-resistant strains and biofilms of *Pseudomonas aeruginosa*. *AntimicrobAgents Chemother*. 2015;59(9):5288-96. doi: AAC.00682-15 [pii];10.1128/AAC.00682-15 [doi].
102. Schaubert J, Svanholm C, Termen S, Iffland K, Menzel T, Scheppach W, et al. Expression of the cathelicidin LL-37 is modulated by short chain fatty acids in colonocytes: relevance of signalling pathways. *Gut*. 2003;52(5):735-41.
103. McGann P, Snesrud E, Maybank R, Corey B, Ong AC, Clifford R, et al. *Escherichia coli* Harboring mcr-1 and blaCTX-M on a Novel IncF Plasmid: First Report of mcr-1 in the United States. *AntimicrobAgents Chemother*. 2016;60(7):4420-1. doi: AAC.01103-16 [pii];10.1128/AAC.01103-16 [doi].
104. OECD. In Vitro Skin Irritation: Reconstructed Human Epidermis Test Method. In: *Guidelines for the Testing of Chemicals*. 2013 [updated 2013].

105. Morales M, Perez D, Correa L, Restrepo L. Evaluation of fibrin-based dermal-epidermal organotypic cultures for in vitro skin corrosion and irritation testing of chemicals according to OECD TG 431 and 439. *ToxicolIn Vitro*. 2016. doi: S0887-2333(16)30139-4 [pii];10.1016/j.tiv.2016.07.010 [doi].
106. Brohem CA, Cardeal LB, Tiago M, Soengas MS, Barros SB, Maria-Engler SS. Artificial skin in perspective: concepts and applications. *Pigment Cell Melanoma Res*. 2011;24(1):35-50. doi: 10.1111/j.1755-148X.2010.00786.x [doi].
107. Mallampati R, Patlolla RR, Agarwal S, Babu RJ, Hayden P, Klausner M, et al. Evaluation of EpiDerm full thickness-300 (EFT-300) as an in vitro model for skin irritation: studies on aliphatic hydrocarbons. *Toxicology in vitro : an international journal published in association with BIBRA*. 2010;24(2):669-76. Epub 2009/09/02. doi: 10.1016/j.tiv.2009.08.019. PubMed PMID: 19720135; PubMed Central PMCID: PMCPMC2947439.
108. Myles IA, Williams KW, Reckhow JD, Jammeh ML, Pincus NB, Sastalla I, et al. Transplantation of human skin microbiota in models of atopic dermatitis. *JCIInsight*. 2016;1(10). doi: 10.1172/jci.insight.86955 [doi].