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14. ABSTRACT

It is the objective of this study to identify the biomarkers associated with the earliest stages of healing in chronic wounds. The findings of this study are intended to facilitate the development a diagnostic tool, which would evaluate the healing process. Samples have been collected from interior and peripheral wound sites over 6 weeks from 17 subjects thus far and analyzed using 2-D page. 161 spots/proteins have been identified that change over time in chronic wounds. The peripheral and interior sites sampled have different proteins present. Some proteins were present only in interior samples and not peripheral samples and vice versa. A series of shadow spots have been identified and linked with healing in chronic wounds. A number of experimental protocols have been developed during this first year including two data analysis methods. The identification of a series of shadow proteins, which appear in wounds healing and disappear during non-healing periods, is the first important step in the identification of biomarkers associated with healing chronic wounds.

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INTRODUCTION

The fact that there are differences in chronic versus normal healing wounds is well documented. What is unknown at this time are the specific biomarkers associated with healing wounds, the role each of these biomarkers play in wound healing, and the biomarkers that can serve as the earliest predictors of healing. It is our hypothesis that specific cytokines, proteases, and growth factors serve as the earliest indicators of healing in chronic wounds. It is the objective of this study to identify the biomarkers associated with the earliest stages of healing in chronic wounds. The findings of this study are intended to facilitate the development a diagnostic tool which would evaluate the healing process.

BODY

Statement of Work

Technical Objective 1: To identify the biochemical changes that occur as a chronic wound begins the healing process.

a. Analyze fluid samples to determine proteins present
b. Identify differences between subjects and subject time points
c. Confirm protein identities

Technical Objective 2: To assess the rate of healing of the wounds analyzed.

a. Measure wound
b. Calculate trajectories of healing for wounds over time

Technical Objective 3: To evaluate the location of the biomarkers assessed.

a. Compare proteins found in different locations using protein analysis

Technical Objective 4: To identify the earliest changing biomarkers occurring in wounds which progressed toward healing.

a. Correlate the changes in wound chemistry with the rate of healing
b. Analyze the earliest biochemical changes present

Technical Objectives 1,2,3, & 4

All four technical objectives rely on the enrollment of human subjects in the trial and collection of samples. Approval to enroll human subjects was received in February 2005 from the Daemen College Human Subjects Research Review Committee and on October 6, 2005 from the Catholic Health System Institutional Review Board. The U.S. Army Medical Research and Materiel Command Human Subject Research Review Board (HSRRB) approved enrollment of human subjects on November 21, 2005.

Sixty (60) subjects have been screened and seventeen (17) subjects have been enrolled. Eighteen (18) subjects were agreeable to discussing the study and were

interested in participating, but three (3) were unable to enroll due to a treatment involving topical growth factors or type of wound present (venous stasis ulcer in five (5) cases and one (1) vascular), four (4) patients did not have sufficient wound drainage, four (4) were discharged to hospital or home, and 1 subject was confused and unable to give consent. Eight (8) subjects gave consent to participate in the study, but were not enrolled. Three (3) of these subjects were nearly healed and did not allow for wound swabbing, one (1) subject's wound was vascular, one (1) subject was discharged, one (1) subject's wound was the site of a previous amputation and the wound was too large for a single photograph and wound measurement employing the software, and two (2) subjects in this group declined after the family had given consent for the subject. Eight (8) subjects discussed the study, but did not give consent. Two (2) subjects agreed to meet, but were hospitalized prior to discussing the study and eight (8) subjects declined to meet the research clinician or discuss the study.

An extension was requested and received to allow for greater enrollment in the study. Also, three additional sites have been added since the beginning of the study and an additonal ten sites are being added pending federal wide assurance numbers approval of the relevant institutional review boards and the U.S. Army Medical Research and Materiel Command Human Subject Research Review Board. Additionally, reimbursement for the subjects participating in the trial ($50) has been approved and will begin with the next enrolled subject. It is anticipated that the addition of more facilities and subject reimbursement will further increase enrollment and allow the study to be completely enrolled.

Technical Objectives 1, 3, &4:

Technical objectives 1, 3, and 4 of the scope of work are dependent on analysis of the subject samples. A number of experimental protocols were developed in order to guarantee consistency between samples. Protocols for protein isolation and purification of wound fluid proteins, protein quantitation, sample storage, isoelectric focusing of wound fluid proteins, second dimension SDS electrophoresis of wound fluid protein, as well as gel staining, image acquisition, and archiving of the resulting data were developed prior to running any experiments on the collected samples. The details of these accomplishments are described in the Key Research Accomplishments section of this report.

Two-dimensional polyacrylamide gel electrophoresis (2D-Page) is a technique enabling separation of thousands of proteins based on molecular weight and isoelectric point. The analysis of the gels provides a unique challenge. Although software for this function is available and used in our laboratory (PDQuest 2-D Analysis Software, version 7.4) it is difficult for the software to correctly identify hundreds of protein spots, and the interaction and subjective input of the operator is critical to the process. The literature has identified and described at length the need to accurately detect spots and remove noise from the images, as well as the possible loss of information as a result of using the software tools (Roy et al, Salmi et al, Woodward et al).

Technical Objectives 1a,b,c,3a,4a & b:

In order to identify the spots of interest, as well as to confirm the identity of the proteins, two distinct methods were developed. Method 1 utilizes a manual technique relying on human operators and Method 2 utilizes the PDQuest 2-D Analysis Software.

Method 1:

A method for analyzing gel images was developed to independently identify and catalogue protein spots as potential biomarkers. This manual method was developed for its simplicity and lack of dependence on the analysis software to filter or analyze gels, which eliminates the potential for loss of spots associated with the software.

The PDQuest 2-D Analysis Software was used to create a standardized template of 12 cropped raw gel images. A matchset was used to align the gel images, but it was not used for any analysis of data. A screenshot of the standard template was taken and printed. Figure 1 shows this template of raw gels. The gels were analyzed via comparison of quadrants of each gel throughout the different time points per subject. To accomplish this in a consistent manner, a second template with 12 distinct 9 quadrant grids was printed on clear transparency and laid over the gel screen shot template. Figure 2 shows the second template. All gels were analyzed first qualitatively (rough spot characteristics and appearance/disappearance of spots) and then quantitatively (numbers of spots per segment). Two operators reviewed gels from all time points for each interior and peripheral samples for all of the subjects enrolled to date. The data was compared on the macro scale to identify spots of interest. Initially, 201 spots of interest were identified.

Figure 1. Template of 12 raw gels from samples from same subject.

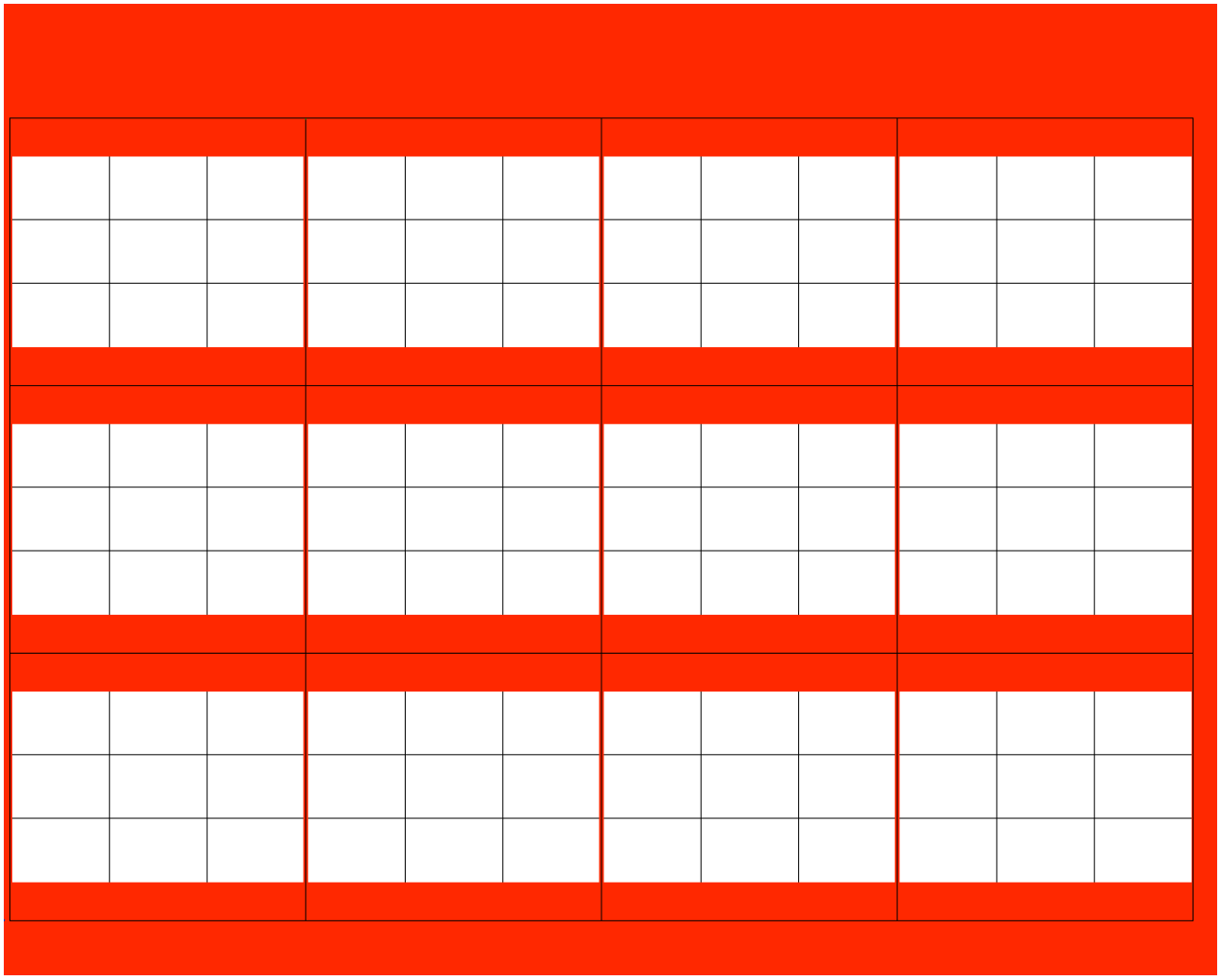

Figure 2. Template used to overlay gel template to provide a visual guide to allow operators to identify changing spots in the gels.

Technical Objectives 1a, c & 3:

After identifying spots of interest on the 2-D PAGE images, it was necessary to identify the proteins within these spots. 2-D gels separate proteins based on isoelectric point (pI) and molecular weight (MW), which can be measured by extrapolating to each axis of the gel. However, because gels must be individually cropped and due to variances in running time and gel distortion, the MW and pI values are not constant on the axes for all gels. To minimize any error in measuring pI and MW, a two-step approach was taken. First, a reference protein common to all gels was identified. Two gels were run. The first contained the Bio-Rad 2-D PAGE protein standard alone. The second gel contained both serum from a subject with a strong reference protein and 2-D PAGE protein standard, with concentrations adjusted for 250 ug total protein.

After imaging, a matchset was made of the two gels to include only the commercial protein standards and subject reference protein. The combination of these

two protein mixtures allowed for a grid to be created that could be normalized to any gel based on location of the reference protein. The MW and pI grid were plotted on the matchset. A graphic of this grid was taken, including a spot indicating the reference protein, and printed out on transparency paper. This transparency was then be laid over experimental gel printouts and used to categorize spots of interest by PI and MW. Figure 3 shows the grid utilized.

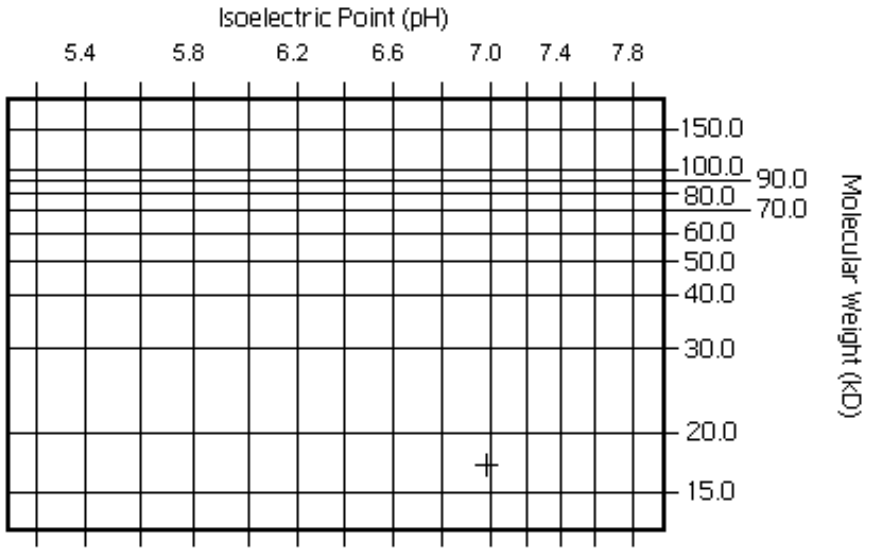

Figure 3. Grid used to overlay gels to identify PI and MW for proteins of interest.
+ indicates reference protein.

Technical Objectives 1b & 4a:

At this time spots and proteins have been compared between time points for the same subjects using Method 1. Initial findings are very interesting in this regard. Figure 4 shows three gels for 3 different time points for 1 subject with the spots of interest outlined in yellow. Figure 5 shows these same spots at a greater magnification where differences are evident. The gel on the right in Figure 5 shows a second set of "shadow" spots evident in subjects when healing has occurred between two sample time points. These shadow spots were evident in a number of subjects that had experienced healing during that particular time frame, but who had not necessarily experienced healing overall or during the entire course of sample collection. As a wound heals, regresses, and then heals again, these shadow spots, only present while a wound is healing, will therefore appear, disappear and then reappear accordingly.

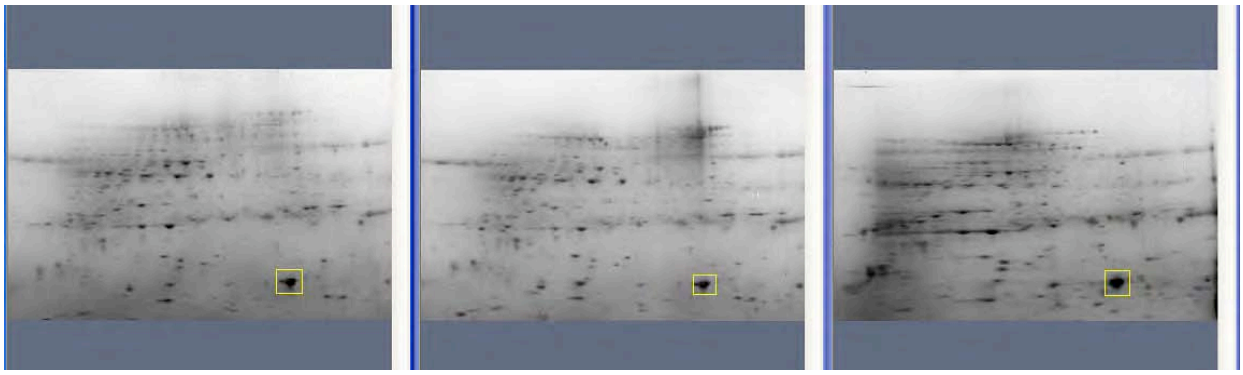

Figure 4. Gels from the same subject at 3 timepoints.

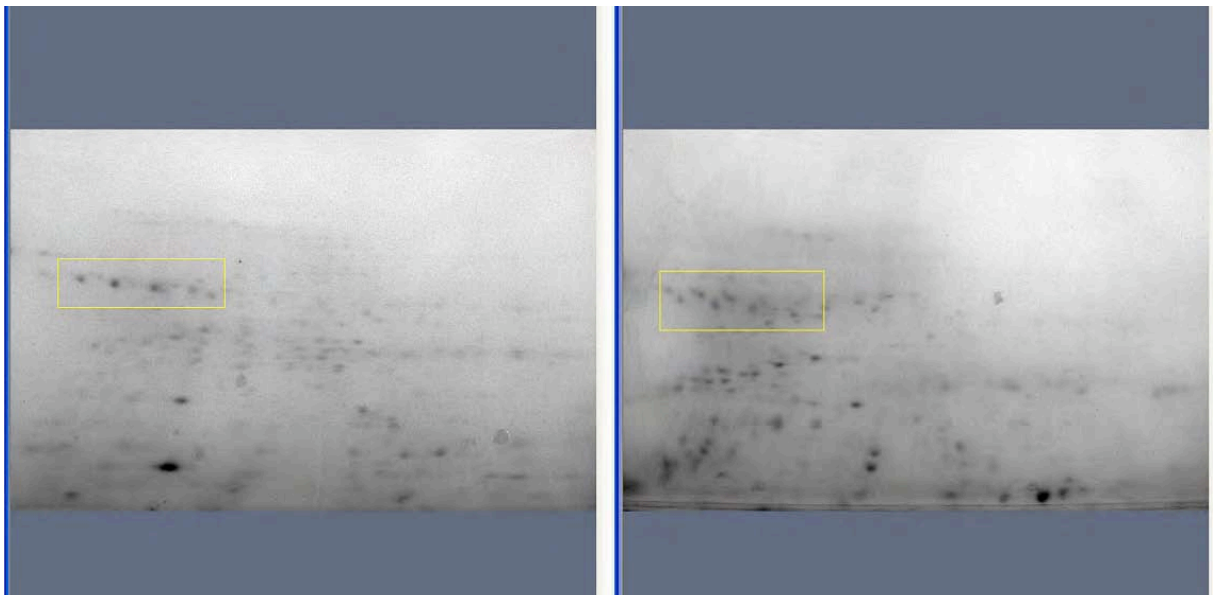

Figure 5. Spots of interest magnified with shadow spots apparent in gel on the right versus gel on the left.

Technical Objective 1a,c

In order to reduce repetition, operators organized a spreadsheet consisting of the arbitrary numbers assigned to each of the original 201 spots of interest and the correlating pI's and molecular weights of each. Operators sorted the information first by ascending pI then by ascending molecular weight to show similarities in point characteristics. The evident similarities indicated possible duplicates in initial point documentation. Operators then manually evaluated points they deemed similar, according to the pI's and molecular weights, to assure the repetition of the suspected points. Operators assigned all duplicates

specific letter names, which will be the points' titles for the remainder of this portion of the study. This, in turn, reduced the number of spots of interest on the list to 161 and eliminated repetition. Figure 6 shows a portion of this database.

New Title	I or P	MW (kD)	pI (pH)
A	P	50.0	5.15
A1	I	19.0	5.49
A2	I	21.2	5.64
A3	I	32.0	5.98
A4	I	48.0	6.57
A5	P	49.9	7.16
A5	P	50.0	7.09
B	P	19.0	5.20
B1	I	21.7	5.49
B1	I	20.0	5.49
B2	I	45.2	5.66
B3	P	19.3	6.05
B4	I	57.0	6.57
B5	I	70.0	7.10
C	I	28.5	5.27
C1	I	28.1	5.49
C2	P	72.0	5.66
C3	I	15.8	6.09
C4	P	16.0	6.58
C5	P	17.3	7.15
D	P	20.0	5.30
D1	I	22.9	5.50

Figure 6. Part of the database of 161 spots of interest. Spots are identified by MW and PI. I indicates interior sample spots and P indicates peripheral sample spots.

The ExPASy TagIdent Tool database (http://www.expasy.ch/tools/tagident.html) was used for each spot identified. Settings used for this database were recorded pI with a range of 0.25 and MW within 1%. All data was sent to an email account created specifically for the project and then copied to the computer. All data was rearranged into spot-specific documents and saved. All search results are being assimilated into a database for easy searching and archiving.

This information then can be used as a precursor to mass spectroscopy for definitive identification of proteins of interest. Based on the combined analysis the proteins/spots of interest will be identified, then cut out and sent for mass spectroscopy identification.

The other method used to analyze the data (Method 2) utilized PDQuest 2-D Analysis Software, version 7.4, build 036.

Method 2:

First-level Matchsets

After image acquisition, all gels were cropped to uniform size using stored settings in the Advanced Crop tool. Gel image files were organized into datasets based on subject number and swab location (i.e. interior vs. periphery). Matchsets were created for each dataset using the Automated Detection and Matching tool. Following spot detection, each gel was reviewed to ensure that only legitimate spots have been marked. Spots resulting from staining artifacts, etc. were removed by the operator. In rare instances spots were added; for example, in the case where a legitimate spot was obscured by a staining artifact. Automated matching was then performed again and any gels (usually those which were faint) that do not match well were selected for spot redetection using more inclusive detection parameters. In this manner two first-level matchsets, one for interior samples and another for periphery samples, have been created for each subject. Analysis of first-level matchsets allows us to see which proteins change over time in each dataset.

Three subjects (BM 002, BM 004, and BM 010) were included in the analysis because of the very high quality datasets available for these subjects. Less-than-perfect gels (i.e. those which are faint, have poor resolution, etc.) are nearly impossible to incorporate into a matchset and poor image quality hinders the quality and accuracy of the analysis. Two gels per sample is the minimum number required for grouping gels using the Replicate Groups feature, which greatly enhances matching efficiency in the software.

Higher-level Matchsets

The organization of first-level matchsets into higher-level matchsets allowed us to make more general analyses using data from multiple subjects. The interior matchsets for subjects BM 002, BM 004, and BM 010 were organized into a single matchset called BM I. This allows us to compare changes that occurred in the interior samples from all three subjects. Another higher-level matchset, BM P does the same for periphery samples. Further, organization of the BM I and BM P higher-level matchsets into yet another higher-level matchset allows us to compare BM I versus BM P and observe differences between interior and periphery samples for all three subjects.

Use of the Analysis Set tool within the BM I and BM P matchsets allowed us to identify spots which are present on all interior gels or all periphery gels. The same Analysis set tool used within the BM I versus BM P matchset allows use of Boolean operators to do analyses such as identifying spots that are present in interior but not periphery samples (I not P), and vice versa (P not I). Another possibility is to identify spots which are present on all interior and all periphery gels (I and P), which represents a set of proteins that may be thought of as being universally expressed in wound fluid. The Spot Review tool is another especially useful tool in that it generates histograms representing the expression levels of each protein in the matchset. This allowed us to view quantitative data for each protein and observe trends in protein expression.

Figure 7 and 8 display a number of spots, which via assessment of the first three subjects analyzed, are found to be present in only interior or only peripheral samples. These data show that there are indeed different biochemical events occurring in the interior and peripheral environments.

MW (kDa)	pI (pH)
18	5.35
21	5.37
60	5.22
60	5.35
32	5.50
33	5.68
30	6.35
38	6.72
25	6.86
22	7.30
44	7.79

Figure 7. Spots which are unique to interior samples for 3 subjects over all time points.

MW (kDa)	pI (pH)
53	5.35
62	5.23
62	5.32
10	5.44
91	5.59
48	5.73
46	5.95
48	6.28
82	6.22
82	6.30
28	6.40
60	6.55
69	6.63
11	6.77
16	6.90
69	6.88
17	7.52
31	7.20
26	7.90

Figure 8. Spots which are unique to peripheral samples for 3 subjects over all timepoints.

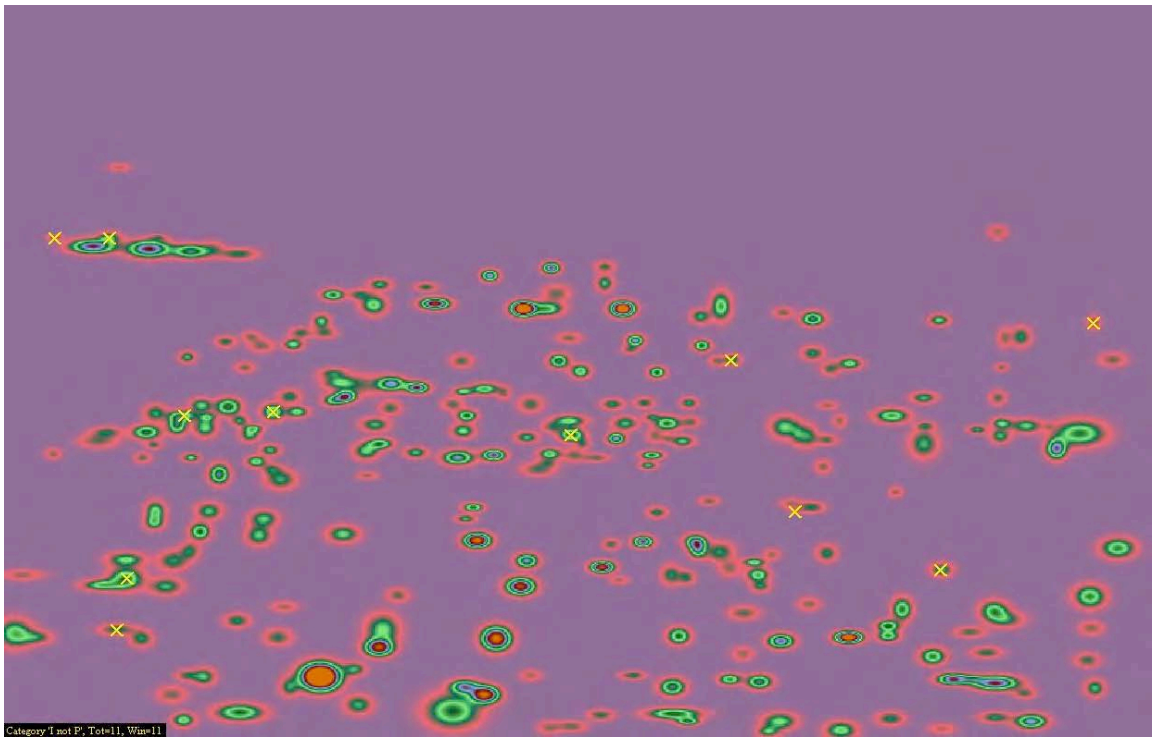

Figure 9. Spots identified with yellow x's are found only in interior samples for 3 subjects analyzed.

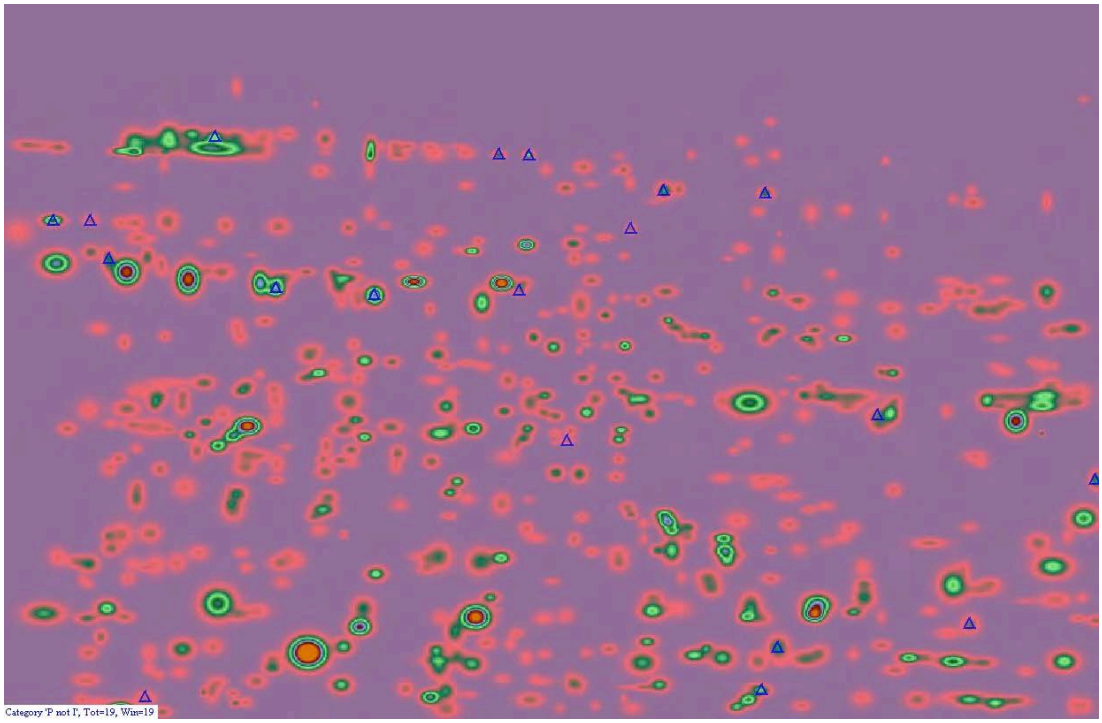

Figure 10. Spots identified with blue triangles found only in peripheral samples for 3 subjects analyzed.

Comparison of Method 1 and Method 2

Method 1 identified many more spots of interest than Method 2. Importantly, all the spots identified in Method 2 were contained in the spots identified in Method 1. These findings support the literature in the findings that there is no comparison for the human eye in identifying spots and selecting patterns in 2-D Gels. Also, it is important to note that there are changes occurring in both interior and peripheral samples. Both Method 1 and Method 2 show that there are different biochemical events occurring in the peripheral versus the interior of the wound bed.

Technical Objective 2:

All wounds were measured at each time point. Appendix 1 contains the measurements and percentages of healing for all subjects to date.

Technical Objective 4:

Completing Technical Objective 4 requires further data analysis and sample collection. Some wounds that healed produced less fluid and thus a fluorescent staining of the gels is necessary. Although we would like to keep the protocols consistent between samples, for samples with low protein it is necessary to use fluorescent staining. Due to the cost this is not a practical technique to be utilized for all samples. As these gels are completed, the analysis will proceed using Method 1. Additionally, further analysis using Method 1 will continue to evaluate changes in proteins identified with healing overall and between subjects.

Ultimately the results obtained from the first year of this project will be utilized to create an expedient system using PDQuest Software to analyze future samples. It is anticipated that information learned using Method 1 will allow the software to be utilized to analyze data in an expedient manner to complete Technical Objectives 1b, 3, and 4b.

The current research is novel with respect to current published research in the field. There are no published studies identifying biomarkers associated with the earliest indicators of healing. The discovery of shadow spots found only during healing is particularly significant since these spots may represent biomarkers linked to healing in chronic wounds. The specific proteins associated with the spots of interest and the shadow have not yet been identified beyond PI and MW, but it is anticipated that the identity of some of these spots will be significant with regard to identification of biomarkers, as well as our understanding of the healing of chronic wounds.

KEY RESEARCH ACCOMPLISHMENTS

- Identification of 161 proteins of interest in peripheral and interior wound samples
- Identification of shadow proteins associated with healing events
- Development of Method 1 to identify spots/proteins of interest
- Development of Method 2 to identify spots of interest
- Comparison of Method 1 and Method 2 (manual and automated) methods
- Protein Isolation

  Swabs made with polyester fiber tips were used in place of the more common cotton tip because polyester is thought to have lower protein binding properties than cotton. Upon collection of the sample, the tips of the swabs are broken off and placed in 2mL cryovials with 150uL saline to prevent dehydration. When samples are brought to the laboratory, 350uL dH_2O are added and the swabs are vortexed for 30 seconds to resuspend proteins and loosen them from the polyester. The tips and all liquid are transferred to clean Eppendorf tubes then centrifuged for 10 minutes at 6,000g to pellet any cellular debris. The supernatants are withdrawn and the samples are again centrifuged for 10 minutes at 6,000g to draw any remaining fluid from the swabs. The supernatants from both centrifugation steps are loaded onto spin columns with 3kDa-cutoff membranes (Millipore Micron YM-3 centrifugal filter). These columns are centrifuged for 99 minutes at 14,000g to concentrate the samples and remove all ionic components from the solutions, as salts can interfere with isoelectric focusing. After centrifugation, 50uL of dH_2O is added to the dry column membranes. Since two swabs are typically collected for each sample, each sample has a final volume of 100uL (50uL dH_2O for rehydration x 2 swabs). The columns are vortexed briefly and allowed to incubate at room temperature for five minutes to resuspend the wound proteins. The columns are then loaded inverted into 1.5mL Protein LoBind Eppendorf tubes (Eppendorf #22 43 108-1) and spun at 1,000g for three minutes to collect the isolated proteins. These tubes are then labeled with the samples' unique ID numbers and the samples proceed to the quantitation assay prior to storage at -80˚C.

- Protein Quantitation & Sample Storage

  The assay is a simple coomassie dye assay with a four point bovine IgG standard curve (1ug/uL, 0.75ug/uL, 0.5ug/ul, 0.25ug/uL). 200uL of diluted coomassie dye (BioRad #500-0006) is added to the wells of a standard 96-well microplate. Ten microliters of sample or IgG standard are added. Because the protein samples are quite concentrated, a dilution of 30:1 in dH_2O is typically required to lower the sample concentration into the standard curve range. The plate is then vortexed for one minute before reading. The microplate reader is controlled by a Dell Optiplex GX520 personal computer loaded with BioRad Microplate Manager software, version 5.1, build 75. The data are collected and stored in digital format on the PC as well as transcribed into a laboratory notebook. After quantitation the protein samples are placed in a -80°C freezer for storage.

- Isoelectric Focusing (IEF) of Wound Fluid Proteins

  Protein samples are thoroughly vortexed (~1minute), then 150ug or 250ug are added to 170uL sample/ rehydration buffer (described below). The sample solutions are vortexed before being pipetted into the lanes of an 11cm IEF focusing tray (BioRad #165-4020). Eleven centimeter pH 5-8 gradient IPG ReadyStrips (BioRad #163-2018) are laid over top of the sample solution. The tray is placed into the Protean IEF cell (BioRad #165-4001), a preprogrammed method is loaded, and the strips are allowed to rehydrate for 12 hours. Mineral oil (BioRad #163-2129) is pipetted over the strips after one hour of rehydration to prevent sample loss by evaporation. After the rehydration step, the cell begins focusing via rapid gradient to a maximum voltage of 8,000V for a total focusing time of 55,000Vhr with a 50mA resistance limit per strip. When focusing is complete (~19hr total run time, including rehydration) strips are either immediately run in the second dimension or are stored at -80°C.

  Development of the protocol for IEF was especially time-consuming due to the large number of options available for different pH gradients, buffer compositions, and ampholyte mixtures. The majority of this developmental work was carried out using the smaller 7cm IPG strips in order to minimize cost. Three IPG strips were tested: 3-10NL from BioRad, 5-8 from BioRad, and 3-10NL from GE Healthcare. Though the 3-10NL gradient strips allows detection of a much greater range of proteins, the 5-8 gradient from BioRad was selected due to its much better resolving power. This strip is an especially good match for the wound fluid samples. Narrower gradient strips (ex. 4.7-5.9, 5.5-6.7, and 6.3-8.3), which can be combined to make very high resolution composite images, were ruled out because of the need to run three gels in order to acquire a single image; due to the large number of samples involved with this project, this was not seen as a practical option.

  Two pre-made sample/rehydration buffers (BioRad #163-2106 and BioRad #163-2083) in combination with various ampholyte mixtures (BioRad Bio-Lyte 5/8 #163-1192, BioRad Bio-Lyte 3/10 #163-1112, and GE Healthcare IPG buffer 3-10NL #17-6000-88) were tested but no combination resulted in an acceptable level of resolution. Several lab-made formulations were tried before one consisting of 8M urea, 2% CHAPS, 0.002% bromophenol blue, 1M dithiothreitol (DTT), and 0.5% GE Healthcare 3-10NL ampholyte was found to work exceptionally well. Thus this is the buffer that is used; it is effective and has the added benefit of being more economical than pre-made buffers. The buffer base (lacking DTT and ampholyte, which are added immediately before use) is made in large quantities and stored at -80°C in 2mL aliquots.

  The first strips run for a sample utilize 150ug of protein, but if the resulting gels are not of sufficient quality a second set utilizing 250ug of protein is run.

- Second Dimension SDS-PAGE of Wound Fluid Proteins

  Briefly, IPG strips are incubated in Equilibration Buffer I (containing dithiothreitol) for twenty minutes, then Equilibration Buffer II (containing iodoacetamide) for

twenty minutes. Twelve Criterion 10-20% Tris-HCl IPG+1 gels (BioRad #345-0107) are unpackaged and placed in the Criterion Dodeca cell (BioRad #165-4130). The strips are placed in the IPG wells of the gels and are cemented in place with molten agarose. The cell is filled with 1x TGS running buffer, and Precision Plus dual color protein standards (BioRad #161-0374) are added to three of the gels in order to monitor the progress of the second dimension separation. The gels are run at 200V for an average time of 3 hours.

As with the IEF method, the majority of the 2D SDS-PAGE method was developed using the smaller 7cm ReadyGel system. Tris-HCl gels consisting of five different gradients (4-20%, 10-20%, 8-16%, 10%, and 10.5-14%) were tested, and the 10-20% gradient was found to result the most ideal separation of the wound fluid samples. These gels have the added benefit of being stronger and thus more resistant to tearing than lower concentration gels. Also as with the IEF method, pre-made buffers (BioRad #163-2107 and #163-2108) were used before a lab-made buffer was found to be superior. The formula for the buffer base that was found to work quite well is 50mM tris base, 6M urea, 30% glycerol, 2% SDS, and 0.002% bromophenol blue. To this is added 65mM dithiolthreitol for Equilibration Buffer I, and 135mM iodoacetamide for Equilibration Buffer II. For overlay agarose: 0.5% Agarose NA (GE Healthcare #17-0554-01) and 0.02% bromophenol blue in 1x TGS running buffer; and for 1x TGS buffer: 25mM tris base, 192mM glycine, 0.1% SDS.

One problem encountered early on in the use of the 12-gel Dodeca apparatus was the issue of heat generation. Although the cell has a built-in cooling coil, running cold tap water through this coil seemed to do little or nothing to keep the cell from becoming warm. It was then discovered that 1x TGS buffer will freeze quite solid in the laboratory freezer set at -10°C. The solution to the heat problem is to pack the cell with frozen buffer. Placement of a standard aquarium thermometer inside of the cell allows constant monitoring of the buffer temperature. Replacement of approximately half of the cell's 5L of TGS buffer with TGS ice keeps the buffer temperature below 5°C for up to two hours when running at 200V. By monitoring the temperature in this manner it is possible to add more ice as needed and thus constantly keep the cell running at a very cool >5°C for the entire ~3hr run time. This has resulted in the consistent production of high quality gels.

- Gel Staining, Image Acquisition, Data Storage

  The standard protocol for gel staining using Bio-Safe colloidal coomassie stain (BioRad #161-0787) is used. Gels are removed from their cassettes and rinsed with rocking at ~40rpm with three changes of 200mL dH_2O for 5 minutes each. Fifty milliliters of Bio-Safe coomassie is added to each gel. Gels are stained for one hour or overnight with rocking at 40rpm, and then destained with 200mL dH_2O for one hour or overnight, again with rocking at 40rpm.

  Gel images are aquired using a GelDoc XR Gel Imaging System and PD Quest 2D analysis software, version 7.4.0, build 036 (all from BioRad), and a Dell Optiplex GX

520 personal computer. Gel images are stored on the computer hard drive and are archived to rewritable DVD disk daily.

REPORTABLE OUTCOMES

- Database of 161 spots/proteins of interest that change over time in healing and non-healing chronic wounds.

CONCLUSION

The development of the experimental protocols in this first year of the project has been critical to sample collection and 2D-Page. Without these techniques the research could not proceed. The development and comparison of Method 1 and Method 2 has identified 161 spots/proteins of importance in the samples taken from subjects over a series of time points and differences present between the interior and peripheral wound environment. These methods are of great importance to the analysis of data and completion of the project overall.

The identification of 161 spots/proteins that change over time in chronic wounds is critical to the completion of this project. As the identity of these proteins is determined through database analysis and mass spectroscopy, the relevance of these proteins with the wound healing model can be assessed. The identification of a series of shadow proteins, which appear in wounds healing and disappear during non-healing periods, is the first important step in the identification of biomarkers associated with healing chronic wounds. The shadow spots are the first spots linked with healing in this project.

The identification of proteins present in interior versus peripheral samples and vice versa is an important result thus far. The wound environment is complex and dynamic and it is unknown whether indicators of healing will be present in the periphery or interior of the wound. The shadow spots seen with healing are present in both interior and peripheral samples. As our research progresses the link between sample location and biomarkers will be clarified.

The development of a diagnostic tool, which can be used to evaluate the healing process, is dependent on the identification of biomarkers, as well as the location of the sample collection.

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Appendix 1.

Subject	0	1	2	3	4	7	8	9	10	11	14	21	28	35	42
BM001	2.01	2.19	2.16	2.42	2.40	2.43	2.90	2.44	2.63	2.32	2.21	3.00	3.36	3.48	3.75
BM002	18.90	22.10	17.30	15.50	19.20	15.80	17.50	19.40	18.60	18.00	17.00	17.00	9.09	13.40	10.10
BM003	9.94	7.35	6.99	5.42	7.07	Hosp.									
BM004	24.50	25.10	23.90	24.40	21.80	28.80	21.30	22.90	20.10	18.90	19.90	14.40	Hosp.		
BM005	0.0180	0.0240	0.0250	0.0057	0.0120	0.0086	0.0410	0.0190	0.0082	0.0091	0.0220	0.0190	0.0140	0.0067	Closed
BM006	0.62	0.71	0.73	0.63	0.33	0.31	0.32	0.38	0.12	0.12	0.055	0.058	0.0082	Closed	
BM007	18.30	20.50	14.30	13.40	22.00	21.20	15.80	21.80	24.00	19.80	23.90	Hosp.			
BM008	3.06	0.86	0.86	0.83	0.67	1.27	0.82	1.50	1.65	1.08	1.03	1.08	0.97	1.07	0.74
BM009	2.51	4.09	3.15	2.93	2.76	2.99	3.04	2.87	Home						
BM010	39.00	29.60	24.20	24.80	26.80	27.10	28.70	15.80	16.90	15.60	11.80	10.20	6.10	6.78	4.51
BM011	1.34	1.17	1.18	1.27	1.15	1.55	1.38	1.66	1.64	1.63	1.58	1.76	1.64	2.10	1.53
BM012	9.78	7.36	8.38	6.59	4.93	4.17	4.36	3.10	4.47	3.90	3.01	1.64	1.07	1.03	0.37
BM013	3.74	3.31	4.31	2.93	Quit										
BM014	1.02	0.95	0.99	0.92	0.96	1.22	0.93	0.72	0.53	0.45	0.39	0.12	Hosp.		
BM015	33.90	36.10	28.30	39.00	33.40	40.50	51.70	34.50	41.30	36.60	33.30	32.50			
BM016	20.70	23.60	26.90	27.50	19.40	22.50	21.30	30.20	27.40	22.00					
BM017	28.80	33.40	28.20	28.60	34.00	26.40	34.90	37.00	33.00	41.20	27.30				

Table 1. Wound measurements in centimeters squared.

Subject	0	1	2	3	4	7	8	9	10	11	14	21	28	35	42
BM001	2.01	0.18	-0.03	0.26	-0.02	0.03	0.47	-0.46	0.19	-0.31	-0.11	0.79	0.36	0.12	0.27
BM002	18.90	3.2	-4.80	-1.80	3.70	-3.40	1.70	1.90	-0.80	-0.60	-1.00	0.00	-7.91	4.31	-3.30
BM003	9.94	-2.59	-0.36	-1.57	1.65										
BM004	24.50	0.6	-1.20	0.50	-2.60	7.00	-7.50	1.60	-2.80	-1.20	1.00	-5.50			
BM005	0.0180	0.006	0.00	-0.02	0.01	0.00	0.03	-0.02	-0.01	0.00	0.01	0.00	-0.01	-0.01	
BM006	0.62	0.09	0.02	-0.10	-0.30	-0.02	0.01	0.06	-0.26	0.00	-0.07	0.00	-0.05		
BM007	18.30	2.2	-6.20	-0.90	8.60	-0.80	-5.40	6.00	2.20	-4.20	4.10				
BM008	3.06	-2.2	0.00	-0.03	-0.16	0.60	-0.45	0.68	0.15	-0.57	-0.05	0.05	-0.11	0.10	-0.33
BM009	2.51	1.58	-0.94	-0.22	-0.17	0.23	0.05	-0.17							
BM010	39.00	-9.4	-5.40	0.60	2.00	0.30	1.60	-12.90	1.10	-1.30	-3.80	-1.60	-4.10	0.68	-2.27
BM011	1.34	-0.17	0.01	0.09	-0.12	0.40	-0.17	0.28	-0.02	-0.01	-0.05	0.18	-0.12	0.46	-0.57
BM012	9.78	-2.42	1.02	-1.79	-1.66	-0.76	0.19	-1.26	1.37	-0.57	-0.89	-1.37	-0.57	-0.04	-0.66
BM013	3.74	-0.43	1.00	-1.38											
BM014	1.02	-0.07	0.04	-0.07	0.04	0.26	-0.29	-0.21	-0.19	-0.08	-0.06	-0.27			
BM015	33.90	2.2	-7.80	10.70	-5.60	7.10	11.20	-17.20	6.80	-4.70	-3.30	-0.80			
BM016	20.70	2.9	3.30	0.60	-8.10	3.10	-1.20	8.90	-2.80	-5.40					
BM017	28.80	4.6	-5.20	0.40	5.40	-7.60	8.50	2.10	-4.00	8.20	-13.90				

Table 2. Change in wound size relative to previous day (cm^2). Red indicates increase in wound size and blue indicates a decrease in wound size.

Subject	0	1	2	3	4	7	8	9	10	11	14	21	28	35	42
BM001		8.96%	-1.37%	12.04%	-0.83%	1.25%	19.34%	-15.86%	7.79%	-11.79%	-4.74%	35.75%	12.00%	3.57%	7.76
BM002		16.93%	-21.72%	-10.40%	23.87%	-17.71%	10.76%	10.86%	-4.12%	-3.23%	-5.56%	0.00%	-46.53%	47.41%	-24.63
BM003		-26.06%	-4.90%	-22.46%	30.44%										
BM004		2.45%	-4.78%	2.09%	-10.66%	32.11%	-26.04%	7.51%	-12.23%	-5.97%	5.29%	-27.64%			
BM005		33.33%	4.17%	-77.20%	110.53%	-28.33%	376.74%	-53.66%	-56.84%	10.98%	141.76%	-13.64%	-26.32%	-52.14%	
BM006		14.52%	2.82%	-13.70%	-47.62%	-6.06%	3.23%	18.75%	-68.42%	0.00%	-54.17%	5.45%	-85.86%		
BM007		12.02%	-30.24%	-6.29%	64.18%	-3.64%	-25.47%	37.97%	10.09%	-17.50%	20.71%				
BM008		-71.90%	0.00%	-3.49%	-19.28%	89.55%	-35.43%	82.93%	10.00%	-34.55%	-4.63%	4.85%	-10.19%	10.31%	-30.84
BM009		62.95%	-22.98%	-6.98%	-5.80%	8.33%	1.67%	-5.59%							
BM010		-24.10%	-18.24%	2.48%	8.06%	1.12%	5.90%	-44.95%	6.96%	-7.69%	-24.36%	-13.56%	-40.20%	11.15%	-33.48
BM011		-12.69%	0.85%	7.63%	-9.45%	34.78%	-10.97%	20.29%	-1.20%	-0.61%	-3.07%	11.39%	-6.82%	28.05%	-27.14
BM012		-24.74%	13.86%	-21.36%	-25.19%	-15.42%	4.56%	-28.90%	44.19%	-12.75%	-22.82%	-45.51%	-34.76%	-3.74%	-64.08
BM013		-11.50%	30.21%	-32.02%											
BM014		-6.86%	4.21%	-7.07%	4.35%	27.08%	-23.77%	-22.58%	-26.39%	-15.09%	-13.33%	-69.23%			
BM015		6.49%	-21.61%	37.81%	-14.36%	21.26%	27.65%	-33.27%	19.71%	-11.38%	-9.02%	-2.40%			
BM016		14.01%	13.98%	2.23%	-29.45%	15.98%	-5.33%	41.78%	-9.27%	-19.71%					
BM017		15.97%	-15.57%	1.42%	18.88%	-22.35%	32.20%	6.02%	-10.81%	24.85%	-33.74%				

Table 3. Percentage change in wound size relative previous day. Red indicates increase in wound size and blue indicates a decrease in wound size.

Subject	0	1	2	3	4	7	8	9	10	11	14	21	28	35	42
BM001		8.96%	7.46%	20.40%	19.40%	20.90%	44.28%	21.39%	30.85%	15.42%	9.95%	49.25%	67.16%	73.13%	86.57%
BM002		16.93%	-8.47%	-17.99%	1.59%	-16.40%	-7.41%	2.65%	-1.59%	-4.76%	-10.05%	-10.05%	-51.90%	-29.10%	-46.56%
BM003		-26.06%	-29.68%	-45.47%	-28.87%										
BM004		2.45%	-2.45%	-0.41%	-11.02%	17.55%	-13.06%	-6.53%	-17.96%	-22.86%	-18.78%	-41.22%			
BM005		33.33%	38.89%	-68.33%	-33.33%	-52.22%	127.78%	5.56%	-54.44%	-49.44%	22.22%	5.56%	-22.22%	-62.78%	
BM006		14.52%	17.74%	1.61%	-46.77%	-50.00%	-48.39%	-38.71%	-80.65%	-80.65%	-91.13%	-90.65%	-98.68%		
BM007		12.02%	-21.86%	-26.78%	20.22%	15.85%	-13.66%	19.13%	31.15%	8.20%	30.60%				
BM008		-71.90%	-71.90%	-72.88%	-78.10%	-58.50%	-73.20%	-50.98%	-46.08%	-64.71%	-66.34%	-64.71%	-68.30%	-65.03%	-75.82%
BM009		62.95%	25.50%	16.73%	9.96%	19.12%	21.12%	14.34%							
BM010		-24.10%	-37.95%	-36.41%	-31.28%	-30.51%	-26.41%	-59.49%	-56.67%	-60.00%	-69.74%	-73.85%	-84.36%	-82.62%	-88.44%
BM011		-12.69%	-11.94%	-5.22%	-14.18%	15.67%	2.99%	23.88%	22.39%	21.64%	17.91%	31.34%	22.39%	56.72%	14.18%
BM012		-24.74%	-14.31%	-32.62%	-49.59%	-57.36%	-55.42%	-68.30%	-54.29%	-60.12%	-69.22%	-83.23%	-89.06%	-89.47%	-96.22%
BM013		-11.50%	15.24%	-21.66%											
BM014		-6.86%	-2.94%	-9.80%	-5.88%	19.61%	-8.82%	-29.41%	-48.04%	-55.88%	-61.76%	-88.24%			
BM015		6.49%	-16.52%	15.04%	-1.47%	19.47%	52.51%	1.77%	21.83%	7.96%	-1.77%	-4.13%			
BM016		14.01%	29.95%	32.85%	-6.28%	8.70%	2.90%	45.89%	32.37%	6.28%					
BM017		15.97%	-2.08%	-0.69%	18.06%	-8.33%	21.18%	28.47%	14.58%	43.06%	-5.21%				

Table 4. Percentage healing versus day 0. Red indicates increase in wound size and blue indicates a decrease in wound size.