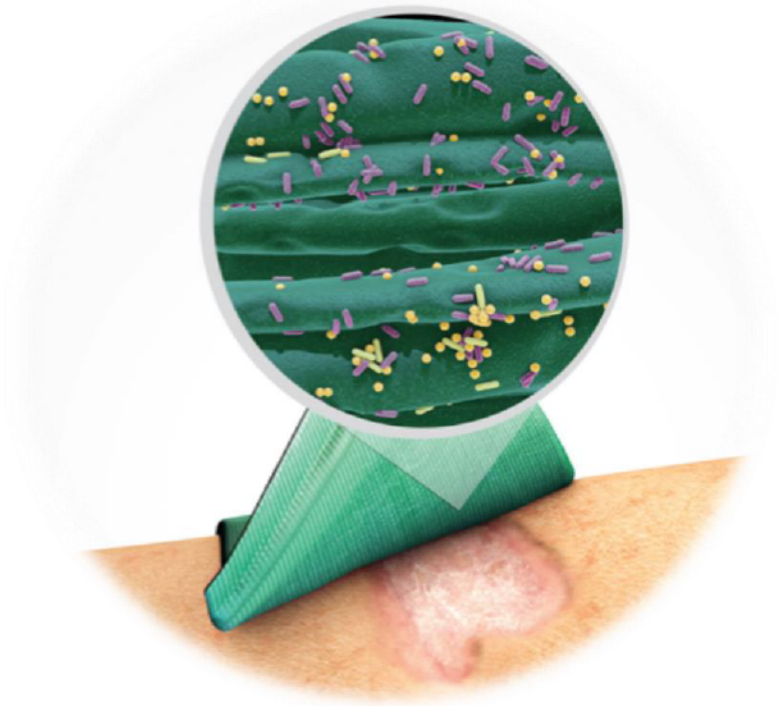
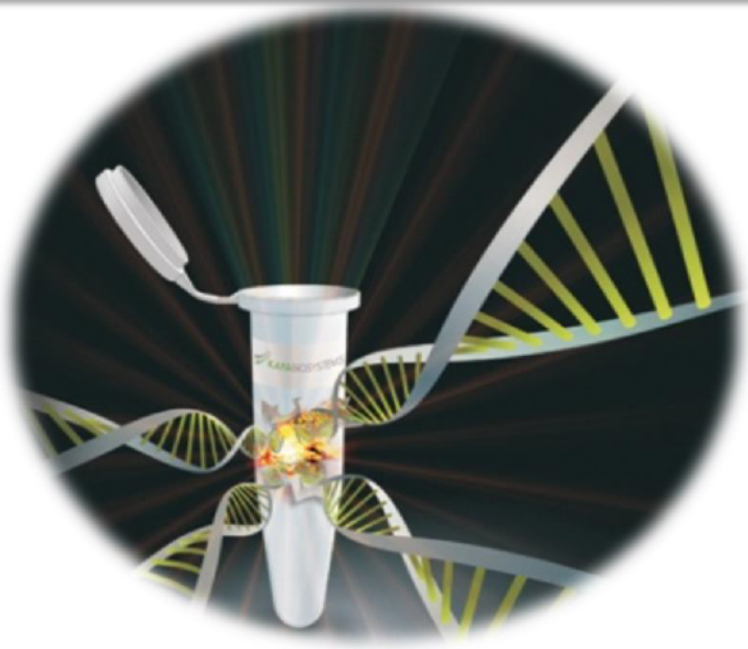
BSN medical



Study Protocol



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Department of Dermatology and Cutaneous Surgery

Wound Healing and Regenerative Medicine Research Program

STUDY PROTOCOL

An exploratory, single center, observer masked, active control, randomized trial to investigate the effectiveness of Dialkylcarbomoyl chloride dressing (Cutimed® Sorbact®) in modifying bacterial load in venous leg ulcers

Hadar Lev-Tov, MD, MAS

Irena Pastar, PhD

Tulay Koru-Sengul, PhD, MHS

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1. EXECUTIVE SUMMARY:

Sponsor: BSN Medical Inc.

Primary investigators:

Hadar Lev-Tov, MD, MAS. Assistant professor of dermatology, University of Miami, FL

Irena Pastar, PhD. Assistant professor of dermatology, University of Miami, FL

Tulay Koru-Sengul, PhD. Associate Professor of Biostatistics, University of Miami, FL

Objective: The goal of the study is to investigate the effectiveness of Cutimed® Sorbact® (Study Device) in modifying bacterial load in venous leg ulcers (VLU).

Design: This is an exploratory study in a single center, observer masked, active control, randomized trial.

Overall, 45 subjects with VLU who have persistent wounds and have been previously treated with silver based therapy will be 1:1 randomized to receive up to four applications over up to four weeks of Study Device or Comparator (silver impregnated contact layer dressing - Acticoat®) with 2 weeks follow up period.

Outcomes: Primary outcome is the change in bacterial load compared to baseline.

Secondary outcomes: Bacterial silver resistance based on genetic and phenotypic characterization over time (see appendix A for molecular microbiology protocol), healing rate, complete healing at 6 weeks, pain, quality of life (QOL) measurements and adverse events.

1.1 Table of assessments and study calendar:

Phase	Screening		Treatment						Follow up
Week	-2	-1	0		+1	+2	+3	+4	+6
Visit	S1	S2	T0 (pre random)	T0(post random)	T1	T2	T3	T4	F1
Window (days)		±2	±2		±2	±2	±2	±2	±2
Informed consent	X								
Eligibility	X	X	X						
Demographics	X								
Medical history	X								
Concomitant medication	X	X	X		X	X	X	X	X
Study ulcer history	X								
Physical exam (focused)	X								X
Vital signs	X								X
Pain (VAS)	X	X	X		X	X	X	X	X
Wound area	X	X	X		X	X	X	X	X
Product application				X	X	X	X		
Tissue collection			X			X	X		
QOL				X				X	X
Randomization				X					
Sharp debridement	X	X		X	X	X	X	X	
Wound photography	X	X		X	X	X	X	X	X
Compression	X	X		X	X	X	X	X	X
Adverse events	X	X	X		X	X	X	X	X
ABI	X								
Venous Doppler	X								
Fitting for stocking								X	X

Table 1. Table of assessments and study calendar

2. BACKGROUND

Wounds are the most common and costly skin disease. Chronic wounds are wounds that fail to progress through the normal process of healing. Briefly, normal healing occurs in four phases that overlap significantly [1]. Immediately following injury damage to endothelial cells exposes collagen and activates platelets to form a plug thus initiating the hemostasis phase. Chemotactic signals from leukocytes and platelets attract immune cells to the wound and activate fibroblasts, smooth muscle cell and endothelial cells. Minutes following injury the first neutrophils arrive to the wound site and help recruit macrophages and together degrade and clear damaged tissue and bacteria. Cytokines and growth factors secreted by macrophages in the wound result in formation of granulation tissue, fibroblast recruitment and keratinocyte migration. Within the first 24 hours growth factors secreted in the wound result in fibroblast activation that begins break down of early fibrin matrix by matrix metalloproteinases, and deposition of new extracellular matrix of collagen, elastin and other extracellular proteins assisting in scar formation and epithelialization. In the weeks and months that follow, fibroblasts transform into myofibroblasts and assist in scar contraction. The predominance of collagen 3 in the scar is slowly replaced by collagen 1 and a final scar is formed estimated to achieve about 80% of the tensile strength of unwounded skin. When a wound fails to progress through these stages of healing it is considered a chronic wound and venous leg ulcers (VLU) represent a specific case of chronic ulcers occurring on the legs. Chronic venous insufficiency and the resulting venous hypertension are typically a result of reflux or outflow failure often due to obstruction. Valvular defects, ineffective calf muscle pump function, deep vein thrombosis and its complications or arteriovenous shunting are the main causes for venous hypertension [2, 3]. The exact pathophysiology of VLU remains unclear. Current hypotheses include the fibrin cuff hypothesis and its extensions of “trap theory” with or without inflammatory cell and cytokine and/or metalloproteinases dysregulation including impaired re-epithelization however, more research is needed to better clarify the exact mechanisms involved [2, 3].

Venous leg ulcers, the problem: Leg ulcers are very common and affect about 1-2% of the American population [2]. Venous leg ulcers (VLU) are wounds that occur in the gaiter area in the setting of chronic venous insufficiency (CVI). VLU account for about 70% of the all chronic leg ulcers and about 20% of all leg ulcers are likely a result on mixed arterial and venous disease. VLU significantly impacts patients’ quality of life [4] and people with VLU are often chronically ill with significantly more comorbid conditions compared to controls [5]. VLU represent an increasingly devastating economical burden. For example, the estimated cost of treating single non healing VLU in 1987 was $10,000 and by 2014 has ballooned to $34,000 [6]. This is also reflected by the 5-fold increase in total cost of VLU treatment from estimated $3 billion to $15 billion over a similar period. The prevalence of VLU is difficult to assess yet recent estimates suggest a significant rise from 0.3% 20 years ago to more recent estimates of 0.6% in adults under 65 years of age and 2.2% in adults over 65 . Considering the aging of the population a concerning picture emerges: more ulcers occurring in sicker patients which are ever more expensive to treat. Standard care for VLU centers around edema reduction via compression and/or leg elevation, meticulous local wound care, control of infection and pain and prevention of recurrence [7]. Advanced therapies include highly absorbent dressing, matrix based

devices with and without cells and negative pressure wound therapy among others. However even with best therapies available up to 50% of VLU fail to heal [8].

Infections play a significant role in the chronicity of VLU. The high wound bacterial burden (>10^5 organisms per gram of tissue), even without the classic signs of infection, is also detrimental to healing [9, 10].

Silver resistance: Silver (Ag) coated dressings are increasingly being used for chronic wounds based on bactericidal properties of released Ag, despite the lack of evidence to support their use [11]. Concerns of widespread Ag resistance in bacterial isolates from chronic wounds have been raised due to the increased utilization of silver based wound dressings [12] [13]. Silver resistant-strains have also been isolated from silver-treated patients at burn centers, where these stains have sometimes caused outbreaks [14]. Furthermore our *in vivo* studies utilizing porcine wound model have shown that silver treatment induces expression of virulence genes important for biofilm formation in clinical isolates of methicillin resistant *Staphylococcus aureus* and *Acinetobacter baumannii* [15]. Although the molecular basis for silver-resistance has been previously characterized [16] [14], to date, expression of silver resistance genes as a response to therapy has not been analyzed in clinical settings.

Here we propose to analyze the presence of silver resistance on genotypic and phenotypic level in response to treatment with Study Device (Cutimed® Sorbact®) and active control (Acticoat®).

The promise of DACC technology. Dialkylcarbomoyl chloride (DACC)- coated dressings are a recent addition to wound care armamentarium. This innovative antimicrobial technology can be imbedded into various dressing and wound care products. Key to its mechanism of action, DACC, a fatty acid derivative is highly hydrophobic. On the other hand the microorganisms, commonly responsible for colonizing chronic VLU, generally have hydrophobic extracellular surfaces, and will therefore irreversibly adhere to the DACC based dressings [17]. Subsequent dressing changes result in the physical removal of large numbers of bacteria and a decreased microbial load. Since the mechanism of antibacterial action is physical binding followed by removal, there is no risk of bacteria developing resistance [17]. Developing non-silver based wound therapies is important due to reports of silver based toxicity [18]. Additionally, this may prove important as evidence exists for the onset of silver resistance in various, clinically relevant microbes [19]. This may be due to possible overuse of silver-based wound care products and is mediated by plasmid acquisition and other genetic mechanisms highlighting the need to better characterize these mechanisms in VLU [18]. Furthermore, silver and other bactericidal agents result in bacterial lysis at the wound bed leading to endotoxin release to the wound bed as well as other proinflammatory mediators that may drive the prolonged inflammatory state of VLU which has been suggested as a mechanism for the chronicity of these wounds [20]. DACC based dressing results in physical removal of whole bacteria avoiding lysis and the subsequent deleterious effects on the wound. Previous pilot studies utilizing standard microbiology culture based methods have shown efficacy of DACC based dressing (Cutimed ® Sorbact®) in reducing bacterial load of VLU when compared to silver based wound dressing [19]. However,

these data are likely underestimating the scope of the problem as only 1-2% of bacterial species can be cultured using culture based methods.

In this study we use advanced molecular and microbiome methods to confirm DACC efficiency in patients with VLUs to help bridge this gap in knowledge. In addition, this exploratory study will add to existing knowledge about silver resistance in VLU and evaluate the effects of DACC treatment on VLU predisposed to be colonized with silver-resistant bacteria.

3. STUDY DESIGN

3.1 Overview:

This is an exploratory, single center, observer masked, active control, 1:1 randomized clinical trial. The goal is to compare the effectiveness of Study Device (Cutimed® Sorbact® wound contact layer) to an active control (silver contact layer Acticoat®)). Both devices are already approved and indicated for treatment of wounds. Standard care selected for use in this trial includes sharp debridement and compression with Jobst Comprifore® 4 layer compression system.

The study consists of three phases: two weeks screening phase (phase-1), up to four weeks treatment phase (phase-2), and two weeks follow up phase (phase-3).

In the screening phase i.e. phase-1, the Investigators will choose Study Ulcer, make baseline measurements and assessments and verify inclusion and exclusion criteria are met. Standard care will be applied without use of Study Device or active control.

The treatment phase i.e. phase-2 may last up to four weeks (if healing did not occur prior) and includes application of study dressings as well as application of standard care and measurements of primary and secondary outcomes. This will be achieved by tissue collection procedures conducted at visits T0, T2, and T3. Dr. Pastar and her research team will conduct the molecular microbiology and standard microbiology assessments of the specimens collected in this study. Gene expression (by qPCR) and standard microbiology will be utilized to characterize the mechanisms through which Cutimed® Sorbact® changes the bacterial load and promotes healing of chronic venous leg ulcers. Understanding the mechanism through which Cutimed® Sorbact® stimulates healing in patients may promote, improve and expand its clinical use.

In the two weeks follow up phase i.e. phase-3 subjects will return for one visit and secondary outcomes will be assessed and standard care provided. The subjects will then be discharged from the study and return to their wound care provider for further management as indicated.

3.2 Study outcomes:

3.2.1 Primary outcomes

Primary outcomes will be the change in bacterial load compared to baseline. (see appendix A – molecular microbiology protocol for details on quantification). Dr. Pastar's laboratory at UM Miller School of Medicine, Department of Dermatology and Cutaneous Surgery will be the coordinating center responsible for tissue sample receipt and

processing. Bacterial load in CFU/g among treatment and control arms will be calculated for each wound at visits T0, T2 and T3. The trajectories of bacterial load for each subjects and average change in bacterial load between groups will be compared.

3.2.2 Secondary outcomes

Secondary outcomes will include:

Microbiological outcomes: bacterial silver resistance characterization on molecular and phenotypic level over time (see appendix A for molecular microbiology protocol).

Clinical wound outcomes: healing rate and percent reduction in area over 6 weeks will be calculated by measuring wound area at each visit and dividing the area change by 6 to calculate average wound healing rate in cm^2/week and the average percent reduction for each study arm.

Complete healing at 6 weeks – the proportion of subjects in each study arm in which the study ulcer achieved complete healing at visit F1.

Pain related outcomes: pain assessments will be collected at every visit using visual analog scale (VAS) and time from visit T0 to clinically meaningful decrease in pain (i.e. 33% reduction from baseline) [21] will be calculated. Additionally, time to first indication of no pain will be calculated (i.e. VAS < 5mm)

Quality of life measurements: the EQ-5D-5L [22] and the Wound Quality of Life (WQoL) [23, 24] instruments will be used and assessments take place at visits T0, T4 and F1.

Safety outcomes: adverse events will be recorded at every visit and type and frequency logged and compared between arms.

4. Study population

4.1 Overview

Subjects for the study will be recruited from the University of Miami (UM) patient population as well as other patients referred to UM for the study. The recruitment goal for this pilot study is 30 subjects. Considering up to 15% drop out rate, the study will enroll up to 35 subjects with the goal of having 15 subjects in each arm complete the study.

4.2 Criteria for inclusion

Criteria for inclusion in the study:

1. Adults, 18 years old and older (i.e. age ≥ 18 years).
2. Venous leg ulcer (VLU) is present on the leg. VLU shall be full thickness but without exposure of deeper tissues (muscle bone or tendon).
3. If more than one ulcer is present, the largest ulcer meeting criteria shall be designated the study ulcer.
4. If more than one ulcer on the study ulcer limb, study ulcer shall be at least one centimeter from other ulcers.
5. Confirmed venous insufficiency, as documented up to 30 days prior to enrollment (Baseline Day 0), by either: a) Duplex ultrasonography, or b) Principal Investigator (PI) clinical assessment to include clinical signs and symptoms of venous ulcerations (e.g., hyperpigmentation of surrounding skin, varicosities, and/or lipodermatosclerosis).
6. Ankle-Brachial Pressure Index (ABI) exam with value of ≥0.80 in the affected limb
7. VLU has been treated with silver based therapy for at least 2 weeks within the previous 6 months.
8. VLU present for at least one month prior to screening visit 1.
9. VLU at least 1 cm in size but not larger than 100 cm^2.
10. After debridement, study ulcer demonstrates a clean wound bed.
11. If subject is a female of childbearing potential, subject must use at least one method of contraception acceptable by PI such as birth control pills, IUD, condoms, or sexual abstinence. At visit 1 urine pregnancy test must be negative.
12. Subject is able to comprehend all study related procedures and adhere to study schedule.
13. Subject is able to provide written informed consent.

4.3 Criteria for exclusion

Criteria for exclusion from the study:

1. Based on investigator medical judgment, ulcer is caused by any etiology exclusive of venous insufficiency.
2. Study Ulcer surface area (post-debridement) has increased or decreased by more than 30% in the period between screening visit 1 and treatment visit 1.

3. Study Ulcer exhibits clinical signs and symptoms of infection in the period between screening visit 1 and treatment visit 1 requiring oral antibacterial therapy.
4. Subject has known allergy to any of the materials used in the study.
5. Subject is unable to tolerate multi-layer compression therapy.
6. Based on investigator medical judgment, the Study Ulcer is suspicious for cancer (e.g. basal cell carcinoma or squamous cell carcinoma).
7. In the month prior to screening visit 1 subject was treated with systemic immunosuppressive medications for more than 2 weeks (e.g. chemotherapy, corticosteroids), and/or it is anticipated subject will require such medications during study period.
8. In the month prior to screening visit 1 subject was enrolled in any other research protocol for treatment of Study Ulcer.
9. The Subject has been diagnosed with malignant disease not in remission over the 5 years immediately preceding screening visit 1. (Except: cervical carcinoma in situ, cutaneous squamous cell carcinoma, cutaneous basal cell carcinoma that have been treated and have no evidence of recurrence or metastases).
10. Study ulcer area has been treated with radiation therapy at any time.
11. In the opinion of PI the subject has a medical condition such as autoimmune, renal, hepatic or hematologic disease that makes the subject an inappropriate candidate for participation in study.
12. In the month preceding screening visit 1 Study Ulcer has been treated with advanced tissue engineered devices matrix based devices (e.g., Apligraf™, Dermagraft™, Oasis™).
13. Subject is diagnosed with New York Heart Association Class III and IV congestive heart.
14. Failure: Class III: Symptoms with moderate exertion or, Class IV: Symptoms at rest.
15. Subject is diagnosed with diabetes mellitus that is poorly controlled and shall be defined as hemoglobin A1C >10%.
16. Study Ulcer is completely or more than 50% of the Study Ulcer is located on the foot (i.e. below the malleolus).
17. Subject is a female of childbearing potential, and refusing to use at least one method of contraception acceptable by PI such as birth control pills, IUD, condoms, or sexual abstinence.
18. Positive pregnancy test in screening visit 1 in a female of childbearing potential or active pregnancy or breast-feeding.
19. In the opinion of the PI the subject is unable to understand or comply with study related protocol including but not limited to providing informed consent.

4.4 Definition of study completion

A subject will be considered as completed the study if:

1. Subject healed during the treatment or follow up phase and completed all visits in the follow up phase.

2. Subject did not heal and completed all visits in the treatment and follow up phase.

3. Subject healed and then wound recurred and completed the follow up phase.

4.5 Subject withdrawal

A subject randomized into a treatment arm that has not completed the study as defined by section 4.4 will be considered incomplete.

Any subject is free to withdraw from the study without explanation and without future consequence on future care. Investigator will make every effort to obtain reason for withdrawal of subject especially eliciting any adverse event.

The PI may suspend or terminate a subject from the trial if deemed medically necessary. The reasons for suspension or termination will be recorded in the patient's study documents.

5. Study procedures

5.1 Subject identification:

After signing informed consent the subject will assigned a subject ID number beginning with BSN2018001, BSN2018002 and so on. All study related materials (e.g. source documents, investigational device etc.) will be labeled with the subject ID a priori.

5.2 Randomization and sequence allocation:

A random sequence of numbers from 1-16 will be generated in two columns (A= Cutimed® Sorbact® and B= Acticoat®) in Excel (Microsoft, Redmond, WA). 8 envelopes with a card labeled Cutimed® Sorbact® and 8 with a card labeled Acticoat® will be created, sealed and assigned the random numbers a priori before randomization of the first subject BSN2018001 by two people who are not part of the research team. Envelopes will be placed in secure location in number order. This procedure will generate a random sequence of 16 subjects and 8 subjects in each arm. This procedure will be repeated for another block of subjects 17-30, and 31-36. The block of 31-36 will only be used if the total randomization does not reach to n=30. Once a subject is randomized, the investigator will open the next envelope in the sequence and write the subject number on the card and include it as part of the record in the source documents. This will be the treatment assignment for the rest of the study.

5.3 Blinding:

Due to the nature of the study and products, investigator blinding is unrealistic and subject blinding is unlikely. Research staff will avoid naming the dressing or suggesting the assignment to the subjects. However, an independent monitor with experience in treating wounds will be blinded to the treatment assignment and will confirm healing outcomes based on images. Dr. Pastar and laboratory personnel will be blinded to the treatment assignments as well. Unblinding will occur when the study is officially closed for statistical analysis at https://clinicaltrials.gov/.

5.3.1 Breaking of blinding

The PI, Dr. Lev-Tov, is not blinded in this study. Dr. Pastar and her laboratory team will be blinded to the intervention. It is not likely that a need for unblinding of the laboratory team will be needed however in such extreme circumstance (e.g. unusual and severe infection in which detailed knowledge of bacterial species in the wound may expedite administration of life saving antibiotic therapy) at his discretion the PI may decide to break blinding. This will be documented and considered in the data analysis.

5.4 Informed consent

On visit S1 each subject will be given an informed consent form (ICF) and ample time to review the information. All questions will be answered by study staff. As detailed in section 8.2 no study procedure should commence prior to ICF signature and subject expression of clear understanding of study procedures.

5.5 Eligibility

Prior to randomization the PI and study staff will ensure subject met all inclusion and exclusion criteria. If all criteria are met, subject will be deemed eligible for participation and proceed to randomization.

5.6 Demographics

Basic demographic data on each subject will be collected at the S1 visit.

5.6.1 Demographic data

The following demographic data to be collected:

a. Name
b. Date of birth
c. Contact information (address, email address, telephone numbers)
d. Gender
e. Race
f. Ethnicity
g. History and current tobacco products use (pack years)
h. Height (in meters)
i. Body weight (in kilograms)

5.7 Quality of life assessments

The EQ-5D-5L and the WQoL instruments will be used and assessments take place at visits T1, F1 and F2. The EQ-5D-5L consists of 2 pages – the EQ-5D-5L descriptive system (page 2) and the EQ Visual Analogue scale (EQ VAS) (page 3). The descriptive system is comprised 5 dimensions: mobility, self-care, usual activities, pain/discomfort, anxiety/depression. Each dimension has 5 levels: no problems, slight problems, moderate problems, severe problems, and extreme problems. The subjects will be asked to indicate his/her health state by checking in the box against the most appropriate statement in each of the 5 dimensions. The EQ VAS records the respondent's self-rated health on a 20 cm vertical, visual analogue scale with endpoints labelled 'the best health you can imagine' and 'the worst health you can imagine'. This information can be used as a quantitative

measure of health as judged by the individual respondents. The instruments and detailed instructions are presented in appendix 9.8.

The WQoL instrument was developed specifically to address health related quality of life outcomes in people with wounds [24]. The WQoL is a short questionnaire measuring health-related quality of life (HRQoL) in chronic wounds and is composed of seventeen items attributed to three subscales on everyday life, body, and psyche.

5.8 Medical history

During the S1 visit pertinent medical history data will be collected from the subjects:

a. List of active medical conditions
b. List other non-active (resolved) medical conditions
c. For each diagnosis the diagnosis name, date of diagnosis and status (i.e. active / non-active) should be listed.
d. New diagnoses arising during study judged by PI as non adverse events will be recorded in the medical history section. Medical histories items should be categorized as follows:

i. Cardiovascular
ii. Dermatologic
iii. Endocrine
iv. Gastrointestinal
v. Genitourinary
vi. Hematologic
vii. Hepatic
viii. Immunologic
ix. Lymphatic
x. Neurological
xi. Other
xii. Psychiatric
xiii. Renal
xiv. Respiratory

5.9 Concomitant medications

All concomitant medication should be recorded and verbally confirmed with the subject during visit S1. Any change in medication (i.e. addition or discontinuation of a medication and any change in dose or frequency) should be recorded during all study visits. Specific attention should be given to pain medications and the following data should be recorded: pain medications prescribed specifically for wound pain versus pain medications written for non-ulcer related pain. For each pain medications specific dose should be listed in visit S1. For PRN pain medications the specific number of pills taken weekly should be estimated with the subject in every visit.

5.10 Study ulcer history

Specific history of Study Ulcer should be documented and the following data recorded:

a. Study ulcer duration. This will be defined as time lapsed since the last time the skin at the ulcer site was completely epithelialized without drainage or a scab.
b. Study ulcer location: Right versus left leg and specific location on the leg: proximal calf OR distal gaiter area, malleolus AND anterior, posterior, lateral OR medial.
c. Non study ulcer number and locations as above as well as shortest distance from Study Ulcer when on the same limb.
d. Current Study Ulcer treatment including cleaning methods, primary and secondary dressing and compression therapy.
e. Study ulcer treatments in the 30 days before visit S1 (record as above)
f. Any other previous Study Ulcer treatments.
g. Age at first VLU ever.
h. Total number of VLU ever.
i. History of VLU recurrence defined as opening of the skin with visible ulcer, drainage or scab in a location of a wound that was previously 100% epithelialized without drainage or scab.
j. History of deep vein thrombosis.
k. Specific history of lipodermatosclerosis, leg dermatitis, itch, edema, varicosities, edema etc.
l. Specific history of any surgical treatment for venous reflux such as sclerotherapy, vein stripping, vein ablation etc. on any limb.

5.11 Vital signs

At every study visit the following vital sign will be assessed: temperature (in Celsius), blood pressure (mmHg), heart rate (beats per minute), respiratory rate (breaths per minute), weight (kilograms), pain (VAS see assessment separately), peri-wound itch (Numeric Rating Scale (NRS), see appendix D), overall body itch (NRS, see appendix D).

5.12 Pain assessment

Pain is a stated outcome in this study. The intensity of pain at the study ulcer is to be assessed at every visit using VAS. The Visual Analog Scale is a validated pain assessment tool.[25] It is critical that pain assessment be conducted prior to removal of any dressing or compression system and prior to any physical manipulation of the limb. Pain assessments are conducted in all phases of the study as detailed in the schedule of assessments.

The VAS scale is 100 mm in length and only original copies placed in the subjects' source docs a priori are to be used. Photocopy of the scale may jeopardize measurements and later data analyses and therefore only the originals should be used. The scale marks the words "no pain" by the left end of the horizontal bar and "The worst pain I can imagine" by the right end of the bar. At each assessment the subject is asked to mark the level of

current pain by marking a notch along the bar. The distance from the left end of the bar to the notch is measured and recorded at each assessment.

For analysis and interpretation the following categories will be used:

0 - <5 mm - no pain
5 - <45 mm - mild pain
45 - <75 mm - moderate pain
75-100 mm - severe pain

5.13 Focused physical exam

The exam will be performed at visits detailed in the table of assessments i.e. the study calendar. Focused exam of the subject's lower extremities will be performed based on the systems described in table 2.

System	Assessment
Vascular	Pulses (femoral, popliteal, dorsalis pedis, tibialis posterior), skin temperature, capillary refill
Neurological	Sensory monofilament testing
Cutaneous	Presence of hyperpigmentation, edema, atrophie blanche, varicosities, scars, dermatitis

Table 2. Focused physical exam

5.14 Wound area measurement

Wound area will be measured at each visit as detailed in the table of assessments. The area is measured in centimeters squared. The area is measured using a ruler and measuring the length of the longest wound axis possible and multiplying by the length of the perpendicular axis.

Additionally, the wound will also be measured using the InSight™ system (eKare Inc.)[26]. Detailed operation instructions are found in appendix E.

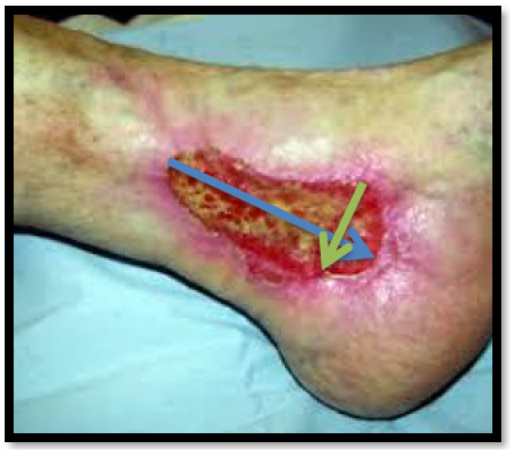


FIGURE 1. Wound area measurement

5.15 Product application

5.15.1 Standard care:

During the screening phase and follow up phase Study Device and Comparator are not applied. Instead, only the secondary dressing is used and applied directly to the wound bed. Dressing decision chart is shown in table 3.

5.15.2 Primary dressing:

Dressings are applied at all visits as indicated by the table of assessments. Study dressing is applied per randomization. After wound cleansing, exam, debridement, measurement and photography, wound dressing is applied to the wound. Dressing should cover the entire wound and overlap up to 1 cm beyond wound edges. Dressing should be in contact with wound bed.

5.15.3 Secondary dressing:

The primary dressing is secured in place by a secondary dressing. Choice of secondary dressing will be based on exudate level as detailed in table 3.

Secondary dressing decision chart: [27]

Classification	Description	Per protocol dressing
Dry	Wound bed is dry; there is no visible moisture and the primary dressing is unmarked; dressing may be adherent to wound.	Cutimed Hydro B

Moist	Small amounts of fluid are visible when the dressing is removed; the primary dressing may be lightly marked.	Cutimed Siltec B
Wet	Small amounts of fluid are visible when the dressing is removed; the primary dressing is extensively marked, but strikethrough is not occurring;	
Saturated	Primary dressing is wet and strikethrough is occurring; periwound skin may be macerated	Cutimed Sorbion
Leaking	Dressings are saturated and exudate is escaping from primary and secondary dressings onto clothes or beyond;	

Table 3. Secondary dressing decision chart

Secondary dressing is secured in place by the first layer of the compression system.

5.15.4 Dressing storage:

All study dressing will be stored safely in a locked space. Products will be stored properly and according the manufacture's storage instructions.

5.16 Tissue collection

Tissue collection will be conducted at visits T0, T2 and T3 as per appendix A. All collection containers are pre-labeled with the subject ID. After collection, the date is written on the container label.

5.17 Sharp debridement

Ulcer debridement is considered standard care. Debridement should be conducted as detailed in the table of assessments. Debridement allows proper wound measurement and assessment. Tissue with eschar, slough or other devitalized tissue will be removed and debridement material collected per appendix A. Sharp debridement will be conducted in this study using a curette, scalpel or other sharp device deemed appropriate by the Investigator.

Careful sharp debridement is to be performed as follows:

1. Study ulcer and the surrounding skin are cleansed with saline.

2. All non-viable tissue is removed from the study ulcer bed and edges using clean technique and avoiding excess debridement.

3. Direct pressure is applied to the wound to achieve hemostasis. Cautery is to be avoided as much as possible and only used if pressure methods failed to achieve hemostasis.

5.18 Compression

Four-layer compression will be standard care for all subjects. The Comprifore® system will be used as described in Appendix B.

5.19 Adverse events

5.19.1 Collecting Adverse Events

Study staff and PI are responsible for soliciting, recording and reporting any adverse event during the study period. In addition to unsolicited reports by subjects, the PI and study staff will open every visit by asking for any change in health or any other complaint since the last visit.

5.19.2 Definition of an adverse event

In this study an adverse event (AE) will be defined based on FDA CFR Title 21: “Adverse event means any untoward medical occurrence associated with the use of a drug in humans, whether or not considered drug related” [28]. Therefore an AE is considered as any unplanned medical occurrence that affects subject’s health and/or well-being. The adverse event need not be directly related to the study device or procedure. As such, an adverse event may include any unintended outcome such as a symptom or a new diagnosis or worsening or improvement of existing medical condition.

Serious adverse event (SAE) is defined according to FDA CFR title 21: “Serious adverse event or serious suspected adverse reaction. An adverse event or suspected adverse reaction is considered "serious" if, in the view of either the investigator or sponsor, it results in any of the following outcomes: Death, a life-threatening adverse event, inpatient hospitalization or prolongation of existing hospitalization, a persistent or significant incapacity or substantial disruption of the ability to conduct normal life functions, or a congenital anomaly/birth defect. Important medical events that may not result in death, be life-threatening, or require hospitalization may be considered serious when, based upon appropriate medical judgment, they may jeopardize the patient or subject and may require medical or surgical intervention to prevent one of the outcomes listed in this definition. Examples of such medical events include allergic bronchospasm requiring intensive treatment in an emergency room or at home, blood dyscrasias or convulsions that do not result in inpatient hospitalization, or the development of drug dependency or drug abuse”.

A SAE is therefore an AE that:

1. Results in death.
2. Is life-threatening at the time of the AE.
3. Results in an inpatient hospitalization or prolongation of existing hospitalization.
4. Results in a persistent or significant incapacity or substantial disruption of the ability to conduct normal life functions.

5. Is a congenital anomaly/birth defect.
6. May require medical or surgical intervention to prevent one of the outcomes listed above.

5.19.3 Documenting and reporting adverse events

All adverse events need to be recorded and reported to the local IRB and the sponsor in a timely manner and in accordance to local regulations.

5.19.4 Assessment of advance event causality

For each AE the PI is responsible for assessing the relationship between study procedure including application of study device and the AE in order to determine causality. The relationship of AE to study activities will be recorded in the AE log. The PI will use the following terms to describe relationship of AE to study activities:

Probable: the AE appears to follow a temporal sequence following study related activities, or has a dose relationship with study activities (i.e. worse when activity increased and visa versa) and cannot be attributed to other causes such as past medical history.

Possible: the AE appears to follow a temporal sequence following study related activities, or has a dose relationship with study activities (i.e. worse when activity increased and visa versa) but could be attributed to other causes such as past medical history and other therapies.

Unlikely: the AE does not appears to follow a temporal sequence following study related activities, and does not demonstrate a dose relationship with study activities (i.e. worse when activity increased and visa versa) and could reasonably be attributed to other causes such as past medical history and other therapies.

Cannot assess: the PI cannot make determination of causality at this time due to insufficient information and/or contradictory or unreliable information. The PI will frequently re-evaluate the AE in order to classify it into one of the classes above.

5.20 Fitting for stocking

Once healed, subjects will be fitted with compression stocking (Jobs Softfit) as per the manufacturer's instructions.

5.21 Instructions for follow up care

Once subjects are identified as healed, they will move into the follow up phase. Subjects who completed the treatment phase (regardless of healing status) will be moved to the follow up stage as well. While in the follow up phase, subjects will be returned to the care provider who referred them to the study and follow up care will consist of appropriate compression therapy, wound care (if needed) and other medically indicated treatments as determined by the PI.

5.22 Ankle- brachial – index (ABI)

ABI should be calculated for all subjects during visit S1. The protocol for measuring and calculating ABI is detailed in appendix C. The result of the test should be documented in the CRF.

6. Study conduct

6.1 Schedule of assessments

Assessments will be conducted as described in the protocol according to the schedule presented in Table 1.

6.2 Handling unscheduled visits

At any point during the study subjects may present for an unscheduled visit or may require an unscheduled visit at the discretion of the PI. Common reasons for unscheduled visits may include (but are not limited to): adjustment or reapplication of compression system, dressing overwhelmed with exudate, adverse event.

In the event of an unscheduled visit, the details of the visit will be recorded in the source documents and adverse events logged as needed.

6.3 Handling missed visits

It is preferred that subjects are seen at regular intervals. However, this may not be possible at times. If a subject needs to miss a visit, every effort should be made to reschedule as close as possible to the original date and then resume following visits at the original intervals. For example, if a subject regularly visits on Wednesdays and will miss a visit and a makeup visit is scheduled for that Friday, the next visit should return to the original intervals and be scheduled for the next Wednesday.

6.4 Stopping rule:

The PI may decide to stop the trial due to safety concerns at any time during the trial. The PI may consult the local Institutional Review Board (IRB) and/or Sponsor for safety related decisions.

7 Statistical methods

This study is a single center randomized longitudinal pilot study to investigate the effectiveness of Cutimed® Sorbact® (Study Device) compared to Comparator (silver impregnated contact layer dressing - Acticoat®) in modifying bacterial load in venous leg ulcers (VLU). Efficacy will be measured as the difference in change in bacterial load compared to baseline between two study arms i.e. Study Device vs. Comparator. This study will also

compare bacterial silver resistance based on genetic and phenotypic characterization over time, healing rate, complete healing at 6 weeks, pain, quality of life (QOL) measurements and adverse events as secondary endpoints.

We expect to study n=30 subjects for both the primary and secondary endpoints. The number of subjects estimated for the study (n=30) is not based on hypothesis driven statistical power calculation. Rather, as a pilot study, a goal for this investigation is to generate effect estimates that will assist in determining an appropriate sample size for future studies.

Prior to performing statistical analyses on quantitative study data, the data will be checked, screened and verified to ensure the integrity of the database. Data screening will focus on several aspects. We will determine the amount, the pattern, and randomness of missing data. We will define outliers as observations that appear to be very high or very low, and decide how to proceed based on the presumed cause. We will identify inconsistencies within a single variable and between variables. Initially, we will perform an explanatory data analysis; visually via graphics/plots and numerically by descriptive statistics with mean, standard deviation for measurements taken on a continuous scale, and frequencies and percentages, various types of cross-tabulations for measurements taken on a categorical scale. Corresponding confidence intervals (CI) for means and proportions will be calculated. To have a better understanding of the relationships among different study measurements, parametric or non-parametric correlation coefficients, bivariate/multidimensional cross-tabulations will be constructed. All the descriptive statistics will be reported for all subjects and by study arm.

The primary endpoint of the study, the bacterial load in CFU/g, will be calculated for each wound at visits T0, T2 and T3 among Study Device and Comparator arms. The difference in means of the bacterial load from T3 to T0 will be calculated and compared between study arms with Student's t-test. To be able to study the trajectories of the bacterial load over time, overall and by study arms, the time series plots will be depicted and repeated measures regression analysis will be performed where time and study arm as the main effects. Types of variance-covariance matrix will be determined by standard statistical procedures. We plan to expand the regression model and include other subject characteristics for additional covariate adjustment. Adjusted regression coefficients and corresponding 95% confidence interval (95%CI) will be reported for the study arm as the main effect in the regression models.

The secondary endpoints such as microbiological outcomes, clinical wound outcomes, pain related outcomes, quality of life measurements, and safety outcomes will be analyzed with similar statistical methods as the primary endpoint.

Due to the nature of a pilot study with limited sample size, regression models might have convergence problem i.e. regression estimates and corresponding 95%CI might not be estimable.

Therefore, we might explore different regression models as a secondary source of modeling approach. Regression model assumptions will be checked and model diagnostics will be done based on the appropriate statistical tools. Transformations of the data in order to meet statistical assumptions will be undertaken when indicated. Type-1 error will be set to 5% (alpha=0.05) and will be adjusted for multiple comparisons. Standard model diagnostic tools will be used. All of the statistical analyses will be carried out using SAS (SAS Institute Inc., Cary, NC) or R (R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/).

8 Regulatory and ethical issues

8.1 protocol institutional approval

The PI will submit the protocol and all related materials (e.g. ICF) for approval by the local Institutional Review Board (IRB) before any study related activity commences. All local and Federal regulatory rules will be followed and a dedicated regulatory monitor will be assigned to the protocol.

8.2 informed consent

During visit S1 and before any study activities are initiated, each subject will be provided with detailed information about the study and ample time to review ICF in order to obtain an informed consent. The subject (or legally authorized representative) will then be given the opportunity to ask any questions about the study and all questions will be answered. The subject (or legally authorized representative) will the sign and date the ICF according to the regulatory and legal requirements. ICF will be signed, dated and retained by the PI as part of the study records. No study related activities or procedures will take place before valid consent is obtained in an ethical manner and without.

In the case of a protocol amendment, the ICF will be revised to reflect said changes as required by IRB. Once revised, the ICF will be signed by all new subjects enrolled from that date forward as well as for all subjects still in the study (i.e. in the screening, treatment or follow up phase) when they are next scheduled to appear for a study visit.

8.3 Data handling, safety and quality assurance

The PI and study staff will be responsible for accurate and complete data collection.

The PI and the study staff will ensure that each subject's anonymity will be maintained and each subject's identity will be protected from unauthorized parties. The investigator will maintain all documents related to this study in strict confidence.

8.3.1 Source documentation

The PI and study staff will create case report forms (CRF) to record all study procedures and outcomes. CRF will be kept in binders dedicated to each subject upon enrollment. PI and study staff will keep all subject-specific materials in the subject binder. All general study related documentation such as study protocol, IRB communications, AE log etc. will be kept in the study binder.

8.3.2 Data safety

All collected data will be filed in study and subject binders. All binders are kept in the study coordinator's office which is locked. The office is located in a building with 24 hours security. Electronic data is not expected to be collected directly during the study except wound imaging. Wound imaging will be collected in a de-identified manner and stored on a secure server maintained by a vendor that is bound by US privacy regulations (See Appendix E). Data analysis will be conducted in a de-identified manner to ensure subject's confidentiality. None of the publications or presentations during and after study end will use any of the subject's personal identifier including images. If relevant, only non-identifiable images of wounds treated in the study will be used in future publications and/or presentations related to this study.

8.3.3 Data quality assurance

The PI and study staff will enter all data in the CRF in an accurate and complete manner. Regulatory personnel designated and authorized by the PI, Sponsor and IRB will periodically review all study materials for completeness and accuracy. They will produce a list of queries to the PI and study staff in order to resolve according the local Standing Operating Procedures.

8.4 Good clinical practice

The PI, study staff and the Sponsor are committed to ensure that all study procedures included in the protocol are carried out to the Good Clinical Practice guidelines set out by the International Conference on Harmonization. Additionally the PI, study staff and Sponsor are committed to abide by all legal requirements set by local agencies.

8.5 Study duration and premature termination

8.5.1 Each subject is enrolled in the study for eight weeks. It is anticipated that the study will be complete (i.e. last subject completed all eight weeks) within one year of first subject recruitment.

8.5.2 It is the intention of all parties to carry out the study to completion. However, if the PI, Sponsor, IRB or any other regulatory authority are made aware of any circumstances that suggest study subjects are at risk if the study continues, the PI or Sponsor, may decide on early termination of the study. Early termination decision will be taken in consultation between both PI and Sponsor.

9 Appendices:

9.1 Appendix A - Molecular Microbiology Protocol (Dr. Pastar's Laboratory)

PURPOSE

The purpose of this protocol is to describe the methods that will be used to evaluate modifying bacterial load and silver resistance in samples from venous leg ulcers (VLU) subjects who have undergone standard of care treatment with Study Device (Cutimed® Sorbact®) and standard of care treatment with active control (Acticoat®) over a 4-week period. Genetic analyses (by qPCR) and standard microbiology will be utilized to characterize the mechanisms through which Cutimed® Sorbact® modifies bacterial load and promotes healing of chronic VLU. Understanding the mechanism through which Cutimed® Sorbact® stimulates healing in patients may promote, improve and expand its clinical use.

INTRODUCTION

The goal of the study will be to investigate the effect and mechanism of action of Cutimed® Sorbact® on bacterial populations and their silver resistance in VLU. Cutimed® Sorbact® products are BSN's proprietary technology dressing device for treating VLU.

Background and Rationale: Our previous *in vivo* studies utilizing porcine wound model have shown that silver treatment induces expression of virulence genes important for biofilm formation in clinical isolates of methicillin resistant *Staphylococcus aureus* and *Acinetobacter baumanni* [15]. Furthermore incidence of silver resistance is an emerging problem in treatment of chronic wound pathogens , [12].

Design: The study is multicomponent involving a clinical study guided by Dr. Hadar Lev-Tov and molecular laboratory component by Dr. Irena Pastar.

The clinical study will enroll 30 patients with VLU who have been exposed to silver based therapy. Following a 2-week run-in phase, 15 subjects will be receiving Cutimed® Sorbact®, while other 15 subjects will receive common wound care product containing silver, Acticoat® in addition to standard of care (SOC). The treatment phase will last 4 weeks followed by a follow up visit 2 weeks later. The standard care involves weekly visits in which wounds will be cleaned and debrided. Debridement tissue from the wounds will be collected for molecular microbiological investigation.

Location: Subjects will be provided standard care at the UM dermatology clinical trials unit. The clinical team led by Dr. Hadar Lev-Tov is pursuing UM IRB approval. Dr. Irena Pastar and her team will conduct the molecular microbiology and standard microbiology assessments of the specimens collected in this exploratory patient outcome study.

RATIONALE

This research protocol will provide information that may further the understanding of Cutimed® Sorbact® treatment in accelerating wound healing via changes in microbial load and prevention of silver resistance. The bacterial load is increased in non-healing

VLUs and promoting a non-healing wound phenotype. We will also evaluate expression of specific set of silver resistance genes whose expression may be stimulated by prolonged Ag-based therapy. [14]

Tissue Collection Procedure

Debridement tissue samples will be taken from the edge of the wound and will be taken at the initial visit and 2 additional points during the trial. The tissue will be transported in a sterile container on ice to Dr. Pastar's lab immediately after collection. One half of the tissue will directly stored into -80˚C freezer for DNA isolation; the second half will be preserved in 80% glycerol for colony isolation using standard microbiology and analyzes of silver resistance on phenotypic level. The initial tissue removed will be considered the baseline and will be noted as T0.

Time Point Definitions

T0 – Pre-Randomization

T2 – Two weeks post-initial application SOC+ Cutimed® Sorbact® or SOC + Acticoat®

T3 – Three weeks post-initial application SOC+ Cutimed® Sorbact® or SOC + Acticoat®

DNA Isolation and qPCR

High bacterial burden (>10^5 organisms per gram of tissue), even without the classic signs of infection, is detrimental to healing [1, 10, 29]. Bacteria are routinely isolated from VLUs by culturing methods. However, standard microbiology techniques present barriers to accurate microflora quantification because many of the chronic wound bacteria are recalcitrant to growth in a culture and are therefore difficult to identify in this manner [30]; [31]; [1]. The availability of molecular PCR based methods has provided researchers with the capability to examine the bacterial composition of human tissue samples in unprecedented detail. This approach would be utilized to further our understanding of mechanism of action Cutimed® Sorbact® on bacterial populations in VLU.

VLU debrided tissue will be collected from patients at the initial clinic visit (T0) and at routine follow-up visits (T2 and T3). Collected tissue will be measured and immediately after preserved at -80˚C. DNA will be extracted from all samples using a solvent and grinding DNA extraction protocol (Bacterial/Fungal DNA isolation kit-Zymo Research). DNA quality and quantity control will be assessed by Real-Time PCR (qPCR) and Quant-It PicoGreen assay (Life Technologies), respectively. All of the methods required for DNA isolation and analysis are well established in Dr. Pastar's laboratory [32]; [33]; [34]. Quantification of all bacterial species in wound samples will be performed based on PCR amplification of 16S rRNA gene. The 16S rRNA gene (rDNA) is a DNA segment in all bacteria and archaea that codes for a structural RNA component of the small ribosomal subunit. The 16S rDNA is commonly used to quantify bacteria from human wound samples. We will employ 16S rDNA multiple hypervariable region to accomplish microbial quantification in VLU. Specifically, the 16S rDNA V1-V3 variable regions will be PCR

amplified to enrich the samples using the degenerate forward 27F.1 and non-degenerate reverse 518R primers that consist of sequences flanking the conserved hypervariable region. Separate PCR reactions will be prepared for each sample, including positive PCR control, and no template control. Real-time PCR will be performed and data will be analyzed using standard procedures established in Dr. Pastar's laboratory. Standard graphs will always be prepared from data accumulated at the same time as the test samples to act as internal controls. All qPCR reactions will be done in triplicate using the CFX96 real-time PCR system (Bio-Rad) and the PerfeCTa SYBR Green SuperMix (Quanta BioSciences). The relative CFUs will be calculated based on the standard curve. Assay results will be expressed as threshold cycle number (Ct) of the 16S rRNA gene copies per sample and tissue mass (mg). Standard curve will be used to determine relative CFUs based on Ct. Relative CFUs for tissue at two-weeks post initial treatment application (T2), and three weeks post treatment application (T3, if the percentage area reduction did not reach 75%). This qPCR based approach allows detection of bacteria which are difficult to cultivate and that would in all practicality remain undetected or underestimated by standard culture microbiology methods.

DNA standards for determining bacterial number by qPCR

Escherichia coli DNA is generally used as the standard for determining bacterial number by qPCR. However, to minimize the effect of variations in rDNA copy number on the calculation of bacterial number, DNA standards will be also prepared from two common VLU isolates, *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

Detection of genes for resistance to silver

Resistance to silver compounds as determined by bacterial plasmids and genes has been defined by molecular genetics. Plasmid pMG101 is a 180-kb silver resistance plasmid that also confers resistance to several antibiotics. The possibility of the horizontal transfer and the increasing use of silver for wound dressing has raised concerns that bacterial resistance to silver might proliferate in a manner analogous to that seen for antibiotics and thereby compromise its clinical utility. The sil operon comprises nine ORFs, seven of which are apparently structural genes (silE, silC, silF, silB, silA, ORF105 and silP) and two of which (silR and silS) encode a putative two-component regulatory circuit. To date, only the function of SilE, SilP and SilS has been determined. SilE, functions as a periplasmic silver-binding protein by restricting the accumulation of silver in the cell through a combination of silver sequestration in the periplasm. SilP is responsible for active efflux as an ATPase transporter, and SilS encodes a regulatory circuit. For detection of these three silver resistance genes, a PCR method will be used. DNA isolated from all tissue samples collected at all time points will be screened for the silE gene silP and silS genes. This PCR base approach allows for detection of silver resistance even in bacteria that may not be grown using standard microbiology approach. Detection of genes encoding for silver resistance will be performed in batches from DNA isolated from 2 samples and more. *E. coli* strain ATCC 25922 (with the complete sil gene cassette) will be included as a positive control. The agarose gel electrophoresis of PCR products for silS, silE, and silP genes will be employed to confirm the expected size of the amplicons.

The PCR products will be confirmed by sequencing and the sequence will be analyzed for homology with the sil genes from pMG101 (NCIB Accession number AG067954) and *E. cloacae* Ag703 (NCIB 679159).

Susceptibility determinations and selection/phenotypic characterization of silver-resistant strains

Once we determine samples positive for the silver resistance genes we will further investigate whether the bacteria possessing Ag-resistant genes express this trait phenotypically. Bacteria will be isolated from the preserved glycerol stocks using standard microbiological methods (culturing on selective media). Tissue samples will be evaluated for the bacterial growth using microbiology methods. Culture condition will include both anaerobic and aerobic growth to enhance the level of bacterial detection. To recover bacteria serial dilutions from preserved stocks will be made from all tissue samples. The extent of microbiological colonization will assessed using the Spiral Plater System. All plates will be incubated both aerobically and anaerobically at 37˚C, after which the number of viable colonies will be counted. After the incubation period, colonies on the plates will be enumerated and the CFU/mL calculated. Similar methods have been used in our laboratories to evaluate the antimicrobial efficacy of various topical agents [33, 34]. All isolates will be tested for growth on LB agar supplemented with 250 µM Ag+ using patch method. Further Ag resistant phenotype will be confirmed for individual isolates using minimal inhibitory concentration (MIC) method.

MIC test to determine susceptibility to silver nitrate

The MIC of silver nitrate will be determined for all isolates capable for growing on LB agar supplemented with 250 µM Ag+. Bacteria will be suspended in Iso-Sensitest broth (Oxoid Ltd., USA). After an overnight incubation at 37°C, 10 µl of the bacterial suspension will be inoculated into a series of tubes with increasing concentrations of silver nitrate (4 to 512 mg/liter) in the same broth. The final bacterial concentration will be 105 CFU/ml. After 24 h of incubation, the MIC will be recorded as the lowest concentration yielding no visible growth. A silver nitrate MIC of >512 mg/liter will classify the bacterium as silver resistant. *E. coli* strain ATCC 25922 will be used as a control. Isolates confirmed for silver resistance will be preserved and further identification of bacteria on a species specific level will be proposed to sponsor.

Data Analyses and Reporting

qPCR Data will be analyzed using the software Prism (Graphpad, La Jolla, CA, USA). Raw data for qPCR will be provided to sponsor in txt format. All results including microbiology analyses, silver resistance and quantification of bacterial load will be presented in the final study report including analysis of data. PCR results will be expressed as threshold cycle number (Ct) of the 16S rRNA or silver resistance gene copies per sample and corresponding CFU/g tissue; the mean and standard deviation will be calculated for each time point. The longitudinal changes in bacterial load as

determined based on qPCR data analyses gene will be examined individually for each tissue. After counting the colonies for the MIC analyses, the data will be tabulated and the Log of colony forming units/g (Log CFU/g) determined. Dr. Pastar will provide final results to include raw data.

9.2 Appendix B – Application of Comprifore system

Comprifore compression system application instructions:

The application of JOBST® Comprifore #1 to #4:

Insure the ankle is dorsoflexed at 90 degrees when applying each bandage (toes to the nose). Place Comprifore #1 at the base of the toes, wrap the foot twice to anchor the bandage firmly.

Cover the heel and proceed up the leg using the spiral wrapping technique at 50% overlap with low tension. Finish just below the knee. Excess bandage can be removed and used as additional padding if required.

Apply Comprifore # 2 bandage with two anchor turns at the base of the toes, cover the heel and proceed with 50% overlap using the spiral technique and just enough tension to fully extend the bandage. Cut off and discard any excess bandage.

Apply Comprifore # 3 light compression with two anchor turns at the base of the toes, cover the heel and proceed with 50% stretch using the Figure of Eight technique to generate the correct pressure and improve pressure distribution. Cut off any excess bandage.

Apply Comprifore # 4 with 2 anchor turns at the base of the toes, cover the heel and proceed with a spiral technique at 50% overlap and 50% extension to the base of the knee. Cut off any excess bandage.

Gently smooth and squeeze the outer layer to smooth and settle the layers and secure the system in place.

9.3 Appendix C – Ankle brachial index measurement

Technique:

1. Check that all needed equipment is available and functioning properly.
2. Explain to the subject what you are about to do and inform them there may be slight discomfort as the cuff inflates.
3. Place the subject in a supine position. Ensure the arms and legs at the same level as the heart.
4. Hold the subject in this position for 10 minutes before measurement.
5. Apply appropriate size blood pressure cuff to the arms and ankles and obtain systolic pressure.

Note: cuff size should be at least 20% greater than the limb diameter

Note: apply the ankle cuff to the leg with the study ulcer, above the malleolus leaving enough room to access the tibialis posterior artery.

6. Obtain systolic pressure on the leg with the study ulcer from the tibialis posterior and the dorsalis pedis arteries.
7. Calculate the ABI using the higher systolic pressure of the arms and ankle using the following formula:

ABI = higher brachial pressure / higher ankle pressure

9.4 Appendix D – itch assessments

Itch Numeric Rating Scale:[35]

Please circle the number that best describes the level of itch around you wound in the last 24 hours:

0	1	2	3	4	5	6	7	8	9	10

No itch — Worst imaginable itch

Please circle the number that best describes the level of itch over your whole body in the last 24 hours:

0	1	2	3	4	5	6	7	8	9	10

No itch — Worst imaginable itch

9.5 Appendix E – Insight wound imaging system

inSight™ 3D Measurement Guide

LL-5005 Rev. B 07/27/2016

Learn how to obtain accurate and consistent 3D measurements

① Start Measurement

Click "Measure Wound" in Wound Summary page

② Point & Shoot

Follow the crosshair guide for optimal imaging distance (40cm, guide turns green), then capture image.

③ Define Wound Border

a. Roughly sketch outside the wound
b. Roughly sketch inside the wound
c. Border is automatically defined
d. Adjust border as necessary or use manual tracing
e. Click "Measure"

④ Define Orientation

a. Rotate compass on upper left to define direction of Length vs. Width
b. Measurements are shown on right
c. Click "Save"

Best Practices

1. Hold the sensor perpendicular to the wound bed
2. Capture image from the optimal distance (follow the crosshair guide)
3. Ensure adequate ambient lighting
4. Do not use sensor during or immediately after charging the sensor (charging iPad is okay)

Know the Limitations

1. Tunneling and Undermining: *we can't measure what we can't see*
2. The tip of a structure (eg. Toes):

What's the "depth" of this wound?

3. Circumferential wounds: *coming soon with inSight™ 2.0*

Need Additional Help?

Log on to portal.ekareinc.com to access the **Help Center** in the main menu.

eKare, Inc. 8280 Willow Oaks Corp. Dr. Suite 600
Fairfax, VA 22031, USA
For more information, visit www.eKareinc.com
info@ekareinc.com 1-844-443-5273

inSight™ Starter Guide

LL-5006 Rev. B 07/27/2016

Welcome to the new era of wound measurement!

What's In the Box?

- iPad tablet
- Structure Sensor
- Battery Charger x2
- Lightening Cable
- Data Cable
- Manuals

inSight™ comes pre-calibrated

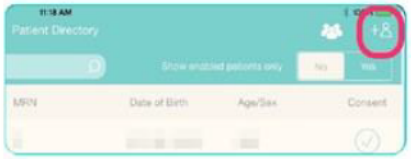
① Getting Started

Connect your Sensor to iPad as shown. Note location of charging port (in red).

Access eKare app on **Home** screen:

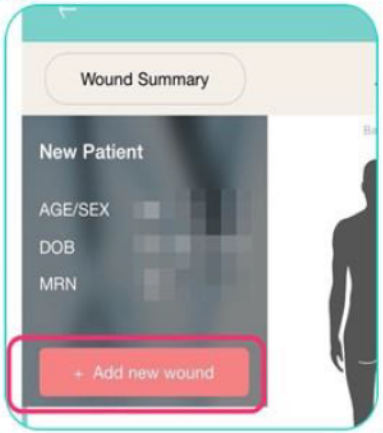
- Ensure good lighting condition
- Ensure stable internet connection

② Create a Patient



Need to obtain patient **consent** to store images.

③ Create a Wound



④ Start 3D Measurement

That's it! You got the 3D measurement.

Need Additional Help?

Log on to portal.ekareinc.com to access the **Help Center** in the main menu.

Alternatively, go to Menu -> [Help] -> [Help Center] in the eKare mobile app.

eKare, Inc. 8280 Willow Oaks Corp. Dr. Suite 600
Fairfax, VA 22031, USA
For more information, visit www.eKareinc.com
info@ekareinc.com 1-844-443-5273

9.6 Appendix F – Acticoat ® application instructions*

Application:
Follow standard protocol to cleanse wound; do not use oil-based cleansing agents.
For heavily exudative wounds:
Remove the ACTICOAT dressing from the package and cut to size.
Apply the dry ACTICOAT dressing to the wound, either side down, as the exudate will be sufficient to activate the dressing.
Cover the ACTICOAT dressing with an absorbent secondary dressing.
Complete the dressing with appropriate gauze wrappings if necessary.

For all other wounds:
Remove the ACTICOAT dressing from package and cut to shape.
Moisten the dressing with sterile water (do not use saline).
Allow the dressing to drain on an absorbent surface in a sterile field for at least 2 minutes.
Apply the ACTICOAT dressing to the wound surface, either side down.
Cover the dressing with a moist absorbent secondary dressing which may be prepared by saturating gauze with sterile water and wringing out the excess water.
Complete the dressing with appropriate gauze wrappings if necessary.
If the dressing dries and adheres to the wound, moisten or soak the dressing prior to removal.
Avoid forceful removal of the dressing and disruption of the healing wound.

* Source: http://www.smith-nephew.com/professional/products/advanced-wound-management/acticoat/acticoat/

9.7 Appendix G

Wound-QoL questionnaire on quality of life with chronic wounds

With the following questions, we aim to find out how your chronic wound(s) affect(s) your quality of life.
Please tick one box per line!

	In the last seven days …	not at all	a little	moderately	quite a lot	very much
1	…my wound hurt	O	O	O	O	O
2	…my wound had a bad smell	O	O	O	O	O
3	…there was a disturbing discharge from the wound	O	O	O	O	O
4	…the wound has affected my sleep	O	O	O	O	O
5	…the treatment of the wound has been a burden to me	O	O	O	O	O
6	…the wound has made me unhappy	O	O	O	O	O
7	…I have felt frustrated because the wound is taking so long to heal	O	O	O	O	O
8	…I have worried about my wound	O	O	O	O	O
9	…I have been afraid of the wound getting worse or of new wounds appearing	O	O	O	O	O
10	…I have been afraid of knocking the wound	O	O	O	O	O
11	…I have had trouble moving about because of the wound	O	O	O	O	O
12	…climbing stairs has been difficult because of the wound	O	O	O	O	O
13	…I have had trouble with day-to-day activities because of the wound	O	O	O	O	O
14	…the wound has limited my leisure activities	O	O	O	O	O
15	…the wound has forced me to limit my activities with others	O	O	O	O	O
16	…I have felt dependent on help from others because of the wound	O	O	O	O	O
17	…the wound has been a financial burden to me	O	O	O	O	O

"Wound-QoL" questionnaire on Health-related Quality of Life in Chronic Wounds ● Augustin et al. 2014; Blome et al. 2014

9.8 Appendix H - EQ-5D-5L instrument

Under each heading, please tick the ONE box that best describes your health TODAY.

MOBILITY

I have no problems in walking about ❑
I have slight problems in walking about ❑
I have moderate problems in walking about ❑
I have severe problems in walking about ❑
I am unable to walk about ❑

SELF-CARE

I have no problems washing or dressing myself ❑
I have slight problems washing or dressing myself ❑
I have moderate problems washing or dressing myself ❑
I have severe problems washing or dressing myself ❑
I am unable to wash or dress myself ❑

USUAL ACTIVITIES *(e.g. work, study, housework, family or leisure activities)*

I have no problems doing my usual activities ❑
I have slight problems doing my usual activities ❑
I have moderate problems doing my usual activities ❑
I have severe problems doing my usual activities ❑
I am unable to do my usual activities ❑

PAIN / DISCOMFORT

I have no pain or discomfort ❑
I have slight pain or discomfort ❑
I have moderate pain or discomfort ❑
I have severe pain or discomfort ❑
I have extreme pain or discomfort ❑

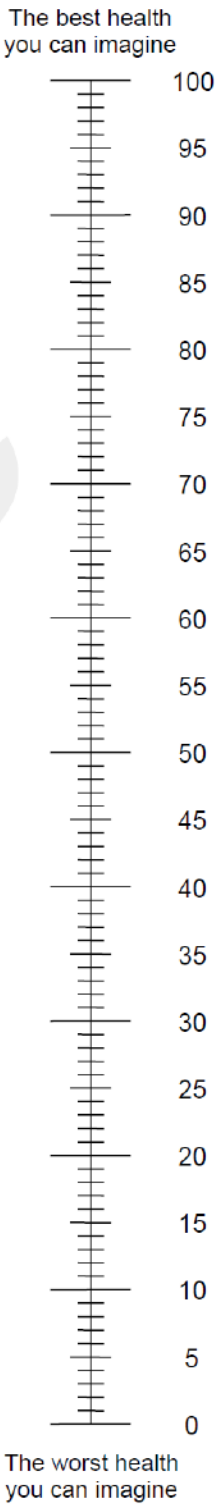
ANXIETY / DEPRESSION

I am not anxious or depressed ❑
I am slightly anxious or depressed ❑
I am moderately anxious or depressed ❑
I am severely anxious or depressed ❑
I am extremely anxious or depressed ❑

2

- We would like to know how good or bad your health is TODAY.
- This scale is numbered from 0 to 100.
- 100 means the best health you can imagine.
  0 means the worst health you can imagine.
- Mark an X on the scale to indicate how your health is TODAY.
- Now, please write the number you marked on the scale in the box below.

YOUR HEALTH TODAY = ☐



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