Immunohistochemical Analysis of Vessel Transformation in Chronic Wounds using Topical Negative Pressure (TNP) Therapy
Gedruckt mit Erlaubnis der
Medizinischen Fakultät der Friedrich-Alexander-Universität
Erlangen-Nürnberg

Dekan: Prof. Dr. med. J. Schüttler
Referent: Prof. Dr. med. R.E. Horch
Korreferent: Prof. Dr. med. G. Schett

Tag der mündlichen Prüfung: 19.03.2012
Meinen Eltern
# Table of contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Abstract</strong></td>
<td>1</td>
</tr>
<tr>
<td><strong>1 Introduction</strong></td>
<td>5</td>
</tr>
<tr>
<td>1.1 Woundhealing as a civilisation problem</td>
<td>5</td>
</tr>
<tr>
<td>1.2 Phases of woundhealing</td>
<td>5</td>
</tr>
<tr>
<td>1.3 Topical negative pressure (TNP) Therapy</td>
<td>6</td>
</tr>
<tr>
<td>1.4 Hypoxia Induced Factor-1α</td>
<td>8</td>
</tr>
<tr>
<td><strong>2 Material and Methods</strong></td>
<td>9</td>
</tr>
<tr>
<td>2.1 Patients characteristic</td>
<td>9</td>
</tr>
<tr>
<td>2.2 Collecting samples</td>
<td>10</td>
</tr>
<tr>
<td>2.3 Experimental groups</td>
<td>12</td>
</tr>
<tr>
<td>2.4 Histopathology and Immunohistochemistry</td>
<td>14</td>
</tr>
<tr>
<td>2.4.1 Common information</td>
<td>14</td>
</tr>
<tr>
<td>2.4.2 CD31 Staining</td>
<td>14</td>
</tr>
<tr>
<td>2.4.3 CD34 Staining</td>
<td>15</td>
</tr>
<tr>
<td>2.4.4 H&amp;E Staining</td>
<td>15</td>
</tr>
<tr>
<td>2.4.5 HIF-1α Staining</td>
<td>15</td>
</tr>
<tr>
<td>2.5 Quantitative and qualitative analysis</td>
<td>16</td>
</tr>
<tr>
<td>2.6 Statistical analysis</td>
<td>16</td>
</tr>
<tr>
<td><strong>3 Results</strong></td>
<td>17</td>
</tr>
<tr>
<td>3.1 Common information</td>
<td>17</td>
</tr>
<tr>
<td>3.2 Immunohistochemistry</td>
<td>19</td>
</tr>
</tbody>
</table>
3.2.1 Vessel density: CD31, CD34 19
3.2.2 Hypoxia: HIF-1α 23
3.2.3 Inflammation: H&E 25

3.3 Patients examples of wound biopsies 27
3.3.1 Patient A -> CD34 27
3.3.2 Patient B -> HIF and H&E 32
   3.3.2.1 HIF-1α 32
   3.3.2.2 H&E 35

3.4 Overall analysis related to the time of treatment 40
3.4.1 Vessel density 40
3.4.2 Hypoxia 40
3.4.3 Inflammation 40
3.4.4 Correlation between vessel density and hypoxia 40
3.4.5 Correlation between vessel density and inflammation 41

4 Discussion 44
4.1 Study design 44
4.2 Patients acquiring 44
4.3 Problems of wound comparison 45
4.4 Vessel proliferation 45
4.5 Hypoxia 46
4.6 Inflammation 47
4.7 TNP and stages of physiological woundhealing 48
4.8 Histological effects of TNP 49

5 Resume 50
Abstract

Purpose: The intention of this prospective clinical study was to observe the effect of TNP on human chronic wounds to gain more profound knowledge about the physiological processes and wound behaviour in general. This prospective clinical study analysed the alteration of vessel density in chronic wounds before and after TNP. Furthermore, we hypothesized to prove a correlation among hypoxia, inflammation, transformation and proliferation of blood vessels related to the duration of treatment.

Methods: 16 tissue biopsies of 15 patients were taken both of the wound edge and the wound bed within different time frames. All patients signed a written informed consent. This particular study was approved by the Ethical Committee of the Friedrich-Alexander-University Erlangen-Nuremberg, Germany (protocol number 3343). All samples were stained with Haematoxylin and Eosin (H&E), Hypoxia Induced Factor-1α (HIF-1α) and endothelial cell marker (CD31, CD34) to follow an alteration of inflammation, hypoxia and vessel proliferation, respectively. Quantitative and qualitative analysis was performed with light microscopy.

Results: In this prospective clinical study TNP was applied to prepare chronic wounds for a following plastic-surgical reconstruction using free or pedicled flaps. 16 chronic wounds of 15 patients (7 female, 9 male) were observed. The patients median age was 57 (35-79) years. The first tissue sample was taken at day 0, the second sample between day two and twelve after TNP. Day zero also
resembled the pre-existing wound conditions before TNP. Patients were divided into three different groups, according to the physiological wound healing process and its constituent stages. Between day five through day eight of treatment a considerable increase of blood vessel density was observed, which reached a maximum of approximately 200% with respect to the vessel density prior to treatment. Even more a rising number of hypoxic and inflammatory cells was noticed in this particular time frame. A treatment time less than five days and more than eight days seems to be inefficient with respect to wound healing.

**Conclusions:**

TNP supports the angiogenesis and the transformation of chronic non-healing wounds towards a physiological wound healing process. The stimulating effect of vessel proliferation is resulting from an increase of hypoxia and inflammation within a treatment between five and eight days.
Zusammenfassung


Schlussfolgerung: TNP unterstützt die Angiogenese und die Transformation einer chronischen, nicht heilenden Wunde in Richtung eines physiologischen Wundheilungsprozesses. Der stimulierende Effekt auf die Gefäßproliferation resultiert aus einem Anstieg der Hypoxie und Inflammation bei einer Behandlungsdauer von 5 bis 8 Tagen.
1 Introduction

1.1 Wound healing as a civilisation problem

Wound healing remains an important issue in times of an ever-increasing number of civilisation diseases, such as diabetes and cardiovascular problems, cancer and demographic changes of a population. Impaired wound healing is a prevalent complication of multimorbid patients [1]. Different treatment options are in common to support physiological wound healing. Among others for instance surgical debridement, autolytic (Hydrogel) and antiseptic, topical negative pressure (TNP), also known as vacuum assisted closure (V.A.C.®, KCI, San Antonio, TX) therapy has become a well-known treatment and an established method in the clinical management of complex wounds [2-4]. Its modality has proven efficacy in treating a variety of wounds [5-8], chronic as well as acute wounds [8], like burns, vascular ulcers, tissue defects as traumas or malignancies and gunshots [3,5]. TNP leads to a decreased healing time and to a reduction of nursing costs [2,6,8]. Additionally, it has been mentioned to be beneficial in the treatment of diabetic wounds and for people with cardiovascular problems, since their time of recovery may be shorter with respect to the conventional therapies [2,9]. There are some clinical observations about the physiological effect of TNP, but fundamental questions about the mechanism of wound healing are not yet satisfactorily clarified and insights are only available from animal studies.

1.2 Phases of wound healing

The period of the different phases of wound healing varies and is dependend on the size of wound and the wound conditions. Major impairments lead to a chronic wound.

- Inflammatory phase:
The first phase of wound healing is the inflammatory phase. It takes place in the first three, four days. Just before it is initiated, coagulation and
hemostasis take place to stop the bleeding process. Debrisd and bacteria are removed and phagocyted. An important role play the monocites and macrophages. They release different signaling molecules \textit{i.e.} growth factors. Furthermore they phagocytise damaged tissue and bacteria. The attraction of macrophages is stimulated by hypoxia in the surrounding tissue. In this tissue of low oxygen enviroment macrophages produce factors, which induce and accelerate angiogenesis \cite{10}. Furthermore they activate other cells to reepithelialize the wound surface and lead to a granulation. At the end of the inflammatory phase inflammation declines and the amount of macrophages will be reduced. In this way the wound healing is transfered into the next phase of wound healing, the proliferative phase.

- Proliferative phase

The second phase of wound healing is the proliferative phase. It takes place between day 4 and 8. The main processes in this phase are the angiogenesis and the formation of tissue granulation. Both did not occur step by step but partially overlap in time. Angiogenesis again is necessary for the migration of fibroblasts and epidermal cells. Through the neovascularisation capillaries are generated to transport oxygen in the hypoxic tissue. This leads to a better perfusion and hypoxia is reduced. So the stimulating factor for macrophages and other growth factor-producing cells is degraded. They reduce the production of angiogenetic factors \cite{10}. No more needed blood vessel die by apoptosis.

- Remodeling phase

The third phase of wound healing is the remodeling phase. It takes place between 9 until 21 days after wounding. Sometimes it can last for years dependent on the wound size and its conditions. In this phase disorganized collagen fibers are rearrangend in longitudinal tension lines. The tensile strenght of the cicatricial tissue increases in the next months to reach approximately the strenght of normal not injured tissue.
1.3 Topical negative pressure (TNP) Therapy

TNP is used in the clinical routine since the 90’s. The first publications take place by Fleischmann et al. [11] and Morykwas and Argenta [5,12]. The application of TNP needs various elements, like a semiocclusive dressing, an open cell polyurethan foam and a tube for a subatmospheric pressure of -125 mmHg. Initially all wounds underwent a surgical sharp debridement in the operating room by a surgeon to diminish the necrotic tissue to improve the conditions for wound healing, before TNP therapy was initiated. The wound cavity was covered with an open cell foam and an adhesive drape. A tube was inserted into the foam and connected with a vacuum pump to apply a continuous subatmospheric pressure of -125 mmHg to create an airtight seal. The extracted fluid was collected in a canister, which was a part of a programmable and portable computer system (V.A.C®, KCI, San Antonio, TX) to keep the subatmospheric surrounding safe.

Schematic way of function [13]:

TNP provides different advantages in comparison to conventional wound therapy as saline gauze assembly or wet wound management [12,14]. It effects vascularity, blood flow, granulation tissue and stimulates cell proliferation [13]. Other developing techniques such as tissue engineering to regenerate defects are promising, but are not yet clinically available [15-21].
They may become additional tools for tissue repair in addition to TNP. Clinical observations show a reduction of edema, increase of granulation tissue and reduction of wound surfaces [13-14,22-24]. Angiogenesis and vasculogenesis are stimulated in animal studies under TNP, as it is described by other research groups [5,7,13,25-27].

**TNP device:**

![TNP device image](source: KCI International)

1.4 Hypoxia Induced Factor-1α

One of the important regulators of angiogenesis is Hypoxia Induced Factor-1α (HIF-1α), which is produced in endothelial cells. In ischaemic tissue an overexpression of HIF-1α occurs as a response of cellular or systemic hypoxia in all mammalian cells. The consequence is a modulation of metabolic key pathways to optimize oxygen utilization in hypoxia [28-30]. This leads to an activation of cascades of several growth factors, which are directly induced by HIF-1α [30-31]. These growth factors accelerate angiogenesis and vasculogenesis tremendously [32]. The intention of this study is to observe the effect of TNP on human chronic wounds to gain more profound knowledge about the physiological processes.
and wound behaviour in general. The aim is to analyse the alteration of vessel density before and after TNP. Furthermore, there is a hypothesis to prove a correlation among hypoxia, inflammation, transformation and proliferation of blood vessels. To prove this idea different stainings were performed, precisely endothelial cell marker (CD31, CD34), HIF-1α and Haematoxylin and Eosin (H&E).
2 Material und Methods

2.1 Patients characteristic

A heterogenous composition of 16 patients with problematic and non-healing wounds was included in this study. All patients signed a written informed consent. This particular study was approved by the Ethical Committee of the Friedrich-Alexander-University Erlangen-Nuremberg, Germany (protocol number 3343). The patients exhibit non-healing, chronic wounds resulting in multimorbid patients, without any physiological regeneration of tissue defects. Wounds existed at least five weeks independent from their origin, pre-existing conditions and patients lifestyle.

Detailed patients characteristic: (Table1).

<table>
<thead>
<tr>
<th>Patients characteristics</th>
<th>Comorbidities</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (wounds)</td>
<td>Diabetes</td>
</tr>
<tr>
<td>Median age</td>
<td>Cardiovascular diseases</td>
</tr>
<tr>
<td>Female/Male</td>
<td>Rheumatic disorders</td>
</tr>
<tr>
<td>Wound duration (mth)</td>
<td>Adiposity (BMI &gt; 30)</td>
</tr>
<tr>
<td>2nd tissue sample (2 until 12 days)</td>
<td>Gastrointestinal diseases</td>
</tr>
<tr>
<td>Smokers</td>
<td>Cancer</td>
</tr>
<tr>
<td></td>
<td>Cirrhosis of the liver</td>
</tr>
<tr>
<td>Wound information</td>
<td>Hypercholesteremia</td>
</tr>
<tr>
<td>Cancer/Radiotherapy</td>
<td>Bacteria in wound smear</td>
</tr>
<tr>
<td>Infection</td>
<td>Staphylococcus aureus</td>
</tr>
<tr>
<td>Ulcera</td>
<td>Pseudomonas aeruginosa</td>
</tr>
<tr>
<td>Localisation:</td>
<td>Saprophytic germs</td>
</tr>
<tr>
<td>Lower extremity</td>
<td>Enterobacterias</td>
</tr>
<tr>
<td>Upper extremity</td>
<td></td>
</tr>
<tr>
<td>Torso</td>
<td></td>
</tr>
<tr>
<td>Medication</td>
<td></td>
</tr>
<tr>
<td>Corticosteroids</td>
<td></td>
</tr>
<tr>
<td>Antibiotics</td>
<td></td>
</tr>
<tr>
<td>Opiode</td>
<td></td>
</tr>
<tr>
<td>Antidepressants</td>
<td></td>
</tr>
<tr>
<td>Heart medication</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Final treatment</td>
<td></td>
</tr>
<tr>
<td>Skin Grafting</td>
<td></td>
</tr>
<tr>
<td>Flap Transfer</td>
<td></td>
</tr>
<tr>
<td>Continuation of TNP</td>
<td></td>
</tr>
</tbody>
</table>

(Protocol number 3343)
2.2 Collecting samples

Initially all wounds underwent surgical debridement to improve the conditions for wound healing, before TNP therapy (polyurethane foams and subatmospheric continuous pressure of -125 mmHg) was initiated. Depending on the wound conditions further surgical debridement and continuation of TNP therapy were necessary. After successful wound bed preparation and treatment with TNP, defect coverage was achieved using pedicled or free flap surgery or skin grafting.

After each debridement two tissue biopsies were taken: one of the wound edge (WE) and one of the wound bed (WB) (Picture 1). For one patient only wound edge was available and for three patients only wound bed. The first tissue sample was taken at day 0, the second sample between day two and day twelve after TNP. Day 0 also resembled the pre-existing wound conditions before TNP.

A computer-controlled vacuum system (V.A.C.®, KCI, San Antonio, TX) was used.
Wound biopsy from WE

Picture 1: Biopsy was taken in the surgery room of the Friedrich-Alexander-University Erlangen-Nuremberg, Germany with the patients agreement.
2.3 Experimental groups

With respect to ethical principles, this study was applied to observe wound conditions and not to create additional wounds for a control group, as it is usually performed in animal experiments. With no control group the main attention was focused on blood vessel density and hypoxia, which effects vessel proliferation. In this context, another interest concerns inflammation, which might also be influential on wound healing. Patients were divided into three different groups.

Criterion for a patient to join one particular group was defined according to the temporal sequences of a physiological wound healing process and its constituent stages. The groups were chosen to reflect the lost physiological phases of wound healing (inflammatory phase, proliferative phase and remodeling phase), which in turn could be recovered in non-healing wounds under TNP.

In Group I all patients with a treatment time of two until four days were included. Group II patients joined in with a treatment time of five until eight days and Group III included all patients with a treatment time between nine and twelve days.

Exclusion criteria were an age younger than 18 years and acute wounds, which existed less than five weeks. One patient exhibited two wounds, one on each lower leg, resulting both wounds documented as two different patients.
Division into three described groups

Table 2:

<table>
<thead>
<tr>
<th></th>
<th>Group I: day 1 to 4</th>
<th>Group II: day 5 to 8</th>
<th>Group III: day 9 to 12</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WB</td>
<td>WE</td>
<td>WB</td>
</tr>
<tr>
<td>patients</td>
<td>5</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>ratio [%]</td>
<td>98.60</td>
<td>99.43</td>
<td>147.66</td>
</tr>
</tbody>
</table>

Table 2: Tissue biopsies of WB and WE divided into 3 groups with main focus of attention on Group II. Ratio is defined as values before (100%) divided by values after treatment.
2.4 Histopathology and Immunohistochemistry

2.4.1 Common information

Wound biopsies were fixed in 10% formalin for one to two days. Subsequently, tissues were embedded in paraffin and cut into 2.5 µm sections, applied on Superfrost slides (Fisher Scientific) and were finally dried in the oven at 60°C overnight. Slices were stained with CD31, CD34, H&E and HIF-1α.

2.4.2 CD31 Staining

For CD31 staining slides were deparaffinized twice in xylene (Carl Roth GmbH, Karlsruhe, Germany) for 15 minutes each, then rehydrated in iso-propanolic solutions (100%, 96%, 70%) (Carl Roth GmbH, Karlsruhe, Germany) for 5 minutes each. Afterwards probes were boiled in citric acid buffer pH = 6.0 (Zytomed Systems GmbH, Berlin, Germany), therefore was used 10,5g citric acid-monohydrat, which was weighed out and filled up to 5 liter with aqua dest. Finally the slides were filled up with 2N NaOH to a volume of V=60 ml. Blocking solution 1 (Zytomed Systems GmbH, Berlin, Germany) was used for 5 minutes before rinsing the slides with a trisbuffer solution (Zytomed Systems GmbH, Berlin, Germany). The antibodies (monoclonal mouse anti-human, CD31, Dako, Hamburg, Germany) were diluted with albumin solution (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) in a 1:300 ratio and incubation was performed overnight. Afterwards, slides were washed three times with the trisbuffer solution before treating with a post block solution (Zytomed Systems GmbH, Berlin, Germany) for 30 minutes, followed by treatment with an AP-Polymer (ZytoChem Plus, AP Polymer System, Zytomed Systems GmbH, Berlin, Germany) for 30 minutes. Again, washing three times with trisbuffer solution, treatment with Fast Red TR salt (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and watering the slides for 2 minutes was performed. The probes were stained with Hämaalaun (Merck KGaA, Darmstadt, Germany) for a short period of time before they were rinsed systematically with water,
destilled water and embedded in Aquatex (Merck KGaA, Darmstadt, Germany).

2.4.3 CD34 Staining

For CD34 staining the same procedure was performed as for CD31, but with CD34 monoclonal antibody (Immunotech, Marseille, France) and an albumin dilution ratio of 1:500.

2.4.4 H&E

Routine histopathology was performed using H&E staining for morphological analysis. Slides were deparaffinized in xylene (Carl Roth GmbH, Karlsruhe, Germany), then rehydrated in iso-propanolic solutions (100%, 96%, 70%) (Carl Roth GmbH, Karlsruhe, Germany). The probes were stained with Mayers Hämaalaun (Merck KGaA, Darmstadt, Germany) for a short period of time before they were rinsed with hydrochloric acid - alcohol for 2 seconds. Afterwards, slides were washed with destilled water for 3-5 minutes. After washing, slides were stained with Eosin (Merck KGaA, Darmstadt, Germany), again rinsing with destilled water for 30 seconds. Then they were rehydrated in iso-propanolic solutions (70%, 96%, 100%) (Carl Roth GmbH, Karlsruhe, Germany) and Xylol (Carl Roth GmbH, Karlsruhe, Germany). Finally probes were embedded in Entellan (Merck KGaA, Darmstadt, Germany).

2.2.5 HIF-1α

“Paraffin sections [...] were dewaxed in xylene and rehydrated in a series of ethanol washes. Slides were coated with 3-aminopropyltriethoxysylane. For detection of HIF [...] monoclonal mouse antihuman HIF-1α antibody (α67; Novus Biologicals, Littleton, CO) [...] was used [...]. Additional primary antibody was monoclonal mouse anti-rat ED-1 (Serotec, Duesseldorf, Germany). Biotinylated secondary anti-mouse or anti-rabbit antibodies (Dako,
Hamburg, Germany) were used. For signal amplification and visualization, a catalyzed signal amplification system (CSA-Kit; Dako) based on a streptavidin-biotin-peroxidase reaction was used according to the manufacturer’s instructions. Antigen retrieval was performed for 6 min in preheated target retrieval solution (Dako), using a pressure cooker. Between incubations, specimens were washed two to three times in buffer (50 mM Tris-HCl, 300 mM NaCl, and 0.1% Tween-20 [pH 7.6]). As chromogen for peroxidase reaction diaminobenzidine was used [...] Terminal deoxynucleotidyl transferase-mediated dUTP nick-end (TUNEL) staining was performed using In Situ Cell Death Detection Kit, Flourescein (Roche, Penzberg, Germany) according to the manufacturer’s instructions.” [33].

2.5 Quantitative and qualitative analysis

All slides were analysed using light-microscopy (Leica System, Bensheim, Germany) and pictures were taken and recorded with a microscopy camera (Leica DFC 420, Bensheim, Germany). Three to six images of the complete biopsy were taken, with respect to the size of the single biopsy. Large biopsies allowed the imaging of six detailed areas. For smaller biopsies only three detailed images were feasible. CD31 and CD34 staining were used for vessel count; HIF-1α and H&E were evaluated by their proportional part of HIF-1α stained cell nuclei or inflammatory cells (macrophages) related to the complete picture detail.

2.6 Statistical analysis

Due to the small sample number, no inferential statistics were applied. Results of repeated measurements were averaged. For each outcome variable the ratio was defined as the value after treatment divided by the value at baseline in percent for each wound. Analogously, group of overall ratios reflected the relation of mean values after versus before treatment. A logarithmic scale on the axes of the graphs was applied, to allow better visual inspection of the results (Figures 1-5, 7). All results refer to an initial value of 100% prior to any treatment.
3 Results

3.1 Common information

Analysis of all patients exhibited an increase of vessel density after TNP treatment. The observed values after treatment refer to the initial number of vessels (=100%). CD31 staining displayed an increase in blood vessel density up to 118% at WB and up to 136% at WE. Similar results were obtained in CD34 staining with an increase up to 108% at WB and up to 135% at WE. On the contrary, a decrease of hypoxia was detected after TNP treatment, i.e. 58% at WB and 83% at WE. Analysis of inflammatory response also showed a decrease of 90% at WB and of 86% at WE after TNP.

For further analysis three groups were classified (vide supra) dependent on the period of wound treatment in order to find differences in the behaviour of vessel proliferation, hypoxia and inflammation. Group I, Group II and Group III included patients with a total TNP treatment time of one until four days, five until eight days and nine until twelve days, respectively (Table 2).
Overall analysis

**Figure 1**: Overall analysis of tissue staining of all treated wounds with TNP is presented. The ratio is defined as the value after treatment divided by the value at baseline in percent for each wound. An increase in the amount of vessel density, a decrease of hypoxic cells (especially at WB) and a decrease of inflammatory cells after TNP is observed.
3.2 Immunohistochemistry

3.2.1 Vessel density: CD31, CD34 (Figure 2: CD31, Figure 3: CD34)

*Group I* included five biopsies from WB and four biopsies from WE with no observable average difference in vessel density. In particular, staining showed no remarkable changes in vessel density, *i.e.* for CD31 99% at WB and at WE, for CD34 99% at WB and 105% at WE (Table 2). Both, CD31 and CD34 staining exhibited a decrease in three wounds and an increase in two wounds at WB. At WE, CD31 staining exhibited a decrease in one wound, no remarkable change in one wound and an increase in two wounds, while CD34 staining exhibited an increase in two wounds and a decrease in two wounds.

*Group II* included eight biopsies from WB and six biopsies from WE with most considerable results in vessel density. An average maximum of >200% was reached (see patients examples below at page 3.3.1).

In particular, staining showed a considerable tendency of increase in vessel density, *i.e.* for CD31 to values of 148% at WB and to values of 202% at WE, for CD34 to values of 124% at WB and to values of 167% at WE (Table 2). CD31 staining exhibited an increase in five wounds, no remarkable change in two wounds and a decrease in one wound at WB and at WE an increase in all six wounds. CD34 staining exhibited an increase in six wounds and a decrease in two wounds at WB and at WE an increase in five wounds and a decrease in one wound.

*Group III* included two biopsies from WB and three biopsies from WE with no observable average difference in vessel density in CD31 staining. In particular, CD31 staining showed no remarkable changes, *i.e.* 106% at WB and 101% at WE, whereas CD34 staining exhibited an alteration in vessel density to values of 90% at WB and to values of 130% at WE (Table 2). However, both CD31 and CD34 staining presented an increase in one wound and a decrease in one wound at WB. At WE, CD31 staining exhibited an
increase in one wound and a decrease in two wounds and CD34 staining exhibited an increase in two wounds and a decrease in one wound.

To summarize, data demonstrated an ascending tendency of vessel proliferation in Group II at WE, which is even more pronounced at WB.
**Figure 2: CD31**

CD31 staining with a classification into three groups dependent on the time of treatment. Each point represents one tissue biopsy, white points represent WE and black points WB. Almost all biopsies disclose an increase of vessel density between day five and day seven. Besides this time frame a balanced proportion in vessel density is noticed.
Figure 3: CD34
CD34 staining with a classification into three groups dependent on the time of treatment. Each point represents one tissue biopsy, white points represent WE and black points WB. Similar results such as in CD31 staining are observed irrespectively values are more spreaded. Most of the biopsies demonstrate an increase of vessel density between day five and day seven. Besides this time frame an almost balanced proportion in vessel density is noticed.
3.2.2 Hypoxia: HIF-1α (Figure 4)

*Group I* included three biopsies from WB and two biopsies from WE with a distinguished decrease of HIF-1α stained cells. In particular, HIF-1α stained cells exhibited a decrease, *i.e.* to values of 30% at WB and to values of 42% at WE (Table 2). These HIF-1α stained cells decreased in all three wounds at WB, while at WE a decrease in one wound and an increase in one wound was observed.

*Group II* included four biopsies from WB and three from WE with an increase of HIF-1α stained cells. In particular, HIF-1α stained cells increased, *i.e.* to values of 103% at WB and to values of 126% at WE (Table 2). However, HIF-1α staining exhibited an increase in three wounds and a decrease in one wound at WB, while at WE an increase in two wounds and a decrease in one wound was observed.

*Group III* included two biopsies from WB and three from WE with a decrease of HIF-1α stained cells, which was more intense at WB (see patients examples below 3.3.2.1). In particular, HIF-1α stained cells decreased, *i.e.* to values of 39% at WB and to values of 96% at WE (Table 2). The decrease was observed in all two wounds at WB, while at WE an increase in two wounds and a decrease in one wound was observed.

To summarize, the data demonstrated an increase of hypoxia until day eight. Starting day nine, a decreasing tendency was noticed, especially at WB.
HIF-1α

Figure 4: HIF-1α
HIF-1α staining with a classification into three groups dependent on the time of treatment. Each point represents one tissue biopsy, white points represent WE and black points WB. An ascending tendency of HIF-1α stained cells is observed, especially at day seven, whereas it is more concise at WB. Besides this time frame decreasing values are observed.
3.2.3 Inflammation: H&E (Figure 5)

*Group I* included five biopsies from WB and four from WE with a decrease of inflammatory cells (macrophages).
In particular, inflammatory cells decreased, *i.e.* to values of 81% at WB and to values of 84% at WE (Table 2). A decrease in three wounds, no remarkable change in one wound and an increase in one wound was observed at WB, while at WE a decrease in two wounds, no remarkable change in one wound and an increase in one wound was observed.

*Group II* included eight biopsies from WB and six biopsies from WE with an increase of inflammatory cells, especially at WB.
In particular, inflammatory cells increased, *i.e.* to values of 125% at WB and to values of 103% at WE (Table 2). Both, WB and WE exhibited an increase in four wounds and in two wounds, respectively and a decrease in four wounds and in two wounds, respectively.

*Group III* included two biopsies from WB and three biopsies from WE with a decrease in inflammatory cells (see patients examples below 3.3.2.2).
In particular, inflammatory cells exhibited a decrease, *i.e.* to values of 46% at WB and to values of 59% at WE (Table 2). An increase in one wound was observed and a decrease in one wound at WB, while at WE a decrease in all three wounds was observed.

Concerning the inflammatory response, an increase of inflammatory cells at WB was detected in *Group II*. Besides this, the other groups notified an ascending tendency of inflammation.
H&E staining with a classification into three groups dependent on the time of treatment. Each point represents one tissue biopsy, white points represent WE and black points WB. An increase of inflammatory cells is observed between day four and day seven. One tissue biopsy at WB on day seven could not be demonstrated in the figure, due to its small value of less than 1%. Besides this time frame decreasing values are observed.
3.3 Patient examples of wound biopsies

3.3.1 Patient A -> CD34

Patient A was a 50 years old female person. Her main diagnosis was a chronic wound pre-patellar on the left knee. As comorbidities she exhibited adiposity (BMI>30) and a depression, furthermore she was a smoker. TNP was applied for five days. The tissue samples were taken from WE before (picture 2) and after (picture 3) TNP treatment. Before TNP application less blood vessels (red stained endothelial cells) were available, in comparison to an increased vessel density after a five day TNP- treatment. A high number of small new generated capillaries was observed, which represent neovascularisation.
CD34 staining of WE before TNP

Picture 2: CD34 pre-TNP staining
The wound tissue was examined with a light microscope with a magnification of 100x. Less vessel density (red stained endothelial cells) was observed. Blue stained tissue marked fibroblasts.
CD34 staining of WE after TNP

The wound tissue was examined with a light microscope with a magnification of 100x. A meaningful increase in vessel density of new generated capillars (red) was noticed.

Picture 3: CD34 post-TNP staining
For better analysis all data of this patient were summarized in one figure (figure 6). HIF-1α staining was not available because of the small sample size. In both endothelial staining an increase in vessel density was observed upon approximately 400% (391% for CD31 and 352% for CD34) compared to the initial amount of vessels at WE. At WB no remarkable increase in vessel density could be noticed at this time (102% for CD31 and 115% for CD34). But at the same time a decrease in inflammatory cells was observed (to 50% at WE and to 82% at WB).
All data of this patient

Figure 6: Summarized data of Patient A: Analysis of all tissue staining of Patient A is presented. The ratio is defined as the value after treatment divided by the value at baseline in percent. Pre-TNP staining is conform to 100% and post-TNP staining refer to this 100%. A remarkable increase in vessel density at WE is observed in both endothelial staining. At the same time inflammatory cells decreased to values of 50% at WE.
3.3.2 Patient B -> HIF-1α and H&E

Patient B was a 69 years old male person. His main diagnosis was an abscess incision of an infected gout tophus at the left heel. As comorbidities he exhibited diabetes, peripheral arterial obstructive disease and chronic venous insufficiency. TNP was applied for twelve days. The tissue samples were taken from WB before and after TNP treatment.

3.3.2.1 HIF-1α

Before TNP application lots of hypoxic cells (brown points) were noticed at WB (picture 4). After a treatment time of twelve days all hypoxic cells were vanished (picture 5).
HIF-1α staining of WB before TNP

Picture 4: HIF-1α pre-TNP staining
To detect the hypoxic cells (brown points) a magnification of 200x was used. Before TNP application a lot of hypoxic cells were displayed in the upper half of the picture.
HIF-1α staining of WB after TNP

Picture 5: HIF-1α post-TNP staining
To detect the hypoxic cells (brown points) a magnification of 200x was used. In the post-TNP picture all hypoxic cells were vanished.
3.3.2.2 Patient B -> H&E

Tissue samples are demonstrated of the same patient described before, diagnosis and comorbidities *vide supra*.
Before TNP lots of inflammatory cells (blue coloured cells) were noticed (Picture 6). After a twelve-day treatment the inflammatory cells were diminished (Picture 7).
H&E staining of WB before TNP

The wound tissue was examined with a light microscope with a magnification of 100x. Before TNP application a lot of inflammatory cells, macrophages (blue coloured cells) were noticed.
H&E staining of WB after TNP

Picture 6: H&E post-TNP staining
The wound tissue was examined with a light microscope with a magnification of 100x. Post-TNP inflammatory cells (macrophages) were diminished.
For better analysis all data of this patient were summarized in one figure (figure 7).

In both endothelial staining an increase in vessel density was observed at WE (168% for CD31 and 157% for CD34) compared to the initial amount of vessels. At WB a decrease of vessel density was noticed at this time (to 76% for CD31 and to 53% for CD34). At the same time a decrease in inflammatory cells was observed (to 84% at WE and to 15% at WB). A correlation between inflammation and hypoxia seemed to be obvious. HIF-1α stained cells decreased in a similar way like the inflammatory cells (to 90% at WE and to 80% at WB).
Figure 7: Summarized data of Patient B: Analysis of all tissue staining of Patient B is presented. The relative ratio is defined as the value after treatment divided by the value at baseline in percent. Pre-TNP staining is conform to 100% and post-TNP staining refer to this 100%. An increase in vessel density can be noticed at WE in both endothelial staining, whereas a decrease at WB is observed. All other staining behave in a similar way with a decrease in inflammatory as well as in hypoxic cells for both, WE and WB.
3.4 Overall analysis related to the time of treatment (Figure 9)

3.4.1 Vessel density

The observation of all patients displayed no change of vessel density in the first four days of treatment. Between a five until eight days TNP treatment a remarkable increase of vessel density is discharged to values of approximately 150% at WB and more pronounced at WE to values of more than 200%. After a treatment time longer than eight days no more alteration in the vessel amount is noticed.

3.4.2 Hypoxia

In the first four days of treatment the amount of HIF-1α stained cells decreased to values less than 40% both, WB and WE. In Group II with a treatment time between five and eight days, there is observed an increase of hypoxic cells especially at WE to values of 125%. The observation of a longer treatment time (> eight days) display a decrease in Hypoxia at WB, but no change in the amount of hypoxic cells at WE.

3.4.3 Inflammation

A similar behaviour of inflammatory cells was noticed. In the first four days of treatment the amount of inflammatory cells decreased to values of 75% both, WB and WE. Between a five until eight days treatment inflammation increased especially at WB, at WE no change was noticed. A treatment time longer than eight days leads to a reduction of inflammatory cells to values of approximately 50%, more pronounced at WB.

3.4.4 Correlation between vessel density and hypoxia

To compare endothelial stained cells with HIF-1α stained cells, no correlation could be demonstrated.
3.4.5 Correlation between vessel density and inflammation (Table 8).

While plotting vessel density against inflammation in the same diagram a correlation between both parameters could be seen. Due to the low number of patients, no statistical test was applicable, however, an correlating tendency of endothelial stained cells and inflammatory cells was clearly observed. CD31 staining in the diagram represents both endothelial staining. In CD34 staining similar results were noticed.
Correlation between vessel density and inflammation

Figure 8: Vessel density was plotted against inflammation. Black points present WB and white points present WE. A correlating tendency could be observed between both parameters, despite the low number of biopsies.
**Overall analysis with division into the three described groups**

![Figure 9](image)

**Figure 9**: Overall analysis of tissue staining and classification into the three described groups. The ratio was defined as the value after treatment divided by the value at the baseline in percent. CD31 represents both endothelial staining. Between day five and day eight a considerable increase of vessel density was observed, simultaneously an increase of hypoxia (WE) and inflammation (WB) in this time frame. Between day one and day four and between day nine and day twelve almost no change in vessel proliferation was mentioned. But TNP leads to a decrease of inflammatory and hypoxic cells, especially between day nine and day twelve.
4 Discussion

4.1 Study design

This study is different to previous trials. It has unique characteristics related to its approach. It reflects a real patients population in hospital and offers a good possibility for wound management despite a high number of co-morbidities. The main attention was focussed on these multimorbide patients to reflect the daily routine in hospital. Reality in hospital does not look like a selected “one-disease” patients population with no risk factors. But exactly these people need a sufficient therapy with less complication rates and a good outcome with less personal risk of therapy.

4.2 Patients acquiring

The aim of this study is to identify how TNP affects chronic wounds at a multimorbide patients population. The patients are chosen as a heterogeneous population with different co-morbidities and medication, as well as diverse causes and locations of wounds. This heterogenous composition is desired, concerning to prove the effect and benefit of TNP at a variety of disease patterns and other circumstances as diseases, physiological and pathological conditions are diverse. Although it is very difficult to compare these different patients with each other in dependence of their diverse starting conditions and co-morbidities. Studies of other research groups describe the advantages of TNP therapy in animal models like a reduction of bacterial load, stimulation of epidermal cell proliferation and fibroblast proliferation and an increase in blood flow [13-14,22-24]. Additionally the angiogenesis and vasculogenesis are stimulated through TNP [5,7,13,25-27]. To keep all this in mind our study was designed in this way. It is a trial to transfer all these animal based knowledge to the human. On the basis of histological tissue analysis the study confirms the benefit of TNP for chronic non-healing wounds of multimorbide patients.
4.3 Problems of wound comparison

Acquiring control experiments are impractical to achieve, because patients would have to exhibit more than one chronic wound at the same time, in order to treat wounds in a different manner. Besides the improbability of new wound creation for control experiments, it is not arguable ethically. Concluding, control experiments have not been performed in this study, which makes comparison of wounds quite challenging.

4.4 Vessel proliferation

The problem described before (4.2) could be one reason, that only a few studies concerning the investigation of proliferation of blood vessels under TNP (and the underlying biological mechanism) on human tissue to date. Herein, an increase of vessel density is presented in 15 patients, which was more pronounced at WE than at WB (Figure 1). This implicates, that vessels are spreading out of WE into the centre of the wound (WB). The proliferation at WE averages an arithmetic mean of 36% in comparison to 18% at WB, which is approximately similar in both endothelial staining. A remarkable result of this study is noticed at a duration time between five until eight days of TNP treatment. This is in accordance with a rabbit model by Chen et al. [25] and another human study by Greene et al. [34]. At that time the vessel density reaches values of approximately 200% in relation to the starting point of vessel amount (Figure 9). This means a doubling of blood vessels especially at WE. A treatment time between one until four days and a treatment time between nine until twelve days has almost no effect on angiogenesis and vasculogenesis (Figure 9). Out of these results it is obviously argued that a treatment time less than five days is too short to stimulate angiogenesis. On the other hand a treatment time longer than eight days leads to an apoptosis of redundant blood vessels. These results are also in accordance with the rabbit model by Chen et al. [25] and Greene et al. [34]. The tissue repair had taken place at a treatment time between five until seven days and the wound switches into the next phase of wound healing. So TNP seems to be most effective in a time frame between five and seven
days. Due to the low number of patients, no statistical test was applicable, however, an increasing tendency in vessel proliferation is clearly observed. These results are different compared to a previous study by Grimm et al. with five post-irradiated wounds [35]. The discrepancy could be related to a different assortment of patients. Our study included only chronic wounds (in comparison to acute and chronic wounds by Grimm et al.), which exist at least five weeks without specific limited disease patterns. All kinds of chronic wounds were included. The only exclusion criteria applied in our study were an age younger than 18 years and an acute wound stage, which means less than five weeks. This fact might be responsible for the variable behaviour of regeneration.

Considering the results of this study, it can be claimed that the most effective TNP treatment time pertaining to blood vessel density is between five until eight days. A treatment time less than four days is too short to induce blood vessel proliferation. Furthermore, a treatment time longer than eight days seems to contain a reduction of vessels and to be a transition point towards apoptosis of redundant blood vessels.

4.5 Hypoxia

This study is a first try to examine if hypoxia plays a role in the wound healing of impaired chronic human wounds. Therefore a few tissue biopsies were taken to stain with HIF-1α. This study demonstrates that in the first four days after initiating TNP treatment HIF-1α stained cells decrease. The same behaviour could be noticed, when TNP application continues longer than eight days. A treatment time between nine and twelve days again displays a reduction of HIF-1α stained cells especially at WB, also at WE, but only in a minor extent. To put main focus of attention on the time frame between five and eight days a remarkable increase of hypoxic cells at day seven is observed. These results are not coincide with a previous study by Elson et al. [28]. One possible reason might be, that they create acute wounds in an animal model in comparison to this study with chronic non-healing human wounds. To compare the wound behaviour in relation to vessel density and hypoxia it can be noticed, that hypoxia and angiogenesis behave in a similar
manner. Especially at day seven this is noticed. This suggests, hypoxia is directly stimulating vessel proliferation at that time up to values higher than 200% (Figure 9, CD31, WE). Other studies support these results and describe hypoxia as a vasculogenetic and angiogenetic factor [28-30]. After several days of treatment an adequate perfusion of tissue occurs. In succession of regenerated vessels and hence neovascularisation, hypoxic cells are vanishing. Unfortunately, a direct correlation between vessel density and hypoxia could not be drawn due to the low number of patients. Besides stimulation of neovascularisation, another important task of hypoxia is the stimulation of macrophages, which plays an essential role in wound healing by secreting angiogenetic factors, which in turn induce and accelerate angiogenesis [10].

4.6 Inflammation

The starting point of treatment in this study is a chronic wound stage. It is associated with less inflammatory activity, but also no tendency towards physiological wound healing. The results of this data do not show a change in inflammation within the first four days of treatment. Wound behaviour could not be modified pertaining the initial situation despite TNP within the first four days. To assume, a longer treatment time of TNP is necessary to effect physiological inflammation, which is a precondition for wound healing. This supposition is approved by the analysis of Group II between a five until eight days TNP treatment. In this time frame a clear increase of inflammatory cells is demonstrated to values up to 125% at WB (Figure 9). These results implicate a proliferation of vessel density at that time, which is equivalent with an increase of gene expression of macrophages and infection defense mechanism [36]. Even more, a tendency of positive correlation between inflammation and vessel density could be seen in this study (Table 8). From day nine on, a decrease of inflammation is detected, which is attributed to an increase of vascularisation and a following stimulated metabolism. In Group III (> nine days TNP treatment) the end of the proliferative phase is reached, which is observed in the decreasing number of blood vessels. The process of
wound healing is already accomplished and consequently inflammatory cells are vanishing.

4.7 TNP and stages of physiological wound healing

Physiological wound healing passes through three phases, i.e. inflammatory phase (day one until day four after wounding), proliferative phase (day five until day eight after wounding) and remodelling phase (> eight days, up to months). The approximate time frames vary depending on the kind of wounds and their size. Naturally, the patient’s post-TNP biopsies are divided into three groups for the evaluation of vessel density, hypoxia and inflammation, referring to the physiological wound healing phases. With this in hand it becomes possible to compare the non-healing wounds with the physiological way of healing. A considerable increase in blood vessel density, particular presented in CD31 staining (Figure 9), reaches a maximum between a day-five and day-eight TNP treatment, with an emphasis at day seven, for both WE and WB. This confirms data based on animal studies [25,37]. Past an eight days TNP-treatment no further alteration of blood vessels could be proofed, which is in concordance to clinical practise and previous observations [5,25,34]. With this in hand, the results of this study relating to the vascularisation present the second phase, which means the proliferative phase of wound healing, which physiologically occurs between day four and day eight. In the following days, months and sometimes years, the remodelling phase of wound healing becomes more relevant. It ultimately leads to a removal of the redundant blood vessels and a subsequent decrease in vessel density, hypoxia and inflammation. This physiological process is observed in both acute [7] and chronic wounds, the latter described in this study. It confirms different stages of wound healing and implicates that TNP is supporting the physiological wound healing process, especially in the proliferative phase.
4.7 Histological effects of TNP

The main histological effect of TNP treatment occurs between day five and day eight. In this time frame, high values of physiological inflammation and hypoxia are noticed, which seem to be initiated and accelerated by TNP. To conclude, a short treatment time (< five days) is inefficient with respect to wound healing, while a longer treatment time (> eight days) histologically shows no further advantages in the healing process. This results suggest that TNP is an effective therapy for chronic wounds at a time frame between a five and eight days treatment. In this time TNP triggers a non-healing wound towards the direction of a physiological wound healing and supports this process at the stage of proliferation according to the results of this study.
5 Resume

The results of this study suggest that TNP leads chronic wounds to a remarkable increase of vessel density in a time frame between five until eight days. At the same time an increase of hypoxia is observed which is described as angiogenetic factor. Hypoxia triggers vasculogenesis and the regeneration of new vessels, which are needed for a physiological wound healing. Even more inflammation increases at that time resulting in an ascending infection defense, which is also needed for wound healing. A therapy less than five days and longer than eight days shows no improvement related to the vascularity in comparison to the starting point of treatment, where TNP was not yet applied.

In conclusion TNP supports a delayed and failed wound healing by stimulating vasculogenesis. This process is supported by an increase of hypoxia and inflammation, which are both associated with angiogenesis and infection defense, respectively. So TNP leads an impaired wound healing into the direction of a physiological wound healing process.
6 References


7 Acknowledgments

I thank Prof. Dr. med. R.E. Horch for his aim oriented help and his encouragement.

I thank Dr. med. Mareike Leffler for mentoring my doctoral theses and the interposition for the different specific departments of the Friedrich-Alexander-University, Erlangen-Nuremberg.

I thank Mrs. Andrea Keller for statistical assistance.

I thank all the doctors of the Department of Plastic and Hand Surgery of the Friedrich-Alexander-University, Erlangen-Nuremberg, for collecting tissue samples in the operating room.

I thank Mrs. Irma Goldberg for making appointments in a straightforward and smooth way.

I thank Dr. med. Wanja Bernhardt and his scientific group, Department of Nephrology of the Friedrich-Alexander-University, Erlangen-Nuremberg, especially Barbara Teschemacher for performing HIF-staining.

I thank Mrs. Ilse Arnold and Mrs. Katja Schubert from the scientific group of the Department of Plastic and Hand Surgery of the Friedrich-Alexander-University, Erlangen-Nuremberg for excellent technical assistance and performing histological staining.

I also thank the attendant Ingo Reijnders for his help in the operating room.

I thank the ladies and gentlemen working in the archive for their help with the patients documents.
I thank Johannes Broichhagen for helping me with all computeral stuff. When everything seems to be lost he always knew how to save it.

I thank all my friends supporting me in reading my theses.

I especially thank my parents Anna-Christine Malsiner and Richard Malsiner, who support my medical studies financially and emotionally at every time.