The Establishment of a Porcine Model of Persistent Inflammation in Chronic Wounds

Master Thesis

For the attainment of the academic degree

Master of Science

from the FH Campus Wien

Submitted by
Nina Bucher

Personal identity code
1510544019

Supervisor
Univ.Prof.Dr.med. Lars-Peter Kamolz, MSc
Division of Plastic, Aesthetic and Reconstructive Surgery
Department of Surgery, Medical University of Graz
Austria

Submitted
19/09/2017
1. Abstract

Although the majority of chronic wounds respond well to conventional treatment, some chronic wounds fail to heal within an appropriate time frame despite the best care. Hence, it was taken advantage of the similarity between human and porcine skin to establish a model mimicking persistent inflammation in chronic wounds as well as providing a cutting-edge technology in personalized medicine, designed to predict the appropriate treatment required by individual patients.

Resiquimod (R-848), a small molecule toll-like receptor (TLR7/8) agonist was investigated to induce a chronic wound status in pigs (n=4). Full thickness wounds were generated with 6mm biopsy punches and were treated with different concentrations of R-848 (0.1% and 0.05%) via topical application of a single daily dose of 50µl for five days consecutively. Propylene glycol (PG), representing the vehicle dissolving R-848, and human serum served as controls. Wound size and inflammation were scored daily for 15 days.

R-848 turned out to be potent to induce persistent inflammation in wounds. Whilst 0.1 % had to be adjusted in concentration to counteract inappropriate wound progression, a constant dose of 0.05 % maintained a persistent inflammation as long as application was continued. Maximum wound severity was reached on day 6 and inflammation started to decline 2 days after treatment was terminated. No significant difference was observed between the progression of wounds treated with PG and human serum respectively, suggesting that a systemic impact and the invasiveness of these probes can be precluded.

Conclusively, we established the first preclinical model for persistent inflammation present in chronic wounds, maintained by the application of R-848. Subsequent adaptations will investigate whether and how a chronic status can be prolonged.
2. Abstrakt

Obgleich die Mehrheit aller chronischen Wunden gut auf konventionelle Therapieformen anspricht, gibt es einzelne Fälle, wo selbst nach einer angemessenen Zeitspanne keine Heilung einzutreten scheint. Darum ist von der Ähnlichkeit zwischen menschlicher- und Schweinehaut Gebrauch gemacht worden, um ein Modell zu entwickeln, welches sowohl eine fortlaufende Entzündung in chronischen Wunden als auch eine innovative Technologie in der personalisierten Medizin darstellen soll, mit der Fähigkeit das optimale Behandlungsverfahren für individuelle Patienten bereitstellen zu können.

Resiquimod (R848), ein niedermolekularer Toll-like Rezeptor (TLR7/8) Agonist war daher im Fokus der Forschung, um einen chronischen Wundstatus in Schweinen (n=4) zu generieren. Dafür wurden Vollhautwunden von 6mm Durchmesser generiert und resultierende Wunden anhand topischer Applikation über fünf aufeinander folgende Tage hinweg mit verschiedenen Konzentrationen an R848 (0,1% und 0,05%) behandelt. Gleichzeitig wurden Propylenglykol (PG), das liquidierende Vehikel, sowie humane Serum Proben als Negativkontrollen eingesetzt. Die Wundgröße und Entzündungsparameter wurden 15 Tage lang beobachtet und aufgenommen.

R848 ist in der Lage einen fortwährenden Inflammationsstatus in Wunden zu erzeugen. Während die Konzentration von 0,1% reduziert werden musste, um einer unverhältnismäßigen Wundprogression entgegenzuwirken, kann eine Konzentration von 0,05% einen chronischen Wundstatus aufrechterhalten, solange die topische Applikation fortgesetzt wird. Das maximale Wundausmaß konnte am Tag 6 beobachtet werden wohingegen bereits 2 Tage nach Behandlungsstopp eine Heilung eintrat. Kein signifikanter Unterschied zwischen der Entwicklung der mit jeweils PG oder humanem Serum behandelten Wunden wurde festgestellt. Diesbezüglich kann eine systemische Entzündungsreaktion sowie die Invasivität jener Proben ausgeschlossen werden.

Diese Forschungsergebnisse repräsentieren das erste präklinische Modell einer fortwährenden Entzündung innerhalb von chronischen Wunden, erzeugt durch R848. Folgende Adaptionen werden klären, ob und wie der chronische Status dieser Wunden aufrechterhalten werden kann.

3. Table of Contents

1. ABSTRACT ........................................................................................................................................... 2

3
Molecular Biotechnology
Nina Bucher
1510544019

2. ABSTRAKT .............................................................................................................. 3

3. TABLE OF CONTENTS .......................................................................................... 4

4. LIST OF FIGURES ................................................................................................... 6

5. LIST OF TABLES ..................................................................................................... 9

6. LIST OF ABBREVIATIONS ...................................................................................... 10

7. INTRODUCTION ..................................................................................................... 11

7.1. WOUND HEALING .............................................................................................. 13

7.1.1. Hemostasis and Inflammation ......................................................................... 14

7.1.2. Proliferation .................................................................................................... 15

7.1.3. Remodeling .................................................................................................... 15

7.2. DEFECTS IN CHRONIC WOUNDS ..................................................................... 16

7.2.1. Proinflammatory cytokines ............................................................................ 18

7.2.2. Cellular level .................................................................................................. 19

7.2.3. Proteases ........................................................................................................ 20

7.2.4. Bacteria and Biofilms ..................................................................................... 20

7.3. ASSESSMENT OF CHRONIC WOUNDS .............................................................. 21

7.3.1. Invasive Classification of Chronic Wounds ...................................................... 21

7.3.2. Optical Classification of Chronic Wounds ....................................................... 21

7.4. CURRENT MODELS FOR THE INVESTIGATION OF CHRONIC WOUNDS ............ 22

7.5. THE PURPOSE OF A PORCINE MODEL OF PERSISTENT INFLAMMATION ............ 24

8. MATERIAL & METHODS ......................................................................................... 25

8.1. MATERIALS, REAGENTS, AND EQUIPMENT ...................................................... 25

8.2. SETUP .................................................................................................................. 26

8.3. COMPOSITIONS OF SOLUTIONS ....................................................................... 28

8.4. SHAVING ............................................................................................................. 29

8.5. WOUND INDUCTION ......................................................................................... 29

8.6. DRESSING ........................................................................................................... 29

8.7. SCORING OF WOUNDS ...................................................................................... 30
8.8. EUTHANASIA ........................................................................................................ 30
8.9. BIOPSY .................................................................................................................. 30
8.10. HEMATOXYLIN & EOSIN STAINING ................................................................. 30
8.11. WOUND ASSESSMENT VIA SCARLETRED®VISION ...................................... 31

9. RESULTS ................................................................................................................. 32

9.1. ESTABLISHMENT OF A SCORING MATRIX ...................................................... 32
9.2. OPTICAL SCORING OF WOUNDS ...................................................................... 37
  9.2.1. Size ................................................................................................................ 38
  9.2.2. Necrosis ......................................................................................................... 39
  9.2.3. Crust ............................................................................................................... 40
  9.2.4. Erythema ...................................................................................................... 41
9.3. SWELLING .......................................................................................................... 43
9.4. WOUND PROGRESSION .................................................................................. 43
9.5. WOUND APPEARANCE .................................................................................... 50

10. DISCUSSION .......................................................................................................... 58

11. OUTLOOK .............................................................................................................. 61

12. REFERENCES ......................................................................................................... 63

13. ACKNOWLEDGEMENT ........................................................................................ 67

14. STATUTORY DECLARATION ............................................................................... 68

15. EIDESSTATTLICHE ERKLÄRUNG .................................................................... 69

4. List of Figures

Figure 1 Clinical features of the four most common wound-healing pathologies: The venous- (1) as well as the arterial ulcer (2) compared to a diabetic foot syndrome (3) and a chronic wound caused by local pressure (4).12 ......................................................... 12
Figure 2 The main phases of acute wound healing: Hemostasis, inflammation, proliferation and remodeling.18 ........................................................................................................... 13
Figure 3 Cellular and molecular mechanisms within distinct healing stages in acute wounds including inflammatory-, proliferative- as well as remodeling phase.59 .......... 16
Figure 4 Comparison of the cellular and molecular difference between chronic- and acute wounds. Imbalances such as elevated protease levels and high inflammatory cytokines may be the cause for chronic wounds.\textsuperscript{26} ................................................................. 17

Figure 5 The molecular pathology in chronic wounds. Including hyperproliferative and nonmigratory epidermis, unresolved inflammation, presence of infection, and biofilm formation, many defects may be found simultaneously in non-healing ulcers.\textsuperscript{28} .............. 18

Figure 6 Comparison of immunohistochemical staining of porcine (left) and human (right) epidermis. (A) is showing Type IV collagen expression in the basement membrane of the epidermis and dermal vessels whereas (C) depicts Keratin 5/6 expression in basal epidermal Keratinocytes. (G) shows the similarities in E-cadherin expression.\textsuperscript{38} ............ 23

Figure 7 Setup of Wound Areas. Each area consisting of 4 biopsy punch wounds with a diameter of 6mm treated according to the assigned substance for 5 consecutive days. ........................................................................................................................................ 26

Figure 8 Timeline of the setup from wound induction (d1) until biopsy collection (d15). ........................................................................................................................................................................... 27

Figure 9 Measurement of wound- and erythema size by SCARLETRED\textregisteredVISION. Area or redness of the wound bed is subtracted from the outer necrotic edge or inflamed area, respectively................................................................. 31

Figure 10 Size score. Representative pictures to ascending scores from zero (A) to 3 (D) depending on the size of a wound. ............................................................................................................................................... 38

Figure 11 Necrosis Score. Score zero for a wound of which the necrotic edge is already covered by a crust (A) as well as when no necrotic edge is observed at all (B). Scores from 1 to 4 are shown in pictures C to F. ........................................................................................................ 39

Figure 12 Crust Score. Score zero for a healed wound/crust fallen off (A), a score of 1 for a wound covering either the wound bed or the necrotic edge only (B, C), a score of 2 for a crust which covers both wound bed as well as necrotic edge (D) and a score of 3 for the absence of a crust (E). .............................................................................................................................................. 40

Figure 13 Erythema score. Representative wounds to ascending scores from zero (A) to 4 (D) as well as a score of +1 (E) for an erythema width greater than 5mm in diameter. .............................................................................................................................................. 41

Figure 14 Comparison of computationally analyzed results of erythema (1) compared to optical assessment (2). ........................................................................................................................................................................ 42

Figure 15 Timeline showing a representative progression of a PG treated wound which was induced on day 1 and observed for 15 consecutive days........................................................................... 43
Figure 16 Wound Progression - Normal/Acute Healing. Representative wound progression of a PG treated wound which was observed for 15 consecutive days...... 44

Figure 17 Comparison between progression of wounds administered with PG as vehicle or a human serum probe, respectively. ................................................................. 45

Figure 18 Mean scores of serum treated wounds of all test subjects calculated for Areas 1 to 4. ......................................................................................................................... 46

Figure 19 Timeline showing a representative progression of an R-848v treated wound which was induced on day 1 and observed for 15 consecutive days......................... 47

Figure 20 Wound progression - Delayed/Chronic Healing. Representative wound progression of a R-848v treated wound which was observed for 15 consecutive days. ................................................................................................................................. 47

Figure 21 Comparison between progression of wounds administered with a variable (0.1-0.01%) or constant dose (0.05%) of R-848, respectively........................................... 48

Figure 22 Optically interpreted scores of wound progression with an acute and fast healing wound status and wounds with a delayed healing phase, respectively. ........ 49

Figure 23 Representative wound progression according to mean scores shown in a healing timeline after termination of R-848 application (day 5). ................................. 50

Figure 24 Hematoxylin and eosin stained tissue sections of biopsied wound beds. On day 15 biopsies were harvested, resulting in wounds in different healing stages due to a time-delayed wound induction setup. .............................................................. 54

Figure 25 H&E stain: Open wound with deep ulceration. Treatment: R-848v day 8. ... 55

Figure 26 H&E stain: Successfully healed wound by reepithelization, showing a clear layer of granulation tissue. Treatment: human serum day 15........................................ 56

Figure 27 Adjusted setup for the establishment of a porcine model of persistent inflammation in chronic wounds comparing topical treatment of 5 and 10 days (A, B) to injections (C, D). The last sedation is followed by the introduction of euthanasia. .... 62
5. **List of Tables**

Table 1 Development of a scoring matrix. Comparison between included parameters prior to study commencement and the parameters included after termination............ 33
Table 2 All wound parameters contained in the scoring matrix applicable for the classification of persistent inflammation in chronic wounds. .......................... 34
Table 3 Starting point for freshly induced 6mm biopsy punch wounds. .................... 35
Table 4 Maximum score arising from the scoring matrix applicable for the classification of persistent inflammation in chronic wounds........................................ 36
Table 5 Maximum area score arising from the scoring matrix applicable for the classification of persistent inflammation in chronic wounds............................ 36
Table 6 Maximum Inflammation score arising from the scoring matrix applicable for the classification of persistent inflammation in chronic wounds......................... 37
6. List of Abbreviations

- APC: Antigen-presenting cell
- CTL: Cytotoxic T-cell
- DC: Dendritic cell
- ECM: Extracellular matrix
- H&E: Hematoxylin and eosin
- MMPs: Matrix metalloproteinases
- MSC: Mesenchymal Stem Cells
- PG: Propylene Glycol
- R-848: Resiquimod
- ROS: Reactive Oxygen Species
- TGF-β: Transforming Growth Factor β
- TF: Tissue Factor
- TLR7/8: Toll-like receptor 7/8
- uPA: Urokinase-type plasminogen activator
7. Introduction

Per definition a chronic wound is classified as a trauma of the skin of which functional and anatomical integrity cannot be restored within three months.\textsuperscript{1,2} The normal phases of wound healing are impaired regarding a timely and orderly manner. Whereas wound healing takes place in overlapping stages in normally healing, so-called acute wounds, chronic wounds fail to appropriately pass through essential healing phases including hemostasis, inflammation, proliferation and remodeling of the skin.\textsuperscript{3–5}

The cellular and molecular mechanisms of wound healing are complex and the impairments in chronic wounds remain poorly understood. Until today, many reasons were discovered which might cause irritations during wound healing such as aging, malnutrition, bacterial infections, diabetes, deficiency of stem cells, local pressure and/or vascular insufficiency, smoking and even psychological stress to name a few.\textsuperscript{6–8} Still, no event in particular was identified to be the direct cause of chronic wounds. Moreover, the majority of chronic wounds respond well to conventional treatment, however in some cases healing does not occur despite the best care. This problem is estimated to affect 15 to 20\% of all chronic wound patients resulting in about 40 million people worldwide.\textsuperscript{9}

Yet, the etiology of chronic wounds is enormously diverse, hindering researchers from the development of a gold-standard therapy.\textsuperscript{9} Moreover, the fact that chronic wounds can be classified in subtypes makes understanding of their mode of formation even more complex. In general, one can distinguish between venous and arterial ulcers, pressure- and diabetic ulcers, of which all generally tend to occur in elderly people. As one can see in Figure 1, their wound appearances can be considered rather similar and most of the defects are located on the lower extremities of the body. Thus, they are often called “lower extremity ulcers”. In general, these are vascular or diabetic in nature, which in turn account for up to 98\% of all lower extremity wounds.\textsuperscript{10–12}
Similar to venous ulcers, which can be associated with deep vein thrombosis, varicose veins and venous hypertension, arterial ulcers can develop as a consequence of atherosclerosis, thrombosis as well as hypertension. Both types of ulcers seem to have some ischemic history in common caused by a lack of blood supply in the respective area. Different to that, diabetic ulcers are a common complication in uncontrolled diabetes mellitus, which can result in impaired immune function, neuropathy and ischemia. These pathologies may then lead to breakage of the skin and can represent the starting point for chronic wounds. Most of these ulcers develop into the so-called diabetic foot syndrome. In contrast to the previously mentioned types of chronic wounds, pressure ulcers are caused by constant pressure and friction from the outside of the body. They can be generated from forces distributed over a localized body surface for a prolonged duration that obstruct blood flow to the tissue. This can lead to injuries of the skin and thus ulceration on the legs or in the area around the tailbone. Therefore, this type of wound is often referred to as decubitus or bed sore. Practically these wounds represent the easiest type of chronic wounds to counteract. This can be attained
by the help of pressure-relieving seat cushions and avoidance of prolonged sitting as far as possible, to ensure an off-load of pressure of the affected area. Yet, all chronic wounds need very diverse and intense care.\textsuperscript{15,16} Suitable approaches might include standard wound care procedures such as adequate debridement and proper dressing. But also endovascular- and open bypass procedures, oxygen therapy and dietary change may be neccessary.\textsuperscript{4,17}

7.1. Wound Healing

However, some chronic wounds cannot be healed yet according to a standard treatment regimen. Therefore, normal, proper healing must be understood to be able to counteract the molecular imbalances in chronic ulcers in the future. Essentially, wound healing takes place in four sequential phases which include hemostasis, inflammation, proliferation and remodeling of the skin as depicted in Figure 2.\textsuperscript{18}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{wound_healing_diagram.png}
\caption{The main phases of acute wound healing: Hemostasis, inflammation, proliferation and remodeling.\textsuperscript{18}}
\end{figure}

7.1.1. Hemostasis and Inflammation

Hemostasis is the physiological process which stops bleeding at the site of an injury while normal blood flow is maintained elsewhere in the circulation.\textsuperscript{19} Since throughout this phase the inflammatory response is also highly stimulated, these two can be
considered interdependent. They may also be referred to as a lag-phase since many cells and factors need to be attracted before bleeding can be stopped and healing is initiated. Upon exposure of tissue factor (TF) from the injured epidermal layer, the extrinsic cascade is initiated while the intrinsic cascade is activated via the encounter of exposed collagen.\textsuperscript{20} The bleeding is limited via vasoconstriction and platelets subsequently form a blood clot in order to close the fissure. It contains fibrin molecules, fibronectin, vitronectin and thrombospondins, forming a matrix that serves as a scaffold structure for the migration of leukocytes, keratinocytes, fibroblasts and endothelial cells. Both platelets and leukocytes produce cytokines and growth factors such as IL-1\textalpha{}, IL-1\textbeta{}, IL-6, IL-8 and TNF-\textalpha{}, stimulate collagen synthesis via release of FGF-2, IGF-1, TGF-\textbeta{}, activate fibroblasts for wound closure via TGF-\textbeta{} and even already support the reepithelialization process via release of EGF, FGF-2, IGF-1 and TGF-\textalpha{}. PDGF which is strongly secreted by macrophages in turn plays an important role in initiating the chemotaxis of neutrophils, monocytes, smooth muscle cells and fibroblasts. Also, TGF-\textbeta{} stimulates cytokine secretion from macrophages and enhances fibroblast and smooth muscle cell chemotaxis. In summary, the presence of PDGF and TGF-\textbeta{} may be one of the hallmarks designating the transition into the next healing phase. Prior to this and in parallel, within the first two to five days after injury, elevated levels of neutrophils can be detected in a wound with the function to kill bacteria via the production of reactive oxygen species (ROS). They also degrade collagen and respective fragments via the production of proteases such as matrix metalloproteinase (MMP)-2 and -9 to eliminate non-viable tissue, which they can in turn phagocytose together with dead bacteria. As soon as neutrophils have fulfilled their function they undergo apoptosis and are removed by macrophages, together with dead body-own cells.
An orderly sequence of these events in the early stage of a wound is crucial since an ongoing inflammation may lead to further tissue damage. In turn, this might represent the preliminary stage of a chronic wound milieu.\textsuperscript{21}

### 7.1.2. Proliferation

An utterly complex proliferative phase takes over approximately three days after injury and may last up to 10 days. Its main objective is closing the wound as well as restoring vascular integrity via the production of collagens, fibronectin and the secretion of cytokines. Keratinocytes, epithelial stem cells, fibroblasts and smooth muscle cells are involved in this process to ensure proper migration of cells via a fibrin/fibronectin-rich provisional extracellular matrix (ECM). Reepithelialization sets in at the wound edges and neovascularization as well as angiogenesis are activated by capillary sprouting.\textsuperscript{21} Collagenases and elastases induce permeability of the membranes which allows migration, so-called shuffling, of keratinocytes over the ECM. This is facilitated by a chemotactic gradient to cover the blood clot, forming a structure consisting of granulation tissue. Simultaneously, small GTPases were found to be the switch for an orchestrated epithelialization process as well as for the termination of migration upon touch of the moving cells. Consequently, a reorganization of the cytoskeleton is initiated and contacts are restored in the newly built tissue.\textsuperscript{21}

### 7.1.3. Remodeling

The last phase of wound healing, namely remodeling phase, is especially important to ensure integrity of the of the newly build skin structure. Thus, this phase may take up to one year after injury. Granulation tissue is removed via apoptosis and the regression of many of the transiently formed capillaries takes place in order to reconstitute a normal vascular density.\textsuperscript{8} This characterizes a mature wound as avascular as well as acellular.\textsuperscript{21} Conclusively, previously produced collagen III within the proliferative phase is removed and replaced by stronger collagen type I. A scar is formed, representing the final event of the four substantial healing phases which are depicted again in Figure 3. Previously described cells as well as secreted factors are shown assigned to the respective phase in a representative way.\textsuperscript{22–24}

### 7.2. Defects in chronic wounds
Despite the advanced understanding of wound healing, chronic wounds still represent a major health burden. Many studies have investigated chronic wounds in order to identify their key steps during development which can be seen in Figure 3. Previous suggestions state that these wounds seem to be stuck either in the inflammatory or the proliferative phase. However, a lot of time and research was invested by then with the evidence that persistent inflammation is a hallmark of chronic wounds.14,23

Figure 3 Cellular and molecular mechanisms within distinct healing stages in acute wounds including inflammatory-, proliferative- as well as remodeling phase.59
Further, it turned out that chronic wounds substantially differ from acute wounds in their environment which was proven via analysis of tissue biopsies as well as wound fluid samples which can be seen in Figure 4.26

![Figure 4 Comparison of the cellular and molecular difference between chronic- and acute wounds. Imbalances such as elevated protease levels and high inflammatory cytokines may be the cause for chronic wounds.](image)

Typically, non-healing wounds display low mitogenic activity in combination with the presence of senescent cells in the surrounding area. Additionally, microbial contamination may enhance prolonged inflammatory stimuli which can trigger an elevated influx of proteases such as MMPs, elastase, plasmin and thrombin. The latter are capable of degrading matrix proteins and growth factors thus reducing proliferation, keratinocyte migration and chemotaxis, which would again activate proteases producing ROS.1,9,26,27 Since growth factors, cytokines, proteases as well as cellular and extracellular elements play important roles in the healing process, the imbalance of one or more of these components may disturb proper transition from one to another healing phase.23 Thus, imbalances within a wound must be cleared without delay in order to escape the advent of a vicious cycle. The complexity of the molecular pathology of chronic wounds is also shown in Figure 5 including unresolved inflammation, presence of infection, biofilm formation, hyperproliferative and nonmigratory epidermis.3,9,28
Proinflammatory cytokines

As a consequence of tissue injury and the bacterial burden, microorganisms and platelet-derived factors can stimulate an influx of immune cells into the wound. This inflammatory state is characterized by neutrophil infiltration. Neutrophils can keep the proinflammatory cytokine cascade sustained, leading to elevated levels of ROS and proteases in the tissue. This process represents a vicious circle, leading to excessive ECM degradation associated with impaired healing. Whereas in acute wounds, proteases are tightly regulated by their inhibitors, in chronic wounds protease levels exceed the ones of their respective inhibitors. This may lead to the destruction of the ECM, growth factors and their receptors. This proteolytic destruction of the ECM not only prevents wound healing but also attracts further inflammatory cells, amplifying the inflammatory cycle.

While IL-1 and TNF-α enhance collagenase secretion, chronic exposure of skin cells to these cytokines is a contributing factor in connective tissue disease. Previous studies

Figure 5 The molecular pathology in chronic wounds. Including hyperproliferative and nonmigratory epidermis, unresolved inflammation, presence of infection, and biofilm formation, many defects may be found simultaneously in non-healing ulcers.28
have shown that levels of IL-1β and TNF-α were approximately 100-fold higher in chronic wound fluids when compared to levels in mastectomy fluids which were drained after surgical procedures, indicating an imbalance of proinflammatory cytokines within the chronic wound.\cite{29,30}

### 7.2.2. Cellular level

Since many cells are involved in the healing process such as keratinocytes, fibroblasts, neutrophils, macrophages, lymphocytes, endothelial cells and stem cells, it can be assumed that defects in one or more of them may be the cause for the development of chronic ulcers.\cite{31} Keratinocytes sitting at the edge of these wounds were found to express a certain gene signature. Upregulation of several cell cycle genes such as cyclins as well as the suppression of cell-cycle-checkpoint regulators including p53 may explain the epidermal hyperproliferation which is seen at the edges of these ulcers. Therefore, it is speculated that either activation or differentiation may be disturbed throughout this cell type, resulting in thick callus-like formations at the edges of venous ulcers as an example.\cite{32} In contrast, fibroblasts present in ulcerated wounds seem to be senescent, have diminished migratory capacity and do not respond to migratory stimulants within the TGF-β pathway. This results in dramatically reduced levels of TGFβR and reduced corresponding signaling cascade components, testified via analysis of biopsies of non-healing ulcer tissue.\cite{14} Also mesenchymal stem cells (MSCs) have been shown to play an important role in wound healing due to the reason that these cells can be recruited into the circulation in response to injury. It was proven that stem cells in animals and patients with diabetes as well as chronic wounds are both deficient and defective.\cite{4} However, compared to these seemingly genetic defects, neutrophils and macrophages are usually active upon bacterial stimulus. In turn, they produce many factors including proteases representing a potential stress factor which might direct a wound towards a chronic state.
7.2.3. Proteases

It is known that several factors are expressed by numerous cell types during different phases of healing as mentioned before. Most importantly, MMPs and serine proteases, including elastase, cathepsin G, and urokinase-type plasminogen activator (uPA), are known to be markedly increased in chronic wound fluids as compared to acute wound fluids. Additionally, MMP levels were shown to decrease in the injured skin area during the healing process.23,29 Further, the degradation and remodeling of the ECM by such proteases, particularly the MMPs, is a key element of tissue repair and plays a role in the influx of leukocytes, angiogenesis, re-epithelialization and clearance of damaged protein.7 In contrast, they are also capable of degrading growth factors and their receptors as well as angiogenic factors hence can have an influence on cellular behavior. Therefore, controlled expression of MMPs is a critical part of normal wound healing whereas elevated and prolonged expression can disrupt the balance between tissue breakdown and repair, leading to excessive ECM degradation associated with impaired healing.29

7.2.4. Bacteria and Biofilms

The presence of bacteria in chronic wounds is unavoidable which makes them omnipresent on the skin as well as in every wound. However, certain bacteria and their corresponding biofilms can delay wound healing and cause serious infections which may extend into the underlying bone, causing systemic septicemia. The most commonly found pathogens in infected chronic wounds are Pseudomonas aeruginosa and Staphylococcus aureus. Whether considered bioburden, biofilm or colonizing infection, the presence of bacteria in a wound is a major issue, hindering chronic wounds from healing via targeting the key inflammatory players in a wound.33 Therefore, the microbial bioburden can be considered a therapeutic target in all non-healing ulcers and the administration of antibiotics is recommended as a precaution.34
7.3. **Assessment of Chronic Wounds**

Since not only bacterial infection and subsequent infiltration of immune cells seems to facilitate the development of chronic wound, many environmental factors are yet to be elucidated in respect to the impaired healing process. Therefore, cellular senescence, cytokines and chemokines, ROS, proteases and many different factors must be assessed in the chronic wound.⁹ To date, a limitation of methods for a standard identification procedure of the key players makes it hard to understand where the defects in a chronic wound primarily result from. Hence, factors directly contributing to a shift towards a chronic state remain unidentified. The most recently developed and frequently used techniques for the evaluation of the chronic wound milieu are explained in the following sections.

7.3.1. **Invasive Classification of Chronic Wounds**

Nowadays, histological and biochemical characterization of biopsied wound tissue is the only reliable method for exact wound assessment. Further, wound fluid can be extracted and analyzed via different approaches. The presence or absence of certain factors in a wound can give indication on the advancement in the healing process and thus tell whether certain essential processes are in progress such as angiogenesis, inflammation, fibroplasia and restoration of the connective tissue matrix, wound contraction and remodeling, epithelialization and differentiation. Additionally, an exact quantitative measurement can give information on the absolute numbers of cells and mediators present in the injured area.³¹ However, these procedures require surgery within defected skin, potentially representing further risks within chronic wounds.

7.3.2. **Optical Classification of Chronic Wounds**

Chronic wounds have a diverse appearance. Attributes such as the size as well as host- and environmental factors which may influence progression should be evaluated in order to provide optimal wound management for each patient.³¹ To date, many tools are available for the classification of the wound status such as the Pressure Ulcer Score of Healing (PUSH), the Wound Healing Scale (WHS) and the Granulometer. Yet these tools are not able to predict healing or measure wound characteristics on the molecular level.³¹ Yet, an advanced tool called DESIGN-R was developed which can serve as a guide for the process of scoring important parameters within wounds. DESIGN-R considers the
depth, exudate, size, inflammation/infection, granulation, necrotic tissue and the pocket of a wound. Nevertheless, the parameters within this scoring systems are enormously complex to interpret and the examination requires high clinical skill as well as experience. This makes grading not only subjective and time-consuming but also not transferable to untrained personnel. Therefore, several noninvasive, optical methods were tested for their potential to characterize physical properties of wounds in vivo. These include laser Doppler perfusion imaging for the measurement of cutaneous blood flow with assessment of wound microcirculation, optical coherence tomography to evaluate tissue structure and a rather new technique called Raman spectroscopy. This innovative technology holds the potential to provide simple and rapid assessment of the biochemistry of a wound bed in situ, making use of the “Raman”-type scattering of photons incident on a material. Thus, it represents an alternative to traditional approaches to the biochemical evaluation of wounds which are based on biopsied. Diseased tissue in patients with breast cancer, atherosclerosis, cervical precancer, and Alzheimer’s disease was already identified using Raman spectroscopy, thus this method may be beneficial for the classification process of chronic wound types in the future. Due to the reason that this technique is very innovative which makes it enormously expensive, its application as a standard method for assessment is not yet possible.

7.4. Current Models for the Investigation of Chronic Wounds

Since the exact origin and sequence of the development of chronic wounds remains a mystery to research, medicine is in need to establish a wound model to mimic and investigate chronic progression features. For this reason, many different in-vivo models were established, yet the difference in wound characteristics can still prevent successful therapy. For example, drugs which work well in non-ischemic wounds may not work in human diabetic wounds due to a lack of a vasculopathy in the model. Therefore, the model must be established as such, to be as similar to the human wound as possible. A rabbit ear ulcer model was developed by suturing off two to three arteries on the ear of the animal in order to generate an ischemic zone by the disruption of blood supply. Thereby, a vasculopathy can be simulated and full-thickness wounds can be induced to test for the appropriate treatment. A similar technique is the skin flap model undermining defined skin areas by silicone sheets thus disconnecting and inhibiting vascularization. Nevertheless, both techniques are time-consuming and require high
skill. Further, considering the heterogeneity and complexity of human chronic wounds, no animal model seems to have the potential to mimic each clinical scenario to the full extent so far.\textsuperscript{14}

Yet, remarkable similarities were found between human skin and the one of the domestic pig, Sus scrofa. It is related closest to the one of man from the perspectives of anatomy and physiology, immunogenicity, cellular composition, and morphology. Immunohistochemical stainings which can be seen in FIGURE depict the similarities in structure of porcine and human skin, clearly showing a distinct delimitation of the different cell types.\textsuperscript{38} This not only renders its skin suitable for studies on wound healing but also on transdermal delivery, dermal toxicology, radiation and UVB effects.\textsuperscript{39–41}

\textbf{Figure 6} Comparison of immunohistochemical staining of porcine (left) and human (right) epidermis. (A) is showing Type IV collagen expression in the basement membrane of the epidermis and dermal vessels whereas (C) depicts Keratin 5/6 expression in basal epidermal Keratinocytes. (G) shows the similarities in E-cadherin expression.\textsuperscript{38} Pig skin displays the greatest thickness in epidermis of all domestic animal species, and can be compared to the thick epidermis of humans, with a striking analogy in the number of cell layers. Additionally, pig collagen is utterly similar to the human one.\textsuperscript{42} Due to all of these parallels, porcine wound healing studies were capable of exhibiting significantly higher concordance with human studies (78%) than in-vitro testing (57%) and even studies in small laboratory animal (53%).\textsuperscript{43,44} Nevertheless, caution is always required
when comparing porcine skin to human skin within a study setup, especially when results are interpreted with regard to the human situation.  

7.5. **The Purpose of a Porcine Model of Persistent Inflammation**

Like already mentioned, the etiology of chronic wounds is enormously diverse. Thus, research has failed to develop a gold-standard therapy for chronic wounds until today. Further, accurate optical classification of chronic ulcers requires experienced personnel and is still a major limitation in providing proper care. Therefore, a new classification system was in the scope of the study, to allow assessment of the status of each wound equally well, unlike other scoring systems. Subsequently, it was made use of the similarity between porcine and human skin to provide a cutting-edge advancement in personalized medicine. Therefore, the potency of Resiquimod (R-848) was elucidated to induce chronic wounds in porcine skin. This Toll-like receptor 7/8 (TLR7/8) agonist was selected since its respective receptor is expressed on macrophages and neutrophils, which account for the major proportion of the infiltrate in human chronic wounds. Thereby, R-848 was found to be able to delay healing in mice and man.

Concludingly, not only a porcine model of persistent inflammation in chronic wounds originated from this study, but also a generalized scoring matrix was developed in parallel.
8. Material & Methods

8.1. Materials, Reagents, and Equipment

- Surgical Clipper 9671 3M
- Skinsept® F ECOLAB Healthcare
- 6mm biopsy punches Henry Schein Medical GmbH
- Forceps
- Scissors
- Cotton swabs sterile Heinz Herenz Medizinalbedarf
- Epinephrine bitartrate salt (E4375) Sigma-Aldrich
- NaCl 0,9% B. Braun, Ecotainer® 1.000 ml B. Braun
- ES-Kompressen unsteril PAUL HARTMANN GES.M.B.H.
- ES gauze swabs (4017254) Hartmann
- Omnifix-F syringes (1ml) B. Braun
- Resiquimod R848 (SML0196) Sigma-Aldrich
- TWEEN 80 (P8074) Sigma-Aldrich
- Human AB Serum (# 35-060-Cl) Corning
- 1,2-Propanediol (82280) Sigma-Aldrich
- Mepilex® Lite foam dressing 10x10 cm Mölnlycke Health Care
- Tegaderm Transparent Film Dressing 10x12cm 3M
- Dog & Cat Body Size M & L Henry Schein Vet GmbH
- Fentanyl 50 µg/h transdermal patch Sandoz
- TWEEN® 80 (63161) Sigma-Aldrich
- (Hydroxypropyl)methyl cellulose HPMC (56340) Sigma-Aldrich
- Leukoplast Waterproof bsn medical
- Histology Containers Thermo Fisher Scientific
- Scalpel blades - Figure 10, BB 510 Henry Schein Dental
- Zolazepam Virbac®
- Source data form Joanneum Research

8.2. Setup
For the series of experiments to establish a model of persistent inflammation in porcine skin, a special setup was derived from previously conducted studies in order to test for the potency of R-848 (Sigma-Aldrich). Four treatment strategies were tested:

- Treatment
  - R-848 variable (0.1-0.01%)
  - R-848 constant (0.05%)
- Negative Controls
  - Vehicle (PG)
  - Human Serum

The induction of 16 wounds per pig was approved by the respective authority (BMWFW-66.010/0111-WF/V/3b/2016, Wien). Five wound pools called Areas were placed on the back of each test subject (n=4). All of them were treated with the same application regimen. The respective scheme can also be seen in Figure 7.

**Figure 7 Setup of Wound Areas. Each area consisting of 4 biopsy punch wounds with a diameter of 6mm treated according to the assigned substance for 5 consecutive days.**
Each Area consisted of four wounds in total. Wound number 1 was treated with propylene glycol (PG, Sigma-Aldrich) which served as the vehicle to resolve the pulverulent R-848. Wound 2 was administered with a human serum sample (Corning) which served as a control in a side study (data not shown). Yet, this control was also useful in this study since it served as a reference for PG. In comparison, wounds for the investigation of R-848 were created (3, 4). R-848 wounds were treated either with a variable dose starting at 0.1% (3) or a constant dose of 0.05% (4), respectively. Different concentrations were examined to elucidate the effect of R-848 when topically applied onto an open wound. The variable dose regimen was planned as such, to enable adjustment to counteract excessive and inappropriate wound progression. The same wound scheme consisting of four different treatment strategies applies to all other areas in the same way.

However, whereas area 1 was induced on day 1, areas 2 to 4 were placed in a time delayed manner. This was done to be able to get wounds in different progression states when harvesting biopsies on day 15. All of the wounds were treated once daily for five consecutive days after wounding. Observation of the wounds was conducted from day 1 and was continued for every test subject and every wound for 15 days in total. The corresponding timeline to the above shown setup can be seen in Figure 8.

Light green squares indicate days which required sedation. Thus the latter are equal to days of punch wound induction (d1, 5, 8 and 10). Euthanasia was introduced after a last sedation on day 15. Yellow squares indicate wound biopsy collection on day 15.

8.3. Compositions of solutions
I. **R-848 variable**

<table>
<thead>
<tr>
<th>Component</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>PG</td>
<td>50%</td>
</tr>
<tr>
<td>dH2O</td>
<td>47,499% - 47,490%</td>
</tr>
<tr>
<td>TWEEN 80</td>
<td>0.5%</td>
</tr>
<tr>
<td>HPMC</td>
<td>2.0%</td>
</tr>
<tr>
<td>R848</td>
<td>0.1 - 0.01%</td>
</tr>
</tbody>
</table>

II. **R-848 constant**

<table>
<thead>
<tr>
<th>Component</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>PG</td>
<td>50%</td>
</tr>
<tr>
<td>dH2O</td>
<td>47,495%</td>
</tr>
<tr>
<td>TWEEN 80</td>
<td>0.5%</td>
</tr>
<tr>
<td>HPMC</td>
<td>2.0%</td>
</tr>
<tr>
<td>R848</td>
<td>0.05%</td>
</tr>
</tbody>
</table>

III. **Vehicle**

<table>
<thead>
<tr>
<th>Component</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>PG</td>
<td>50%</td>
</tr>
<tr>
<td>dH2O</td>
<td>47.5%</td>
</tr>
<tr>
<td>TWEEN 80</td>
<td>0.5%</td>
</tr>
<tr>
<td>HPMC</td>
<td>2.0%</td>
</tr>
</tbody>
</table>
IV. Human Serum

98 % human Serum
2 % HPMC

8.4. Shaving

Pigs were shaved with a surgical clipper on each side of the back. A rectangle of 10 x 30 cm is sufficient for wound placement.

8.5. Wound induction

Pig skin was cleaned and disinfected with Skinsept® F (Ecolab Healthcare) prior to wounding. Anesthesia of the test subject was induced by 3-6 mg/kg body weight Zolazepam (Virbac®). 6mm biopsy punches (Henry Schein Medical GmbH) were used for the induction of full-thickness wounds. Punched skin was carefully pulled out by the help of forceps and excized. Bleeding was stopped by gently wiping the wound bed with a sterile cotton swab (Heinz Herenz Medizinalbedarf) coated with 0.01% epinephrine diluted in NaCl (Sigma-Aldrich). Thereafter, wounds were rinsed with NaCl to generate a clean wound as an appropriate and equal starting point for treatment.

1ml syringes (B.Braun) were used in order to transfer one drop of the respective substance into each wound with a volume of about 50µl. Wound edges were wiped in order to avoid transfer of the respective substance into another wound.

8.6. Dressing

To ensure proper coverage of every wound pool consisting of four wounds each, a quarter of a Mepilex® Lite foam dressing 10x10 cm (Mölnlycke Health Care) was used. All of the patches were covered with 3M™ Tegaderm™ Transparent Film Dressing 10x11.5cm sparingly in order to assure that no patch can come loose and treatment is not transferred to other wounds. Dog&Cat bodies (Henry Schein Vet GmbH) were used as additional precaution. For postsurgical pain control a fentanyl patch 50µg/h was applied.

8.7. Scoring of Wounds
Scoring took place starting from day 2 since no reaction can be noticed directly after the first treatment. Bandages were taken off beforehand and all of the parameters concerning the wound appearance were gathered on the respective source data form.

8.8. Euthanasia

Before specimen collection, test subjects were sedated and sacrificed via intravenous administration of Pentobarbital-Natrium (Exagon®) by a veterinarian.

8.9. Biopsy

Wounds were excised in squares using scalpel blades (Henry Schein Dental). All of the affected tissue around the wound was collected. The skin was transferred into pre-filled histology containers with 4 % formalin (Thermo Fisher Scientific).

8.10. Hematoxylin & Eosin Staining

H&E staining was conducted by the institute of pathology at the Medical University Graz via a standard regimen.
8.11. Wound Assessment via SCARLETRED®VISION

Additional to optical scoring of the wound parameters, wounds were also assessed by SCARLETRED®VISION. Measurement of wound- and erythema size was conducted as depicted in Figure 9. Further, the intensity of the redness was evaluated via objective skin imaging algorithms.47

![Figure 9 Measurement of wound- and erythema size by SCARLETRED®VISION. Area or redness of the wound bed is subtracted from the outer necrotic edge or inflamed area, respectively.](image-url)
9. Results

Until today, chronic wounds represent a public health problem affecting more than 40 million people worldwide. Therefore, the need for a better understanding of the imbalances causing improper wound progression is greater than it has ever been. An estimation suggests that about 1 to 2% of the population will experience some form of a chronic wound during their lifetime which makes a personalized model for the selection of individual therapy very attractive. This is reinforced by the fact that no gold standard has been discovered yet which is potent of treating all types of chronic wounds equally well. Nowadays, wound assessment, holistic management and direct wound management seem to be the only options after classifying a wound failing to heal. Hence, a classification system was the primary objective of this study which should apply to all wounds and predict optimal treatment. Subsequently, the goal was the establishment of a porcine model of persistent inflammation in chronic wounds which shall not only give a better insight into the pathophysiology of these ulcers but also serve as template to find the best therapy for every individual patient.

9.1. Establishment of a Scoring Matrix

The establishment of a standardized scoring matrix which applies to every wound appearance was derived from the parameters included in the Novartis and the DESIGN-R scoring tool. Since it was not the interest of the study to score for pressure ulcers or the healing process in particular, to state some examples, but to classify the wound stage as exact as possible, parameters of these scoring systems were combined and modified in order to find the best way to categorize each wound. DESIGN-R includes parameters in the score such as depth, exudate, size, inflammation/infection, granulation, necrotic tissue and the pocket of a wound. Due to this reason, it seemed to represent a potent instruction to get a good overview of the wound status. Additionally, single parameters are weighed to result in higher scores according to their severity. Different wounds thus may have the same score despite showing different appearances. However, it was assumed that the outcome might still be too superficial, therefore the tool was refined in collaboration with Novartis before project commencement.

Table 1 shows the development of the final scoring matrix from draft to final version.
Table 1 Development of a scoring matrix. Comparison between included parameters prior to study commencement and the parameters included after termination.

<table>
<thead>
<tr>
<th>Parameters Before Study Conduct</th>
<th>Parameters After Study Conduct</th>
</tr>
</thead>
<tbody>
<tr>
<td>Open/moist</td>
<td>Dry/moist</td>
</tr>
<tr>
<td>Closed/dry</td>
<td>Size</td>
</tr>
<tr>
<td>Crust</td>
<td>Wound content</td>
</tr>
<tr>
<td>Wound content</td>
<td>Pus</td>
</tr>
<tr>
<td>Pus</td>
<td>Crust</td>
</tr>
<tr>
<td>Erythema</td>
<td>Erythema</td>
</tr>
<tr>
<td>Erythema &gt;5mm</td>
<td>Erythema Width ≥5mm</td>
</tr>
<tr>
<td>Swelling</td>
<td>Swelling</td>
</tr>
<tr>
<td></td>
<td>Necrosis</td>
</tr>
<tr>
<td></td>
<td>Maximal sum 20</td>
</tr>
<tr>
<td></td>
<td>Maximal Inflammation Score 8</td>
</tr>
<tr>
<td></td>
<td>Maximal Area Score 13</td>
</tr>
</tbody>
</table>

New parameters and extensions were incorporated in the new system since a better classification of the wound status was predicted. Inclusion of the size of the wound was necessary since it can correlate with the severity or healing of the wound. Further, wound contraction can be an indicator for healing whereas the extension of a wound is a sign for worsening wounds.\textsuperscript{50} The same applies for the development of necrotic wound edges as an indicator for cell death, thus also having an impact on the size of the wound. Crusting and thus healing can take place either starting from the wound edges or the necrotic edge if present. For each scenario, a new score applies which is explained in the following sections.
Consequently, a new maximum score emerged, which was split up into an inflammation score as well as an area score, to give further information on wound progression. In Table 2 all new scoring parameters are listed together with the corresponding score.

**Table 2 All wound parameters contained in the scoring matrix applicable for the classification of persistent inflammation in chronic wounds.**

<table>
<thead>
<tr>
<th>Criterium</th>
<th>Explanation</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Wound</strong></td>
<td>Dry</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Moist</td>
<td>1</td>
</tr>
<tr>
<td><strong>Size</strong></td>
<td>≤2mm</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>≤5mm</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>6mm</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>≥8mm</td>
<td>3</td>
</tr>
<tr>
<td><strong>Wound content</strong></td>
<td>Full</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Half</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Empty</td>
<td>2</td>
</tr>
<tr>
<td><strong>Pus</strong></td>
<td>Not present</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Present</td>
<td>1</td>
</tr>
<tr>
<td><strong>Crust</strong></td>
<td>Fallen off</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Coating wound</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Extended</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>3</td>
</tr>
<tr>
<td><strong>Erythema</strong></td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Intense</td>
<td>4</td>
</tr>
<tr>
<td><strong>Erythema width</strong></td>
<td>&lt;5mm</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>≥5mm</td>
<td>1</td>
</tr>
<tr>
<td><strong>Swelling</strong></td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Intense</td>
<td>2</td>
</tr>
<tr>
<td><strong>Necrosis</strong></td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Present</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Extended 2-3mm</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Extended ≥3mm</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Eschar</td>
<td>4</td>
</tr>
</tbody>
</table>

As an example, the score for a freshly induced 6mm punch wound is shown in Table 3. With a diameter of 6 mm, being empty and moist when freshly induced, this appearance
is the starting point within the project. With a score of 9, every wound was prepared for treatment.

Table 3 Starting point for freshly induced 6mm biopsy punch wounds.

<table>
<thead>
<tr>
<th>Criterium</th>
<th>Explanation</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wound</td>
<td>Moist</td>
<td>1</td>
</tr>
<tr>
<td>Size</td>
<td>6mm</td>
<td>2</td>
</tr>
<tr>
<td>Wound content</td>
<td>Empty</td>
<td>3</td>
</tr>
<tr>
<td>Pus</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>Crust</td>
<td>None</td>
<td>3</td>
</tr>
<tr>
<td>Erythema</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>Erythema width</td>
<td>≥5mm</td>
<td>0</td>
</tr>
<tr>
<td>Swelling</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>Necrosis</td>
<td>Eschar</td>
<td>0</td>
</tr>
</tbody>
</table>

**Sum 9**

In general, the lower the wound score (towards or zero itself) the less severe the appearance of the wound. Compared to this, a high value with a maximum score of 20 indicates a very severe wound. Yet, one must bear in mind that the total score cannot exceed a value of 20 due to the reason that a wound cannot be classified empty in presence of pus. The explanation for this can be seen in Table 4 showing the maximum achievable score of a wound.
### Table 4 Maximum score arising from the scoring matrix applicable for the classification of persistent inflammation in chronic wounds.

<table>
<thead>
<tr>
<th>Criterium</th>
<th>Explanation</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wound Moist</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Size ≥8mm</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Wound content Half</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Pus Present</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Crust None</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Erythema Intense</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>Erythema width ≥5mm</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Swelling Intense</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Necrosis Eschar</td>
<td></td>
<td>4</td>
</tr>
</tbody>
</table>

**Sum 20**

However, as previously mentioned, the total score is too general for explaining the inflammation state of a wound. Hence, the maximum inflammation score as well as the maximum area score of 13 and 8 respectively, are listed in Table 5 and Table 6.

### Table 5 Maximum area score arising from the scoring matrix applicable for the classification of persistent inflammation in chronic wounds.

<table>
<thead>
<tr>
<th>Criterium</th>
<th>Explanation</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wound Moist</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Size ≥8mm</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Wound content Empty</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Crust None</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Necrosis Extended</td>
<td>+Eschar</td>
<td>4</td>
</tr>
</tbody>
</table>

**Sum 13**
Table 6 Maximum Inflammation score arising from the scoring matrix applicable for the classification of persistent inflammation in chronic wounds.

<table>
<thead>
<tr>
<th>Criterium</th>
<th>Explanation</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pus</td>
<td>Present</td>
<td>1</td>
</tr>
<tr>
<td>Erythema</td>
<td>Intense</td>
<td>4</td>
</tr>
<tr>
<td>Erythema width</td>
<td>≥5mm</td>
<td>1</td>
</tr>
<tr>
<td>Swelling</td>
<td>Intense</td>
<td>2</td>
</tr>
<tr>
<td>Sum 8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

For visualization of score/wound relationships, many different wound appearances are shown in sections 9.4. and 9.5. However, no pictures representing maximum scores can be shown, since hyperstimulation was avoided to prevent inappropriate wound progression.

9.2. Optical Scoring of Wounds

The general setup as well as scoring was conducted in collaboration with Novartis. This was done in respect to their previously published study.\(^{46}\) It includes fastening of the test subject in a specially designed hammock, placing biopsy punch wounds in the correct area and in the appropriate distance from each other, dressing as well as scoring of the wound status.

A major issue throughout the project was, that visual assessment of parameters of a wound is very subjective and may even fluctuate from day to day. Most of the variations arise from delusions, since the eye might associate a broader halo with a greater intensity of redness at the same time as an example. Consequently, one may overrate the total score of the wound. Also, light conditions and/or direct lighting can influence color and thus appearance of the wound. Therefore, wounds were photographed and scores were taken down daily.
9.2.1. Size

Since the size can give a very good indication on whether a wound is declining or healing, Figure 10 shows pictures of possible applicable scores. A score of zero (A) represents a strongly contracted and thus almost healed wound at a diameter of around or below 2 mm. A score of 1 (B) indicates a wound which has entered the healing phase and possesses a diameter of about 4 mm. Additionally, this is supported by the fact that the wound has dried out and starts to crust. Wound C shows a diameter of 6 mm and is still moist which represents the starting point within the experiment. A score of 9 is assigned as mentioned before. A wound with a size score of 3 (D) is around or exceeding 8 mm in diameter and thus can be considered worsening.

Comparison of all the pictures in Figure 10, picture D is the only wound which showed a aggravating appearance. Whereas wounds in picture A and B already dried out and their diameter diminished, wound D showed necrotic wound edges and an increase in size. For wounds with an appearance like in picture C, one can not yet say whether they will enter the healing phase or decline over the next days when looking at them out of context. This is dependant on the respective treatment.
9.2.2. Necrosis

Different types of necrosis are shown below in Figure 11, ranging from zero to three from left to right. Wound (A) obviously did possess a necrotic edge before the healing process started, however it was covered by a crust. Therefore, the necrosis is not scored anymore (score zero). Picture (B) does not show a necrotic wound edge and was scored with a zero as well. Pictures (C) and (D) both were assigned with a score of 1 due to a small necrotic edge in picture (C) and a dark, eschar-like coloration in picture (D). From pictures (E) to (F) the scores increase up to a score of two and three, respectively. A score of four which applies for a necrotic edge in combination with eschar without being covered by a crust was not observed throughout this project.

![Figure 11 Necrosis Score. Score zero for a wound of which the necrotic edge is already covered by a crust (A) as well as when no necrotic edge is observed at all (B). Scores from 1 to 4 are shown in pictures C to F.](image-url)
9.2.3. Crust

Pictures corresponding to crust formation can be seen in Figure 12, ranging from zero to three from left to right. They account for a crust which has already fallen off (A) with a score of zero, to a coverage of either only the wound or the necrotic edge (B, C) with a score of 1. An extended crust covering wound area as well as the necrotic edge (D) is assigned with a score of 2 and an open wound without a crust (E) is assigned with a score of 3.

For scoring of the crust, it is especially important to keep the wound size in mind. As an example, it is very hard to distinguish wounds B and D on photos. Via optical scoring their status can only be measured via the size and diameter. Since the wound in picture B did not possess a necrotic edge, its diameter remained in the range of 6mm and the crust is only covering the wound bed. Therefore, this wound is scored with 1. A necrotic edge, which was present before crusting in picture D, results in an extension of the wound. Thus, the crust did not only cover the wound bed but also a necrotic halo. This results in a score of 2 for the wound.

Additionally, it was observed that the parameters Size, Necrosis and Crusting are highly interlinked.
9.2.4. Erythema

Figure 13 shows each of the possible intensities of redness from a score of zero to an intensity of four from left to right. An additional score of 1 is given for a red halo which is greater than 5mm (E).

![Figure 13 Erythema score. Representative wounds to ascending scores from zero (A) to 4 (D) as well as a score of +1 (E) for an erythema width greater than 5mm in diameter.](image)

Scoring of the redness of a wound is especially hard due to very small differences between single wounds. Thus, the score was not only evaluated in the group but additionally via the SCARLETRED®VISION program. This innovative technology was invented to counteract subjective skin assessment and the lack of standardization in dermatology.\textsuperscript{51} In this setup it was used to provide exact measurements of the wound size and redness.

The results for the measurement of the redness can be seen in Figure 14. Due to the reason that different computational languages were used for building both graphs, the results seem to differ from each other. However, rather equal wound progression was measured by both methods. As already described in section 8.11., the redness within the punch wound was subtracted from the redness of the outer halo. Though, since some wounds did not possess a red halo at the beginning of the experiment, the value dropped below zero. Still, the results in picture 1 and 2 are very similar. Negative controls show almost identical lines in picture 1 and 2 with a peak on day 5. A small deviation within the vehicle probe in picture 2 was evened out by the program, resulting in a slightly lower curve.
Also, the R-848 probes show very similar progression in picture 1 and picture 2. Redness reaches a maximum around day 8 and decreases rather fast towards day 10. The high standard deviation of the R-848v treated wounds on day 7 was evened out by ScarletRed indicating a slight inconsistency in optical scoring on the respective day. However, one can state that the program can confirm the accuracy of optical scoring which hence is proven to be successfully designed.

9.3. Swelling

Assessment of a swollen area represents a very difficult process due to being heavily dependent on touch as well as high technical skill. Many different types of swollen areas
were observed throughout the study. Wounds can exhibit an equally swollen perimeter whereas others may be swollen on one side only. Some wounds possess thickened wound edges whereas others are elevated with a width of up to one centimeter. However, due to the reason that this parameter cannot be detected and evaluated on footage, no pictures are shown in this section.

9.4. Wound Progression

16 full-thickness wounds with a diameter of 6mm were placed on the back of each of the four test subjects. Additionally, wounds were induced at different time points to generate wounds in different healing states for biopsy. On days 1, 5, 8 and 10, four wounds were induced, respectively. Each including one wound for treatment with the vehicle PG, human serum, R-848v and R-848c probes. After a monitoring time of 15 days in total, biopsies from wounds in different healing stages were collected. Figure 15 depicts an acute and thus normal healing process. The wound was treated with a negative control (vehicle) which did not cause an immune reaction. Pictures are shown starting from day 2, since day 1 represents a freshly induced wound which does not show a certain reaction yet.

![Figure 15 Timeline showing a representative progression of a PG treated wound which was induced on day 1 and observed for 15 consecutive days.](image)
As one can see, after biopsy punch wound induction the wound edges start to contract towards day 6. Already on day 8 a crust can be observed which is covering the entire wound area. The transition of the above wound, treated with PG, can also be depicted graphically in terms of the score. Figure 16 shows the respective curve starting at a score of 9. After a short drop in the score, a slope was detected due to redness on day 5. However, thereafter the wound declined back to a dry appearance on day 7.

Also, a mean value for wounds treated with either PG or human serum was calculated including all test subjects. Figure 17 shows the comparison of both negative controls. While the wounds treated with human serum immediately decreased back towards a score of zero, the mean score of the vehicle probe showed a minimal slope resulting in a plateau between day 4 and 5. This may be caused by a light immune reaction caused by PG. However, both vehicle and human serum treated wounds showed a fast healing process and thus no chronic wound status was achieved.

Figure 16 Wound Progression - Normal/Acute Healing. Representative wound progression of a PG treated wound which was observed for 15 consecutive days.
Yet, further analyses were giving the impression that a systemic immune reaction might have advanced. Towards Area 4 and a later stage of the experiment, an increased inflammation score was observed which can be seen in Figure 18.

Figure 17 Comparison between progression of wounds administered with PG as vehicle or a human serum probe, respectively.
Whilst curves with almost similar progression in score were expected, the score of the serum treated wounds, was elevated in area 4 throughout all subjects. Directly followed by elevated levels within area 2 instead of area 3, inconsistencies were observed analyzing the respective graph. Compared to area 1, the values of area 4 were found to be substantially higher than the ones for area 4.

Figure 18 Mean scores of serum treated wounds of all test subjects calculated for Areas 1 to 4.
In comparison to acute/normal wound progression, wounds treated with R-848 can be seen in Figure 19. These wounds develop a very severe appearance within only a few days.

![Figure 19 Timeline showing a representative progression of an R-848v treated wound which was induced on day 1 and observed for 15 consecutive days.](image)

In comparison to Figure 16, Figure 20 shows a representative wound progression curve treated with R-848v. This wound reaches a maximum severity on day 5 which is maintained until day 7. Despite a small drop, the plateau remains almost constant until day 9. Healing did rapidly take place after that day 9 which means that after termination of R-848v a wound can be maintained for about 4 days.

Also, conclusions about the impact of different R-848 concentrations were drawn from the results. Since it was expected that a topically applied dose of 0.1% R-848 would result in a very strong wound progression, a variable dose regimen was planned to
counteract any inappropriate wound progression. Hence, wounds treated with a constant dose of 0.05% R-848 were induced additionally to compare it to the variable dose starting at 0.1%. Interestingly, no difference between the impact of 0.1% and 0.05% R-848 was observed. This was due to the reason that the R-848v dose was adjusted to show an even wound progression. In turn, wound progression was very similar to the reaction of the wounds treated with R-848c. Both curves can be seen in Figure 21. The appearance of R-848v and R-848c treated wounds developed in a parallel manner with a minimally higher score for the R-848v treated wounds. Yet, R-848v and R-848c were both capable of maintaining a chronic wound state for four days after termination of application with R-848.

Figure 21 Comparison between progression of wounds administered with a variable (0.1-0.01%) or constant dose (0.05%) of R-848, respectively.
In conclusion, all of the above-mentioned results are combined in Figure 22 which perfectly shows the difference in score between the wounds which were guided towards a chronic wound state (R-848v and R-848c) whereas the negative controls (PG and human serum) show a rather acute wound behavior healing rapidly after control treatment was discontinued.

As previously described, R-848 treated wounds showed a much higher slope than control treated ones. Via topical treatment of the immune modulator R-848, a greater immune reaction can be caused in wounds compared to the negative controls. Generally, one can say that the severe wounds which developed within the model were delayed in wound healing. Comparing chronic and acute wound curves in Figure 22, one can see that both curves show the same healing behavior. The only differences are the intensity of immune reaction at the beginning of the study and thus a later initiation of the healing phase within R-848 treated wounds. Thereby, a short plateau of four days was maintained in these wounds. This was also demonstrated by the representative delayed/chronic wound progression curve shown in Figure 20.

9.5. Wound Appearance
To show the appearance of wounds matching the mean scores presented in Figure 22, Figure 24 shows representative pictures at days 6, 8, 11 as well as day 15. The parameters observed in each wound are listed in the following sections.

<table>
<thead>
<tr>
<th>Day 6</th>
<th>Day 8</th>
<th>Day 11</th>
<th>Day 15</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-848v</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
<td>R-848c</td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
<td><img src="image7.png" alt="Image" /></td>
</tr>
<tr>
<td>PG</td>
<td><img src="image9.png" alt="Image" /></td>
<td><img src="image10.png" alt="Image" /></td>
<td><img src="image11.png" alt="Image" /></td>
</tr>
<tr>
<td>hSerum</td>
<td><img src="image13.png" alt="Image" /></td>
<td><img src="image14.png" alt="Image" /></td>
<td><img src="image15.png" alt="Image" /></td>
</tr>
</tbody>
</table>

*Figure 23: Representative wound progression according to mean scores shown in a healing timeline after termination of R-848 application (day 5).*
9.5.1.1. Wounds treated with variable R-848 (0.1%-0.01%)

- Day 6: Necrosis, increase in size
  Erythema
  Swelling
  Pus, moist half empty wound bed
- Day 8: Necrosis, increase in size
  Erythema
  Swelling
  Pus, moist, half empty wound bed
- Day 11: Increase in size
  Crust
- Day 15: Increase in size
  Crust

9.5.1.2. Wounds treated with constant R-848 (0.05%)

- Day 6: Necrosis, increase in size
  Erythema
  Swelling
  Moist, half empty wound bed
- Day 8: Crust, increase in size
  Erythema
  Dry, half empty wound bed
- Day 11: Crust, increase in size
  Dry, full wound bed
• Day 15: Crust, increase in size
  Dry, full wound bed

9.5.1.3. Wounds treated with the vehicle PG

• Day 6: Necrosis, increase in size
  Erythema
  Dry, half empty wound bed

• Day 8: Crust
  Dry, full wound bed

• Day 11: Crust
  Dry, full wound bed

• Day 15: Crust
  Dry, full wound bed

9.5.1.4. Wounds treated with the human Serum

• Day 6: Dry, half empty wound bed
  Erythema

• Day 8: Crust
  Dry, full wound bed

• Day 11: Crust
  Dry, full wound bed

• Day 15: Crust fallen off
Dry, full wound bed

Healing was initiated in a fast manner for wounds treated with PG and human serum after termination of application on day 5. Whereas wounds started to dry out on day 6 and granulation tissue was found, on day 8 both wounds were fully covered by a crust. Towards day 15, the serum treated wound even lost its crust which indicated a successful healing process. Even the potential of PG to irritate open wounds to some extent was proven to be no problem in this setup. Wound progression looks almost identical to the human serum treated wounds which indicates that the results of the R-848 probes are not manipulated by mixing them with PG.

In comparison, R-848 treated wounds showed a very severe inflammatory response on day 6. Also on day 8 pus, reddening and a strong swelling was found in the R-848v treated wound, whereas the R-848c wound was already dried out. Yet, a rather strong reddening of the area half-covered by a crust was detected. In these wounds, a chronic plateau was maintained for three (R-848v) and two days (R-848c), respectively. They represent wounds with the closest scores to the mean scores of all wounds treated with either substance such as shown in Figure 20. On days 11 as well as day 15, both wounds showed a crusted appearance of the wound bed as well as previously necrotic edges, indicating that healing was in process.
In comparison, Figure 24 shows the respective cross sections of to Figure 24. Via a hematoxylin and eosin (H&E) staining the wound healing process can be assessed accurately, additionally to the wounds’ optical appearance.

Many different stages of wounds were found as a result of a successful time-delayed setup. Deep, ulcerated wound beds were found in comparison to already healed ones. As an example for a rather severe case of inflammation Figure 25 is shown. In this picture, a deep wound bed as well as deep ulceration can be found, marked by a
roundish violet coloration. Further, granulation tissue formation can be observed at the necrotic edges of the wound.

Figure 25 H&E stain: Open wound with deep ulceration. Treatment: R-848v day 8.

Compared to Figure 25 showing an ongoing and severe inflammation, Figure 26 showed a healing wound marked by reepithelization and granulation tissue formation. This is marked by a clear dark violet layer covering the healed wound bed. It was found that the epithelialization process is impaired in all types of chronic wounds. Additionally, a wound cannot be considered healed in the absence of re-epithelialization. Yet, this particular wound treated with human serum, was able to reach remodeling phase until project end.
According to Figure 24, a very representative healing process was also observed from wounds treated with PG between day 6 and 8. Due to the reason that the pictures result from the same test subject, one can see that healing is initiated from the wound edges until full closure via keratinocyte migration is reached.55

All in all, one can say that healing was delayed in the R-848 treated wounds compared to the wounds treated with PG and human serum. Whereas the latter showed strong reepithelization on day 8 already, the R-848 treated wounds showed an excess of granulation tissue. The R-848c treated wound even showed that the wound bed was still half empty on day 11 whereas the R-848v treated wound was fully filled. This is because the wounds were representatively selected to match the mean scores in Figure 22. Same applies for the difference found between the PG treated wounds on day 11 and 15. Although looking seemingly identical in Figure 23, the histological samples have proven the difference. On the picture representing the mean score for day 11, the reepithelization process has formed an even skin layer covering the wound.

In contrast, the picture representing day 15 showed that granulation tissue was still covering the closed wound. The difference results from the healing process, which has progressed differently for pig 4 (day 11) and pig 3 (day 15).
A very interesting picture was observed for the serum wound in the picture on day 8. Whereas a new skin layer was already built to cover the wound under a crust, the wound was still hollow on the inside. This indicates that the healing process was not yet fully completed. However, since the wound was covered and has already dried out on the outside by that time, one can say that the score was not falsified by this appearance.

Concludingly, a successful reepithelization process was observed in all representative wounds on day 15. This states that, R-848v and R-848c treated wounds as well as PG and human serum treated ones did heal within 10 days of observation. Yet, the results for R-848 again show that the experimental setup must be modified to generate a longer chronic plateau.
10. Discussion

Since the etiology of chronic wounds is enormously diverse, some chronic wounds fail to heal within an appropriate time frame despite the best care. These critical wounds represent a public health problem affecting more than 40 million people worldwide. Hence, a porcine model of persistent inflammation in chronic wounds was in the focus of this study. Despite many different in-vivo models have been established until today, the difference in wound characteristics can still prevent successful therapy. Therefore, this new wound model to mimic chronic progression features could not only give new insights into the pathophysiology of chronic wounds but also enable direct testing of new therapies. Following up previous studies, it was derived from and in collaboration with Novartis.

Moreover, unlike previously designed scoring matrices, a new tool was developed which seemed to be potent to classify each type of chronic wound in the same way. Whereas others like the PUSH tool can only be used to assess pressure ulcers in particular, this modified scoring matrix applies to every wound. Parameters of both, the Novartis- and the DESIGN-R scoring tool, were combined as well as weighed according to their relevance concerning inflammation. To mention some, these include the size of the wound, redness, swelling and many more.

Additionally, due to a special setup, wounds in different healing stages were generated towards project termination on day 15, via a time-delayed wound induction. Four wound pools consisting of four wounds, respectively resulted in wound biopsies on day 6, 8, 11, and 15. From the induction dates, wounds where applied with the respective treatment for five consecutive days and scored from day 2 until day 15. Since on day 1 no inflammation was visible, scoring was omitted on days where wound induction took place.

For the establishment of a chronic wound status, the potential of R-848 was investigated. This molecule is a TLR7/8 agonist of which the respective receptor is expressed on macrophages and neutrophils. These types of immune cells account for the major proportion of the infiltrate in human chronic wounds. Further, already existing results have shown, that R-848 can cause non-infectious inflammation which can result in a delay in healing in mice and man.
These results were reproduced in porcine skin via the topical application of R-848. Compared to the impact of PG and human serum, remarkable potency to induce a delayed, chronic-like wound status was found. Whereas PG served as the vehicle to dissolve the pulverulent R-848, human serum was used as part of a side study (data not shown). However, additionally it served as a control and for comparison with PG in this study.

A variable dose regimen regarding R-848 treatment was utilized to avoid inappropriate wound progression. Yet both, a variable (0.1-0.01%) as well as a constant concentration of 0.05% R-848 resulted in similar score curves. Both concentrations induced an immune response which could be maintained up to four days after termination of application (day 5). However, after this plateau, both R-848v and R-848c treated wounds entered a healing phase. This indicated that topically applied R-848 for five days is not potent to induce a longer chronic wound state than four days. Although, a chronic phase could be maintained for this time frame, a longer period was of interest within this study. This is due to the reason that the model shall serve for the investigation of new therapies to cure chronic wounds. To extend this phase, an idea might be causing a faster and/or greater reaction, possibly by applying R-848 for more than 5 days. The resulting score curve is expected to have a much sharper slope due to a more intense inflammatory response within the wound. After a rather steep slope until a chronic plateau is reached, healing should not set in within 5 days after termination of application.

In general, the reproduced results via the use of R-848 were inconsistent with the results of previous studies, which stated the presence of inflammatory signs for at least five days after the last R-848 application. These slight variations might be the result of different classification systems. It will be in the focus of upcoming studies to clarify the origin of these differences in cooperation with Novartis.

Compared to the results of R-848, PG resulted a very similar wound appearance like the ones which were treated with human serum. Additionally, it is possible to observe a very fast transition from hemostasis and inflammation into proliferative and remodeling phase. Hence, the experiment showed that the latter take much longer in the healing process than inflammation.

Still, the invasiveness of PG when topically applied can be disproven within this study. It can be said that the results generated via topical application of R-848 were not
manipulated by the addition of PG. Yet, the results for the applied human serum seemed to indicate that a systemic immune reaction might have advanced.

Towards a later stage of the experiment meaning the curve for area 4, elevated immune parameters were found. Since this wound pool was not located next to especially invasive substances varying from the normal setup, a manipulation from other wounds can be precluded. A possible explanation could be physical as well as psychological stress of the test subjects towards project end. Interestingly however was, that the second highest curve was not the one depicting the score for area 3 but the score for area 2 representing an unexpected pattern. Nevertheless, one can say that these results are not significant in difference throughout all areas. Thus, the variance might be caused by a slight inconsistency in scoring, which is accentuated by a high standard deviation for area 4 in particular. Therefore, it is most likely that this figure is a parade example for the fluctuations within the frame of this project. Nevertheless, the mean scores did not display these deviations. As a precaution for upcoming experiments, blood samples could be drawn at regular intervals to analyze immune parameters of the test subjects.

Concludingly, one can say that the target-aimed setup of this project resulted in wounds within different healing stages from inflammation to remodeling phase. Optical evaluation, pictures, SCARLETRED®VISION analysis as well as H&E stains gave a great insight into the healing process of each wound, which allowed a detailed assessment of their status. A clinically induced chronic plateau was maintained for about four days after termination of application with R-848. Nevertheless, since testing of new therapies was one objective of this model, a period of four days seems too short. Thus, the question emerges whether the chronic plateau can be prolonged. In order to reach this goal, a longer administration period of R-848 may be potent to extend a chronic plateau of four days after termination of application. To do so, the concentration of the applied R-848 can be restricted to a constant dose of 0.05% which has turned out to be effective in causing a delay in wound healing.
11. **Outlook**

This study represents the first steps within the establishment of the first clinical chronic wound model. So far, the results indicated that the model was potent of reaching and maintaining a chronic wound state for about four days only. Further investigation was instigated to be able to extend this plateau in the future. This is due to the reason that a chronic period of an appropriate duration is essential to test for the potential of new treatments.

Since the topical application of 0.05% R-848 over five consecutive days only showed a short chronic plateau in the total- and in the inflammatory wound score, a pilot study was initiated to clarify whether an extension of the topical administration of R-848 is potent to induce a longer chronic wound status. Still, this method does not seem perfectly reproducible because some of the applied R-848 can be lost due to movement of the test subject during treatment. Thus, less substance may be present in the wound or even a higher concentration after reapplication. This can cause undesired inconsistencies between single wounds treated with the same substance and dose respectively. Further, a method which is potent to induce a chronic wound state after one treatment only, would be beneficial. This could reduce physical as well as psychological stress for the test subject to a minimum. Therefore, a new setup was established to counteract these problems. One pilot animal was requested to compare the known application method to a prolonged treatment of ten days and a newly designed method using wound injection.

Again, four wound pools per test subject consisting of four wounds each will be induced. One area treated topically with a constant dose of 0.05% R-848 for five days will be compared to an application period of 10 days. Additionally, two pools were planned, testing for the potency of the intralesional injection of 0.05% R-848. Four injections per wound will be placed in equal distances from each other within the wound edge. Not only a loss but also the transfer of substance within different wounds may be prevented. Further, by that a more direct and stronger immune reaction will be caused which may be sufficient to restrict application and thus sedation to one day only while still leading to an extended chronic plateau. A scheme of the planned setup can also be seen in Figure 27.
Figure 27 Adjusted setup for the establishment of a porcine model of persistent inflammation in chronic wounds comparing topical treatment of 5 and 10 days (A, B) to injections (C, D). The last sedation is followed by the introduction of euthanasia.
12. References


Molecular Biotechnology

Nina Bucher
1510544019


13. Acknowledgement

I kindly want to thank Univ.Prof. Dr Lars-Peter Kamolz, MSc for giving me the great opportunity to do research in the division of Plastic, Aesthetic and Reconstructive Surgery at the Regional & University Hospital in Graz.

I also want to give my thanks to DI Dr Birngruber who incorporated me into his research team at the HEALTH department at Joanneum Research and especially Dr Joanna Adamczak who represented a great supervisor.

Concluding, I am very glad that the University of Applied Sciences Campus Vienna gave me the opportunity to absolve another practical semester as part of my studies since I believe that these experiences are the key to success in my future career.
14. Statutory declaration

I hereby declare that the submitted Master thesis was written by myself and that I did not use any aids other than those indicated, none of which are unauthorized.

I assure that I have not previously submitted this Master thesis or its contents in any form for assessment as part of an examination either in Austria or abroad.

Furthermore, I assure that all copies submitted by myself (electronic and printed) are identical.

Date

Signature

19/09/2017

Nina Bucher
15. **Eidesstattliche Erklärung**

Ich erkläre, dass die vorliegende Masterarbeit von mir selbst verfasst wurde und ich keine anderen als die angeführten Behelfe verwendet bzw. mich auch sonst keiner unerlaubter Hilfe bedient habe.

Ich versichere, dass ich diese Masterarbeit bisher weder im In- noch im Ausland (einer Beurteilerin/einem Beurteiler zur Begutachtung) in irgendeiner Form als Prüfungsarbeit vorgelegt habe.

Weiters versichere ich, dass die von mir eingereichten Exemplare (ausgedruckt und elektronisch) identisch sind.

Datum: 19.09.2017

Unterschrift: Nina Bucher