Phd Thesis

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Peptides and their effect on wound healing

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Preface and acknowledgements

This thesis is submitted to partially fulfill the requirements for obtaining a PhD degree at Roskilde University. The presented work has been carried out at the department of Science and Environment at Roskilde University, Denmark from March 2016 to March 2019. Parts of the research have also been carried out at the faculty of Public Health, Section of Environmental Health, University of Copenhagen, Copenhagen, Denmark from September 2016 to January 2017, and at the Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal from February to April and from October to November 2018. This project was financed by the Danish Council for Independent Research, Technology and Production (grant #4005-00029). Travel expenses were funded by the Lundbeck foundation and an Erasmus grant.

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Finally, my unconditional love and gratitude for my husband Kristian Mouritzen, without whom I would never have gotten to this point.

Abstract

The incidence of non-healing wounds is increasing because of an aging population and because of the prevalence of diseases, such as diabetes increases. Host defense peptides (HDPs) have gained attention because of their dual properties of having both antimicrobial and immunomodulatory effects. HDPs have been demonstrated to have a positive effect on wound healing, especially for chronic wounds, as they can eradicate bacterial biofilms and alter pro-inflammatory cytokines, which is some of the main problems linked to diabetic wounds. The aim of this study was to test the wound healing effect of known peptides, such as the substance P, neurotensin, LL-37, bovine lactoferricin (LFcinB), Nisin A, and IDR-1018. These peptides have different origins coming either from humans (substance P, neurotensin, LL-37, and lactoferricin), bovines (lactoferricin), or bacterial isolates (Nisin A), but also synthetic peptides (IDR-1018) have been studied. Here, these studies demonstrated that the peptides enhanced migration of keratinocyte (**Paper I-VII**) and endothelial cell lines (**Paper III-V**), inhibited pro-inflammatory cytokines (**Paper II, III, V**), and altered chemokine secretion levels of MCP-1 (**Paper II-V)**. Interestingly, the peptides inhibited LPS-induced TNFα from peripheral blood mononuclear cells (**Paper III-V**). The lead peptides were further investigated in an *ex vivo* porcine model (**Paper III-V**), but only LFcinB significantly increased migration and proliferation (**Paper V**). Bacterial inhibition studies were performed *in vitro* (**Paper III and IV**), and *in vivo* in the *Galleria mellonella* larva, where Nisin A enhanced survival (**Paper III**). A murine model was used to demonstrate healing kinetics, angiogenesis, bacterial diversity, and mRNA levels of TNFα in the wounds (**Paper IV, V**). The experiments demonstrated increased healing in the diabetic mouse models, but not in the healthy model (**Paper V**). Simultaneously,

angiogenesis was increased in the diabetic skin, while cytokine levels for TNFα and the number of bacterial strains were decreased when treated with peptides. Additionally, analogues of IDR-1018 (**Paper IV**) were investigated for improvements of biological activity when altering length, charge, and hydrophobicity. The analogues were evaluated for bacterial activity, toxicity, and migratory effect. Furthermore, optimization of peptides demonstrated that altering their sequences could increase their biological activity, but that shortening them too much decreases their activity. Together, these data demonstrate some of the biological processes influenced by the peptides that can potentially lead to enhanced wound healing.

Resume

Forekomster af ikke-helende sår er tiltagende, både på grund af den aldrende befolkning og på grund af en hyppigere forekomst af sygdomme som eksempelvis diabetes. Vært-forsvarspeptider har fået øget opmærksomhed på grund af deres dobbelte egenskaber med både antimikrobielle og immunomodulatoriske effekter. Forsvarspeptider har vist at have en positiv effekt på sårheling, særligt kroniske sår, eftersom de kan fjerne bakteriel biofilm og ændre pro-inflammatoriske cytokiner, der er nogle af de centrale problemer relateret til diabetiske sår. Formålet med dette studie var at teste, hvordan sårheling bliver påvirket af kendte peptider, som substance P, neurotensin, LL-37, bovine lactoferricin (LFcinB), Nisin A, og IDR-1018. Disse peptider stammer fra henholdsvis mennesker (substance P, neurotensin, LL-37, og lactoferricin), kvæg (lactoferricin), og bakterieisolater (Nisin A), men inkluderer også syntetisk designede peptider (IDR-1018). Peptiderne viste sig at forbedre migrationen af keratinocyt- (**Paper I-VII**) og endotel-cellelinjer (**Paper III-V**), hæmme pro-inflammatoriske cytokiner (**Paper II, III, V**), og forøge kemokinsekretionsniveauer af MCP-1 (**Paper II-V)**. Interessant nok, hæmmede peptiderne LPS-inducerede TNFα fra mononuklear perifert blod (**Paper III-V**). De førende peptider blev yderlige undersøgt i en *ex vivo*-svinemodel (**Paper III-V**), men kun LFcinB havde en øget migration og proliferation (**Paper V**). Studier i bakteriel hæmning blev udført *in vitro* (**Paper III and IV**) og *in vivo* i *Galleria mellonella*-larve, hvor Nisin A forøgede larvernes overlevelse (**Paper III**). En musemodel blev brugt til at undersøge helende kinetik, angiogenese, bakteriediversitet, og mRNA-niveauerne af TNFα i sårene hos henholdsvis diabetiske og raske mus (**Paper IV, V**). Forsøgene viste en forøget heling hos diabetiske mus, men ikke hos de raske. Samtidig blev angiogenese forøget i den

diabetiske musehud, mens cytokinniveauet for TNFα og antallet af bakteriestammer faldt efter peptidebehandlingen. Derudover blev IDR-1018 (**Paper IV**) testet for at undersøge om længde, ladning, eller hydrofobicitet forbedrede biologisk aktivitet. Desuden undersøgtes analogernes bakterielle aktivitet, toxicitet, og migratoriske effekt. Endvidere viste en optimering af peptiderne, at en ændring af deres sekvenser kan forøge deres biologiske aktivitet, men at en for stor forkortelse formindskede deres aktivitet. Samlet set viser disse data nogle af de biologiske processer, som peptider har indflydelse på og som potentielt kan forbedre sårheling.

Hypothesis and experimental goals

The hypothesis for the study was that topical treatment with peptides would positively affect wound healing, due to their antimicrobial activity and immunomodulatory effects. Peptides with different origins were subjected to testing and evaluation of their wound healing properties, as potential treatments for chronic diabetic foot ulcers. Different *in vitro* models, and later *ex vivo* and *in vivo* models, were used and evaluated for high-throughput testing and for modeling human chronic wounds. Optimization studies of peptides were also performed to test the importance of length and charge for their biological activity.

List of publications

Paper I: **Mouritzen, M. V.**, Jenssen, H. "Optimized scratch assay for *in vitro* testing of cell migration with an automated optical camera". Journal of Visualized Experiments, vol. 138 (2018). DOI:10.3791/57691.

Paper II: **Mouritzen, M.V.**, Abourayale, S. Ejaz, R., Ardon, C. B., Carvalho, E., Dalgaard, L. T., Roursgaard, M., Jenssen, H. "Neurotensin, substance P, and insulin enhance cell migration". Journal of peptide science, vol. 24 no. 7 (2018). DOI: 10.1002/psc.3093.

Paper III: **Mouritzen, M. V.**, Andrea, A., Qvist, K., Poulsen, S. S., Jenssen, H. "Immunomodulation potential of Nisin A with application in wound healing". Submitted manuscript and accepted with revision at journal of wound repair and regeneration (WRR-18-12-0351).

Paper IV: **Mouritzen, M. V.**, Petkovic, M., Molchanova, N., Qvist, K., Saporito, P., Poulsen, S. S., Alarico, S., Hansen. P. R., Roursgaard, M., Leal, E. C., Empadinhas, N., Carvalho. E., Jenssen, H. "Synthetic peptide IDR-1018 anti-inflammatory and microbiome modulatory properties positively impact wound healing". Submitted manuscript to journal of biochimica et biophysica acta – molecular disease (BBADIS-19-141).

Paper V: **Mouritzen, M. V.**, Petkovic, M., Qvist, Poulsen, S. S., Alarico, S., Leal, E. C., Empadinhas, N., Carvalho. E., Jenssen, H. "Bovine Lactoferricin alters cellular movement, cytokine profile, microbiome leading to increased wound healing *ex vivo* and *in vivo*". Manuscript submitted to journal for dermatological science.

Additional papers and patent application:

Saporito, P., **Mouritzen, M. V.**, Løbner-Olesen, A, Jenssen, H. "LL-37 fragments have antimicrobial activity against *Staphylococcus epidermidis* biofilm, and wound healing potential in HaCaT cell line". Journal of peptide science, vol. 24 no. 7 (2018). DOI: 10.1002/psc.3080.

Saporito, P., **Mouritzen, M. V.**, Løbner-Olesen, A, Jenssen, H. "Anti-biofilm and wound healing properties of single amino acids substitutions in LL-37 derived peptide fragments" Manuscript in preparation.

Desriac, F., **Mouritzen, M. V.**, Jenssen, H. Upton M., “Epidermicin wound healing properties” Patent application, publication pending on a patent application at University of Plymouth.

Table of Contents

Abbreviations

AMP	Antimicrobial peptide
cDNA	Complement DNA
CFU	Colony forming units
DAMP	Damage-associated molecule patterns
DFU	Diabetic foot ulcer
DMEM	Dulbecco's modified eagle medium
ECM	Extracellular matrix
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EPS	Extracellular polymeric substance
FBS	Fetal bovine serum
HaCaT	Immortalized human keratinocyte cell line
HUVEC	Human umbilical vein endothelial cells
HDPs	Host defense peptides
IDR	Innate defense regulator
IL	Interleukin
LPS	Lipopolysaccharides
MCP-1	Monocyte chemoattractant protein-1
MHB	Mueller hinton broth
MIC	Minimal inhibition concentration
MMP	Matrix metalloproteinase
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt
NT	Neurotensin
ON	Overnight

PAMP	Pathogen-associated molecules patterns
PBMC	Peripheral blood monocyte cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
qPCR	Quantitative PCR
RBC	Red blood cells
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
SD	Standard deviation
SP	Substance P
TIMP	Tissue inhibitor of metalloproteinase
TNFα	Tumor necrosis factor α
VEGF	Vascular endothelial growth factor

Chapter 1: Background

1.1. Wound healing

The skin is the largest organ of the body and it forms a physical and chemical barrier that protects the body from microorganisms, ultraviolet radiation, and mechanical tears[1]. The skin is a constant renewing, dynamic organ separated into three layers: the epidermis, dermis, and subcutis layer. The epidermis is the outermost layer of the skin, and the subcutis layer is the innermost layer. The epidermis mainly consists of keratinocytes and is further separated into layers, depending on the maturation level of keratinocytes. This maturation process of keratinocytes is terminal, as it leads to the loss of the nucleus of the keratinocytes, called corneocytes[2]. As the keratinocytes mature they move from the basal membrane, containing stem cells, toward the outer layer, where they are shed[3]. The keratinocytes in the skin are tightly bound by desmosomes, which together form the anatomical barrier[4]. The epidermis does not have its own blood supply, and is dependent on the dermis for transportation of nutrition and oxygen[5]. The subcutis layer is mainly a layer of fat, important for anchoring the upper layers to the muscle, protecting the bones and muscles, and maintaining temperature[6].

The chemical barrier of the skin is important, as the skin is naturally inhabited by a diverse collection of microorganisms, such as bacteria, fungi, and viruses[7]. The chemical barrier functions through pH-regulation and host defense peptides (HDPs)[4, 8], where pH regulation is important for activation of various enzymes and to decrease bacterial growth[9, 10]. HDPs are secreted through the skin as one of the first lines of defense because of their anti-pathogenic activity[11]. In the skin, the keratinocytes produce both chemokines, cytokines, and HDPs, such as MCP-1[12], IL-6, IL-8, tumor necrosis factor α (TNFα)[13], and LL-37[14]. These molecules are a part of the innate immune response that recruits leukocytes, neutrophils, and

monocytes[15, 16]. When a breach occurs in the skin, these molecules are crucial for initiating the healing process, and ultimately to prevent loss of fluids and opportunistic bacteria from infecting the wound by healing the breach[17].

The wound healing process can be divided into four phases: hemostasis, inflammation, migration and proliferation, followed by tissue remodeling[18] (**Figure 1**). The phases occur overlappingly in a timely fashion[19].

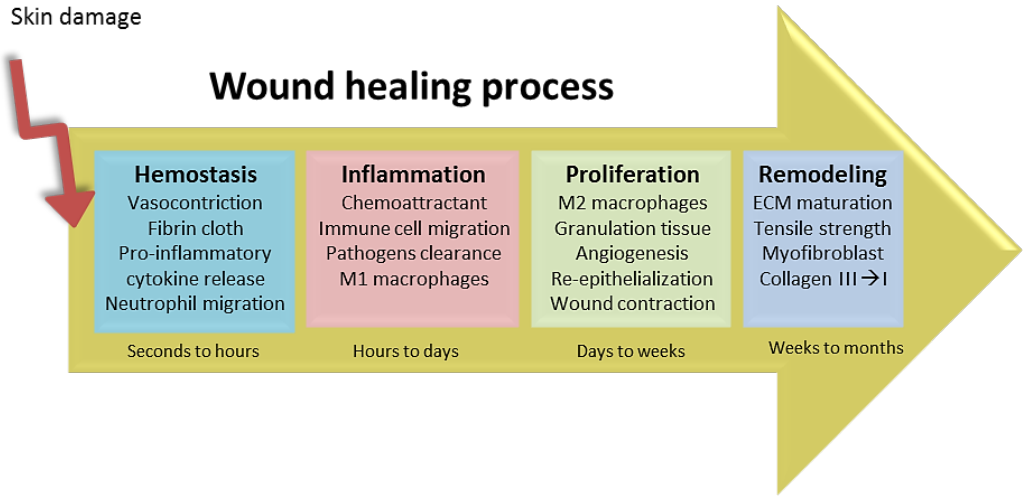

Figure 1: Wound healing phases – Wound healing can be separated into four different phases: Hemostasis, inflammation, proliferation and migration, and remodeling. The first phase starts immediately and it is characterized by clotting of the blood, while the second phase is initiated to clean the wound. Here, infiltration and activation of immune cells are the main characteristics. The third phase starts after some days where the cells proliferate, migrate, and differentiate. This is especially seen for epithelial, endothelial, and fibroblast cells to rebuild the new tissue. The final phase is maturation, where strengthening and remodeling of the newly formed tissue can take from weeks to months.

1.1.1 Hemostasis

The hemostasis phase starts immediately after wounding occurs, where constriction of the vessels are initiated to stop bleeding and a fibrin clot is formed to seal the wound[20]. The fibrin clot is formed by platelets that bind to the exposed collagen creating an unstable clot. The clot is further strengthened by clotting

factors that lead to fibrin formation[21]. Wounding of the skin leads to the release of growth factors, pro-inflammatory cytokines, and chemokines[22]. The release of chemokines initiates migration of immune cells, such as neutrophils and monocytes into the wounded area, and the secretion of pro-inflammatory molecules starts the inflammatory phase[22, 23].

1.1.2 Inflammation

The inflammatory phase is characterized by neutrophil, lymphocyte, and macrophage infiltration of the wound site to eliminate pathogens and cellular debris[24].

Neutrophils are among the first immune cells that are recruited to the site of injury. The neutrophil infiltration depends on pro-inflammatory cytokines[25] and damage-associated molecule patterns (DAMP), such as DNA, RNA, and other cellular content[26]. At the site of wounding, the neutrophils kill bacteria by phagocytosis, resulting in further release of cytokines, growth factors, reactive oxygen species (ROS), proteolytic enzymes, and HDPs[27], enabling degradation and killing of pathogens[28]. These factors are secreted from numerous cells such as keratinocytes, endothelial cells, and fibroblasts and the release of cytokines further activates other immune cells[29]. Macrophages are among the most predominant immune cells in wounds and can be divided into two subgroups; the M1 and M2 macrophages, depending on the cytokine secretion profile[30]. In the inflammatory phase, DAMPs, pathogen-associated modifying proteins (PAMPs), and pro-inflammatory cytokines affect the phenotype of the macrophages to become M1 macrophages[31]. M1 macrophages are characterized by their secretion of pro-inflammatory cytokines, such as IL-6, IL-8, and TNFα, which are important for attracting additional immune cells to the injury site[32].

The role of the immune cells in this phase is to eliminate intracellular pathogens, but the macrophages are also important for phagocytosis of apoptotic neutrophils. Controlled elimination of old neutrophils is crucial for continued healing. This is ensured by inhibiting neutrophil degradation and thereby the release of phlogistic material. Without controlled phagocytosis, neutrophils release DAMPs, leading to further inflammation and tissue damage[33]. Once the neutrophils are removed, the environment changes will initiate polarization of the M1 macrophages altering their phenotype to M2 macropahges[34]. The M2 macrophages secrete anti-inflammatory cytokines, such as IL-10, which is characteristic for the initiation of the proliferation and migration phase[35].

1.1.3 Proliferation and migration

The proliferative and migratory phase is divided into three different parts: re-epithelialization, neovascularization, and formation of granulation tissue. Characteristic of this phase is the migration and proliferation of keratinocytes, endothelial cells, and fibroblasts[20]. Together these close the epithelial layer, revascularize the damaged area, and replace the tissue in a fibroblast-mediated manner[36]. These three parts occur by overlapping one another.

Re-epithelialization of the epithelial layer occurs under the fibrin clot by migration of the keratinocytes initiated by epidermal growth factor (EGF). Migration of the cells is a dynamic process performed by actin filaments, causing the cells to protrude, adhere, contract, and detach[37]. This process is initiated by polymerization of the actin in the leading edge, resulting in a protrusion that is stabilized to the surface by adhesion proteins[38]. The cells contract by actin-myosin polymerization[39], leading to a forward motion[40]. In order for the forward

motion to occur, the cells are detached from the matrix by enzymes such as matrix metalloproteinase (MMPs)[41].

Angiogenesis is important for proper wound healing because oxygen and nutrition are essential for energy production[42]. After wounding, more energy is needed for various processes such as cell proliferation, migration, and production of collagen. These processes are increased and without oxygen the mitochondria's ATP synthesis will be reduced, resulting in stalled wound healing[43]. Angiogenesis in new tissue is an invasive process, which can be divided into four steps: degradation of the matrix, migration toward angiogenic stimuli, proliferation of the endothelial cells, and reorganization. The sprouting angiogenesis is guided by the tip cell, which responds to vascular endothelial growth factor (VEGF) stimuli[44]. The tip cell consists of filopodia that secret proteolytic enzymes[45], required to degrade the basement membrane of the endothelial cells to sprout into new tissue areas[46].

Granulation tissue is the new connecting tissue that is formed if a wound penetrates the dermis. This tissue is characterized by the presence of fibroblasts, synthesizing extracellular matrix (ECM), and collagen to strengthen the new tissue. In the granulation tissue, collagen III is one of the major proteins present[47]. However, the newly formed skin is weaker compared to unwounded skin, until remodeling has occurred to strengthen the tissue.

1.1.4 Remodeling

The remodeling phase can take up to several years before the new tissue is matured and resembles the old tissue[48]. This phase is characterized by re-arranging the collagen and de-vascularizing of the new tissue. Furthermore, the differentiated form of fibroblasts, called myofibroblasts, reduces the size of the

newly formed tissue by binding to collagen at the wound edges and contracting the wound area[49]. The maturation of the tissue is a delicate process, where key components of the ECM are degraded and replaced, as seen for collagen III that is replaced by collagen I[49, 50]. This alteration of collagen increases tension and strengthens the wound area. After the contraction of the wound is completed, the myofibroblast undergoes apoptosis[51], the number of immune cells decreases[52], and the vessels reside[53]. The newly formed skin differs from unwounded skin, as it has thicker fibers and the collagen is disorganized. During the remodeling phase, the fibers become more organized and inter-linked to further increase strength of the tissue[54].

1.1.5 Chronic wounds

Chronic wounds are defined as wounds that do not heal naturally within three months. In the Scandinavian countries, the cost of treating chronic wounds accounts for 2-4% of health care services[55]. Wounds that have difficulties into healing are subcategorized to four groups: venous, pressure, diabetic, and arterial insufficient ulcers[56]. In this study, only diabetic wounds will be the focus.

Diabetes mellitus is a disease caused by insulin deficiency, where the patient is either intolerant of insulin, or produces insulin in too low an amount[57, 58]. If not treated properly, insulin deficiency leads to hyperglycemia. Prolonged periods with hyperglycemia lead to damaged and dysfunctioning organs and tissues in the body[59]. The most common complications are vascularity and neuropathy, leading to complications such as loss of sensation in the feet, renal disease, and retina problems, among others[60]. It is estimated that 15% of diabetic patients suffer from diabetic foot ulcers (DFUs)[61], and 60% will have recurring ulcers after three years[62]. Diabetic patients have an increased risk of infection, compared

to the non-diabetic population[63], because of hyperglycemia[64–66]. Patients with DFU have a 15-time higher risk of lower limb amputation compared to healthy individuals[61], as a result of non-healing DFU.

Factors affecting wound healing can be categorized into local and systemic factors. Systemic factors are present at all times in the individual, as seen in diseases such as diabetes, stress, obesity, and age, while local factors include factors, such as infection, oxygenation, and foreign bodies, which directly influence the wound locally[24]. The local factors lead to high levels of pro-inflammatory cytokines and ROS, impaired cell and protease function, and lack of growth factors, among others[67] (**Figure 2**).

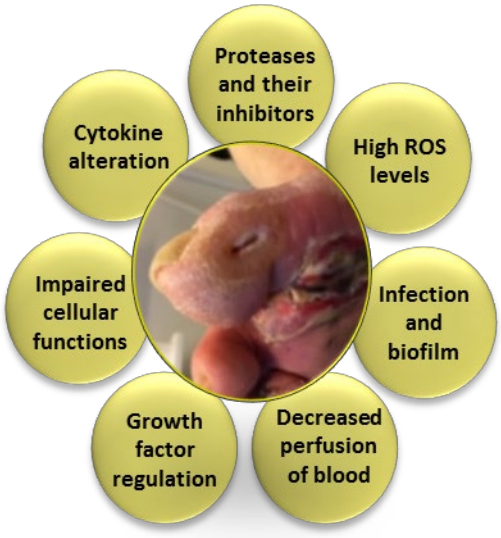

Figure 2: Factors affecting healing of chronic wounds – Chronic wounds have been shown to have deficiencies. Some of the most reported are increased levels of pro-inflammatory cytokines, proteases, and reactive oxygen species. Furthermore, a decrease of blood flow, growth factors, lack of cell response, and protease inhibitors are often seen in chronic wounds. The picture is from Steno diabetes center.

Diabetes itself is a systemic factor, but diabetic patients have also been shown to have chronically increased levels of pro-inflammatory cytokines[68]. The enhanced

levels of these cytokines affect the transition of M1 macrophage phenotypes to M2, stalling the macrophages as M1, prolonging the inflammatory phase and thereby slowing the healing of the wound[69]. Prolongation of the inflammatory phase has also been shown to be a result of decreased growth factor activity, local hypoxia, increased levels of proteases, and infections, which lead to an altered pathophysiology[70]. The high levels of pro-inflammatory cytokines form a negative loop, where more immune cells are attracted to the wound site, as seen for neutrophils and M1 macrophages. Deficiency of immune cells has been demonstrated in diabetes, as altered chemotactic phagocytosis, response to growth factors, and diminished antimicrobial activity[71, 72]. Together, this makes chronic wounds more prone to infection. Additionally, the levels of ROS and proteases are increased. Due to the lack of specificity of ROS, they cause degradation of the ECM[73], inactivation of growth factors, increase of proteases[74], and senescence of cells[75], all of which can eventually harm the host[29]. Growth factor deregulation is also a major factor in wound healing delay[70]. VEGF, a factor crucial for angiogenesis, is significantly down-regulated in chronic wounds preventing the formation of new capillaries[76]. It has also been demonstrated that keratinocytes produce less growth factors, and that they have lower migration potential compared to keratinocytes from healthy skin[77]. Likewise, fibroblasts from chronic wounds have an impaired response to hypoxia and an impaired migrational potential[78]. Finally, it has been demonstrated that there is an increased level of MMPs in chronic wounds, as well as a decreased level of their inhibitors, called tissue inhibitor of metalloproteinase (TIMP)[79]. Bacteria in the wound further disrupt healing, by the secretion of their own proteases, as well as their effect on host cells, leading to secretion of pro-inflammatory cytokines[80].

Bacterial infection of wounds is one of the main reasons for non-healing wounds. The skin is inhabited by various bacteria that are not harmful for the host. However, once a wound occurs, opportunistic bacteria can colonize the wound and form a biofilm[81]. A biofilm is a community of bacteria that forms a matrix of polysaccharides which adheres to a surface, and is usually formed within 5-10 hours *in vitro*[82, 83]. Formation of biofilm is separated into four stages: adhesion, proliferation, maturation, and dispersion (**Figure 3**).

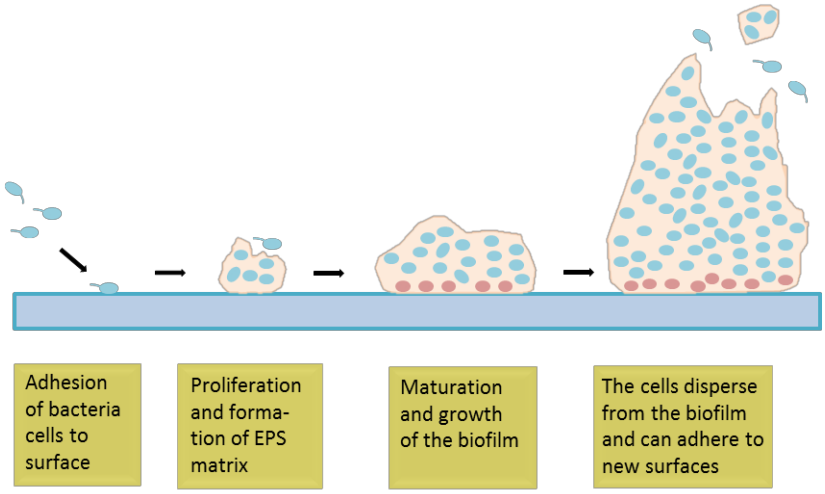

Figure 3: Biofilm formation stages – Biofilm formation is initiated once planktonic cells adhere to a surface. On the surface, the bacteria will proliferate and form an EPS matrix. The growing biofilm will adapt to the flow of nutrition, enabling differentiation of the cells in the biofilm, where some will slow down their metabolism (red) and other will continue to proliferate (blue).

Adhesion of the bacterial cell is dependent on the different surfaces. Once the bacteria are adhered, they start to form an extracellular polymeric substance (EPS) matrix and proliferate in a nutrient dependent manner resulting in a growing biofilm[84, 85]. The biofilm can disperse due to mechanical force or as a result of environmental changes, such as low nutrition, oxygen, or toxic

products[86]. As the biofilm grows, a nutrition gradient will form and result in different growth rates of the bacterial cells. The innermost cells of the biofilm will slow down their growth rate, whereas the cells with easy access to nutrition will have a steady growth rate (red and blue cells, respectively **Figure 3**)[87].
Once biofilm is formed, the immune response of the host is insufficient, as the bacteria cannot be engulfed by the phagocytic immune cells. As a result, leukocytes bind to the biofilm, die, and release enzymes that, in combination with the enzymes released from the bacteria, prolong the inflammatory phase of wound healing[88]. When bacteria enter the host, the host has multiple receptors to recognize intruders known as pattern recognition receptors (PRRs)[89]. The PRRs recognize conserved molecular patterns PAMPs, such as peptidoglycan from Gram-positive and lipopolysaccharides (LPS) from Gram-negative bacteria[90]. Recognition of PAMPs by PRRs will initiate a downstream signaling pathway resulting in secretion of cytokines, chemokines, and HDPs[91, 92]. However, the bacteria in the biofilm communities are different from planktonic bacteria with regards to gene expression, antibiotic sensitivity, and interaction with the host. Bacteria can develop resistance against antibiotics by producing efflux pumps, altering the target site, and synthesizing degrading enzymes[93,94]. The resistance gene can be transferred vertically or horizontally, by gene mutation, conjugation, transduction, or transformation[95]. It has been demonstrated that the bacteria in a biofilm are 10 to 1000-fold more resistant to conventional treatments[96].

In the clinics, treatment of chronic wounds starts with an evaluation of the blood perfusion, neuropathy, and infection as the three predominant contributors of non-healing wounds. DFUs are treated with surgery to remove non-viable tissue and visible infection and to improve blood flow, if possible. Antibiotics are

prescribed to clear remaining infection, but most importantly, the wound is off-loaded by specialized shoes, to minimize pressure on the wound[97, 98]. However, if blood flow cannot be improved, and if the infection affects deep-tissue, surgical removal can be necessary[99].

Treatment with antibiotics has long been a growing problem, as the amount of resistant bacteria is on the rise[100, 101]. Resistance has emerged as a consequence of extensive use of antibiotics, thereby applying an increased selective pressure that increases the rate of gene mutations[102]. The mutations have resulted in alteration in the target site of the antibiotics, inhibiting their effect and over the last decade, an increase of resistant strains has emerged. This has led to a search for new treatment option, where HDPs have gained interest over the years due to their dual effect of antimicrobial activity and immunomodulatory effect.

1.2. Host defense Peptides

HDPs are conserved molecules found in all kingdoms of life. In humans, they are naturally secreted in areas where the environment of the body meets microbes, for instance on the skin, in saliva, and in the gut[103]. HDPs, also known as antimicrobial peptides (AMP), are mostly studied for the anti-pathogenic effect against bacteria[104], viruses[105], fungi[106, 107], and parasites[108], but have also shown a positive effect against cancer[109]. The term HDP was later introduced as it became clear that the AMPs not only have antimicrobial activities, but that they also affect the immune cells of the host. The structure of HDPs differs, since they can form α-helixes, β-sheets, loop peptides, or extended structures rich in proline, among others[110]. Most often, they are amphipathic molecules having a positive charge ranging from +2 to +9, a short length of 10-50 amino acids, and contain up to 50 % hydrophobic residues[111, 112].

In the body, HDPs are produced in all organs by various cells, but in the skin the major production of HDPs is located in the keratinocytes[113]. Many of the natural peptides are secreted as pre-peptides and are post-transcriptionally modified, such as demonstrated for neurotensin (NT)[114], and substance P (SP)[115], and bacterial nisin[116–118]. There are more than 20 different peptides identified in the skin[119], and the production of HDPs occurs in the stratum granulosum, the uppermost layer of nucleated keratinocytes. The HDPs are packed into lamellar bodies and transported upwards to the stratum corneum, but are also secreted in fluids such as sweat[4, 120]. Additionally, HDPs are stored in the granules of phagocytic cells[121]. Some HDPs are constitutively produced in low levels, whereas others are induced locally upon infection or by degranulation of phagocytes[122, 123], as demonstrated for the HDPs defensins and cathelicidins[124]. The transcription of some HDPs is altered according to need, and once an infection is detected in the skin, the levels of HDPs rise[125, 126].

HDPs have been studied for a variety of purposes both for use in the food industry and for therapeutic applications[123, 127]. More recently, studies have investigated the structure of known peptides to both determine sequence-specific effects and to optimize the peptides. A group of synthetic peptides called innate defense regulators (IDRs), designed to enhance the immunomodulatory effects, are based on bactenecin (Bac2A) isolated from bovine neutrophil granules[128]. Bac2A was used as a template to make a library of 12-mer peptides through substitutions, deletions, and scrambling, which led to the discovery of the IDR peptides[129]. Of the IDRs, IDR-1018 was found superior in comparison with the other IDRs because of its ability to influence cytokine levels and chemotaxis crucial for

wound healing[129]. Furthermore, design of shorter synthetic peptides that contain less residues is more cost-efficient[130, 131].

1.1.6 Antimicrobial activity of peptides

The antibacterial function of HDPs is mainly a result of their positive charge and hydrophobicity. Positively charged peptides bind to negatively charged surfaces, such as the phospholipid membranes of bacteria, due to the electrostatic interaction[132]. Peptides have both hydrophobic and hydrophilic residues, which enables them to fold to an amphipathic secondary structure on the membrane. Their mode of action is still unknown, but several mechanisms of how the HDP perturbs the microbial membrane have been proposed[133]. Some of the proposed modes of action are the carpet, barrel-stave, or toroidal pore model[134]. In all the mentioned models, the peptides are associate with the polar head groups of the lipids by an electrostatic interaction and then form a hole[135], disrupting the membrane and thus killing the bacteria directly. The three models differ in regard to the lipid structure and stability of the formed pore[136]. Other peptides work intracellularly by inhibiting the synthesis of DNA, RNA, or proteins[137]. Still other peptides function by recruiting immune cells and thus killing the bacteria indirectly[119, 135]. Positively charged HDPs are efficient therapeutics against bacteria, as they target negatively charged membranes, and thereby do not target mammalian host cells, which have a neutral charge[126, 138].

Peptides have been demonstrated to eliminate bacteria not only *in vitro*[139], but also *in vivo*[140]. However, resistance has been shown *in vitro* when growing bacteria in sub-minimal inhibition concentration (MIC) with peptides, altering the MIC from 1 μg/mL to >500 μg/mL[141]. Resistance has also been observed for a β-defensin[142] and LFcinB[143] *in vitro*. Although only very few *in vitro* studies,

and no *in vivo* studies, have demonstrated resistance, it is important to ensure resistance does not occur for HDPs if they are to be used as future therapeutics. However, the rapid antimicrobial activity of peptides is thought to make resistance less likely to occur[144]. A compelling argument for using peptides is that some can kill antibiotic resistant bacteria[145]. The use of peptides in combination with antibiotics could be beneficial. When tested against biofilms alone or in combination with conventional antibiotics, peptides have demonstrated complete eradication of the biofilm and the ability to work synergistically with antibiotics[96, 146].

1.1.7 Immunomodulatory effect of peptides

HDPs are one of the first lines of defense of the body and they are therefore produced in areas where the outer environment meets the environment of the body, such as the gastrointestinal tract, eyes, ears, and the skin[147]. However, HDPs have a broad range of effects on the body beside antimicrobial activity, such as inducing angiogenesis[113,148], increasing the migratory potential of cells[149], and inhibiting apoptosis[150]. The immunomodulatory properties of peptides have been demonstrated by their ability to regulate expression of cytokines[151], chemokines[152], proteases[153], growth factors[154], and immune cell activity[112, 155, 156]. One of the most reported immunomodulatory effects is alteration of cytokines[157].

Cytokines function as messenger molecules and the different cytokines can have similar or completely opposite effects[158]. HDPs have been proven to alter the expression level of various anti-inflammatory[152, 159, 160] and pro-inflammatory cytokines[157, 161], as well as the level of their receptors[162]. Most studied of the cytokines is of the interleukin (IL) family, where especially IL-6, IL-8, and IL-

10 have been studied. In wound healing, the HDPs has been shown to decrease the levels of pro-inflammatory and increase the anti-inflammatory cytokines, and they can protect against sepsis by influencing cytokines[163]. HDPs have been shown to affect chemokines in the same way as shown for cytokines[162, 164, 165] and to have direct chemotaxis effect on immune cells[166, 167]. Furthermore, it has been shown that peptides also influence growth factors, such as EGF, VEGF, and their respective receptors[168, 169]. The activation of the receptor of EGF has further been shown to increase migration of the keratinocyte cell line HaCaT[169]. A study demonstrated, that topical treatment with peptides enhanced the levels of ERK and Akt[170]. ERK is a part of a complex phosphorylation pathway where activated ERK is translocated to the nucleus and activates gene transcription, which leads to proliferation or differentiation[171,172]. Akt is an activator of VEGF, and thereby important for angiogenesis[173]. In diabetic wounds, Akt is found to be expressed in a lower amount compared to non-diabetic wounds, which could explain the low healing and altered vasculature in diabetic patients.

The shown immunomodulatory properties of HDPs and their ability to kill bacteria makes them ideal as therapeutics for wound healing[110]. However, not all peptides have all the reported effects, and the mode of action of the immunomodulatory effects remains unclear. For synthetic peptides, it is unknown where they end up or where they bind. It is hypothesized that the peptides, being soluble, bind to the membrane and translocate to the nucleus, where they bind intracellular receptors[174]. Studies have investigated specific receptors for the natural occurring HDPs, such as the receptor for SP[175], NT[176], and insulin[177]. Nevertheless, knockout studies have yet to be performed in regard to wound healing properties, to determine the importance of these receptors.

In this study, a variety of peptides were used. In **Table 1**, the peptides are listed in alphabetical order showing origin, number of amino acids, net charge, and sequence of the peptides.

Chapter 2: Material and methods

All experiments were performed three independent times in triplicates unless indicated otherwise in the description of the method. Furthermore, all experiments have been performed with 25 µg/mL of the peptides.

2.1. Peptides

A variety of peptides (**Table 1**) was used for the performed experiments. Bovine lactoferrin was purchased from Sigma-Aldrich and human lactoferrin was kindly donated from Cappel/Organon Teknika Corporation (NC, USA). Both bovine and human lactoferricin were purchased from the Centre for Food Technology (Brisbane, Australia) (**Paper I and V**). Insulin was from Sigma-Aldrich, and neurotensin and substance P from Bachem (**Paper II**). Nisin A was isolated and purified from *Lactococcus lactis* as previously described[178] (**Paper III**). IDR-1018 and the library of analogues were all synthesized by solid phase peptide synthesis by Natalia Molchanova at Roskilde University (**Paper IV**). LL-37 and the 12-mer were purchased from Genscript[139] (appendix). All the peptides were dissolved in milliQ H_2O and stored at -20°C.

Table 1: Peptides are listed with information of origin, number of amino acids, net charge, and sequences – Charge and molecular weight (MW) are calculated using Innovagen website (pepcal.com) at pH 7. Disulfide bridges and their binding are indicated with numbers. The lactoferrin protein sequence is not mentioned, as it is too long. In Nisin A, special amino acids are given in three letter codes and in colors: Green: dehydrobutyrine (Dhb), red: dehydroalanine (Dha), and purple: 2-aminobutyric acid (Abu).

Name	Origin	Amino acids	Net charge pH 7	MW g/mol	Sequence	Ref

IDR-1018	Synthetic	12	+5	1536	VRLIVAVRIWRR-$CONH_2$	[111]
Insulin	Human	51	+5	5808	α:GIVEQC_1C_2TSIC_1SLYQLEN YC$_3$N β:VNQHLC_2GSHLVQ-ALYLVC_3GERGFFYTPKT	[177]
LFcinB 17-41	Bovine	25	+8	3126	FKC_1RRWQWRMKKLGAPSITC$_1$ VRRAF	[105, 179]
LFcinH 18-42	Human	25	+6	3022	TKCFQWQRNMRKVRGPPVSCIK RDS	[105, 179]
IDR-1018	Synthetic	12	+5	1536	VRLIVAVRIWRR -$CONH_2$	[111]
Neuro-tensin	Human	13	0	1676	ELYENLPRRPYIL	[180, 181]
Nisin A	*Lactococcus lactis*	34	+5	3354	IDhbA$_1$IDhaLA$_1$Abu$_2$PGA$_2$KAbu$_3$GALMGA$_3$NMKAbu$_4$AAbu$_5$A$_4$HA$_5$SIHVDhaK	[182]
Substance P	Human	1	+2	1349	RPKPQQFFGLM	[183]

2.2. *In vitro* assays

2.1.1 Cell lines and human blood cells

The immortalized human cell line of keratinocytes (HaCaT)[184] were kindly provided by Bispebjerg Hospital (Denmark). The HaCaT cells were grown in Dulbecco's modified eagle medium (DMEM) with glutamax (ThermoFisher Scientific), 10% fetal bovine serum (FBS) (Gibco), 100 units/mL penicillin and streptomycin (Sigma-Aldrich). The primary cell line of human umbilical vein endothelial cells (HUVEC) was purchased from Fisher Scientific. The HUVEC cells were grown in endothelial cell basal medium (Cell applications Inc.), supplemented with endothelial cell growth supplement (Cell applications Inc), and 100 units/mL penicillin and streptomycin. Additionally, 0.1% gelatin

(Specialty media) was used to pre-coat flasks and plates to enable cell adhesion. The macrophage cell line J7741A was kindly provided by Anne Mette Hvid Larsen. The cells were grown in DMEM without glutamax (ThermoFisher Scientific), supplemented with 10% FBS and 100 units/mL penicillin and streptomycin.

All three cell lines were sub-cultured approximately twice a week, by washing with phosphate buffered saline (PBS), before trypsinization (Sigma-Aldrich) to detach the cells. The cell lines were used for migration, proliferation, and viability assays. HaCaT cells were additionally used for quantitative polymerase chain reaction (qPCR) and ELISA, while the HUVEC cells were used for angiogenesis assays.

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy blood donors. The blood samples were washed with PBS and transferred slowly on top of Ficoll-Paque™ PLUS (GE Healthcare) for separation of the PBMCs. The isolated PBMCs were washed 2× in PBS and kept in Roswell Park Memorial Institute (RPMI-1640) media supplemented with 10% FBS and 100 units/mL penicillin and streptomycin. The primary cells were used for ELISA.

2.1.2 Migration assay

The scratch assay was used as a simple *in vitro* wound healing model to test wound closure by cell migration. Briefly, the cells were transferred to a 48-well plate with 7.5×10^4 cell per well with 250 µL of media. The cells were incubated at 37ºC and 5% CO_2 overnight (ON), reaching an approximate confluency of 95%. The cells were treated with 10 µg/mL mitomycin C, to prevent proliferation of the cells, for two hours. The cells were washed with 2× with 250 µL PBS and

in the second wash, a scratch was made manually with a 200 µL pipet tip. The stimuli were added and the plate was incubated in an optical camera system called the oCelloScope (BioSense Solutions ApS, Denmark) placed in an incubator at 37ºC and 5% CO_2. The imaging and measurements of cell migration were automatically performed by the Uniexplorer software (version 8.0.0.7144 (RL3)) of the oCelloScope, capturing pictures of the fixed scan area in the plate. EGF, media, and free amino acids were used as positive and negative controls, respectively. The free amino acid control consisted of amino acids found in the whole peptides in the same mole/ratio. The results were calculated as percentage migration of the initial scratch and compared with the untreated control. The data were normalized to the untreated control (set to 1) or given as percentage of migration.

2.1.3 Proliferation assay

Proliferation was tested by seeding 1×10^4 cells per well to a 48-well plate in 250 µL media, left in a conventional incubator ON to adhere to reach a confluency of approximately 10%. The following day, the peptide stimuli were added and the plate was incubated in an oCelloScope as previously described.

EGF, media, and free amino acids were used as positive and negative controls, as described for migration. The results were calculated as the percentage of increased number of cells, normalized to the untreated control (set to 1), and compared with the untreated control.

2.1.4 Hemolysis

Blood samples from healthy donors were collected in a K_2-EDTA coated vacutainer tube (BD Vacutainer). The red blood cell (RBC) layer was isolated

using Ficoll-Paque and dilution in 0.9% NaCl (saline) to 20%. The diluted RBCs were transferred to a round bottomed 96-well plate, stimuli were added, and the plate was incubated at 37ºC and 5% CO_2 for 24 hours. The following day, 20 µL of the supernatant were diluted in 100 µL of saline, after centrifugation at 1200×g, and the plate was read at 546 nm in a plate reader (Bio-TEK, Synergy HT). 10% Triton-X-100 and saline were used as positive and negative controls, respectively. The data were normalized to Triton-X-100 (set to 100%).

2.1.5 Viability assay

The viability of the cells were tested after 24 hours of incubation with peptide stimuli and assessed by measuring the degraded form of tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) (Promega). Briefly, 2×10^4 cells were seeded per well in a 96-well plate and left to adhere ON at 37ºC and 5% CO_2. The cells were washed with PBS, treated with peptide stimuli for 24 hours. MTS-phenazine methosulfate solution compound was added to each well and incubated for 4 hours. The absorbance of the plate was measured in a spectrophotometer at 490 nm. As controls, media or 10% Triton-X-100 were used as positive and negative controls, respectively. The results were calculated as percentage viability compared with the untreated control. The data were normalized to the untreated control (set to 100%).

2.1.6 RNA isolation

Total RNA was isolated from the cultivated cells and tissue samples. The cultivated cells were harvested using TRIreagent (Sigma-Aldrich) and the tissue samples were cut into small pieces and homogenized in QIAzol. The samples

were resuspended in chloroform (MERCK), centrifuged at 12,000×g for 10 minutes, and RNA isolation was precipitated with isopropanol. The RNA was washed with 75% ethanol and the resulting pellet was air-dried for 5-10 minutes. The RNA was resuspended in 20 µL nuclease-free H_2O and the concentration was measured at 260 nm in a Spectrophotometer (NanoDrop® ND-1000).

2.1.7 cDNA synthesis and quantitative PCR

The high capacity cDNA reverse transcription kit (Applied BioSystem) was used to synthesize complement DNA (cDNA) from 1 µg RNA. The RNA was mixed as described by the manufacturer with RT buffer and RT enzyme mix, and run on a thermal cycler for 60 minutes at 37ºC, 5 minutes at 95ºC, and kept at 4ºC. The resulting cDNA products were verified with on an agarose gel (Sigma-Aldrich) after PCR with RPLP0. qPCR was performed with QuantiTectSYBR PCR kit (Qiagen) according to the protocol of the manufacturer. The cDNA samples were diluted 10×, mixed with 5 µL 2×QuantiTect-SYBR® green, 1 µL of each of the primers, and 2 µL H_2O. In **Table 2** the used primer sequences can be seen. The samples were measured on a Stratagene MX3005P (Agilent) and run for 45 cycles. The data were normalized to the untreated control according to the ΔΔCt method[185]. PBMCs and H_2O were used as the positive and negative controls, respectively.

Table 2: Primer sequences used for qPCR – Annealing temperature was 60ºC for all primer sets, except for RPLP0, which was 52ºC.

Primers	Sequence (5'→3')
RPLP0 forward	GCTTCCTGGAGGGTGTCC
RPLP0 reverse	GGACTCGTTTGTACCCGTTG
IL-6 forward	TACATCCTCGACGGCATCTC

IL-6 reverse	ACCAGGCAAGTCTCCTCATTG
IL-8 forward	CACCGGAAGGAACCATCTCA
IL-8 reverse	TTGGGGTGGAAAGGTTTGGAG
TNFα forward	TTCCTGATCGTGGCAGGC
TNFα reverse	GAGCTGCCCCTCAGCTTG

2.1.8 Enzyme-linked immunosorbent assay of MCP-1 and TNFα

The supernatant from PBMCs and HaCaT cells were stimulated with peptides alone or in combination with LPS (*Pseudomonas aeruginosa* Pa01) for 24 hours, following centrifuged of the plate for 5 minutes at 300×g, were used for enzyme-linked immunosorbent assay (ELISA). ELISA was performed to quantify monocyte chemoattractant protein-1 (MCP-1) and TNFα (Invitrogen) according to the protocol of the manufacturer. Briefly, a 96-well plate was pre-coated with caption antibody (1:250) and incubated at 4ºC ON. The wells were washed 3× with wash buffer (1×PBS with 0.05% Tween-20 (Merck)), blocked 1 hour with 1× ELISA/ELISPOT, and incubated with supernatant at 4ºC ON. Washing was performed 3× with wash buffer, followed by a 1 hour incubation at room temperature with detection antibody (1:250). Afterward, thorough washing of 6× was performed, followed by incubation with HRP for 30 minutes at room temperature. Finally, TMB solution was added for 15 minutes and the plate was measured on a spectrophotometer at 450 nm. A read of 570 nm was performed to eliminate background noise. The data were normalized to the untreated control and expressed as fold changes from the untreated control (set to 1). A standard curve was used as a positive control.

2.1.9 Angiogenesis assay

The endothelial HUVEC cell line was used to investigate the angiogenic effect of the peptides using the endothelial tube-formation assay kit (Cell BioLabs) according to the protocol provided by the manufacturer. Briefly, the 96-well plate (TPP) was pre-coated with the extracellular matrix gel and incubated 30 minutes in a 37ºC and 5% CO_2 incubator before use, 2×10^4 cells were seeded along with the peptide stimuli, and incubated for 4 hours at 37ºC. The cells were washed with 100 µL staining buffer and 1 µM calcein AM was incubated for 30 minutes to stain the tube-formations. The wells were washed 2× with 100 µL PBS to remove the staining, and the endothelial tubes were examined on a Leica fluorescence microscope at 380 nm. Images were acquired using a 10× objective and angiogenesis was quantified by counting the branches from each center point from three independent experiments. Media and 100 ng/mL recombinant vascular endothelial growth factor $(VEGF)_{165}$ were used as the negative and positive controls, respectively.

2.1.10 Bacterial strains

The clinical isolates for **paper II** were collected by culturing punch biopsy and a swab from patients with chronic inflammation at the Dermatological Department of Roskilde Hospital, (Denmark). For identification, the strains were grown in enriched broth at 35ºC with 5% CO_2 for three days and sub-cultured in 5% blood and chocolate agar plates for up to 10 days. The bacterial species were identified by colony morphology, antibiogram, and MALDI-TOF mass spectroscopy. In **paper II**, two identified *Staphylococcus lugdunensis* (*S. lugdunensis*) strains from healthy individuals, and the reference strain (ATCC 49576), were used to

compare MIC in Mueller hinton broth (MHB) media. The collection of punch biopsies was approved by the Regional Scientific Ethics committee.

Bacterial strains from **paper IV and V**, were gathered using bacterial swabs (Sarstedt) from murine wounds at day 0, before shaving the mice, and at day 5 and 10, before treatments were added topically to the wounds. The bacteria were identified using MALDI-TOF mass spectroscopy. The bacterial swabs were incubated in 1 mL brain heart infusion broth (Becton Dickinson) for 3 hours and recovered on three plates: lysogeny broth, chocolate agar (blood agar Nº2 Base), and mannitol salt agar plates. After 24-48 hours of incubation at 37ºC with 5% CO_2, colonies of different morphology (size, color, or texture) were collected for analysis. A single colony of a bacterial isolate was smeared on the sample plate and overlaid with galaxy HCCA matrix (Bruker). Samples unidentified on the MALDI-TOF, were re-run, but were overlaid with a combination of 70% formic acid and galaxy HCCA matrix to break the bacterial membrane and optimize ionization. Strains identified with a value of 1.70 or above were used, as previously described[186, 187]. The collection of bacterial swabs was approved by the Institutional and Governmental Research Ethical Board. All bacteria were stored in 15% glycerol at -80ºC.

2.1.11 Minimal inhibitory concentration

Bacteria were grown in 5 mL MHB media at 37ºC and a final concentration of 5×10^5 colony forming units (CFU)/mL was plated in a round bottom 96-well plate (Costar) with peptide stimuli for 24 hours. MHB medium and rifampicin (Sigma-Aldrich) or Nisin A were used as negative and positive controls, respectively. MIC was determined as the minimal concentration needed to inhibit visible growth of the bacteria. Experiments were performed twice in triplicates.

2.3. *In vivo* and *ex vivo* models

2.1.12 Porcine wound healing model

Ears from freshly slaughtered pigs were used to investigate migration and proliferation potential of peptide treatment *ex vivo*. The ears were thoroughly washed under tap water before the skin was separated from the cartilage. On ice, a 2 mm punch biopsy was used to mark the epithelial layer, and the epithelial and small part of the dermis was removed with a scalpel. The skin was cut into 1 cm^2 pieces and kept on sterile gaze in DMEM media with peptide stimuli in a 12-well plate. The media were changed daily, and the samples were collected at day 0, 1, 3, and 5 post-wounding and stored in 4% paraformaldehyde. The skin was analyzed by histology staining with ki67 (1:800, Abcam). For that purpose, the skin was divided in the center of the wound, cast in paraffin, and cut on a microtome. The sections were mounted on slides, boiled 1 hour at 60ºC, and the paraffin removed using tissue-clear (Sakura). The sections were further hydrated with washing in decreasing percentage of ethanol (96-70%). Previous to staining, the slides were boiled in T-EG buffer (pH 9), blocked in 2% bovine serum albumin and washed 3× in PBS. Incubation with the secondary antibody, biotin-conjugated (1:200, Vector laboratories), and HRP was performed, following signal amplification with biotinylated horseradish peroxidase macromolecular complex (Vector laboratories) and Avidin-Biotin Complex Kit (Vector laboratories). For visualization, 3,3-diaminobenzine (Vector laboratories) was used.

2.1.13 *Galleria mellonella* bacteria model

The greater wax moth *Galleria mellonella* (*G. mellonella*) was purchased from Mini Zoo (Denmark). The larvae were stored at 13°C without food in a conventional incubator. For the study, healthy larvae, with no dark spots, and of 0.20-0.30 grams weight were used. Previous to the experiments, the larvae were acclimatized for one day at 37°C. The larvae were injected 10 µL of 1-5×10^8 CFU/mL in the left proleg using a 0.3 mL syringe (31G, Pic solutions) and incubated at 37°C for 1 hour. Next, a second injection with peptide stimuli at the right proleg was given, and the larvae were placed in 9 cm petri dishes without food. The larvae were kept at 37°C and the survival of the larvae was monitored every 24 hours for three days. The larvae were considered dead when they were unresponsive to touch. Non-infected larvae, receiving PBS as first injection were used as controls. The controls were further separated into groups receiving peptide treatment or PBS at the second injection. Experiments were repeated twice with 10 replicates in each treatment group.

2.1.14 Murine wound healing model

From Charles River Corporation Inc. (Barcelona, Spain), male C57BL/6 mice of 25-30g were obtained. The mice were matched in age, housed in a room at 22-24°C with a 12 hours light/night cycle, and had free access to food and water. The mice were used for wound healing kinetic for LFcinB and IDR-1018, and bacterial diversity studies. The tissue samples were additionally used for q PCR, western blot, and immunohistochemistry.

One group of mice was made diabetic by intraperitoneally injections with streptozotocin (Sigma-Aldrich) at 50 mg/kg body weight for five consecutive days. All the treated mice were diabetic with a blood glucose of >250 mg/dL.

The mice were housed six weeks previous to the studies and given 0.1–0.2 units of isophane insulin (Novo Nordisk), subcutaneously, to avoid weight loss. On wounding day, analgesia was injected subcutaneously with 0.1 mg/kg of buprenorphine 30 minutes previous to anesthesia, and then every 6-8 hours for the following 48 hours. Anesthesia of isoflurane combined with oxygen was inhaled in a chamber with a flow rate of 0.5 L/minute, increasing from 2-5% until the mice were unresponsive to touch. Then, the mice were moved to individual masks with 2.5% anesthesia during the remaining procedure. Before the wounding, the dorsal hair was removed, the skin sterilized with betadine, and two full-thickness wounds were made. A 6 mm punch biopsy was used to mark the area and by lifting the skin with a tweezer, the wounded area was cut with a surgical scissor. For LFcinB, wounds were made side-by-side, whereas wounds were made top-and-bottom for IDR-1018. The wound size was monitored daily by drawing the wound area on acetate paper, and topical treatment was added twice a day the first two days and then once daily the remaining days. At day 10 post-wounding, the mice were sacrificed and biopsies of the wounds were harvested for analysis. Saline was used as a negative control and the experiments were performed with four mice per treatment with two wounds each.

The skin sections were preserved in OCT for fluorescent microscopy and cut in 10 μm sections on a microtome. Platelet endothelial cell adhesion molecule 1 (PECAM-1) (1:100, Millipore) was used as the primary antibody and anti-rat antibody (1:500, conjugated to Alexa Fluor 568, Invitrogen) as the secondary antibody. Both were diluted in PBS containing 1% and 5% bovine serum albumin with 0.1 vol.% Triton-X-100 (pH 7.4), respectively. The skin sections were fixated 10 minutes in ice cold acetone, blocked with 5% bovine serum for 30 minutes, and incubated at 4°C with the primary antibody ON. Next, the sections

were washed in PBS, incubated with secondary antibody and DAPI for 1 hour at room temperature, and images were captured using a Karl Zeiss Axio Observer Z1 microscope.

2.4. Statistics

Statistical analyses were performed with statistical package for social sciences version 24 and GraphPad version 6 to determine statistical significance between samples with one-way ANOVA, with post-hoc comparisons using the method of Tukey. A general linear model (univariate, main effects) and multiple linear regression was used to determine the variance components and correlations with multiple treatments for wound closure (Pearson correlations), respectively. GraphPad Prism version 6 was used for all the graphs. The significance level was set to $p<0.05$.

Chapter 3: Results and discussion

Non-healing wounds are characterized by their altered cellular functions, prolonged inflammation, and proneness to infection. Diabetic patients are in high risk of developing chronic DFU, because of their increased risk of neuropathy, vascular alterations, and infection[188].

The aim of this study was to evaluate HDPs with different origins for their wound healing effects and as potential topical treatment for non-healing wounds. Later, optimization studies were performed to evaluate the importance of different lengths and charges for biological activity. For this, several *in vitro* experiments were performed, as these studies are preferable for initial testing of new compounds. *In vitro* models were used to investigate migration, proliferation, angiogenesis, cytokine and chemokine levels, and bacterial growth. However, these studies have little value for complex physiological responses, making the

use of animal models crucial for testing the full effect of compounds. Therefore, more complex *ex vivo* and *in vivo* models were used, both for a more complete understanding of the effects of peptides, but also to investigate the accuracy of the *in vitro* models. The studies were performed using seven different peptides with human, bovine, or bacterial origin and varying in length from 11 to 51 amino acids and a net charge from 0 to +8. The peptides are summarized in **Table 1**.

The key findings in the papers are summarized in **Table 3**, and analogues are summarized later in **Table 4.**

Table 3: Key findings of paper I to VII – The peptides were investigated for migration, proliferation, angiogenesis, expression levels of cytokines (TNFα, IL-6, IL-8), the chemokine MCP-1, and their ability to dampen LPS-induced TNFα from PBMCs (10 ng/mL). The porcine model was used for migration (percentage) and proliferation (significance) (day 5), while the murine model was used for wound healing kinetics, compared to saline treatment. 0 = neutral effect (not different from control), + = p<0.05, ++ = p<0.01, +++ = p<0.001, - = p<0.05, --- = p<0.001, ND = not detected. [a] Bacterial activity from the murine wound model at 25 µg/wound,[b] Bacterial activity from *Galleria mellonella* model at 25 µg/mL

	IDR-1018	Insulin	LFcinB	Nisin A	Neuro-tensin	Substance P
Migration						
HaCaT	–	+++	+	+	+++	+++
HUVEC				++		
Proliferation						
HaCaT	0		0	0	0	0
HUVEC				0		
Angiogenesis						
In vitro	+++	+++			+++	+++
In vivo	++		++			
mRNA						
TNFα		---	---	---	---	---
IL-6		0		ND	0	+++
IL-8		+++	+	---	+++	0
PBMCs MCP-1						
+ LPS	+++		+++	+++		
- LPS	0		0	0		
Combination	+++		+++	+++		

HaCaTs MCP-1						
+ LPS	+++		+++	+++		
- LPS	0	++	0	0	+++	0
Combination	+++		+++	0		
PBMCs TNFα						
+ LPS	+++	+++	+++	+++	+++	+++
- LPS	0	0	0	0	0	0
Combination	0		0	0		
Porcine skin						
Migration%	80%		95%	97%		
Proliferation	0		+++	0		
Murine healing	+		+			
Anti-bacterial activity	+++[a]		++[a]	+++[b]		

First, a high-throughput screening model was optimized for determining migratory effects (**Paper I**). All the peptides were investigated for migratory potential (**Paper I-VII**) and most for their proliferative effects (**Paper III-V**). Migration and proliferation were further investigated using an *ex vivo* porcine skin model (**Paper IV-VI**). Cytokine levels were investigated in the HaCaT and PBMC cells (**Paper II-V**), and skin from pig and mice models (**Paper IV and V**). Changes in angiogenesis were likewise demonstrated *in vitro* (**Paper II and IV**) and in the murine wound healing models (**Paper IV and V**). Additionally, wound healing kinetics and bacterial diversity were investigated in diabetic mice compared to healthy mice (**Paper IV-V**), where bacterial properties likewise were investigated. *G. mellonella*, the greater wax moth larvae, was used for *in vivo* modeling of bacterial clearance (**Paper III**). Finally, optimization studies on IDR-1018 (**Paper IV**) were performed on analogues by investigating them for migratory effects, MIC, and anti-bacterial activity.

3.1. Migration assay as a screening tool

The HaCaT cells are an immortalized cell line, but have been proven to mimic primary keratinocyte cells[189, 190], HaCaT cells more reliable for keratinocytes found in wounds. A simple, cheap, and high-throughput migration screening assay was implemented using the optical camera system called the oCelloScope (**Paper I**). Several optimizations of the scratch assay were tested to detect migration (**Figure 4**). First, the FBS was reduced from 10%, as the standard growth medium, to 1% to minimize proliferation of cells (**Paper II**). Here, the untreated control closed the gap with approximately 35% (**Figure 4A and B**). Alternatively, mitomycin C was used before scratching to inhibit proliferation of the cells[191], resulting in a reduction of migration in the control to 20% (**Figure 4C and D**). Mitomycin C enables the investigation of migration alone and was therefore used as a standard (**Paper I, III-VII**), reporting the true migratory potential of the compounds.

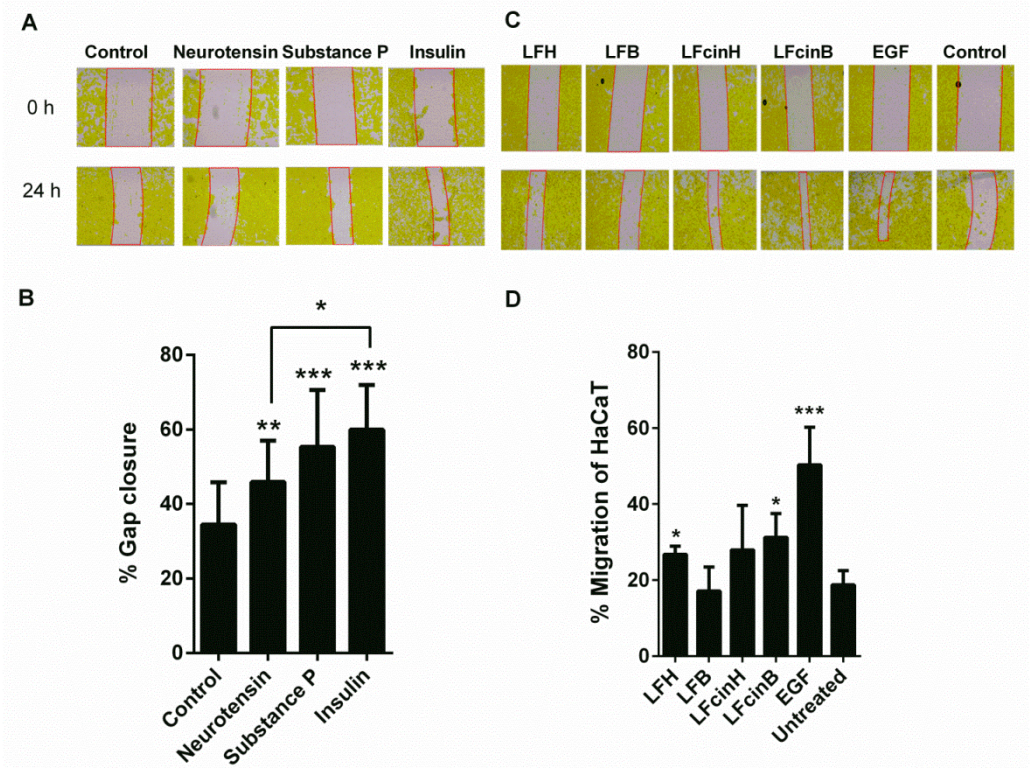

Figure 4: The cell migration of HaCaT cells stimulated by various peptides in an *in vitro* set-up with or without the use of mitomycin C – A) The gap closure effect of 25 µg/mL neurotensin, substance P or insulin was tested with a low percentage of FBS (1%) and compared to the untreated control. **B)** Quantification of percentage of gap closure from the initial scratch. **C)** The migratory effect of lactoferrin (LF) and lactoferricin (LFcin), from bovine and human origin tested on cells pre-treated with mitomycin C (10 µg/mL). **D)** Quantification of percentage of migration from the initial scratch. Graphs represent mean +SD of three independent experiments *= $p<0.05$, **= $p<0.01$, ***= $p<0.001$.

As mentioned, before keratinocytes can migrate the cells have to remodel the actin-myosin cytoskeleton[192]. Cells migrate by forming a protrusion in their leading end, adhering under the protrusion, disrupting the adhesion in the rear end, and contracting, resulting in cell migration[193]. However, little is known about the mechanism activating remodeling of the cytoskeleton, but it has been shown that re-epithelialization is driven by growth factors, such as EGF[194]. EGF was therefore used as the positive control for migration, resulting in 50% closure of the gap at 100 ng/mL after 48 hours (**Figure 4D**). The concentration of EGF

was tested at 500 ng/mL demonstrating a 80% closure over a period of 24 hours (data not shown)(**Paper III**). The oCelloScope was likewise used to detect proliferation, but none of the peptides had a significant effect (data not shown).

Migration was reported for two proteins, seven peptides, and numerous analogues (**Section 3.7**). Surprisingly, in the progress of this project, the screening of migratory potential of the various peptides demonstrated that only IDR-1018 (**Paper IV**) and LL-37[139] (appendix) had an inhibitory effect on migration. This data corresponded with a previous report result from IDR-1018[195].To insure that the demonstrated effects were indeed a result of peptide treatment, and not the addition of amino acids, an additional control of free amino acids was later included. The control of free amino acids was a mix of the amino acids found in the whole peptide in the same mole-ratio and demonstrated no significant migratory effects on HaCaT cells (**Paper III-V**).

The neuropeptides NT and SP are naturally secreted in the body and circulates between the brain on the peripheral organs[71]. These peptides have mostly been studied with regard to their role in the brain and central nervous system. However, neuropeptides have specific receptors found expressed on various immune cells, but also on epithelial keratinocytes, therefore they have been associated with migration[196], proliferation[197], and differentiation of keratinocytes[71]. The screening of migratory effects demonstrated that SP and NT enhanced migration of HaCaT cells (**Figure 5A**). However, no study has been performed to investigate the relationship between these peptides (**Paper II**). Co-treatment of SP and NT led to the surprising find that SP and NT have a negative correlation when used in combination (**Figure 5B-E**).

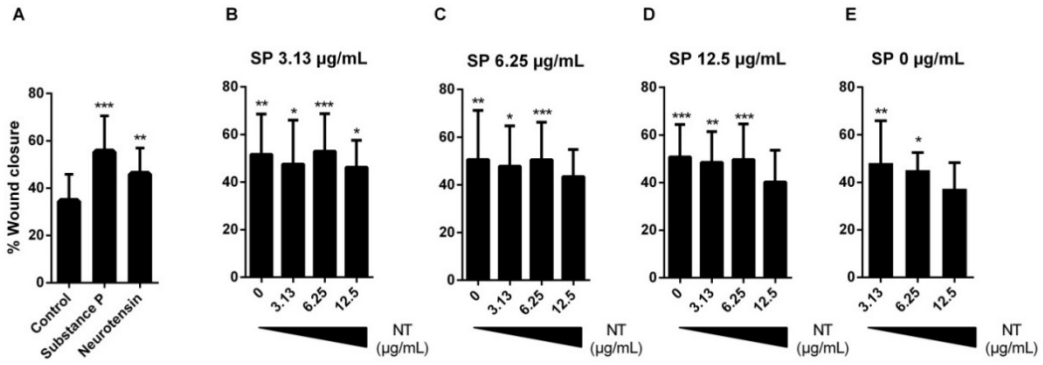

Figure 5: **Wound closure of HaCaT cells stimulated by different concentrations of neurotensin and substance P in a wound healing model –** A scratch was made in the monolayer of cells, and the cell migration was measured on an oCelloScope platform over 24 hours. The wound closure effect of neurotensin (NT), and substance P (SP) was tested on HaCaT cells alone or in combination ranging from 3.13-12.5 µg/mL and compared to the untreated control. **A)** Untreated (control), NT, and SP at 25 µg/mL. NT was tested at 3.13-12.5 µg/mL together with **B)** SP at 3.13 µg/mL **C)**, SP 6.25 µg/mL **D)** or 12.5 µg/mL. **E)** NT alone in concentrations ranging from 3.13-12.5 µg/mL. The graph demonstrates the mean +SD of four independent experiments performed in duplicates. *p<0.05, **p<0.01, ***p<0.001

Even though all the peptides alone increased migration, it was shown that some peptides can influence the migratory effect of each other. The mechanism behind this potential inhibition is unclear, and more studies are needed to explain these results.

Migration of HUVEC cells was tested in a 2D model (**Paper III and IV**) and 3D model (**Paper II and IV**). The 2D model was performed as for the HaCaT cells in a standard 48-well plate. However, the HUVEC cells cannot adhere to the surface of a plate without pre-coating with 0.1% gelatin. This led to problems with the detection of the cells for the oCelloScope. To evaluate the migratory and proliferative potential of the HUVEC cells, pictures taken with the oCelloScope were manually quantified. This process was slower compared to the data treatment with the non-coated cells but still enabled relatively high-throughput screening. However, the free amino acid control for Nisin A resulted in a

significant increase (**Paper III**), demonstrating that this model is not good for proliferation. Additionally, contradicting results were gathered when comparing migration of HUVEC cells in the 2D versus the 3D model for angiogenesis.

3.2. Angiogenesis

In the 3D model of angiogenesis, the cells migrated and proliferated in a matrix gel, enabling the cells to form tubes. This enabled an evaluation of angiogenic potential of peptides. All the peptide treatments increase angiogenesis at 25 µg/mL, and thereby increased migration and proliferation. The best angiogenic effect was detected for NT, closely followed by SP and the positive control $VEGF_{165}$. Interestingly, an *in vivo* study of NT demonstrated that it did not affect 3D tube formation[198], contradicting findings presented here (**Figure 6**). However, the study used different concentrations (17 and 170 µg/mL) and a different set-up, which could explain the differences[198]. These results for SP[199–201] and insulin[202] were confirmed by previous findings *in vitro*, as well as *in vivo*.

The lowest treatment effect was with insulin, but this difference could potentially be due to the high molecular weight (5808 g/mol) of insulin, compared to the smaller peptides (~1500 g/mol).

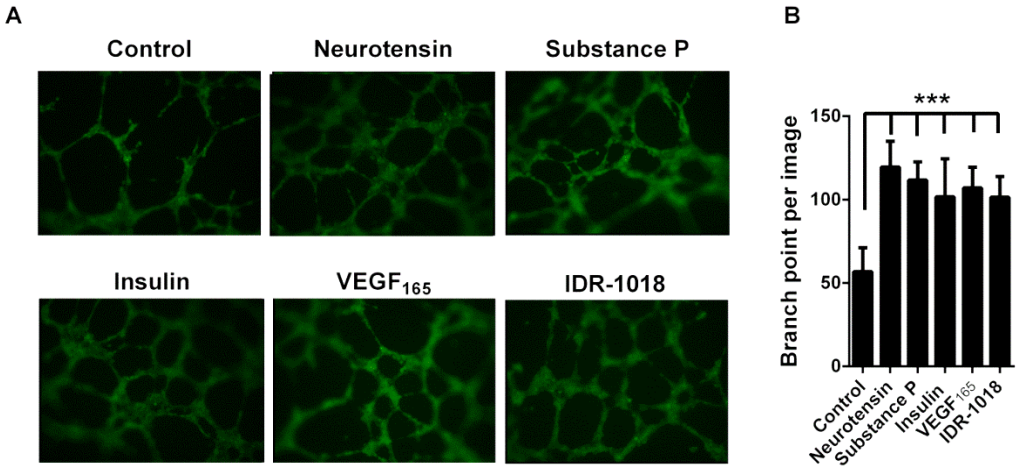

Figure 6: Migration and proliferation in angiogenesis assay, demonstrating the effect of neurotensin, substance P, insulin, and IDR-1018 – A) Tube-formation in HUVEC cells after treatment with 25 µg/mL neurotensin, substance P, insulin or IDR-1018. Calcein AM (1 µM) was used to stain the tube-forming cells with detection at 385 nm. Pictures were taken using a 10× objective on a Leica fluorescent microscope. **B)** Quantification of angiogenic branching points, determined by counting each branch point from each center point. Untreated HUVECs were used as a negative control and $VEGF_{165}$ (100 ng/mL) treated cells were used as a positive control. The graph represents the mean +SD of three independent experiments performed in duplicates. Of each duplicate, three representative images were used. *** = $p<0.001$.

In the scratch assay, an inhibitory effect was demonstrated for treatment with IDR-1018. However, in the 3D model for angiogenesis, IDR-1018 significantly enhanced tube formation with the HUVEC cells, indicating an increase of both proliferation and migration of the cells. The importance of 3D models have previously been demonstrated for cancer models, where the 2D model deviated from the 3D model, as the nature of the HUVEC cells were not met in the 2D model[203]. The same is demonstrated here, where the cells in a 2D model are flat and sheet-like in their morphology, whereas the 3D model enables natural shapes and structures[204]. Furthermore, the 3D model alters the cell motility as they are surrounded, giving rise to more complex cell-cell interactions[205] and dynamic migration[206]. Together, this potentially makes the 3D models a better indicator of how the effect will be *in vivo*.

The angiogenic potential of IDR-1018, as well as LFcinB, was also investigated *in vivo* in a murine wound healing model (**Figure 7**). The wounds were treated with either a low or high concentration of 12.5 or 25 µg/wound, based on a previous study with IDR-1018 demonstrating wound healing effects at these concentrations[207]. Histology sections of the murine wounds were evaluated with immunohistochemistry using platelet endothelial cell adhesion molecule 1 (PECAM-1) to detect dividing endothelial cells (**Paper IV and V**). The treatment with LFcinB was used on both healthy (**Figure 7A**) and diabetic mice (**Figure 7B**), while IDR-1018 was only used on the diabetic mice (**Figure 7C**).

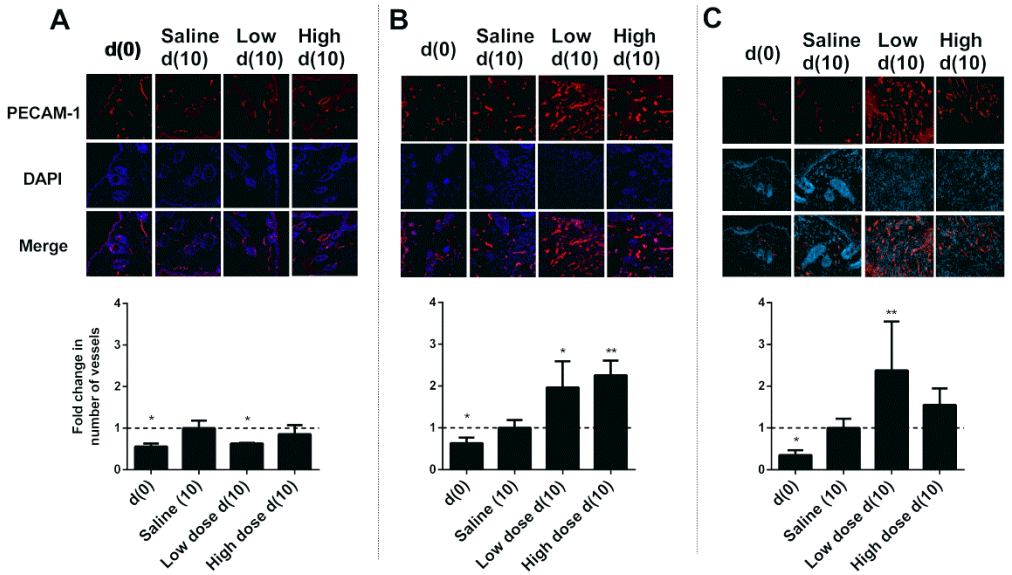

Figure 7: Angiogenesis on wounded murine skin samples after LFcinB and IDR-1018 treatment – Murine wound healing models were used to evaluate topical treatment with LFcinB and IDR-1018 in a low and high concentration (12.5 and 25 µg/wound, or saline. **A)** Healthy mice treated with LFcinB, **B)** Diabetic mice treated with LFcinB, and **C)** Diabetic mice treated with IDR-1018. Representative pictures of immunohistochemistry analysis of PECAM-1 of wounded murine skin samples and quantification of mice skin samples stained for PECAM-1 in FIJI software are shown. The graphs demonstrate the mean +SD of five replicates of each the four mice in the three groups. * = $p < 0.05$, ** = $p<0.01$.

The treatments were topically applied to the wounds for 10 days and compared to saline treatment. The treatment with IDR-1018 significantly enhanced angiogenesis at the low concentration, while LFcinB enhanced angiogenesis at

both concentrations. For the non-diabetic mice, treatment with LFcinB had a negative effect at the low concentration but a neutral effect was seen for the high concentration. For the diabetic mice a significant increase was shown with both concentrations of LFcinB and the low concentration of IDR-1018, where the high concentration demonstrated a tendency to increase angiogenesis. The 3D model supported the *in vivo* data for IDR-1018 treatment to some degree as both models demonstrated a positive effect.

Only few studies have investigated the angiogenic effect of IDR-1018, but a study demonstrated that IDR-1018 enhanced the level of mRNA of VEGF 75-fold[195]. It is therefore possible that IDR-1018 does not affect angiogenesis directly by increasing migration and proliferation, but indirectly by altering VEGF levels. LFcinB only increased healing for diabetic mice, which might be connected with the increased angiogenesis. However, LFcinB has previously been demonstrated to inhibit both VEGF and fibroblast growth factor *in vitro*[208], as well as inhibit angiogenesis in tumor tissue[209]. LFcinB could therefore have an effect on processes altered in diabetic mice. Furthermore, investigation of the effect of peptides on mRNA or protein level on key growth factor as VEGF could give a better insight in the demonstrated effects. Further research is needed to explain this find.

3.3. Cytokine and chemokine levels with qPCR and ELISA

Cytokines in the skin barrier are important for repair[210]. The cytokines work through the specific receptors which mediate their biological effect. In this thesis, the expression levels of the cytokines TNFα, IL-6, and IL-8, and the chemokine MCP-1 were investigated in HaCaT cells and PBMCs. However, expression

levels of IL-10 were also investigated, but were not detectable with qPCR, probably because IL-10 is normally expressed in very low levels.

On mRNA level, all of the peptides treatment decreased the level of TNFα (**Figure 8C, Paper III, IV, and V**). For IL-6, the mRNA levels were either decreased (**Paper III**), unchanged or increased (**Figure 8A**), or undetected (**Paper V**). Different levels of IL-8 mRNA were demonstrated and were either decreased (**Paper III or V)** or unchanged (**Figure 8B**) after the peptide treatment. The secretion levels of the chemokine MCP-1 were investigated from both HaCaT cells and PBMCs.

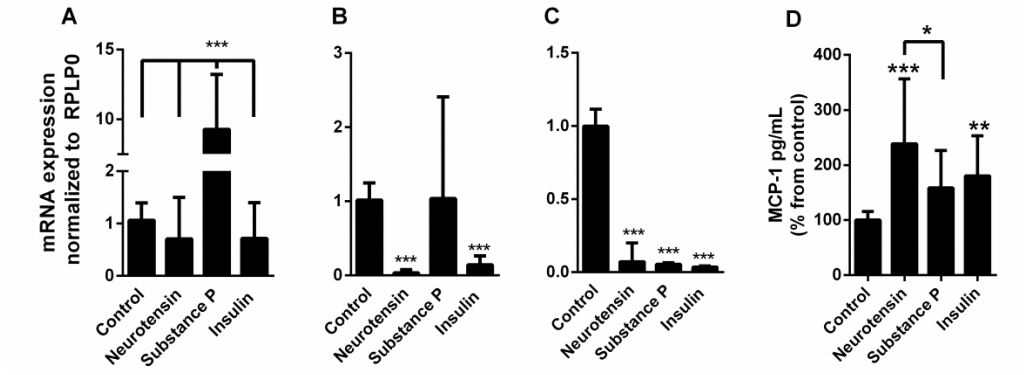

Figure 8: Relative mRNA expression levels quantified from HaCaT cells after 24 hours of treatment and secretion levels of MCP-1 from PBMCs – HaCaT cells and PBMCs were treated with neurotensin, substance P, and insulin (25 µg/mL). **A)** The mRNA level of IL-6 was significantly increased when treated with substance P, but when treated with neurotensin and insulin it was slightly decreased. **B)** The mRNA levels of IL-8 were significantly decreased when treated with neurotensin and insulin, but substance P treatment demonstrated a tendency of increasing the level of IL-8 compared to the control. **C)** All the treatments significantly decreased the mRNA levels of TNFα. **D)** PBMCs were treated, and the supernatants were investigated for MCP-1 with ELISA. Treatment with neurotensin and insulin significantly enhanced the level of MCP-1, while substance P treatment showed no difference from the control. The graph represents the mean +SD of experiments from three different healthy donors * = p<0.05,** = p<0.01,*** = p<0.001

For the HaCaT cells, stimulation with the majority of the peptides alone did not alter the levels significantly (**Figure 8D, Paper III, IV, and V**), while NT and insulin enhanced the levels significantly. Surprisingly, a significant difference was demonstrated between NT and SP. The main difference of NT and SP is the

charge, where NT is neutral and SP has a net charge of +2. However, insulin has a charge of +5, further underlining the dissimilar mode of action between the two neuropeptides (**Figure 5**). In chronic wounds, SP could be the better choice as MCP-1 attracts immune cells[211].

The mentioned pro-inflammatory cytokines are primarily secreted from immune cells as a response to PAMPs. Of the PAMPs, LPS is one of the most studied of the Gram-negative bacteria[212]. LPS is recognized by TLR4 found on various cells, such as keratinocytes[213] and on PBMCs[214], which leads to gene transcription of TNFα, IL-6, and IL-8[215–217]

TNFα is critical in early wound repair, as it is a key molecule to signal invasion of pathogens and to start the healing process[218]. However, prolonged increased expression can result in apoptotic effects, stalling wound healing[219]. IL-6 and IL-8 on the other hand have both pro- and anti-inflammatory properties[220]. IL-6 is important for wound healing, demonstrated in a IL-$6^{-/-}$ knockout mice model, where the lack of IL-6 was connected with decreased vessel formation due to decreased levels of VEGF[221, 222]. IL-8 has likewise been shown to have angiogenic properties, but also chemotactic properties[223]. After migration of neutrophils through the endothelium, IL-8 exerts a strong chemotactic response, that accelerates neutrophils migration toward the wound area[224]. However, no clear connection between the various effect of the peptide treatments and angiogenic effect was seen. Potentially, the decrease of some of the pro-inflammatory cytokine could theoretically affect the transition of M1 macrophages to M2. The macrophage cell line J7741A was used to investigate migration potential of the peptides, but the experiments were unsuccessful (data not show). It would have been interesting to investigate if stimulation of the

macrophage cell line with peptides alone, or in combination with LPS, could alter polarization of the M1 and M2.

The secretion levels of MCP-1 and TNFα from HaCaT and PBMC cells were investigated. MCP-1 was also increased as a result of PAMP recognition[225, 226] and combination studies were likewise performed (**Paper III-V**). LPS alone stimulated a significant enhancement of MCP-1 for HaCaT cells (**Figure 9A**), but for TNFα, the secretion levels were undetectable for all treatments (data not shown). On PBMCs, LPS significantly enhanced the level of MCP-1 (**Figure 9B**) and TNFα (**Figure 9C**). When using co-treatment of LPS to stimulate an infection on HaCaT cells, Nisin A was the only treatment that decreased the levels of LPS-induced MCP-1. This might be due to the ability of Nisin A to bind lipid A of LPS. For the MCP-1 levels secreted from PBMCs, the peptides did not alter the levels (**Figure 9B**). For TNFα secretion levels from PBMCs, all the tested peptides diminished the LPS-induced levels of TNFα to levels no different from the untreated control, except for the highest concentration of LPS (100 ng/mL) for LFcinB and the analogue A11 (**Figure 9C**). Interestingly, IDR-1018 diminished the effect of LPS for all concentrations, while the analogue A11, with lower charge but three amino acids shorter (VRL in the N-terminal end, see **table 4** for full sequence), had less effect with the higher concentration. However, A11 still decreased the level of TNFα for all the tested concentrations.

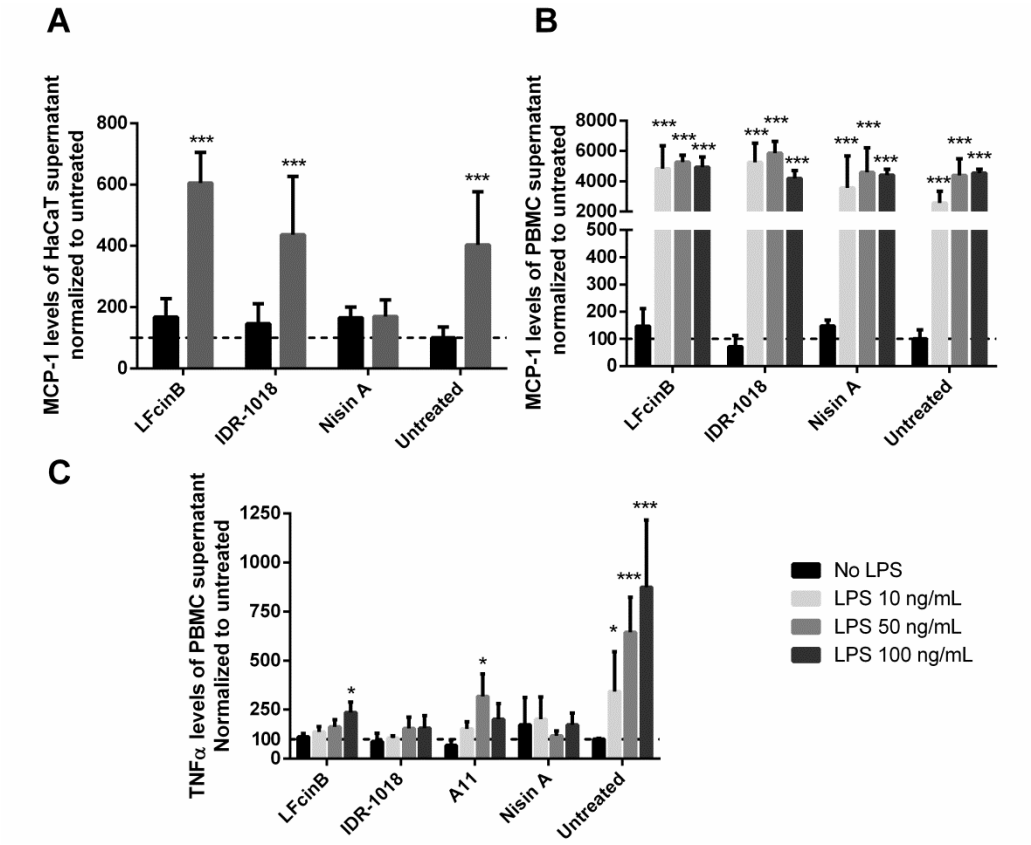

Figure 9: LPS-induced levels of TNFα and MCP-1 after treatment with LFcinB, IDR-1018, A11, or Nisin A from HaCaT and PBMC cells – The cells were treated with LPS (*Pseudomonas aeruginosa*) alone (10, 50, or 100 ng/mL) or in combination with LFcinB, IDR-1018, or Nisin A (25 µg/mL). **A)** HaCaT cells had significantly increased levels of MCP-1 when stimulated with LPS. When co-stimulated with peptide, only Nisin A inhibited the LPS-induced levels of MCP-1. Stimulation with peptide alone demonstrated a slight increase. **B)** PBMCs had significantly increased levels of MCP-1 when stimulated with LPS, and co-stimulation did not alter the MCP-1 levels. On the other hand, co-stimulation increased the levels, compared to PBMCs stimulated with LPS alone. **C)** PBMCs had significantly increased levels of TNFα when stimulated with LPS in a dose-dependent manner. Co-stimulation with peptides demonstrated that LFcinB inhibited the LPS-induced levels of TNFα in a dose-dependent manner, but not for the highest concentration of LPS. IDR-1018 and Nisin A inhibited LPS for all concentrations, whereas the IDR-1018 analogue A11 did not inhibit LPS at 50 ng/mL. The graph represents the mean +SD of experiments from three different healthy donors * =p<0.05, ** = p<0.01,*** = p<0.001

The effect demonstrated on the TNFα levels were not due to binding of the LPS, as previous studies have demonstrated that IDR-1018 does not block LPS by

binding to it directly[167], as might be the case for Nisin, as it is known to bind lipid A[227]. LFcinB on the other hand has been speculated to have an different binding site than lipid A[228], as a consequence of its different structure changing from α-helix to a β-sheet[229]. The effects shown here might be due to immunomodulation of the PBMCs. For treatment of chronic wounds, Nisin A seems the best candidate, as it neutralized pro-inflammatory cytokines, LPS-induced MCP-1 and TNFα levels.

To investigate the wound healing effects of treatments, many models have been used. The most reported are mouse, rat, pig, and dog models[230], and less traditionally the *Drosophila* and zebrafish models[194]. Here, pigs and mice were used to evaluate wound healing properties in more complex models.

3.4. Porcine wound healing model

The porcine skin model has been considered the best model for human skin, because of its similarities[231], having similar anatomy, skin composition[232], absorption of drugs over the skin[233–236], and immune cell responses[237], such as mast cells, dendritic cells, and neurotrophils[238, 239]. Additionally, the skin of the pigs closely resembles the human skin, since it has tightly connected skin layers, whereas rodents have loosely connected skin. Pigs have similar HDPs, such as LF and LFcin, but they also have peptides not found in humans[240, 241]. A review of 25 studies revealed a consensus between pigs and humans for 78% of *in vivo* studies, while only 53% was consistent between murine and humans[242]. The porcine model is therefore an important model but is less attractive because of its high cost, need of space, and long gestation period[243].

Finding an ideal model to investigate chronic wounds is still an ongoing quest[244]. In this study, the migration and proliferation were tested in porcine skin in an *ex vivo* model, using Nisin A (**Paper III**), IDR-1018 (**Paper IV**), and LFcinB (**Paper V**). Migration and proliferation were quantified from histological sections collected at day 0, 1, 3, and 5 post-wounding by staining with ki67 (**Figure 10**). Migration was measured over the wound bed and reported as a percentage of re-epithelialization (**Figure 10B**) and proliferative cells were given per amount of cells in the epithelium layer (**Figure 10C**). All the treatments lead to a steady increase of re-epithelialization, but only LFcinB treatment enhanced migration and proliferation significantly from day 1 to day 3. No significant proliferative or migratory effects were seen for IDR-1018 or Nisin A treatments. However, all the treatments had a tendency of improving the re-epithelialization compared to the control. Interestingly, the *ex vivo* model demonstrated different results compared to the *in vitro* data. For migration, Nisin A significantly enhanced migration *in vitro* (**Paper III**), but this was not seen *ex vivo.*

The enhancement demonstrated by LFcinB, and not the others, could potentially be because of its higher charge. However, the different effects of the peptides might be explained by their different modes of action. IDR-1018 has been proven to have antimicrobial activity against various Gram-positive and Gram-negative strains, both in planktonic and biofilm state[111, 129, 146, 245], but mostly it is studied for its immunomodulatory effects[165, 167, 246]. Previously, the wound healing effect of IDR-1018 was documented on pigs, where healing of full-thickness wounds were observed after 10 days[207]. In the *ex vivo* model, the tested skin had no blood or oxygen supply as is the case for some DFUs[247].

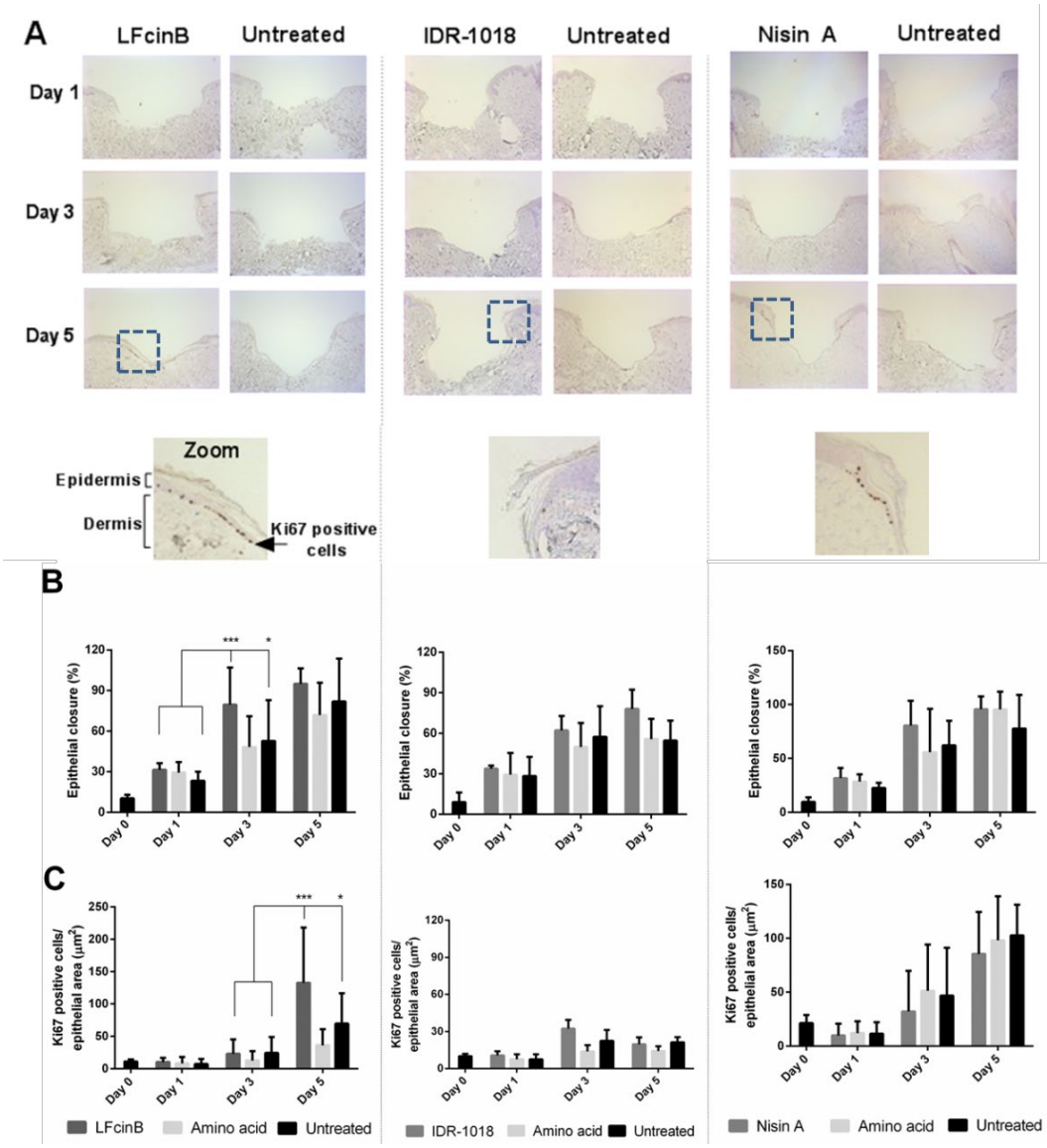

Figure 10: Treatment with LFcinB, IDR-1018 or Nisin A on an *ex vivo* porcine wound healing model - Ears from freshly slaughtered pigs were treated with the peptides LFcinB, IDR-1018 or Nisin A. Free amino acids or media were used as controls. The skin samples were treated for five continuous days and samples were collected at day 0, 1, 3, and 5 for histological analysis. **A)** Representative pictures after ki67 staining. The zoom shows a clear separation of the epithelium and dermis layers, where the ki67 positive cells are located in the epithelial layer **B)** Quantification of epithelial closure calculated as percentage of closure compared to the size of the wound bed. **C)** Quantification of ki67 positive cells in the epithelial layer of the wound bed. The graphs demonstrate the mean +SD of two independent experiments performed with 8 replicates of each treatment for each collection day. * = $p<0.05$, *** = $p<0.001$

Consequently, the chemotaxis property of IDR-1018 will diminish, as immune cells, such as neutrophils have a half-life of 6-8 hours[248]. Nisin A only has antimicrobial activity against Gram-positive bacteria[249, 250], and it is therefore possible that Gram-negative bacteria can have had a negative effect on the re-epithelialization. Finally, LFcinB is active against both Gram-positive and Gram-negative bacteria[251, 252]. LFcinB binds bacterial membranes, making holes in the membranes, but does not disintegrating them, which has led to the hypothesis that LFcinB has an intracellular target, beside its ability to target the bacterial membranes[253, 254]. The different modes of action are therefore important to include in the analysis of the data, as aspects can be neglected in some models.

This porcine model has not been described previously in the literature and several optimization steps were tested in regard to wounding method and washing of the skin (not shown). The *ex vivo* model had previously been used for antimicrobial testing[255], and different washing methods were investigated[256]. From the study, a milder washing with 70% ethanol was chosen to ensure epithelial survival. However, a harder method should still be tested. The *ex vivo* model presented here still needs optimization, as big variations were observed and the skin were prone to undesired infections. Here, the media of the skin samples were changed daily, but still infections were a recurring problem. Infection was especially a problem for control treatments of media and amino acid treatments (data not shown). Thereby, the antimicrobial effects of the peptide treatments were indirectly shown. The differences in mode of action could be confirmed by evaluating the pathogens seen in the infection, as the differences could be due to lack of anti-pathogen activity against specific microorganisms found on the porcine skin.

The *ex vivo* model seems a good model for lead peptides, because it is mimics chronic wounds and it is ideal for further investigation of bacterial effects at a later point. A combination study of peptides could be interesting, as synergy between treatments is often reported[252].

3.5. *Galleria mellonella* bacterial clearance model

The *Galleria mellonella* (*G. mellonella*) is a great wax moth that, unlike mammals, only has an innate immune system. However, this system consists of cellular and humoral responses, similar to that of mammals[257]. The *G. mellonella* model was used both to investigate the bacterial clearance potential of Nisin A *in vivo*, but also to demonstrate potential immunomodulatory effects (**Paper III**). Two bacterial strains were used, the Gram-positive *S. epidermidis* and the Gram-negative *E. coli*. As Nisin A only has antimicrobial activity against Gram-positive bacteria[249, 250], it was hypothesized that if treatment could alter survival of the *E. coli* treated larvae, it would be due to immunomodulation. However, the treatment only increased survival of the larva injected with the Gram-positive strain (data not shown). Thereby, Nisin A did not enhance immunomodulation in *G. mellonella*, but it significantly enhanced survival against *S. epidermidis* compared to the control.

The potential of *G. mellonella* larvae as a model to study immunomodulation has not been explored. This model is a very simple and easily managed *in vivo* model for studying planktonic infection, but also has a great potential to study pre-formed biofilm in future studies. The simplicity of the model makes it good for screening bacterial clearance *in vivo*.

3.6. *In vivo* murine wound healing model

Rodents are most often used as a model for wound healing, because they are cheap, have a short gestation period, and are easily maintained and genetically manipulated[258]. In this study, a healthy and a diabetic mice model were used to investigate IDR-1018 and LFcinB (**Paper V and VI**). Wounds were made manually, and the wound healing kinetics of the treatments were followed for 10 days, where bacterial swabs were collected at day 0, 5 and 10 (**Figure 11**). The healthy mouse model was treated with LFcinB (**Figure 11A**), while the diabetic model was pre-treated with streptozotocin to ablate the β-cells in the pancreas, and then treated with either LFcinB (**Figure 11B**) or IDR-1018 (**Figure 11C**). Treatments with both LFcinB and IDR-1018 enhanced wound healing in the diabetic mouse model, but not in the non-diabetic treated with LFcinB. The enhancements were mostly seen for the later days, where the effect of LFcinB was seen at day 5, and 7-9, while IDR-1018 was not affected until day 9 and 10. Additionally, bacteria with different morphology were identified to species level from the wounds and given as percentage of distribution. The wounds of the healthy mice demonstrated a low bacterial distribution, whereas the diabetic mice had a greater diversity. Once the wounds were treated, the diversity in the wounds decreased. This was especially seen for the IDR-1018 treatment, where treatment with 25 μg/wound diminished growth of all but *Staphylococcus aureus* (*S. aureus*) and *Staphylococcus xylosus* (*S. xylosus*). However, the three experiments were performed at different times, but in the same laboratory facilities. Additionally, the wounding on the diabetic mice were different (side-by-side for LFcinB and top-and-bottom for IDR-1018), making the comparison between the treatments difficult. The alteration was made, as the top wound for IDR-1018 closed fast, compared to the bottom wound.

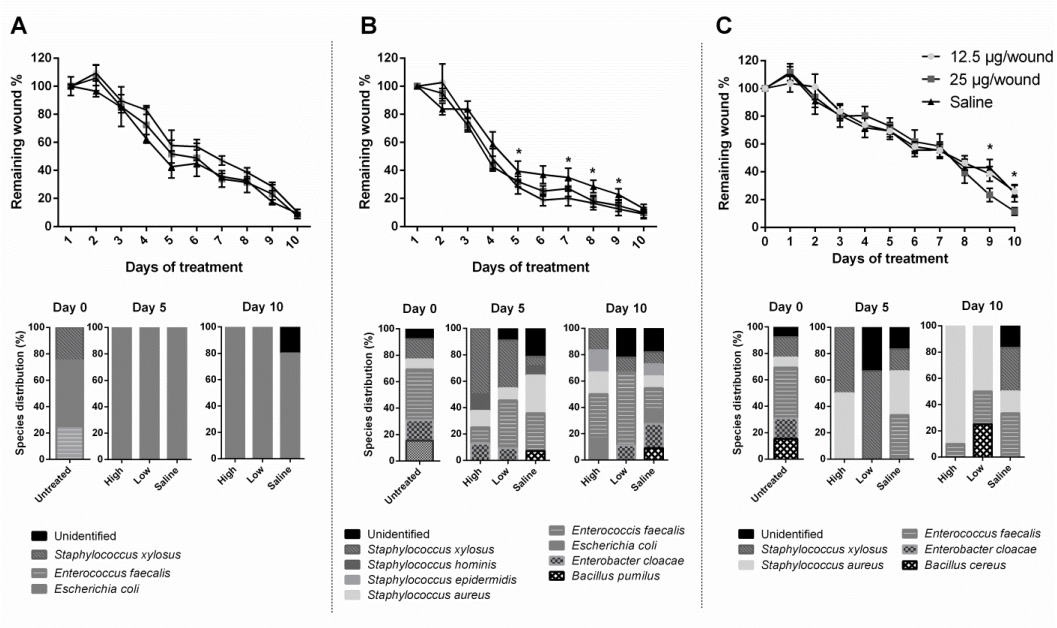

Figure 11: Peptide treatment enhances wound healing for diabetic mice and alters the microbiomes in the wounds – After wounding, mice were separated into three treatment groups: Low or high concentration of bovine lactoferricin, IDR-1018 (12.5 or 25 µg/wound), or saline. The mice were treated for 10 consecutive days, with two treatments the first two days and hence once per day. **A)** Healthy mice treated with LFcinB demonstrated no significant effect in wound closure after the treatments. Bacteria identified in the wound decreased over the treatment periods, but the effect was not due to the treatment **B)** Diabetic mice treated with LFcinB demonstrated a significant increase in wound healing at day 5 and 7-9, when treated with high the concentration of LFcinB, compared to saline treatment. The bacterial diversity in the wounds decreased slightly with treatment. **C)** Diabetic mice treated with IDR-1018 demonstrated enhanced healing at day 9 and 10, compared to saline treatment. Furthermore, the bacterial diversity of the wound decreased over the treatment and a significant decrease was seen at day 5 and 10. The graph represent mean +SD of an experiment performed with four mice with two wounds per mice for each treatment. * = $p<0.05$.

Nevertheless, all the treatments decreased bacterial strains and enhanced wound healing. Assessing the bacterial load in the wounds would have been of great interest, as the treatment could have affected the diversity without altering the total load in the wounds. As with the porcine wound model, LFcinB showed to be the best treatment by increasing wound healing over most days in the diabetic model, compared to IDR-1018. However, IDR-1018 has previously been shown to significantly enhance healing in healthy mice in lower concentration than used

here[207]. Murine wounds treated with saline had higher bacterial diversity, and did not affect wound healing kinetics. Regardless, the saline treatment had the same overall effect on day 10, where 10% of the wounds still remained open as also seen for the peptide treated wounds. However, the skin of the rodents and the primary wound healing method differs that this of humans. The rodents have skin loosely attached to the subcutis with consecutive hair that is replaced every third week, and heal mainly by contraction of the wound[258, 259]. Humans on the other hand, have tight skin attachment and heal by granulation tissue formation and re-epithelialization[260]. The formation of granulate tissue has been demonstrated to be largely dependent on angiogenesis. Therefore, angiogenesis of the wounds was investigated as previously shown (**Figure 7)**.

As mentioned, one of the major problems related to chronic wounds is high levels of pro-inflammatory cytokines. This was confirmed, using streptozotocin treated murine models, which simulated type I diabetes, showing an enhanced level of TNFα that was 3-fold higher for diabetic mice compared to the healthy mouse model (**Figure 12**).
Surprisingly, treatments with LFcinB heightened the level of TNFα on mRNA level at day 10 compared to saline treatment, while LFcinB significantly decreased TNFα levels on the healthy mice. However, the high dose gave different results for the different mice in the treatment group, resulting in a high standard deviation, but the effect was not significant. It would have been interesting to see how the levels were in earlier phase of healing, as the wounds were closed on day 10, to see if the treatments altered the levels in the inflammatory versus the migrative phase.

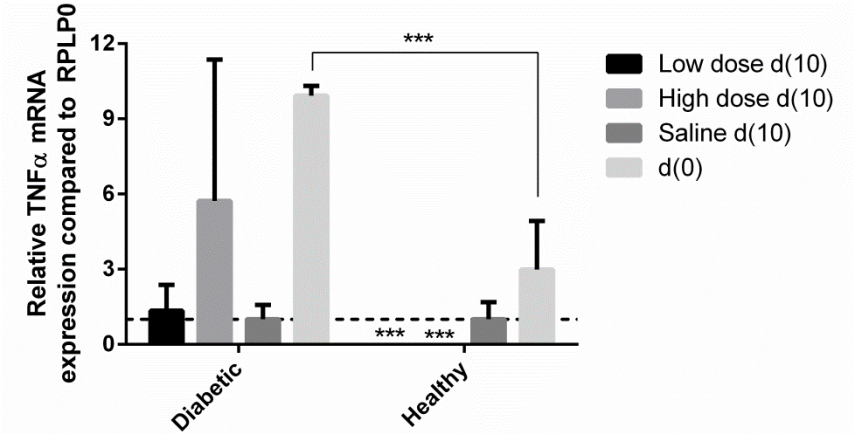

Figure 12: mRNA cytokine levels in murine skin are altered in diabetic mice compared to healthy mice, when treated with LFcinB – The mRNA levels of the pro-inflammatory cytokines TNFα were measured in skin samples collected at day 0 and at day 10 for diabetic and healthy mice. For both diabetic and healthy mice, the mice were separated into three treatments groups: Low and high dose of bovine lactoferricin (12.5 and 25 µg/wound, respectively), or saline. *** = p<0.001.

Overall the murine model demonstrated differences for diabetic versus non-diabetic mice and the treatment increased healing with peptide treatments. However, at day 10, no matter the treatment type, the wounds were closed in an equal manner. This indicates that either the treatments were not good enough, or that the model is not ideal. The differences in healing and skin composition, between mice and humans further raise questions regarding the usefulness of the murine wound healing model. As previously mentioned, the translatability of the murine model to human is low compared to the porcine models. The porcine model could potentially become a better model, as the skin itself is more comparable to human skin. The murine model is good for initial studies, as it is easier and cheaper than live porcine models, but conclusions should not be made using murine studies alone.

3.7. Optimization of peptides

Optimization studies are most often performed to either increase activity and/or to shorten large peptides, to make it more cost-efficient to synthesize peptides. Here, the IDR-1018 was chosen, since it affected migration. Furthermore, investigation of the structure-activity relationship was performed on libraries of analogues by investigating MIC, toxicity, viability, and migration compared to the parent peptides. For the IDR-1018 library, one amino acid from the C- or N-terminal was removed one at a time to shorten the length down to a 4-mer (**Paper IV**). The analogues were used for evaluating charge and length (**Table 4**).

The IDR-1018 library consisted of 16 analogues (A1-A16), where the parent peptide IDR-1018 has a net charge of +5, MIC of 16 µg/mL against *S. epidermidis*, no toxic effects, and inhibits migration. Characteristic for the terminal ends of IDR-1018 is a cluster of the positively charged amino acid arginine.

Shortening IDR-1018 from the C-terminal demonstrated no alteration in antimicrobial activity whether the charge was +5 or +4 (A1). However, when amino acids were removed from the N-terminal, the analogue A9 had a 4-fold increase in antimicrobial activity, compared to IDR-1018, even though it had the same net charge (**bold in Table 4**). When the length was further shortened from the C-terminal end, the antimicrobial activity decreased, correlating with loss of charge. When the length was shortened with four amino acids or more (A4-A8), the antimicrobial activity was lost at the tested concentrations.

Table 4: Amino acid sequence, MIC, hemolysis, viability, and migratory effects of analogues of IDR-1018 – All data represent treatments of 100 µg/mL. MIC was against *Staphylococcus epidermidis*, and hemolysis

was tested on red blood cells (RBC) isolated from healthy donors. Viability was tested on HaCaT and HUVEC cells, as well as migratory effect on HaCaT cells.

Name	Sequence	Charge pH 7	MIC µg/mL	Hemolysis RBC %	Viability HaCaT HUVEC	Migration fold from control
IDR-1018	VRLIVAVRIWRR	+5	16	2.5 ± 1.6	109 ± 3 199 ± 15	0.6 ± 0.3
A1	VRLIVAVRIWR	+4	16	1.8 ± 0.6	98 ± 6 134 ± 12	0.7 ± 0.2
A2	VRLIVAVRIW	+3	32	2.0 ± 0.9	115 ± 1 107 ± 8	1.4 ± 0.1
A3	VRLIVAVRI	+3	32	1.8 ± 0.6	112 ± 1 103 ± 15	1.1 ± 0.3
A4	VRLIVAVR	+3	>64	1.8 ± 0.6	104 ± 3 127 ± 19	0.7 ± 0.2
A5	VRLIVAV	+2	>64	1.9 ± 0.7	98 ± 3 216 ± 8	1.2 ± 0.3
A6	VRLIVA	+2	>64	1.8 ± 0.5	86 ± 2 171 ± 12	1.4 ± 0.4
A7	VRLIV	+2	>64	3.7 ± 5.6	146 ± 6 187 ± 33	1.3 ± 0.4
A8	VRLI	+2	>64	1.8 ± 1.9	111 ± 2 169 ± 18	1.0 ± 0.4
A9	RLIVAVRIWRR	+5	**4**	1.8 ± 1.0	126 ± 3 124 ± 11	0.5 ± 0.3
A10	LIVAVRIWRR	+4	16	1.9 ± 1.1	113 ± 3 169 ± 30	0.9 ± 0.4
A11	IVAVRIWRR	+4	>64	1.7 ± 0.9	119 ± 8 189 ± 41	**1.6 ± 0.3**
A12	VAVRIWRR	+4	>64	1.9 ± 1.1	111 ± 9 166 ± 34	1.0 ± 0.4
A13	AVRIWRR	+4	>64	1.8 ± 0.8	129 ± 23 177 ± 40	1.1 ± 0.4
A14	VRIWRR	+4	>64	1.8 ± 0.7	117 ± 7 162 ± 12	1.2 ± 0.2
A15	RIWRR	+4	>64	1.8 ± 0.8	120 ± 3 159 ± 23	1.4 ± 0.4
A16	IWRR	+3	>64	2.6 ± 1.0	115 ± 6 154 ± 20	1.0 ± 0.5

The antimicrobial activity was greatly reduced once three amino acids were removed from the N-terminal, despite having a net charge of +4. This might indicate that the amino acids at the N-terminal are important for stabilizing or folding the peptide, and that an optimal length for IDR-1018 is nine amino acids. However, more research is needed to support this data.

The analogues were also tested for their migratory properties, normalized to the untreated control, and compared to IDR-1018. Many of the analogues had an increased migratory effect of the HaCaT cells, but only A2 and A11 had a significant effect (**bold in Table 4**). Both analogues have a net charge of +4 and were 11-mer and 9-mer, respectively. Here however, charge should not affect their ability to influence the biological effect, as the lipids in eukaryotic cells have no charge[126]. Finally, the analogues were investigated for cytotoxic effects. IDR-1018 had no cytotoxic effect against RBC, HaCaT, or HUVEC cells. Unsurprisingly, the shortened analogues had no toxic effects either. It would have been interesting to test the effects of the analogues on cytokine levels, as done for IDR-1018 to compare the immunomodulatory activities. Furthermore, a combination study using A9 and A11 would be of high interest, as they have enhanced antimicrobial activity and migratory effect, respectively.

3.8. Problems with peptides as therapeutic agents

Several aspects need to be addressed before peptides can be used in the clinic, such as proteolytic degradation issues, high cost of synthesis, and resistance[261]. Stability of the peptides is a problem, as they are prone to proteolytic degradation and thereby have a short half-life. Regulation of naturally secreted peptides is maintained by proteases, such as peptidases, which are found in the membranes of keratinocytes and fibroblasts among others[262]. Treatment of wounds with peptides therefore requires an optimal dose to reach therapeutic effect before the peptides are degraded. The proteolytic issue of peptides has been addressed by altering amino acids in the peptides from L-amino acids to D-amino acids, or by altering the backbone of the peptide, termed peptoids[263]. The peptoids were

demonstrated to have antimicrobial activity comparable to the mimicked peptides[264]. However, degradation and toxicity in regard to systemic application still needs to be clarified for peptoids. High cost of synthesizing peptides has also been highlighted as an issue of implementing them as therapeutic agents. Hence, optimization studies to decrease the length have been given an increased focus as demonstrate in this study, with a minimum of nine amino acids for the tested IDR-1018 analogues compared to the full parent peptide. HDPs have been reported as good antimicrobial agents with activity against various resistant strains in low concentration *in vitro*. However, the activity of the peptides decreases *in vivo*[265]. This has been shown to be a result of the altered structure of the peptides in different solvents, which are hypothesized to alter their effects[111]. Troublingly, salt and serum alter the secondary structure of the peptides, and their activity[266]. Hence, physiological environments could explain the differences demonstrated *in vitro* and *in vivo*. As mentioned, resistance has been shown *in vitro* when a peptide was cultured at sub-MIC levels. Use of the peptide alone could potentially lead to development of resistance. Therefore, using a cocktail of peptides could be beneficial, as to decrease the possibility of resistance as well as to increase the biological effect.

The use of peptides as therapeutics for treatment of difficult wounds seems ideal, as they can inhibit pathogens, improve the immune response, and may have desired effect in non-healing wounds, such as increased angiogenesis and migration, and decrease bacterial diversity and pro-inflammatory cytokines. Peptides as therapeutic agents have come a long way over the last decade with several peptides entering clinical phase trials and some getting approved for the marked[267].

Concluding remarks

The tested peptides demonstrated to have various cellular effects, desired for topical treatment of difficult non-healing wounds, such as DFUs.

Several methods were used to evaluate these effects: migration, proliferation, angiogenesis, cytokine levels, MIC, viability, and toxicity, and despite of the differences of the peptides, all of them increased migration and angiogenesis, but not proliferation. Furthermore, TNFα mRNA levels as well as secretion levels of LPS-induced TNFα were diminished when treated with peptides. Few of the peptides increased the secretion levels of the chemokine MCP-1, but none affected the LPS-induced levels. In the more complex models, several of the *in vitro* data were confirmed, as shown for angiogenesis in both the 3D model and *in vivo*. Most interestingly, LFcinB increased both migration and proliferation in the porcine *ex vivo* model, whereas treatment with IDR-1018 and Nisin A did not, despite all having similar mode of action. However, IDR-1018 enhanced healing in the murine model, potentially by indirectly increasing angiogenesis and decreasing the number of bacterial strains, an effect that would be insignificant in the porcine model. These opposing results underline the importance of using accurate models and of including the different modes of action when choosing treatments, and not relying on *in vitro* data alone.

Together, these data demonstrate that using peptides as treatment has good potential, but several aspects such as stability and resistance need to be addressed. Therefore, combination treatment should be further investigated, for treating DFUs.

References

1. Zaidi, Z., Lanigan, S. Skin: structure and function. *Dermatology in Clinical Practice*. 1–15, doi: 10.1097/IPC.0b013e3181660bf4 (2010).
2. Matsui, T., Amagai, M. Dissecting the formation, structure and barrier function of the stratum corneum. *International Immunology*. **27** (6), 269–280, doi: 10.1093/intimm/dxv013 (2015).
3. Cichorek, M., Wachulska, M., Stasiewicz, A., Tymińska, A. Skin melanocytes: Biology and development. *Postepy Dermatologii i Alergologii*. **30** (1), 30–41, doi: 10.5114/pdia.2013.33376 (2013).
4. Afshar, M., Gallo, R.L. Innate immune defense system of the skin. *Veterinary Dermatology*. **24** (1), 32–38, doi: 10.1111/j.1365-3164.2012.01082.x (2013).
5. Burr, S., Penzer, R. Promoting skin health. *Nursing Standard*. **19** (36), 57–65, doi: 10.7748/ns2005.05.19.36.57.c3871 (2005).
6. Lai-Cheong, J.E., McGrath, J.A. Structure and function of skin, hair and nails. *Medicine (United Kingdom)*. **45** (6), 347–351, doi: 10.1016/j.mpmed.2017.03.004 (2017).
7. Schommer, N.N., Gallo, R.L. Structure and function of the human skin microbiome. *Trends in Microbiology*. **21** (12), 660–668, doi: 10.1016/j.tim.2013.10.001 (2013).
8. Bäsler, K., Bergmann, S., Heisig, M., Naegel, A., Zorn-Kruppa, M., Brandner, J.M. *The role of tight junctions in skin barrier function and dermal absorption*. *Journal of Controlled Release*. **242**, doi: 10.1016/j.jconrel.2016.08.007. Elsevier B.V. (2016).
9. Schmid-Wendtner, M.H., Korting, H.C. The pH of the skin surface and its impact on the barrier function. *Skin Pharmacology and Physiology*. **19** (6), 296–302, doi: 10.1159/000094670 (2006).
10. Fluhr, J.W., Elias, P.M. Stratum corneum pH: Formation and function of the "acid mantle." *Exogenous Dermatology*. **1** (4), 163–175, doi: 10.1159/000066140 (2002).
11. Jenssen, H., Hamill, P., Hancock, R.E.W. Peptide antimicrobial agents. *Clinical Microbiology Reviews*. **19** (3), 491–511, doi: 10.1128/CMR.00056-05 (2006).
12. Mai, W., Lu, D., Liu, X., Chen, L. MCP-1 produced by keratinocytes is associated with leucocyte recruitment during elicitation of nickel-induced occupational allergic contact dermatitis. *Toxicology and Industrial Health*. **34** (1), 36–43, doi: 10.1177/0748233717738633 (2018).
13. Tüzün, Y., Antonov, M., Dolar, N., Wolf, R. Keratinocyte Cytokine and

Chemokine Receptors. *Dermatologic Clinics*. **25** (4), 467–476, doi: 10.1016/j.det.2007.06.003 (2007).
14. Erdag, G., Morgan, J.R. Interleukin-1alpha and interleukin-6 enhance the antibacterial properties of cultured composite keratinocyte grafts. *Ann Surg*. **235** (1), 113–124, doi: 10.1097/00000658-200201000-00015 (2002).
15. Dorschner, R.A. *et al.* Cutaneous injury induces the release of cathelicidin anti-microbial peptides active against group A streptococcus. *Journal of Investigative Dermatology*. **117** (1), 91–97, doi: 10.1046/j.1523-1747.2001.01340.x (2001).
16. Gröne, A. Keratinocytes and cytokines. *Veterinary Immunology and Immunopathology*. **88** (1–2), 1–12, doi: 10.1016/S0165-2427(02)00136-8 (2002).
17. Grice, E. a, Segre, J. a The skin microbiome. *Nature reviews. Microbiology*. **9** (4), 244–53, doi: 10.1038/nrmicro2537 (2011).
18. Harper, D., Young, A., McNaught, C.E. The physiology of wound healing. *Surgery (United Kingdom)*. **32** (9), 445–450, doi: 10.1016/j.mpsur.2014.06.010 (2014).
19. Thiruvoth, F., Mohapatra, D., Sivakumar, D., Chittoria, R., Nandhagopal, V. Current concepts in the physiology of adult wound healing. *Plastic and Aesthetic Research*. **2** (5), 250, doi: 10.4103/2347-9264.158851 (2015).
20. Ellis, S., Lin, E.J., Tartar, D. Immunology of Wound Healing. *Current dermatology reports*. **7** (4), 350–358, doi: 10.1007/s13671-018-0234-9 (2018).
21. Dryden, S. V., Shoemaker, W.G., Kim, J.H. Wound management and nutrition for optimal wound healing. *Atlas of the Oral and Maxillofacial Surgery Clinics of North America*. **21** (1), 37–47, doi: 10.1016/j.cxom.2012.12.008 (2013).
22. Beldon, P. Basic science of wound healing. *Surgery*. **28** (9), 409–412, doi: 10.1016/j.mpsur.2010.05.007 (2010).
23. Kurahashi, T., Fujii, J. Roles of Antioxidative Enzymes in Wound Healing. *Journal of Developmental Biology*. **3**, 57–70, doi: 10.3390/jdb3020057 (2015).
24. Guo, S., DiPietrio, L. Factors Affecting Wound Healing. *Journal of Dental Research*. **89** (3), 219–229, doi: 10.1177/0022034509359125 (2010).
25. Futosi, K., Fodor, S., Mócsai, A. Neutrophil cell surface receptors and their intracellular signal transduction pathways. *International Immunopharmacology*. **17** (3), 638–650, doi: 10.1016/j.intimp.2013.06.034 (2013).

26. Kono, H., Rock, K.L. How dying cells alert the immune system to danger. *Nature Reviews Immunology*. **8** (4), 279–289, doi: 10.1038/nri2215 (2008).
27. Zhao, R., Liang, H., Clarke, E., Jackson, C., Xue, M. Inflammation in Chronic Wounds. *International Journal of Molecular Sciences*. **17** (12), 2085, doi: 10.3390/ijms17122085 (2016).
28. Borregaard, N., Sørensen, O.E., Theilgaard-Mönch, K. Neutrophil granules: a library of innate immunity proteins. *Trends in Immunology*. **28** (8), 340–345, doi: 10.1016/j.it.2007.06.002 (2007).
29. Wilgus, T.A., Roy, S., Mcdaniel, J.C. Neutrophils and Wound Repair : Positive Actions and Negative Reactions. **2** (7), 379–388, doi: 10.1089/wound.2012.0383 (2013).
30. Koh, T.J., DiPietro, L.A. Inflammation and wound healing: the role of the macrophage. *Expert Reviews in Molecular Medicine*. **13** (2008), e23, doi: 10.1017/S1462399411001943 (2011).
31. Kotwal, G.J., Chien, S. Macrophage Differentiation in Normal and Accelerated Wound Healing. *Results Probl Cell Differ.* 353–364, doi: 10.1007/978-3-319-54090-0_14 (2017).
32. Mosser, D.M. The many faces of macrophage activation. *Journal of Leukocyte Biology*. **73** (2), 209–212, doi: 10.1189/jlb.0602325 (2003).
33. Greenlee-Wacker, M.C. Clearance of apoptotic neutrophils and resolution of inflammation. *Immunological Reviews*. **273** (1), 357–370, doi: 10.1111/imr.12453 (2016).
34. Bratton, D.L., Henson, P.M. Neutrophil clearance: When the party is over, clean-up begins. *Trends in Immunology*. **32** (8), 350–357, doi: 10.1016/j.it.2011.04.009 (2011).
35. Landén, N.X., Li, D., Ståhle, M. Transition from inflammation to proliferation: a critical step during wound healing. *Cellular and Molecular Life Sciences*. **73** (20), 3861–3885, doi: 10.1007/s00018-016-2268-0 (2016).
36. Midwood, K.S., Williams, L.V., Schwarzbauer, J.E. Tissue repair and the dynamics of the extracellular matrix. *International Journal of Biochemistry and Cell Biology*. **36** (6), 1031–1037, doi: 10.1016/j.biocel.2003.12.003 (2004).
37. Ilina, O., Friedl, P. Mechanisms of collective cell migration at a glance. *Journal of Cell Science*. **122** (18), 3203–3208, doi: 10.1242/jcs.036525 (2009).
38. Webb, D.J., Parsons, J.T., Horwitz, A.F. Adhesion assembly, disassembly and turnover in migrating cells – over and over and over again. *Nature*

Cell Biology. **4** (4), E97–E100, doi: 10.1038/ncb0402-e97 (2002).
39. Li, B., Wang, J.H.-C. Fibroblasts and myofibroblasts in wound healing: Force generation and measurement. *Journal of Tissue Viability*. **20** (4), 108–120, doi: 10.1016/j.jtv.2009.11.004 (2011).
40. Hopkinson, S.B., Hamill, K.J., Wu, Y., Eisenberg, J.L., Hiroyasu, S., Jones, J.C.R. Focal contact and hemidesmosomal proteins in keratinocyte migration and wound repair. *Advances in Wound Care*. **3** (3), 247–263, doi: 10.1089/wound.2013.0489 (2014).
41. Ronfard, V., Barrandon, Y. Migration of keratinocytes through tunnels of digested fibrin. *Proceedings of the National Academy of Sciences*. **98** (8), 4504–4509, doi: 10.1073/pnas.071631698 (2001).
42. DiPietro, L.A. Angiogenesis and wound repair: when enough is enough. *Journal of Leukocyte Biology*. **100** (November), doi: 10.1189/jlb.4MR0316-102R (2016).
43. Schreml, S., Szeimies, R.M., Prantl, L., Karrer, S., Landthaler, M., Babilas, P. Oxygen in acute and chronic wound healing. *British Journal of Dermatology*. **163** (2), 257–268, doi: 10.1111/j.1365-2133.2010.09804.x (2010).
44. Betz, C., Lenard, A., Belting, H.-G., Affolter, M. Cell behaviors and dynamics during angiogenesis. *Development*. **143** (13), 2249–2260, doi: 10.1242/dev.135616 (2016).
45. Chappell, J.C., Wiley, D.M., Bautch, V.L. Regulation of blood vessel sprouting. *Seminars in Cell and Developmental Biology*. **22** (9), 1005–1011, doi: 10.1016/j.semcdb.2011.10.006 (2011).
46. Van Hinsbergh, V.W.M., Koolwijk, P. Endothelial sprouting and angiogenesis: Matrix metalloproteinases in the lead. *Cardiovascular Research*. **78** (2), 203–212, doi: 10.1093/cvr/cvm102 (2008).
47. Tracy, L.E., Minasian, R.A., Caterson, E.J. Extracellular Matrix and Dermal Fibroblast Function in the Healing Wound. *Advances in Wound Care*. **5** (3), 119–136, doi: 10.1089/wound.2014.0561 (2016).
48. Velnar, T., Bailey, T. The Wound Healing Process : an Overview of the Cellular and Molecular Mechanisms. *The Journal of International Medical Research*. **37** (5), 1528–1542 (2009).
49. Darby, I.A., Laverdet, B., Bonté, F., Desmoulière, A. Fibroblasts and myofibroblasts in wound healing. *Clinical, Cosmetic and Investigational Dermatology*. **7**, 301–311, doi: 10.2147/CCID.S50046 (2014).
50. Gonzalez, A.C. de O., Costa, T.F., Andrade, Z. de A., Medrado, A.R.A.P. Wound healing - A literature review. *Anais Brasileiros de Dermatologia*. **91** (5), 614–620, doi: 10.1590/abd1806-4841.20164741 (2016).

51. Desmouliere, A., Redard, M., Darby, I., Baggiani, G. Apoptosis mediates the decrease in cellularity during the transition between granulation tissue and scar. *American Journal of Pathology*. **146** (1), 55–66, doi: 10.1016/S0921-5107(97)00214-6 (1995).
52. Hinz, B., Gabbiani, G. Cell-matrix and cell-cell contacts of myofibroblasts: Role in connective tissue remodeling. *Thrombosis and Haemostasis*. **90** (6), 993–1002, doi: 10.1160/TH03-05-0328 (2003).
53. DiPietro, L.A. Angiogenesis and scar formation in healing wounds. *Current Opinion in Rheumatology*. **25** (1), 87–91, doi: 10.1097/BOR.0b013e32835b13b6 (2013).
54. Xue, M., Jackson, C.J. Extracellular Matrix Reorganization During Wound Healing and Its Impact on Abnormal Scarring. *Advances in Wound Care*. **4** (3), 119–136, doi: 10.1089/wound.2013.0485 (2015).
55. Sen, C.K. *et al.* Human Skin Wounds: A Major Snoballing Threat to Public Health and Economy. *Wound Repair Regen. 2009*. **17** (6), 763–771, doi: 10.1111/j.1524-475X.2009.00543.x.Human (2010).
56. The Wound Healing Society *Chronic Wound Care Guidelines: Abridged Version*. *Wound Repair and Regeneration*. **14** (6), at <http://www.woundheal.org/assets/documents/final pocket guide treatment.pdf>. (2006).
57. Scheen, A.J. Pathophysiology of type 2 diabetes. *Acta Clinica Belgica*. **58** (6), 335–341, doi: 10.1179/acb.2003.58.6.001 (2003).
58. Gillespie, K.M. Type 1 diabetes: Pathogenesis and prevention. *Cmaj*. **175** (2), 165–170, doi: 10.1503/cmaj.060244 (2006).
59. Alam, U., Asghar, O., Azmi, S., Malik, R.A. *General aspects of diabetes mellitus*. *Handbook of Clinical Neurology*. **126**, doi: 10.1016/B978-0-444-53480-4.00015-1. Elsevier B.V. (2014).
60. Deshpande, A.D., Harris-Hayes, M., Schootman, M. Epidemiology of Diabetes and Diabetes-Related Complications. *Physical Therapy*. **88** (11), 1254–1264, doi: 10.2522/ptj.20080020 (2008).
61. Yazdanpanah, L., Nasiri, M., Adarvishi, S. Literature review on the management of diabetic foot ulcer. *World Journal of Diabetes*. **6** (1), 37–53, doi: 10.4239/wjd.v6.i1.37 (2015).
62. Armstrong, D.G., Boulton, A.J.M., Bus, S.A. Diabetic Foot Ulcers and Their Recurrence. *New England Journal of Medicine*. **376** (24), 2367–2375, doi: 10.1056/NEJMra1615439 (2017).
63. Muller, L.M. a J. *et al.* Increased risk of common infections in patients with type 1 and type 2 diabetes mellitus. *Clinical Infectious Diseases*. **41** (3), 281–288, doi: 10.1086/431587 (2005).

64. Casqueiro, J., Casqueiro, J., Alves, C. Infections in patients with diabetes mellitus: A review of pathogenesis. *Indian journal of endocrinology and metabolism*. **16 Suppl 1** (Dm), S27-36, doi: 10.4103/2230-8210.94253 (2012).
65. Butler, S.O., Btaiche, I.F., Alaniz, C. Relationship between hyperglycemia and infection in critically ill patients. *Pharmacotherapy*. **25** (7), 963–976, doi: 10.1592/phco.2005.25.7.963 (2005).
66. Shah, B.R., Hux, J.E. Quantifying the risk of infectious diseases for people with diabetes. *Diabetes Care*. **26** (2), 510–513, doi: 10.2337/diacare.26.2.510 (2003).
67. Frykberg, R.G., Banks, J. Challenges in the Treatment of Chronic Wounds. *Advances in wound care*. **4** (9), 560–582, doi: 10.1089/wound.2015.0635 (2015).
68. Chen, L., Chen, R., Wang, H., Liang, F. Mechanisms Linking Inflammation to Insulin Resistance. *International Journal of Endocrinology*. **2015**, doi: 10.1155/2015/508409 (2015).
69. Landén, N.X., Li, D., Ståhle, M. Transition from inflammation to proliferation: a critical step during wound healing. *Cellular and Molecular Life Sciences*. **73** (20), 3861–3885, doi: 10.1007/s00018-016-2268-0 (2016).
70. Demidova-Rice, T.N., Hamblin, M.R., Herman, I.M. Acute and Impaired Wound Healing. *Advances in Skin & Wound Care*. **25** (7), 304–314, doi: 10.1097/01.ASW.0000416006.55218.d0 (2012).
71. da Silva, L., Carvalho, E., Cruz, M.T. Role of neuropeptides in skin inflammation and its involvement in diabetic wound healing. *Expert opinion on biological therapy*. **10** (10), 1427–39, doi: 10.1517/14712598.2010.515207 (2010).
72. Loots, M.A.M. *et al.* Fibroblasts derived from chronic diabetic ulcers differ in their response to stimulation with EGF, IGF-I, bFGF and PDGF-AB compared to controls. *European Journal of Cell Biology*. **81** (3), 153–160, doi: 10.1078/0171-9335-00228 (2002).
73. Schultz, G.S., Ladwig, G., Wysocki, A. Extracellular matrix: Review of its roles in acute and chronic wounds. *World Wide Wounds*. **2005** (January 2017) (2005).
74. Lauer, G. *et al.* Expression and proteolysis of vascular endothelial growth factor is increased in chronic wounds. *Journal of Investigative Dermatology*. **115** (1), 12–18, doi: 10.1046/j.1523-1747.2000.00036.x (2000).
75. Ben-Porath, I., Weinberg, R.A. The signals and pathways activating

cellular senescence. *The international journal of biochemistry & cell biology*. **37** (5), 961–76, doi: 10.1016/j.biocel.2004.10.013 (2005).
76. Stojadinovic, O. *et al.* Deregulation of keratinocyte differentiation and activation: A hallmark of venous ulcers. *Journal of Cellular and Molecular Medicine*. **12** (6B), 2675–2690, doi: 10.1111/j.1582-4934.2008.00321.x (2008).
77. Usui, M.L., Mansbridge, J.N., Carter, W.G., Fujita, M., Olerud, J.E. Keratinocyte Migration, Proliferation, and Differentiation in Chronic Ulcers From Patients With Diabetes and Normal Wounds. *Journal of Histochemistry and Cytochemistry*. **56** (7), 687–696, doi: 10.1369/jhc.2008.951194 (2008).
78. Lerman, O.Z., Galiano, R.D., Armour, M., Levine, J.P., Gurtner, G.C. Cellular dysfunction in the diabetic fibroblast: Impairment in migration, vascular endothelial growth factor production, and response to hypoxia. *American Journal of Pathology*. **162** (1), 303–312, doi: 10.1016/S0002-9440(10)63821-7 (2003).
79. Cook, H., Stephens, P., Davies, K.J., Harding, K.G., Thomas, D.W. Defective extracellular matrix reorganization by chronic wound fibroblasts is associated with alterations in TIMP-1, TIMP-2, and MMP-2 activity. *Journal of Investigative Dermatology*. **115** (2), 225–233, doi: 10.1046/j.1523-1747.2000.00044.x (2000).
80. McCarty, S.M., Percival, S.L. Proteases and Delayed Wound Healing. *Advances in Wound Care*. **2** (8), 438–447, doi: 10.1089/wound.2012.0370 (2013).
81. Metcalf, D., Bowler, P. Biofilm delays wound healing: A review of the evidence. *Burns & Trauma*. **1** (1), 5, doi: 10.4103/2321-3868.113329 (2013).
82. Harrison-Balestra, C., Cazzaniga, A.L., Davis, S.C., Mertz, P.M. A wound-isolated Pseudomonas aeruginosa grows a biofilm in vitro within 10 hours and is visualized by light microscopy. *Dermatologic Surgery*. **29** (6), 631–635, doi: 10.1046/j.1524-4725.2003.29146.x (2003).
83. Mancl, K.A., Kirsner, R.S., Ajdic, D. Wound biofilms: Lessons learned from oral biofilms. *Wound Repair and Regeneration*. **21** (3), 352–362, doi: 10.1111/wrr.12034 (2013).
84. Garrett, T.R., Bhakoo, M., Zhang, Z. Bacterial adhesion and biofilms on surfaces. *Progress in Natural Science*. **18** (9), 1049–1056, doi: 10.1016/j.pnsc.2008.04.001 (2008).
85. Nadell, C.D., Drescher, K., Wingreen, N.S., Bassler, B.L. Extracellular matrix structure governs invasion resistance in bacterial biofilms. *ISME*

Journal. **9** (8), 1700–1709, doi: 10.1038/ismej.2014.246 (2015).
86. Kostakioti, M., Hadjifrangiskou, M., Hultgren, S.J. Bacterial Biofilms: Development, Dispersal, and Therapeutic Strategies in the Dawn of the Postantibiotic Era. *Cold Spring Harbor Perspectives in Medicine*. **3** (4), a010306–a010306, doi: 10.1101/cshperspect.a010306 (2013).
87. Serra, D.O., Hengge, R. Stress responses go three dimensional - The spatial order of physiological differentiation in bacterial macrocolony biofilms. *Environmental Microbiology*. **16** (6), 1455–1471, doi: 10.1111/1462-2920.12483 (2014).
88. Vyas, K.S., Wong, L.K. Detection of Biofilm in Wounds as an Early Indicator for Risk for Tissue Infection and Wound Chronicity. *Annals of Plastic Surgery*. **76** (1), 127–131, doi: 10.1097/SAP.0000000000000440 (2016).
89. Medzhitov, R. Recognition of microorganisms and activation of the immune response. *Nature*. **449** (7164), 819–826, doi: 10.1038/nature06246 (2007).
90. Medzhitov, R., Janeway, C.A. Decoding the patterns of self and nonself by the innate immune system. *Science (New York, N.Y.)*. **296** (5566), 298–300, doi: 10.1126/science.1068883 (2002).
91. Takeda, K., Akira, S. Toll-like receptors in innate immunity. *International Immunology*. **17** (1), 1–14, doi: 10.1093/intimm/dxh186 (2005).
92. Kawai, T., Akira, S. Toll-like Receptors and Their Crosstalk with Other Innate Receptors in Infection and Immunity. *Immunity*. **34** (5), 637–650, doi: 10.1016/j.immuni.2011.05.006 (2011).
93. Anaya-López, J.L., López-Meza, J.E., Ochoa-Zarzosa, A. Bacterial resistance to cationic antimicrobial peptides. *Critical Reviews in Microbiology*. **39** (2), 180–195, doi: 10.3109/1040841X.2012.699025 (2013).
94. Wright, G.D. Q&A: Antibiotic resistance: Where does it come from and what can we do about it? *BMC Biology*. **8**, doi: 10.1186/1741-7007-8-123 (2010).
95. Munita, J.M., Arias, C.A. Mechanisms of Antibiotic Resistance. *Virulence Mechanisms of Bacterial Pathogens, Fifth Edition*. **37** (2), 481–511, doi: 10.1128/microbiolspec.VMBF-0016-2015 (2004).
96. de la Fuente-Núñez, C., Reffuveille, F., Haney, E.F., Straus, S.K., Hancock, R.E.W. Broad-Spectrum Anti-biofilm Peptide That Targets a Cellular Stress Response. *PLoS Pathogens*. **10** (5), doi: 10.1371/journal.ppat.1004152 (2014).
97. Armstrong, D.G., Nguyen, H.C., Lavery, L.A., Van Schie, C.H.M.,

Boulton, A.J.M., Harkless, L.B. Off-loading the diabetic foot wound: A randomized clinical trial. *Diabetes Care*. **24** (6), 1019–1022, doi: 10.2337/diacare.24.6.1019 (2001).
98. Joseph, W.S., Lipsky, B.A. Strategies to Prevent and Heal Diabetic Foot Ulcers : Medical Therapy of Diabetic Foot Infections. **100** (5), 395–400 (2010).
99. Fisher, T.K., Scimeca, C.L., Bharara, M., Mills, J.L., Armstrong, D.G. A Stepwise Approach for Surgical Management of Diabetic Foot Infections. *Journal of the American Podiatric Medical Association*. **100** (5), 401–405, doi: 10.7547/1000401 (2010).
100. Freire-Moran, L. *et al.* Critical shortage of new antibiotics in development against multidrug-resistant bacteria - Time to react is now. *Drug Resistance Updates*. **14** (2), 118–124, doi: 10.1016/j.drup.2011.02.003 (2011).
101. Woodford, N., Ellington, M.J. The emergence of antibiotic resistance by mutation. *Clinical Microbiology and Infection*. **13** (1), 5–18, doi: 10.1111/j.1469-0691.2006.01492.x (2007).
102. Bassetti, M., Merelli, M., Temperoni, C., Astilean, A. New antibiotics for bad bugs: Where are we? *Annals of Clinical Microbiology and Antimicrobials*. **12** (1), 1, doi: 10.1186/1476-0711-12-22 (2013).
103. Wang, G. Human antimicrobial peptides and proteins. *Pharmaceuticals*. **7** (5), 545–594, doi: 10.3390/ph7050545 (2014).
104. Malanovic, N., Lohner, K. Gram-positive bacterial cell envelopes: The impact on the activity of antimicrobial peptides. *Biochimica et Biophysica Acta - Biomembranes*. **1858** (5), 936–946, doi: 10.1016/j.bbamem.2015.11.004 (2016).
105. Jenssen, H. Anti herpes simplex virus activity of lactoferrin/lactoferricin - An example of antiviral activity of antimicrobial protein/peptide. *Cellular and Molecular Life Sciences*. **62** (24), 3002–3013, doi: 10.1007/s00018-005-5228-7 (2005).
106. Lohner, K., Leber, R. Antifungal Host Defense Peptides. *Host Defense Peptides and Their Potential as Therapeutic Agents*. 27–55, doi: 10.1007/978-3-319-32949-9_2 (2016).
107. Lee, W., Lee, D.G. Fungicidal mechanisms of the antimicrobial peptide Bac8c. *Biochimica et Biophysica Acta - Biomembranes*. **1848** (2), 673–679, doi: 10.1016/j.bbamem.2014.11.024 (2015).
108. Mor, A. Multifunctional host defense peptides: Antiparasitic activities. *FEBS Journal*. **276** (22), 6474–6482, doi: 10.1111/j.1742-4658.2009.07358.x (2009).

109. Papo, N., Shai, Y. Host defense peptides as new weapons in cancer treatment. *Cellular and Molecular Life Sciences*. **62** (7–8), 784–790, doi: 10.1007/s00018-005-4560-2 (2005).
110. Hancock, R.E.W., Sahl, H.-G. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nature Biotechnology*. **24** (12), 1551–1557, doi: 10.1038/nbt1267 (2006).
111. Wieczorek, M. *et al.* Structural studies of a peptide with immune modulating and direct antimicrobial activity. *Chemistry and Biology*. **17** (9), 970–980, doi: 10.1016/j.chembiol.2010.07.007 (2010).
112. Choi, K.Y., Chow, L.N.Y., Mookherjee, N. Cationic host defence peptides: Multifaceted role in immune modulation and inflammation. *Journal of Innate Immunity*. **4** (4), 361–370, doi: 10.1159/000336630 (2012).
113. Mangoni, M.L., McDermott, A.M., Zasloff, M. Antimicrobial peptides and wound healing: biological and therapeutic considerations. *Experimental Dermatology*. **25** (3), 167–173, doi: 10.1111/exd.12929 (2016).
114. St.-Gelais, F., Jomphe, C., Trudeau, L.É. The role of neurotensin in central nervous system pathophysiology: What is the evidence? *Journal of Psychiatry and Neuroscience*. **31** (4), 229–245 (2006).
115. Wong, M., Jeng, A.Y. Posttranslational modification of glycine-extended substance P by an alpha-amidating enzyme in cultured sensory neurons of dorsal root ganglia. *Journal of Neuroscience Research*. **37** (1), 97–102, doi: 10.1002/jnr.490370113 (1994).
116. Sen, A.K. *et al.* Post-translational modification of nisin. The involvement of NisB in the dehydration process. *European Journal of Biochemistry*. **261** (2), 524–532, doi: 10.1046/j.1432-1327.1999.00303.x (1999).
117. Kluskens, L.D. *et al.* Post-translational modification of therapeutic peptides by NisB, the dehydratase of the lantibiotic nisin. *Biochemistry*. **44** (38), 12827–12834, doi: 10.1021/bi050805p (2005).
118. Moll, G.N., Kuipers, A., De Vries, L., Bosma, T., Rink, R. A biological stabilization technology for peptide drugs: Enzymatic introduction of thioether-bridges. *Drug Discovery Today: Technologies*. **6** (1–4), e13–e18, doi: 10.1016/j.ddtec.2009.03.001 (2009).
119. Lai, Y., Gallo, R.L. AMPed Up immunity: how antimicrobial peptides have multiple roles in immune defense. *Trends Immunol*. **30** (3), 131–141, doi: 10.1016/j.it.2008.12.003.AMPed (2009).
120. Hata, T.R., Gallo, R.L. Antimicrobial Peptides, Skin Infections, and Atopic Dermatitis. *Seminars in Cutaneous Medicine and Surgery*. **27** (2),

144–150, doi: 10.1016/j.sder.2008.04.002 (2008).
121. Bals, R. Epithelial antimicrobial peptides in host defense against infection. *Respiratory Research*. **1** (1), doi: 10.1186/rr25 (2000).
122. Gallo, R.L., Nakatsuji, T. Microbial Symbiosis with the Innate Immune Defense System of the Skin. *J Invest Dermatol*. **131** (10), 1974–1980, doi: 10.1038/jid.2011.182.Microbial (2012).
123. Yeung, A.T.Y., Gellatly, S.L., Hancock, R.E.W. Multifunctional cationic host defence peptides and their clinical applications. *Cellular and Molecular Life Sciences*. **68** (13), 2161–2176, doi: 10.1007/s00018-011-0710-x (2011).
124. Yang, D., Chertov, O., Oppenheim, J.J. The role of mammalian antimicrobial peptides and proteins in awakening of innate host defenses and adaptive immunity. *Cellular and molecular life sciences : CMLS*. **58** (7), 978–989, doi: 10.1007/PL00000914 (2001).
125. Kaiser, V., Diamond, G. Expression of mammalian defensin genes. *Journal of leukocyte biology*. **68** (6), 779–784 (2000).
126. Zasloff, M. Antimicrobial peptides of multicellular organisms. *Nature*. **415** (0028–0836 (Print)), 389–395, doi: 10.1038/415389a (2002).
127. Cleveland, J., Montville, T.J., Nes, I.F., Chikindas, M.L. Bacteriocins: Safe, natural antimicrobials for food preservation. *International Journal of Food Microbiology*. **71** (1), 1–20, doi: 10.1016/S0168-1605(01)00560-8 (2001).
128. Hilpert, K., Volkmer-Engert, R., Walter, T., Hancock, R.E.W. High-throughput generation of small antibacterial peptides with improved activity. *Nature Biotechnology*. **23** (8), 1008–1012, doi: 10.1038/nbt1113 (2005).
129. Mansour, S.C., De La Fuente-Núñez, C., Hancock, R.E.W. Peptide IDR-1018: Modulating the immune system and targeting bacterial biofilms to treat antibiotic-resistant bacterial infections. *Journal of Peptide Science*. **21** (5), 323–329, doi: 10.1002/psc.2708 (2015).
130. Haney, E.F., Hancock, R.E.W. Peptide design for antimicrobial and immunomodulatory applications. *Biopolymers*. **100** (6), 572–583, doi: 10.1002/bip.22250 (2013).
131. Haney, E.F., Mansour, S.C., Hilchie, A.L., de la Fuente-Núñez, C., Hancock, R.E.W. High throughput screening methods for assessing antibiofilm and immunomodulatory activities of synthetic peptides. *Peptides*. **71**, 276–285, doi: 10.1016/j.peptides.2015.03.015 (2015).
132. Yeaman, M.R., Yount, N.Y. Mechanisms of antimicrobial peptide action and resistance. *Pharmacological reviews*. **55** (1), 27–55, doi:

10.1124/pr.55.1.2 (2003).
133. Salwiczek, M. *et al.* Emerging rules for effective antimicrobial coatings. *Trends in Biotechnology*. **32** (2), 82–90, doi: 10.1016/j.tibtech.2013.09.008 (2014).
134. Bahar, A.A., Ren, D. Antimicrobial peptides. *Pharmaceuticals*. **6** (12), doi: 10.3390/ph6121543 (2013).
135. Brogden, K. a Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? *Nature Reviews Microbiology*. **3** (3), 238–250, doi: 10.1038/nrmicro1098 (2005).
136. Galdiero, S., Falanga, A., Cantisani, M., Vitiello, M., Morelli, G., Galdiero, M. Peptide-Lipid Interactions: Experiments and Applications. *International Journal of Molecular Sciences*. **14** (9), 18758–18789, doi: 10.3390/ijms140918758 (2013).
137. Bechinger, B., Gorr, S.U. Antimicrobial Peptides: Mechanisms of Action and Resistance. *Journal of Dental Research*. **96** (3), 254–260, doi: 10.1177/0022034516679973 (2017).
138. Peschel, A. *et al.* Staphylococcus aureus Resistance to Human Defensins and Evasion of Neutrophil Killing via the Novel Virulence Factor Mprf Is Based on Modification of Membrane Lipids with l-Lysine. *The Journal of Experimental Medicine*. **193** (9), 1067–1076, doi: 10.1084/jem.193.9.1067 (2001).
139. Saporito, P., Vang Mouritzen, M., Lobner-Olesen, A., Jenssen, H. LL-37 fragments have antimicrobial activity against Staphylococcus epidermidis biofilms and wound healing potential in HaCaT cell line. *Journal of Peptide Science*. (March), e3080, doi: 10.1002/psc.3080 (2018).
140. Som, A., Navasa, N., Percher, A., Scott, R.W., Tew, G.N., Anguita, J. Identification of synthetic host defense peptide mimics that exert dual antimicrobial and anti-inflammatory activities. *Clinical and Vaccine Immunology*. **19** (11), 1784–1791, doi: 10.1128/CVI.00291-12 (2012).
141. Perron, G.G., Zasloff, M., Bell, G. Experimental evolution of resistance to an antimicrobial peptide. *Proceedings of the Royal Society of London B*. **273** (1583), 251–256, doi: 10.1098/rspb.2005.3301 (2006).
142. Shelburne, C.E., Coulter, W.A., Olguin, D., Lantz, M.S., Lopatin, D.E. Induction of β-defensin resistance in the oral anaerobe Porphyromonas gingivalis. *Antimicrobial Agents and Chemotherapy*. **49** (1), 183–187, doi: 10.1128/AAC.49.1.183-187.2005 (2005).
143. Samuelsen, Ø. *et al.* Induced resistance to the antimicrobial peptide lactoferricin B in Staphylococcus aureus. *FEBS Letters*. **579** (16), 3421–3426, doi: 10.1016/j.febslet.2005.05.017 (2005).

144. Zhang, L., Rozek, A., Hancock, R.E.W. Interaction of Cationic Antimicrobial Peptides with Model Membranes. *Journal of Biological Chemistry*. **276** (38), 35714–35722, doi: 10.1074/jbc.M104925200 (2001).
145. Dürr, M., Peschel, A. Chemokines meet defensins: The merging concepts of chemoattractants and antimicrobial peptides in host defense. *Infection and Immunity*. **70** (12), 6515–6517, doi: 10.1128/IAI.70.12.6515-6517.2002 (2002).
146. Reffuveille, F., De La Fuente-Nűnez, C., Mansour, S., Hancock, R.E.W. A broad-spectrum antibiofilm peptide enhances antibiotic action against bacterial biofilms. *Antimicrobial Agents and Chemotherapy*. **58** (9), 5363–5371, doi: 10.1128/AAC.03163-14 (2014).
147. Nizet, V. *et al.* Innate antimicrobial peptide protects the skin from invasive bacterial infection. *Nature*. **414** (6862), 454–457, doi: 10.1038/35106587 (2001).
148. Bardan, A., Nizet, V., Gallo, R.L. Antimicrobial peptides and the skin. *Expert opinion on biological therapy*. **4** (4), 543–549, doi: 10.1517/eobt.4.4.543.29795 (2004).
149. Niyonsaba, F. *et al.* Antimicrobial peptides human beta-defensins stimulate epidermal keratinocyte migration, proliferation and production of proinflammatory cytokines and chemokines. *The Journal of investigative dermatology*. **127** (3), 594–604, doi: 10.1038/sj.jid.5700599 (2007).
150. Chamorro, C.I., Weber, G., Grönberg, A., Pivarcsi, A., Ståhle, M. The human antimicrobial peptide LL-37 suppresses apoptosis in keratinocytes. *Journal of Investigative Dermatology*. **129** (4), 937–944, doi: 10.1038/jid.2008.321 (2009).
151. Capparelli, R. *et al.* New perspectives for natural antimicrobial peptides: application as antinflammatory drugs in a murine model. *BMC immunology*. **13** (1), 61, doi: 10.1186/1471-2172-13-61 (2012).
152. Scott, M.G. *et al.* An anti-infective peptide that selectively modulates the innate immune response. *Nature Biotechnology*. **25** (4), 465–472, doi: 10.1038/nbt1288 (2007).
153. Wiegand, C., Schönfelder, U., Abel, M., Ruth, P., Kaatz, M., Hipler, U.C. Protease and pro-inflammatory cytokine concentrations are elevated in chronic compared to acute wounds and can be modulated by collagen type i in vitro. *Archives of Dermatological Research*. **302** (6), 419–428, doi: 10.1007/s00403-009-1011-1 (2010).
154. Koczulla, R., Bals, R. Cathelicidin AMP modulate Angiogenesis. **49** (0),

191–196 (2007).
155. Huang, H.J., Ross, C.R., Blecha, F. Chemoattractant properties of PR-39, a neutrophil antibacterial peptide. *Journal of leukocyte biology*. **61** (5), 624–629, doi: 10.1002/jlb.61.5.624 (1997).
156. Welkos, S. *et al.* Humanized θ-defensins (retrocyclins) enhance macrophage performance and protect mice from experimental anthrax infections. *Antimicrobial Agents and Chemotherapy*. **55** (9), 4238–4250, doi: 10.1128/AAC.00267-11 (2011).
157. da Silva, L., Miguel Neves, B., Moura, L., Cruz, M.T., Carvalho, E. Neurotensin Decreases the Proinflammatory Status of Human Skin Fibroblasts and Increases Epidermal Growth Factor Expression. *International Journal of Inflammation*. **2014**, 1–9, doi: 10.1155/2014/248240 (2014).
158. Zhang, J.-M., An, J. Cytokines, Inflammation and Pain. *Int Anesthesiol Clin.* **45** (2), 27–37, doi: 10.1097/AIA.0b013e318034194e.Cytokines (2007).
159. Mookherjee, N. *et al.* Systems biology evaluation of immune responses induced by human host defence peptide LL-37 in mononuclear cells. *Molecular BioSystems*. **5** (5), 483–496, doi: 10.1039/b813787k (2009).
160. Nijnik, A. *et al.* Synthetic cationic peptide IDR-1002 provides protection against bacterial infections through chemokine induction and enhanced leukocyte recruitment. *Journal of immunology (Baltimore, Md. : 1950)*. **184** (5), 2539–2550, doi: 10.4049/jimmunol.0901813 (2010).
161. Turner-Brannen, E. *et al.* Modulation of interleukin-1β-induced inflammatory responses by a synthetic cationic innate defence regulator peptide, IDR-1002, in synovial fibroblasts. *Arthritis Research and Therapy*. **13** (4), 1–14, doi: 10.1186/ar3440 (2011).
162. Scott, M.G., Davidson, D.J., Gold, M.R., Bowdish, D., Hancock, R.E.W. The Human Antimicrobial Peptide LL-37 Is a Multifunctional Modulator of Innate Immune Responses. *The Journal of Immunology*. **169** (7), 3883–3891, doi: 10.4049/jimmunol.169.7.3883 (2002).
163. Torossian, A., Gurschi, E., Bals, R., Vassiliou, T., Wulf, H.F., Bauhofer, A. Effects of the antimicrobial peptide LL-37 and hyperthermic preconditioning in septic rats. *Anesthesiology*. **107** (3), 437–441, doi: 10.1097/01.anes.0000278906.86815.eb (2007).
164. Castellani, M.L. *et al.* Stimulation of CCL2 (MCP-1) and CCL2 mRNA by substance P in LAD2 human mast cells. *Translational Research*. **154** (1), 27–33, doi: 10.1016/j.trsl.2009.03.006 (2009).
165. Choe, H. *et al.* Immunomodulatory Peptide IDR-1018 Decreases Implant

Infection and Preserves Osseointegration. *Clinical Orthopaedics and Related Research*. **473** (9), 2898–2907, doi: 10.1007/s11999-015-4301-2 (2015).
166. Soehnlein, O. *et al.* Neutrophil secretion products pave the way for inflammatory monocytes. *Blood*. **112** (4), 1461–1471, doi: 10.1182/blood-2008-02-139634 (2008).
167. Niyonsaba, F., Madera, L., Afacan, N., Okumura, K., Ogawa, H., Hancock, R.E.W. The innate defense regulator peptides IDR-HH2, IDR-1002, and IDR-1018 modulate human neutrophil functions. *Journal of leukocyte biology*. **94** (1), 159–70, doi: 10.1189/jlb.1012497 (2013).
168. Mookherjee, N., Hancock, R.E.W. Cationic host defence peptides: Innate immune regulatory peptides as a novel approach for treating infections. *Cellular and Molecular Life Sciences*. **64** (7–8), 922–933, doi: 10.1007/s00018-007-6475-6 (2007).
169. Tokumaru, S. *et al.* Induction of keratinocyte migration via transactivation of the epidermal growth factor receptor by the antimicrobial peptide LL-37. *Journal of immunology (Baltimore, Md. : 1950)*. **175**, 4662–4668, doi: 175/7/4662 [pii] (2005).
170. Lima, M.H.M. *et al.* Topical insulin accelerates wound healing in diabetes by enhancing the AKT and ERK pathways: A double-blind placebo-controlled clinical trial. *PLoS ONE*. **7** (5), 1–14, doi: 10.1371/journal.pone.0036974 (2012).
171. Mccain, J. The MAPK (ERK) Pathway. *journal for formulary management*. **38** (2) (2013).
172. Yoon, S., Seger, R. The extracellular signal-regulated kinase: Multiple substrates regulate diverse cellular functions. *Growth Factors*. **24** (1), 21–44, doi: 10.1159/000094762 (2006).
173. Somanath, P.R., Chen, J., Byzova, T. V. Akt1 is necessary for the vascular maturation and angiogenesis during cutaneous wound healing. *Angiogenesis*. **11** (3), 277–288, doi: 10.1007/s10456-008-9111-7 (2008).
174. Haney, E.F., Wu, B. (Catherine), Lee, K., Hilchie, A.L., Hancock, R.E.W. Aggregation and Its Influence on the Immunomodulatory Activity of Synthetic Innate Defense Regulator Peptides. *Cell Chemical Biology*. **24** (8), 969–980.e4, doi: 10.1016/j.chembiol.2017.07.010 (2017).
175. Rosso, M., Muñoz, M., Berger, M. The role of neurokinin-1 receptor in the microenvironment of inflammation and cancer. *TheScientificWorldJournal*. **2012**, 381434, doi: 10.1100/2012/381434 (2012).
176. Sarret, P., Kitabgi, P. Neurotensin and Receptors. *Encyclopedia of*

Neuroscience. **20** (July), 1021–1034, doi: 10.1016/B978-008045046-9.01469-8 (2009).
177. Hrynyk, M., Neufeld, R.J. Insulin and wound healing. *Burns*. **40** (8), 1433–1446, doi: 10.1016/j.burns.2014.03.020 (2014).
178. Kindrachuk, J. *et al.* Manipulation of innate immunity by a bacterial secreted peptide: lantibiotic nisin Z is selectively immunomodulatory. *Innate immunity*. **19** (3), 315–27, doi: 10.1177/1753425912461456 (2013).
179. Arias, M., McDonald, L.J., Haney, E.F., Nazmi, K., Bolscher, J.G.M., Vogel, H.J. Bovine and human lactoferricin peptides: Chimeras and new cyclic analogs. *BioMetals*. **27** (5), 935–948, doi: 10.1007/s10534-014-9753-4 (2014).
180. Carraway, R., Leeman, S.E. The Amino Acid Sequence of a Hypothalamic Neurotensin *. *Biological Chemistry*. (5), 1907–1911 (1975).
181. Tyler-McMahon, B.M., Boules, M., Richelson, E. Neurotensin: Peptide for the next millennium. *Regulatory Peptides*. **93** (1–3), 125–136, doi: 10.1016/S0167-0115(00)00183-X (2000).
182. Field, D., Cotter, P.D., Ross, R.P., Hill, C. Bioengineering of the model lantibiotic nisin. *Bioengineered*. **5979** (June 2015), 37–41, doi: 10.1080/21655979.2015.1049781 (2015).
183. Carraway, R., Leeman, S.E. The Amino Acid Sequence of Bovine Hypothalamic Substance P. *The Journal of Biological Chemistry*. **254** (8), 2944–2945, doi: 10.1021/ja01596a029 (1979).
184. Wilson, V.G. Growth and Differentiation of HaCaT Keratinocytes. *Methods in Molecular Biology*. (1341), 33–41, doi: 10.1007/7651_2013_42 (2013).
185. Schmittgen, T.D., Livak, K.J. Analyzing real-time PCR data by the comparative CT method. *Nature Protocols*. **3** (6), 1101–1108, doi: 10.1038/nprot.2008.73 (2008).
186. Van Veen, S.Q., Claas, E.C.J., Kuijper, E.J. High-throughput identification of bacteria and yeast by matrix-assisted laser desorption ionization-time of flight mass spectrometry in conventional medical microbiology laboratories. *Journal of Clinical Microbiology*. **48** (3), 900–907, doi: 10.1128/JCM.02071-09 (2010).
187. Han, H.W., Chang, H.C., Hunag, A.H., Chang, T.C. Optimization of the score cutoff value for routine identification of Staphylococcus species by matrix-assisted laser desorption ionization-time-of-flight mass spectrometry. *Diagnostic Microbiology and Infectious Disease*. **83** (4), 349–354, doi: 10.1016/j.diagmicrobio.2015.08.003 (2015).

188. Escandon, J., Vivas, A.C., Tang, J., Rowland, K.J., Kirsner, R.S. High mortality in patients with chronic wounds. *Wound Repair and Regeneration*. **19** (4), 526–528, doi: 10.1111/j.1524-475X.2011.00699.x (2011).
189. Colombo, I. *et al.* HaCaT Cells as a Reliable In Vitro Differentiation Model to Dissect the Inflammatory / Repair Response of Human Keratinocytes. *Med*. **2017**, 1–12, doi: 10.1155/2017/7435621 (2017).
190. Micallef, L. *et al.* Effects of extracellular calcium on the growth-differentiation switch in immortalized keratinocyte HaCaT cells compared with normal human keratinocytes. *Experimental Dermatology*. **18** (2), 143–151, doi: 10.1111/j.1600-0625.2008.00775.x (2009).
191. Tomasz, M. Mitomycin C: small, fast and deadly (but very selective). *Chemistry and Biology*. **2** (9), 575–579, doi: 10.1016/1074-5521(95)90120-5 (1995).
192. Peplow, P. V., Chatterjee, M.P. A review of the influence of growth factors and cytokines in in vitro human keratinocyte migration. *Cytokine*. **62** (1), 1–21, doi: 10.1016/j.cyto.2013.02.015 (2013).
193. Ponti, A. Two Distinct Actin Networks Drive the Protrusion of Migrating Cells. *Science*. **305** (5691), 1782–1786, doi: 10.1126/science.1100533 (2004).
194. Martin, P., Nunan, R. Cellular and molecular mechanisms of repair in acute and chronic wound healing. *British Journal of Dermatology*. **173** (2), 370–378, doi: 10.1111/bjd.13954 (2015).
195. Marin-Luevano, P. *et al.* Induction by innate defence regulator peptide 1018 of pro-angiogenic molecules and endothelial cell migration in a high glucose environment. *Peptides*. **101** (November 2017), 135–144, doi: 10.1016/j.peptides.2018.01.010 (2018).
196. Wollina, U., Huschenbeck, J., Knöll, B., Sternberg, B., Hipler, U.C. Vasoactive intestinal peptide supports induced migration of human keratinocytes and their colonization of an artificial polyurethane matrix. *Regulatory Peptides*. **70** (1), 29–36, doi: 10.1016/S0167-0115(97)00011-6 (1997).
197. Pincelli, C. *et al.* Substance P Is Diminished and Vasoactive Intestinal Peptide Is Augmented in Psoriatic Lesions and These Peptides Exert Disparate Effects on the Proliferation of Cultured Human Keratinocytes. *Journal of Investigative Dermatology*. **98** (4), 421–427, doi: 10.1111/1523-1747.ep12499846 (1992).
198. Busby, J.E., Shih, S.J., Yang, J.C., Kung, H.J., Evans, C.P. Angiogenesis is not mediated by prostate cancer neuropeptides. *Angiogenesis*. **6** (4),

289–293, doi: 10.1023/B:AGEN.0000029409.94626.64 (2003).
199. Burssens, P., Steyaert, A., Forsyth, R., van Ovost, E.J., Depaepe, Y., Verdonk, R. Exogenously administered substance P and neutral endopeptidase inhibitors stimulate fibroblast proliferation, angiogenesis and collagen organization during Achilles tendon healing. *Foot & ankle international / American Orthopaedic Foot and Ankle Society [and] Swiss Foot and Ankle Society*. **26**, 832–839, doi: 901887 [pii] (2005).
200. Marion-Audibert, A. *et al.* Effects of endocrine peptides on proliferation, migration and differentiation of human endothelial cells. *Gastroenterol Clin Biol.* **1218** (6), 607–692, doi: doi.org/GCB-06-2000-24-6-0399-8320-101019-ART9 (2000).
201. Kant, V. *et al.* Topically applied substance P enhanced healing of open excision wound in rats. *European Journal of Pharmacology*. **715** (1–3), 345–353, doi: 10.1016/j.ejphar.2013.04.042 (2013).
202. Liu, Y., Petreaca, M., Martins-Green, M. Cell and molecular mechanisms of insulin-induced angiogenesis. *Journal of Cellular and Molecular Medicine*. **13** (11–12), 4492–4504, doi: 10.1111/j.1582-4934.2008.00555.x (2009).
203. Duval, K. *et al.* Modeling Physiological Events in 2D vs. 3D Cell Culture. *Physiology*. **32** (4), 266–277, doi: 10.1152/physiol.00036.2016 (2017).
204. Edmondson, R., Broglie, J.J., Adcock, A.F., Yang, L. Three-Dimensional Cell Culture Systems and Their Applications in Drug Discovery and Cell-Based Biosensors. *ASSAY and Drug Development Technologies*. **12** (4), 207–218, doi: 10.1089/adt.2014.573 (2014).
205. Baker, B.M., Chen, C.S. Deconstructing the third dimension – how 3D culture microenvironments alter cellular cues. *Journal of Cell Science*. **125** (13), 3015–3024, doi: 10.1242/jcs.079509 (2012).
206. Gjorevski, N., Piotrowski, A.S., Varner, V.D., Nelson, C.M. Dynamic tensile forces drive collective cell migration through three-dimensional extracellular matrices. *Scientific reports*. **5** (February), 11458, doi: 10.1038/srep11458 (2015).
207. Steinstraesser, L. *et al.* Innate defense regulator peptide 1018 in wound healing and wound infection. *PLoS ONE*. **7** (8), 1–7, doi: 10.1371/journal.pone.0039373 (2012).
208. Mader, J.S., Smyth, D., Marshall, J., Hoskin, D.W. Bovine Lactoferricin Inhibits Basic Fibroblast Growth Factor- and Vascular Endothelial Growth Factor165-Induced Angiogenesis by Competing for Heparin-Like Binding Sites on Endothelial Cells. *The American Journal of Pathology*. **169** (5), 1753–1766, doi: 10.2353/ajpath.2006.051229 (2006).

209. Li, H.Y., Li, M., Luo, C.C., Wang, J.Q., Zheng, N. Lactoferrin Exerts Antitumor Effects by Inhibiting Angiogenesis in a HT29 Human Colon Tumor Model. *Journal of Agricultural and Food Chemistry*. **65** (48), 10464–10472, doi: 10.1021/acs.jafc.7b03390 (2017).
210. Hänel, K.H., Cornelissen, C., Lüscher, B., Baron, J.M. Cytokines and the skin barrier. *International Journal of Molecular Sciences*. **14** (4), 6720–6745, doi: 10.3390/ijms14046720 (2013).
211. Deshmane, S.L., Kremlev, S., Amini, S., Sawaya, B.E. Monocyte Chemoattractant Protein-1 (MCP-1): An Overview. *Journal of Interferon & Cytokine Research*. **29** (6), 313–326, doi: 10.1089/jir.2008.0027 (2009).
212. Matsuura, M. Structural modifications of bacterial lipopolysaccharide that facilitate Gram-negative bacteria evasion of host innate immunity. **4** (May), 1–9, doi: 10.3389/fimmu.2013.00109 (2013).
213. Köllisch, G. *et al.* Various members of the Toll-like receptor family contribute to the innate immune response of human epidermal keratinocytes. *Immunology*. **114** (4), 531–541, doi: 10.1111/j.1365-2567.2005.02122.x (2005).
214. Pu, D.A.N., Wang, W.E.I. Toll - like receptor 4 agonist , lipopolysaccharide , increases the expression levels of cytokines and chemokines in human peripheral blood mononuclear cells. *Experimental and therapeutic medicine*. **8**, 1914–1918, doi: 10.3892/etm.2014.2025 (2014).
215. Matsushima, K., Oppenheim, J.J. Interleukin 8 and MCAF: novel inflammatory cytokines inducible by IL 1 and TNF. *Cytokine*. **1** (1), 2–13, doi: 10.1016/1043-4666(89)91043-0 (1989).
216. Sedger, L.M., McDermott, M.F. TNF and TNF-receptors: From mediators of cell death and inflammation to therapeutic giants - past, present and future. *Cytokine and Growth Factor Reviews*. **25** (4), 453–472, doi: 10.1016/j.cytogfr.2014.07.016 (2014).
217. PIER, G. Pseudomonas aeruginosa lipopolysaccharide: A major virulence factor, initiator of inflammation and target for effective immunity. *International Journal of Medical Microbiology*. **297** (5), 277–295, doi: 10.1016/j.ijmm.2007.03.012 (2007).
218. Ritsu, M. *et al.* Critical role of tumor necrosis factor-α in the early process of wound healing in skin. *Journal of Dermatology & Dermatologic Surgery*. **21** (1), 14–19, doi: 10.1016/j.jdds.2016.09.001 (2017).
219. Tang, X., Marciano, D.L., Leeman, S.E., Amar, S. LPS induces the interaction of a transcription factor , LPS-induced TNF- ␣ factor , and STAT6 (B) with effects on multiple cytokines. *PNAS*. **102** (14), 2–7, doi:

10.1073/pnas.0501159102 (2005).
220. Scheller, J., Chalaris, A., Schmidt-Arras, D., Rose-John, S. The pro- and anti-inflammatory properties of the cytokine interleukin-6. *Biochimica et Biophysica Acta - Molecular Cell Research*. **1813** (5), 878–888, doi: 10.1016/j.bbamcr.2011.01.034 (2011).
221. Cohen, T., Nahari, D., Cerem, L.W., Neufeld, G., Levin, B.Z. Interleukin 6 induces the expression of vascular endothelial growth factor. *Journal of Biological Chemistry*. **271** (2), 736–741, doi: 10.1074/jbc.271.2.736 (1996).
222. Lin, Z.-Q., Kondo, T., Ishida, Y., Takayasu, T., Mukaida, N. Essential involvement of IL-6 in the skin wound-healing process as evidenced by delayed wound healing in IL-6-deficient mice. *Journal of Leukocyte Biology*. **73** (6), 713–721, doi: 10.1189/jlb.0802397 (2003).
223. Li, A. *et al.* IL-8 Directly Enhanced Endothelial Cell Survival, Proliferation, and Matrix Metalloproteinases Production and Regulated Angiogenesis. *Journal of Immunology*. **170**, 3369–76, doi: 10.4049/jimmunol.170.6.3369 (2003).
224. Gillitzer, R., Goebeler, M. Chemokines in cutaneous wound healing. *J Leukoc Biol*. **69** (4), 513–521, doi: 10.1189/jlb.69.4.513 (2001).
225. Conti, P., DiGioacchino, M. MCP-1 and RANTES are mediators of acute and chronic inflammation. *Allergy and asthma proceedings : the official journal of regional and state allergy societies*. **22** (3), 133–137, doi: 10.2500/108854101778148737 (2001).
226. Chu, W.-M. Tumor necrosis factor. *Cancer Letters*. **328** (2), 222–225, doi: 10.1016/j.canlet.2012.10.014 (2013).
227. Lanne, A.B.M. *et al.* Molecular recognition of lipopolysaccharide by the lantibiotic nisin. *Biochimica et Biophysica Acta - Biomembranes*. **1861** (1), 83–92, doi: 10.1016/j.bbamem.2018.10.006 (2019).
228. Farnaud, S. *et al.* Interactions of lactoferricin-derived peptides with LPS and antimicrobial activity. *FEMS Microbiology Letters*. **233** (2), 193–199, doi: 10.1016/j.femsle.2004.01.039 (2004).
229. Hwang, P.M., Zhou, N., Shan, X., Arrowsmith, C.H., Vogel, H.J. Three-Dimensional Solution Structure of Lactoferricin B, an Antimicrobial Peptide Derived from Bovine Lactoferrin †. *Biochemistry*. **37** (12), 4288–4298, doi: 10.1021/bi972323m (1998).
230. Ignacio, G., El-Amin, I., Mendenhall, V. *Animal Models for Wound Healing*. *Skin Tissue Engineering and Regenerative Medicine*. doi: 10.1016/B978-0-12-801654-1.00019-X. Elsevier Inc. (2016).
231. Sullivan, T.P., Eaglstein, W.H., Davis, S.C., Mertz, P. The pig as a model

for human wound healing. *Wound repair and regeneration*. **9** (2), 66–76, doi: 10.1046/j.1524-475x.2001.00066.x (2001).
232. Godin, B., Touitou, E. Transdermal skin delivery: Predictions for humans from in vivo, ex vivo and animal models. *Advanced Drug Delivery Reviews*. **59** (11), 1152–1161, doi: 10.1016/j.addr.2007.07.004 (2007).
233. Kong, R., Bhargava, R. Characterization of porcine skin as a model for human skin studies using infrared spectroscopic imaging. *Analyst*. **136** (11), 2359–2366, doi: 10.1039/c1an15111h (2011).
234. Vardaxis, N.J., Brans, T.A., Boon, M.E., Kreis, R.W., Marres, L.M. Confocal laser scanning microscopy of porcine skin : implications for human wound healing studies. *J Anat*. **190**, 601–611 (1997).
235. Tfaili, S., Gobinet, C., Josse, G., Angiboust, J.-F., Manfait, M., Piot, O. Confocal Raman microspectroscopy for skin characterization: a comparative study between human skin and pig skin. *The Analyst*. **137** (16), 3673, doi: 10.1039/c2an16292j (2012).
236. Schmook, F.P., Meingassner, J.G., Billich, A. Comparison of human skin or epidermis models with human and animal skin in in-vitro percutaneous absorption. *International Journal of Pharmaceutics*. **215** (1–2), 51–56, doi: 10.1016/S0378-5173(00)00665-7 (2001).
237. Meurens, F., Summerfield, A., Nauwynck, H., Saif, L., Gerdts, V. The pig: A model for human infectious diseases. *Trends in Microbiology*. **20** (1), 50–57, doi: 10.1016/j.tim.2011.11.002 (2012).
238. Mair, K.H. *et al. The porcine innate immune system: An update*. *Developmental and Comparative Immunology*. **45** (2), doi: 10.1016/j.dci.2014.03.022. Elsevier Ltd. (2014).
239. Summerfield, A., Meurens, F., Ricklin, M.E. The immunology of the porcine skin and its value as a model for human skin. *Molecular Immunology*. **66** (1), 14–21, doi: 10.1016/j.molimm.2014.10.023 (2015).
240. Sang, Y., Blecha, F. Porcine host defense peptides: Expanding repertoire and functions. *Developmental and Comparative Immunology*. **33** (3), 334–343, doi: 10.1016/j.dci.2008.05.006 (2009).
241. Agerblrth, B. *et al.* Amino acid sequence of PR-39 Isolation from pig intestine of a new member of the family of proline-arginine-rich antibacterial peptides. *Eur. J. Blochem*. **202**, 849–854 (1991).
242. Rittié, L. Cellular mechanisms of skin repair in humans and other mammals. *Journal of Cell Communication and Signaling*. 103–120, doi: 10.1007/s12079-016-0330-1 (2016).
243. Rothkötter, H.J., Sowa, E., Pabst, R. The pig as a model of developmental immunology. *Human & Experimental Toxicology*. **21** (9–10), 533–536,

doi: 10.1191/0960327102ht293oa (2002).
244. Nunan, R., Harding, K.G., Martin, P. Clinical challenges of chronic wounds: searching for an optimal animal model to recapitulate their complexity. *Disease Models & Mechanisms*. **7** (11), 1205–1213, doi: 10.1242/dmm.016782 (2014).
245. Rivas-Santiago, B. *et al.* Ability of Innate Defence Regulator Peptides IDR-1002, IDR-HH2 and IDR-1018 to Protect against Mycobacterium tuberculosis Infections in Animal Models. *PLoS ONE*. **8** (3), e59119, doi: 10.1371/journal.pone.0059119 (2013).
246. Pena, O.M. *et al.* Synthetic Cationic Peptide IDR-1018 Modulates Human Macrophage Differentiation. *PLoS ONE*. **8** (1), 1–10, doi: 10.1371/journal.pone.0052449 (2013).
247. Catrina, S.-B., Zheng, X. Disturbed hypoxic responses as a pathogenic mechanism of diabetic foot ulcers. *Diabetes/Metabolism Research and Reviews*. **32** (1), 179–185, doi: 10.1002/dmrr.2742 (2016).
248. Summers, C., Rankin, S.M., Condliffe, A.M., Singh, N., Peters, A.M., Chilvers, E.R. Neutrophil kinetics in health and disease. *Trends in Immunology*. **31** (8), 318–324, doi: 10.1016/j.it.2010.05.006 (2010).
249. Wiedemann, I. *et al.* Specific binding of nisin to the peptidoglycan precursor lipid II combines pore formation and inhibition of cell wall biosynthesis for potent antibiotic activity. *Journal of Biological Chemistry*. **276** (3), 1772–1779, doi: 10.1074/jbc.M006770200 (2001).
250. de Kruijff, B., van Dam, V., Breukink, E. Lipid II: A central component in bacterial cell wall synthesis and a target for antibiotics. *Prostaglandins Leukotrienes and Essential Fatty Acids*. **79** (3–5), 117–121, doi: 10.1016/j.plefa.2008.09.020 (2008).
251. Murata, M., Wakabayashi, H., Yamauchi, K., Abe, F. Identification of milk proteins enhancing the antimicrobial activity of lactoferrin and lactoferricin. *Journal of Dairy Science*. **96** (8), 4891–4898, doi: 10.3168/jds.2013-6612 (2013).
252. Sánchez-Gómez, S. *et al.* Structural features governing the activity of lactoferricin-derived peptides that act in synergy with antibiotics against Pseudomonas aeruginosa in vitro and in vivo. *Antimicrobial Agents and Chemotherapy*. **55** (1), 218–228, doi: 10.1128/AAC.00904-10 (2011).
253. Liu, Y., Han, F., Xie, Y., Wang, Y. Comparative antimicrobial activity and mechanism of action of bovine lactoferricin-derived synthetic peptides. *BioMetals*. **24** (6), 1069–1078, doi: 10.1007/s10534-011-9465-y (2011).
254. Ulvatne, H., Haukland, H.H., Olsvik, O., Vorland, L.H. Lactoferricin B causes depolarization of the cytoplasmic membrane of Escherichia coli

ATCC 25922 and fusion of negatively charged liposomes. *FEBS Letters*. **492** (1–2), 62–65, doi: 10.1016/S0014-5793(01)02233-5 (2001).
255. Davis, S.C., Ricotti, C., Cazzaniga, A., Welsh, E., Eaglstein, W.H., Mertz, P.M. Microscopic and physiologic evidence for biofilm-associated wound colonization in vivo. *Wound Repair and Regeneration*. **16** (1), 23–29, doi: 10.1111/j.1524-475X.2007.00303.x (2008).
256. Yang, Q. *et al.* Development of a novel ex vivo porcine skin explant model for the assessment of mature bacterial biofilms. *Wound Repair and Regeneration*. **21** (5), 704–714, doi: 10.1111/wrr.12074 (2013).
257. Müller, U., Vogel, P., Alber, G., Schaub, G. The innate immune system of mammals and insects. *Contributions to Microbiology*. **15**, 21–44, doi: 10.1159/000135684 (2008).
258. Dorsett-Martin, W.A., Wysocki, A.B. Rat models of skin wound healing. *Source Book of Models for Biomedical Research*. 631–638, doi: 10.1007/978-1-59745-285-4_65 (2008).
259. Chen, L., Mirza, R., Kwon, Y., DiPietro, L.A., Koh, T.J. The murine excisional wound model: Contraction revisited. *Wound Repair and Regeneration*. **23** (6), 874–877, doi: 10.1111/wrr.12338 (2015).
260. Wong, V.W., Sorkin, M., Glotzbach, J.P., Longaker, M.T., Gurtner, G.C. Surgical Approaches to Create Murine Models of Human Wound Healing. *Journal of Biomedicine and Biotechnology*. **2011** (January), 1–8, doi: 10.1155/2011/969618 (2011).
261. Kang, S.-J., Kim, D.-H., Mishig-Ochir, T., Lee, B.-J. Antimicrobial peptides: Their physicochemical properties and therapeutic application. *Archives of Pharmacal Research*. **35** (3), 409–413, doi: 10.1007/s12272-012-0302-9 (2012).
262. Gabrilovac, J., Čupić, B., Breljak, D., Zekušić, M., Boranić, M. Expression of CD13/aminopeptidase N and CD10/neutral endopeptidase on cultured human keratinocytes. *Immunology Letters*. **91** (1), 39–47, doi: 10.1016/j.imlet.2003.10.006 (2004).
263. Mojsoska, B., Zuckermann, R.N., Jenssen, H. Structure-Activity Relationship Study of Novel Peptoids That Mimic the Structure of Antimicrobial Peptides. *Antimicrobial Agents and Chemotherapy*. **59** (7), 4112–4120, doi: 10.1128/AAC.00237-15 (2015).
264. Mojsoska, B., Carretero, G., Larsen, S., Mateiu, R.V., Jenssen, H. Peptoids successfully inhibit the growth of gram negative E. coli causing substantial membrane damage. *Scientific Reports*. **7** (February), 1–12, doi: 10.1038/srep42332 (2017).
265. Doern, G. V., Brecher, S.M. The clinical predictive value (or lack thereof)

of the results of in vitro antimicrobial susceptibility tests. *Journal of Clinical Microbiology*. **49** (9 SUPPL.), 11–14, doi: 10.1128/JCM.00580-11 (2011).
266. Mohanram, H., Bhattacharjya, S. Salt-resistant short antimicrobial peptides. *Biopolymers*. **106** (3), 345–356, doi: 10.1002/bip.22819 (2016).
267. Kaspar, A.A., Reichert, J.M. Future directions for peptide therapeutics development. *Drug Discovery Today*. **18** (17–18), 807–817, doi: 10.1016/j.drudis.2013.05.011 (2013).

Chapter 4: Papers

Paper I: Optimized scratch assay for *in vitro* testing of cell migration with an automated optical camera

Re-print is in agreement with the journal of visualized experiments

Paper II: Neurotensin, substance P, and insulin enhance cell migration

Re-print is in agreement with the journal of peptide sciences

Paper III: The effect of Nisin A on wound healing *in vivo*, *in vitro*, and *ex vivo*

Paper IV: Synthetic peptide IDR-1018 anti-inflammatory and microbiome modulatory properties positively impact wound healing

Paper V: Bovine Lactoferricin alters cellular movement, cytokine profile, microbiome leading to increased wound healing *ex vivo* and *in vivo*

Appendix: Preliminary results

Anti-biofilm and wound healing properties of single amino acids substitutions in LL-37 derived peptide fragments