

Defining a New Diagnostic Assessment Parameter for Wound Care: Elevated Protease Activity (EPA), an Indicator of Non-healing, for Targeted Protease- modulating Treatment

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Abstract

It is widely accepted that elevated protease activity (EPA) in chronic wounds impedes healing. However, little progress has occurred in quantifying the level of protease activity that is detrimental for healing. The aim of this study was to determine the relationship between inflammatory protease activity and wound healing status, and to establish the level of EPA above which human neutrophil elastase (HNE) and matrix metalloproteases (MMP) activities correlate with non-healing wounds.

Chronic wound swab samples (n=290) were collected from 4 wound centers across the USA to measure HNE and MMP activity. Healing status was determined according to percentage reduction in wound area over the previous 2-4 weeks; this was available for 211 wounds.

Association between protease activity and non-healing wounds was determined by receiver operating characteristic analysis (ROC), a statistical technique used for visualizing and analyzing the performance of diagnostic tests.

ROC analysis showed that area under the curve (AUC) for HNE were 0.69 for all wounds and 0.78 for wounds with the most reliable wound trajectory information, respectively. For MMP, the corresponding AUC values were 0.70 and 0.82. Analysis suggested that chronic wounds having values of HNE >5 and/or MMP ≥ 13 , should be considered wound healing impaired.

EPA is indicative of non-healing wounds. Use of a diagnostic test to detect EPA in clinical practice could enable clinicians to identify wounds that are non-healing, thus enabling targeted treatment with protease modulating therapies.

Introduction

It has been recently estimated that there are over 40 million chronic wounds worldwide;¹ this represents a major global problem that not only causes considerable morbidity and mortality, but also reduces quality of life and has a substantial financial burden for individuals, health care systems and economies.²⁻⁵ Unfortunately, the prevalence of chronic wounds is forecasted to increase,¹ largely as a result of the growing elderly population and the rising prevalence of chronic diseases, such as diabetes, that may contribute to wound formation, perpetuation and amputation rates. At least 15% of diabetic patients are predicted to develop chronic foot ulcers during their lifetime and of these it is estimated that 20% will require amputation.^{6,7} However, it is speculated that more than 80% of these amputations could be prevented with improved patient education on foot care, more effective treatments for diabetic foot ulcers and better prevention of ulcer recurrence.⁸

While many advances have been made in developing new therapies for chronic wounds, including growth factors, cellular and tissue-based products,⁹ they have had limited clinical adoption. However, in many of the clinical studies it appears that while these treatments have had minimal efficacy over moist wound healing on the whole population they have been effective for a sub-population.¹⁰ This has led to the hypothesis that there are sub-populations of chronic wounds that fail to heal due to different underlying biochemical defects and it is only when suitable solutions are utilized to correct a particular defect that healing ensues. Therefore, the key to developing more efficacious treatments for these wounds is in understanding the pathophysiology of chronic wounds and using a targeted approach to ensure that the correct therapy is used on the right wound. This personalized approach, however, is dependent upon our ability to stratify patients by biochemical defect as opposed to etiology, which requires the need

for predictive diagnostic markers in order to know how to target products effectively. While this approach is completely new to wound care, it has been validated in oncology and has led to both improved clinical efficacy and reduced overall treatment costs.¹¹

In this study, we have tested this hypothesis using one such biochemical defect: the presence of uncontrolled proteolysis, which has been reported and established as a pathogenic factor in non-healing wounds. Publications over the last 30 years have reported on various serine and matrix metalloproteases (MMPs) comparing levels and activity in acute and chronic wounds. Total MMP activity in chronic wound fluid has been reported to be 30 times higher than that in acute wound fluid.¹² More specifically, the activities of MMP-2, MMP-8, MMP-9 and human neutrophil elastase (HNE) have been found to be considerably higher in chronic wound fluid than in fluid from healing wounds.¹³⁻¹⁶

Even though the detrimental effect of elevated protease activity in chronic wounds has been recognized for some years, there has been little progress in identifying the level of protease activity above which wound healing is delayed and intervention is necessary. However, defining elevated protease activity (EPA) is not straightforward. This is in part due to the complexity of healing and inflammatory processes, and the multiplicity of proteases and mediators involved. The proteases involved in wound healing interact with each other (for example, HNE activates MMP-9), often have substrates in common (for example, HNE and MMP-9 can both degrade elastin), and may act synergistically (for example, both MMP-8 and MMP-9 are needed to digest collagen type I).¹⁷⁻¹⁹ This means that while a wound that has apparently low levels of one or more proteases may also have high levels of others. Consequently, measurement of multiple protease activities is necessary to truly reflect the proteolytic state of the wound.

To provide a reliable indication of the proteolytic status of the wound, we decided to measure both MMP and HNE activity. Measuring MMP levels by ELISA techniques can lead to incorrect results as they do not differentiate between latent and active enzyme; activity assays are more appropriate as they provide a measure of what is actually causing damage. These assays, for total MMP activity (including MMP-8 and MMP-9 activity) and HNE activity, collectively give a measure of inflammatory protease activity, recognized as the predominant proteases in the chronic wound *milieu*.^{15,16,20}

For detection of elevated protease activity levels to be clinically useful, the clinician needs to know when protease activity is affecting healing status and consequently when interventions to modulate protease activity are most likely to be beneficial. This paper reports for the first time, research that was conducted to clarify the relationship between inflammatory protease activity and healing status, defined by percentage wound area reduction during the first 4 weeks of treatment. We have defined the level of protease activity above which delayed healing in chronic wounds is highly probable. By establishing this relationship between protease activity and non-healing wounds, we provide a new objective diagnostic parameter to aid clinicians in the assessment and treatment of chronic wounds. This study may therefore represent a true revolutionary step forward in the treatment of chronic wounds. The use of a diagnostic test to assess protease activity in clinical practice could enable clinicians to identify wounds that are non-healing due to EPA. This could in turn aid treatment decisions and enable the targeted use of advanced dressings.

Methods

Settings

The study was carried out in four wound healing centers located in Erie, PA, Kittanning, PA, Dayton, OH and Orangeburg, SC.

Patient eligibility

Patients over the age of 18 years with a chronic wound, who were capable of giving consent, were assessed by the clinical investigator. To be eligible for study enrollment, the wound was required to be present for at least 4 weeks. It was agreed by a panel of wound healing experts that chronicity is not necessarily dependent on the time since the wound was first formed.²¹ A chronic wound was defined as a wound that fails to progress through a normal, orderly, timely sequence of repair and where comorbidities interfere with the normal healing process.^{22,23} This encompasses wounds described as delayed, stalled, hard to heal, recalcitrant, difficult, complex or failing to respond and could include acute wounds that have healing problems. Wounds evaluated in the study included diabetic foot ulcers, pressure ulcers, venous leg ulcers, arterial ulcers, surgical and traumatic wounds. Patient recruitment occurred between May 16, 2011 and January 16, 2012.

Statement on informed consent

The study was conducted in accordance with the clinical protocols and was in full compliance with FDA regulatory requirements: the basic principles outlined in 21 CFR Parts 11, 50, 54, 56 and 812, the ICH-Guidelines for Good Clinical Practice as published in the Federal Register on May 9, 1997, and the Declaration of Helsinki 1975. All subjects completed informed consent.

Healing status

The percentage reduction in wound area over the previous 2-4 weeks prior to swabbing was used to determine if the wound was in a healing or non-healing trajectory.²⁴ For diabetic foot ulcers, a reduction of 50% or more over a 4 week time period was used to define a wound on a healing trajectory²⁴ while for arterial/venous leg ulcers and pressure ulcers a wound area reduction of 20-40% is predictive of healing,²⁴⁻²⁶ but for pragmatic reasons a threshold of 30% was used for all other chronic wounds recruited in this study. Any wounds not meeting this percentage reduction in wound area over the 4-week time period were classified as non-healing. If a wound's surface area was not determined 24-28 days prior to the measurement when the swab was taken, the surface area determined on the next closest date within 14-23 days was used to assess the healing status.

Sample collection

Wounds were swabbed with a foam swab (Puritan Medical, Guilford, ME, USA) using a new technique (Serena Technique[®], Table 1) developed for the optimal assessment of protease activity in wound fluid. Once a wound fluid sample was collected, the foam swabs were frozen to -70°C as soon as possible and typically within 1 hour of collection. These swabs were then transported within 72 hours of collection on dry ice to the laboratory for analysis (Alere, Scarborough, ME, USA).

Extraction of wound fluid from swab

Wound fluid was eluted from the foam swabs by submerging the swab and vigorously mixing in 110 µL assay buffer (50 mM Tris/HCl, pH 7.4 containing 10 mM calcium chloride dehydrate, 100 mM sodium chloride, 50 µM zinc chloride, 0.025% Brij 35 and 0.09% sodium azide). To ensure that the maximum recovery of fluid was obtained from the swab, the swab

head was centrifuged (5415D, Eppendorf, Westbury, NY, USA) in a 0.45 µm filter at 930 relative centrifugal force for 2 minutes. The swab extracts were used immediately for protease testing.

Human neutrophil-derived elastase (HNE) activity assay

The activity levels of neutrophil-derived elastase present in the wound fluid samples were measured spectrofluorimetrically using a substrate activity assay. The substrate comprises a short peptide synthesized to mimic the enzyme cleavage site and contains a fluorescent reporter group, which is released upon hydrolysis: MeOSuc-Ala-Ala-Pro-Val-AMC (BaChem Ltd, UK). Enzyme activity was determined by measuring the rate of production of the fluorimetric compound, 7-amino 4-methyl coumarin (AMC). Activity was measured as the change in relative fluorescence units with time (RFU/min) and converted to mU HNE activity per 110 µL swab extract using a standard curve plotting RFU/min and HNE with known activities (mU) obtained from Innovative Research, Inc (Novi, MI, USA). Each sample was tested in duplicate and the mean calculated. The substrate was prepared at a 10mM-stock concentration, and diluted to a working concentration of 0.2 mM in the assay buffer. The reaction mixture, combined in a microtiter well (black, flat bottomed) comprised 5 µL wound fluid, and 195 µL assay buffer containing substrate (0.2 mM). The microtiter plate was read immediately at 455 nm (excitation 383 nm) and fluorescence monitored at timed intervals over the next 30 minutes using a microtiter plate fluorimeter; during this time the plate was incubated at room temperature.

Total MMP activity assay

Total MMP activity present in wound fluid was measured spectrofluorimetrically using a substrate activity assay. This fluorogenic substrate, Mca-Lys-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂ (Enzo Life Sciences, Inc., Farmingdale, NY, USA), is cleaved by a broad spectrum of

MMPs and is therefore appropriate for measuring total MMP activity present in a biological sample. Activity was measured as the change in relative fluorescence units with time (RFU/min) and converted to U MMP activity per 110 μ L swab extract using a standard curve plotting RFU/min and MMP catalytic domain with known activities (U) obtained from Enzo Life Sciences. Each sample was tested in duplicate and the mean calculated. The substrate was prepared at a 10mM-stock concentration and diluted to a working concentration of 5 μ M in the assay buffer. The reaction mixture, combined in a microtiter well (black, flat bottomed) comprised 5 μ L wound fluid, and 195 μ L assay buffer containing substrate (5 μ M). The microtiter plate was read immediately at 400 nm (excitation 328 nm) and fluorescence monitored at timed intervals over the 30 minutes using a microtiter plate fluorimeter; during this time the plate was incubated at room temperature. MMP-9 was measured using the SensoLyte[®] Plus 520 MMP-9 Assay Kit (Anaspec, CA, USA).

Database creation

The data from four separate studies conducted from May 16, 2011 through January 16, 2012 were first combined. Five entries were deleted from the combined dataset because no wound types were recorded leaving 290 swabs to be analyzed.

A reliability value for healing/non-healing was also calculated as follows: 4-week area data used, highest reliability [1]; only 2-week data used, lower reliability [2].

Statistical analysis

Continuous variables were reported as means (SD; standard deviation) if data were normal or median and range (and interquartile range if relevant) if data were non-normal as demonstrated by the Wilk-Shapiro (WS) test. Some exceptions were made for marginal test

violation as the WS tends to be overly sensitive for large samples and inspection of histograms can provide a better assessment.

Categorical variables were reported as proportions (percentages). Correlations between continuous variables (e.g., MMP and HNE) were conducted using Spearman's correlation. Binary classification of healing/non-healing wounds (yes or no) was conducted using ROC curve analysis in which optimal classification was based on the best compromise between sensitivity and 1 – specificity, assuming it was better than a line of no discrimination.

All statistical analysis was conducted using SPSS Statistics 21 (IBM, Chicago, IL). An alpha of 0.05 was considered statistically significant. All tests were two-sided.

Results

A total of 290 wounds were included in the analysis: diabetic foot ulcers (DFUs; n=86; 29.7%); venous leg ulcers (VLUs; n=92; 31.7%); pressure ulcers (PUs; n=43, 14.8%); arterial ulcers (n=14, 4.8%); surgical wounds (n=13; 4.5%); traumatic wounds (n=6; 2.1%); and other wound types (n=36, 12.4%). Although the median HNE value for all wounds was 2.6 mU/110 μ L, it was considerably higher for some wound types—5.0 for PUs, and 11.1 for arterial ulcers (Table 2; Figure 1). The median MMP value for all wounds was 12.6 U/110 μ L, with less variation between wound types, although it was also much higher for arterial ulcers (23.5; Table 2; Figure 2). A similar pattern was evinced for MMP9, in which the median value was 1.5 U/110 μ L (Table 2; Figure 3). A moderately strong linear correlation between HNE and MMP was also found (Spearman: 0.626; $p = 1.4 \times 10^{-32}$).

While about a third of all wounds (71/211; some wounds were missing healing information) were considered to be on a healing trajectory at the time the swab was taken based on the area algorithm (i.e. healing wounds), wound etiology had considerable influence: only

67% of surgical wounds were healing, followed by other wound types: 42%; VLU: 38%; traumatic wounds: 33%; PUs: 27%; DFUs: 21%; and arterial ulcers: 13%.

To examine the classification of non-healing wounds in the context of HNE and MMP values, ROC curve analysis was performed. The area under the curve (AUC) for HNE was 0.69 with best classification at 0.35 mU/110 μ L: sensitivity: 0.755; specificity: 0.588; (n=207; some wounds were missing HNE values) (Figure 2). When the highest reliability for non-healing determination was used the AUC was 0.78 with best classification at 5.5: sensitivity: 0.667; specificity: 0.857; (n=40) (Figure 3). Similar results were obtained for MMP: AUC (all wounds): 0.70 with best classification at 6.9: sensitivity: 0.820; specificity: 0.574; (n=207; some wounds were missing MMP values); AUC (highest reliability for non-healing determination): 0.82 with best classification at 12.9: sensitivity: 0.806; specificity: 0.714; (n=40). While there are far fewer results that only included those wounds with highest reliability for non-healing determination, the ROC analysis suggests that chronic wounds having values of HNE and MMP > 5 and/ or ≥ 13 , respectively, should be considered wound healing impaired and treated accordingly. Based on these thresholds, it was calculated that about 66% of non-healing chronic wounds tested had EPA.

Discussion

This is the first study to provide a relatively large sampling of chronic wounds in which HNE, Total MMP and MMP-9 were measured in an attempt to define thresholds above which such wounds should be considered healing impaired due to excessive protease activity. A standardized specimen collection protocol (Serena Technique[®]), was used to ensure optimal collection of wound fluid for assessment of protease activity. The Serena Technique[®] uses a

polyester swab (Puritan, Guilford, ME) that absorbs a consistent amount of wound fluid (50 μ L) permitting for a reliable measurement of EPA.²⁷

The results established that chronic wounds having values of HNE >5 and/or MMP ≥ 13 , should be considered wound healing impaired. One of the intriguing findings was that arterial ulcers showed the highest activity of HNE and overall MMP. While the underlying mechanism for this higher elevation is not well understood, the results indicate that these ulcers could possibly have the greatest level of inflammation.

This study provided an insight into the level of inflammatory protease activity above which MMPs and elastase activities correlate with non-healing wounds, however, it did not assess protease activity at weekly interval over the 4 weeks of treatment. Since the level of MMP and elastase may vary over a period of time, future work should explore correlation between amount of healing and level of protease activity at regular intervals.

Previous studies have been restricted to a smaller sampling of specific wound etiologies without defining thresholds for individual proteases linked to impaired wound healing.^{12,15,16,28} This has limited the utility of testing chronic wounds for excessive proteases in clinical practice despite the acknowledged potential that such diagnostic tests might have in selecting treatment plans.^{23, 24} For example, a non-healing wound with EPA could be treated with protease-modulating agents such as doxycycline,^{29,30} or collagen/oxidized regenerated cellulose dressings.^{31,32}

The development of specific diagnostic tests for proteases in wound care is an ongoing process and this study fulfils the first step by defining thresholds for some specific elevated proteases. A limitation of the thresholds reported here is that while such thresholds identify non-healing wounds with EPA, they do not identify all wounds which are non-healing.

ROC analysis employed in this study is a standard approach to evaluating medical diagnostic tests in which a gold standard is available for determining the disease status of each patient. In the simplest case it takes the form of a binary classification (patient has the disease or not) in which sensitivity is plotted against $1 - \text{specificity}$ for different values of the test to determine the best test value for a cut-off value or break point. A major limitation of our study is that there is no gold standard at present that can be utilized to predict if a chronic wound will heal early in its healing trajectory. Consequently, based on our look back to judge healing/non-healing wounds, some of our classification may be in error and thus our proposed breakpoints for HNE and MMP—thresholds at which we would judge wound healing impairment based on an elevated protease level—have a range of uncertainty. An estimate of this uncertainty can be understood by looking at the breakpoint differences in the full datasets versus the truncated datasets based on more reliable data—for example, 6.9 versus 12.9 for MMP values.

Next steps include improvements in creating a specific test for users, which can easily show whether the chronic wound being tested has high enough levels of proteases that could interfere with healing. However, it is important to understand that excessive levels of proteases are not the only mechanism by which wound healing can be impaired: the balance between proteases and tissue inhibitors of metalloproteinases (TIMPs),³⁰ comorbidities, ischemia, infection, certain medications and other factors can all play a role.²⁴ Thus, sensitivity, which is the ability to correctly identify non-healing chronic wounds in this context, and specificity, which is the ability to correctly identify healing wounds, will have some limitations based on our classification analysis, because some chronic wounds have other causes for non-healing. Other limitations of this study include problems with correctly determining wound healing trajectories

at 4 weeks, which affects sensitivity/specificity; that the protease activity is measured from a swab after a freeze-thaw cycle resulting in comparative, but potentially lower activities compared to results from “fresh” swabs; and that current healing trajectories are based on wound area and not depth, which for some wound types, such as pressure ulcers, may lead to incorrect classifications.

In conclusion, this study presents data showing that high levels of HNE and MMP activity can be correlated with non-healing of chronic wounds; classification analysis suggests where the breakpoints or cut-off value for HNE and MMP would indicate wound healing impairment. Further work is required to validate or rationalize the use of diagnostics in the treatment of chronic wounds; however this work supports the need and utility of diagnostic tests to identify non-healing wounds based on protease activity in clinical practice.

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Table 1 Specimen collection protocol (Serena Technique[®]).

Using the sterile swab provided, collect the wound fluid sample by swabbing the surface of the wound:

1. Prior to swabbing, gently cleanse the wound with sterile saline to remove all loose debris, remains of therapeutic agents (e.g enzymatic debriders, gels, dressings, etc.) and necrotic tissue. Do not perform sharp wound debridement prior to sample collection.
2. Ensure that complete haemostasis has been achieved before obtaining the specimen.
3. Apply additional saline to the wound area to be swabbed, such that the area is visibly moist. Care should be taken not to flood the wound with excessive saline. Avoid pooling of saline.
4. Avoid swabbing areas that contain blood, necrotic material or thick slough or fibrinous tissue.
5. Press the head of the swab flat against the base of the wound and gently roll it back and forth several times while applying pressure. Continue rolling the swab head until it is fully coated and discoloured (tan/yellow) by wound fluid.

Table 2. Values of HNE, Total MMP, and MMP9 by wound type.

Wound Type	HNE (mU/110 μ L)			Total MMP (U/110 μ L)			MMP9 (U/110 μ L)		
	N=286			N=286			N=140		
	Median	Range	IQR*	Median	Range	IQR*	Median	Range	IQR*
Diabetic foot ulcer	2.0	0-97	17	11	0-476	30	13	0-25	5
Venous leg ulcer	2.0	0-80	8	14	0-329	20	0.5	0-50	6
Pressure ulcer	5.0	0-34	15	13	0-360	31	3.3	0-35	10
Arterial ulcer	11.1	0-55	15	23.5	11-138	38	3.6	0-12	4
Surgical wound	4.5	0-108	12	16.1	1-423	21	—	—	—
Traumatic wound	3.6	0-35	27	8.5	4-33	10	1.8	1-3	—
Other wound/ulcer	1.6	0-52	9	9.5	0-92	16	4.4	0-22	5
All	2.6	0-108	12	12.6	0-476	23	1v5	0-50	6

*IQR: interquartile range

Figure Legends

Fig1. Plot of HNE values (spread out on the X axis for better visualization).

Fig 2. Plot of MMP values (spread out on the X axis for better visualization).

Fig 3. Plot of MMP9 values (spread out on the X axis for better visualization).

Fig 4. ROC curve for HNE (all wounds).

Fig 5: ROC curve for HNE (wounds with highest reliability of non-healing determination).

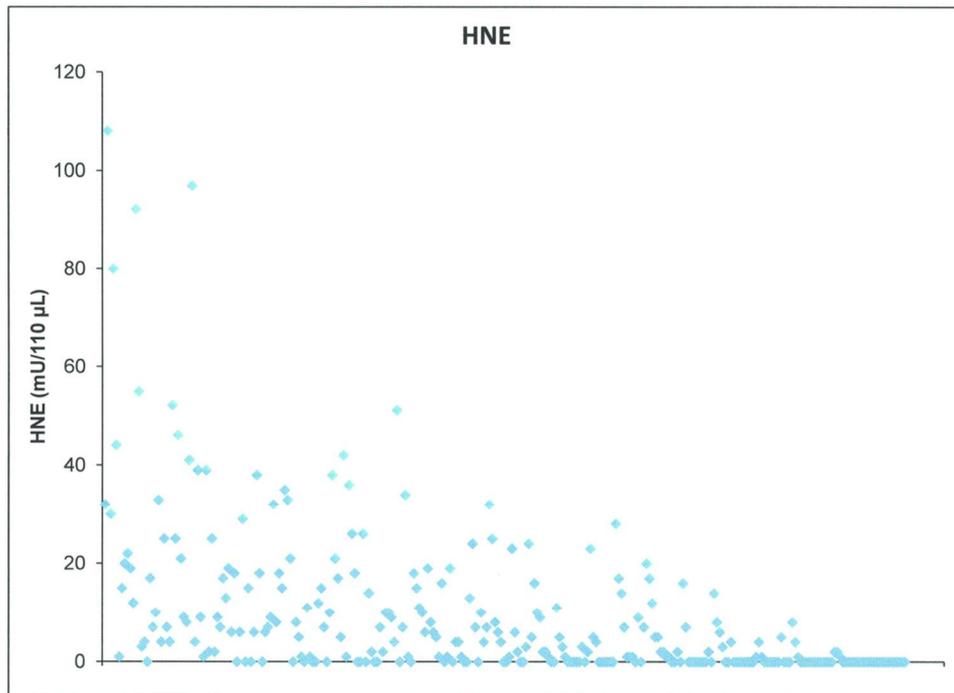


Fig1. Plot of HNE values (spread out on the X axis for better visualization).
93x68mm (300 x 300 DPI)

Accept

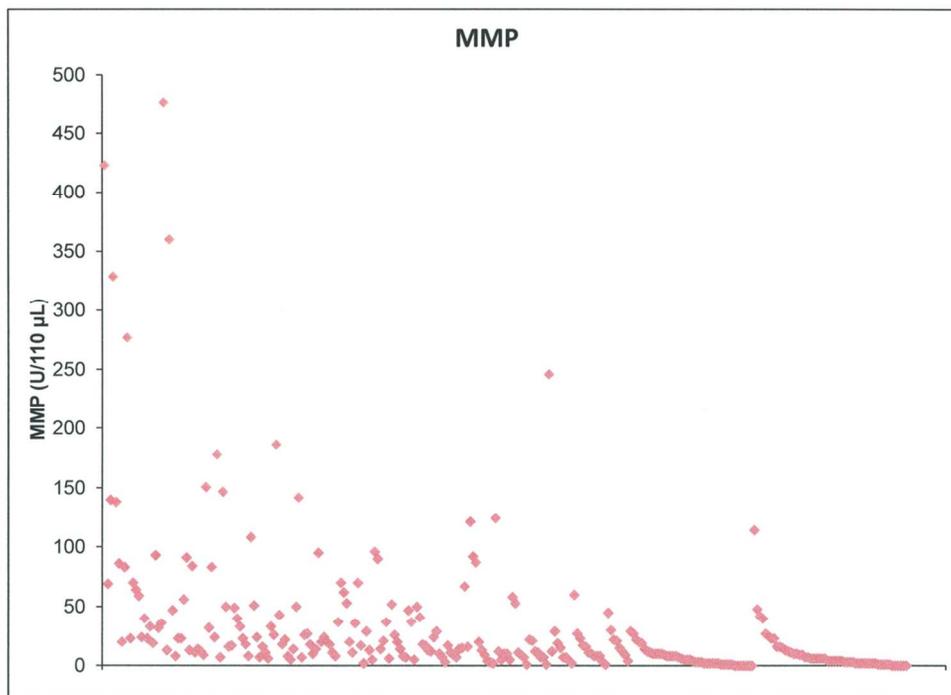


Fig 2. Plot of MMP values (spread out on the X axis for better visualization).
94x70mm (300 x 300 DPI)

Accept

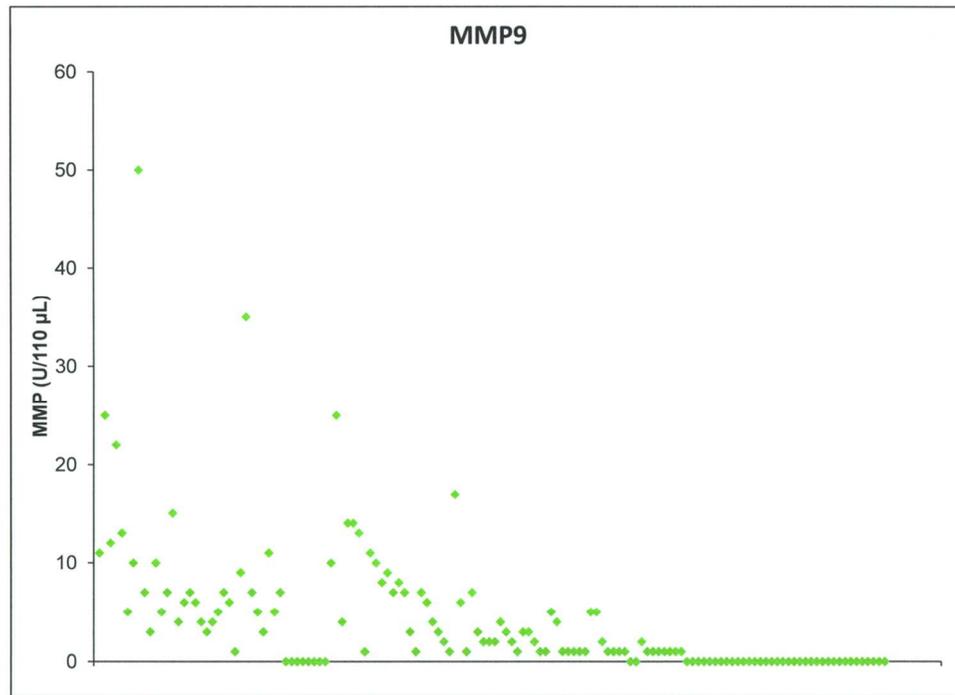
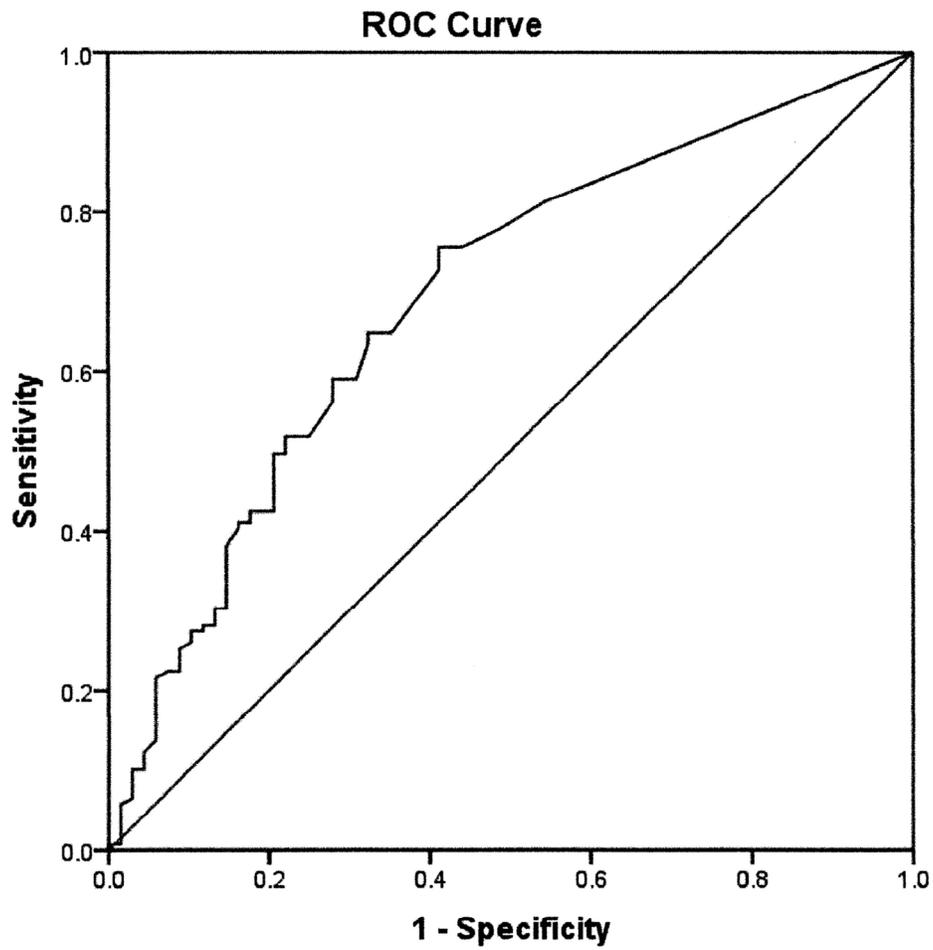


Fig 3. Plot of MMP9 values (spread out on the X axis for better visualization).
93x68mm (300 x 300 DPI)

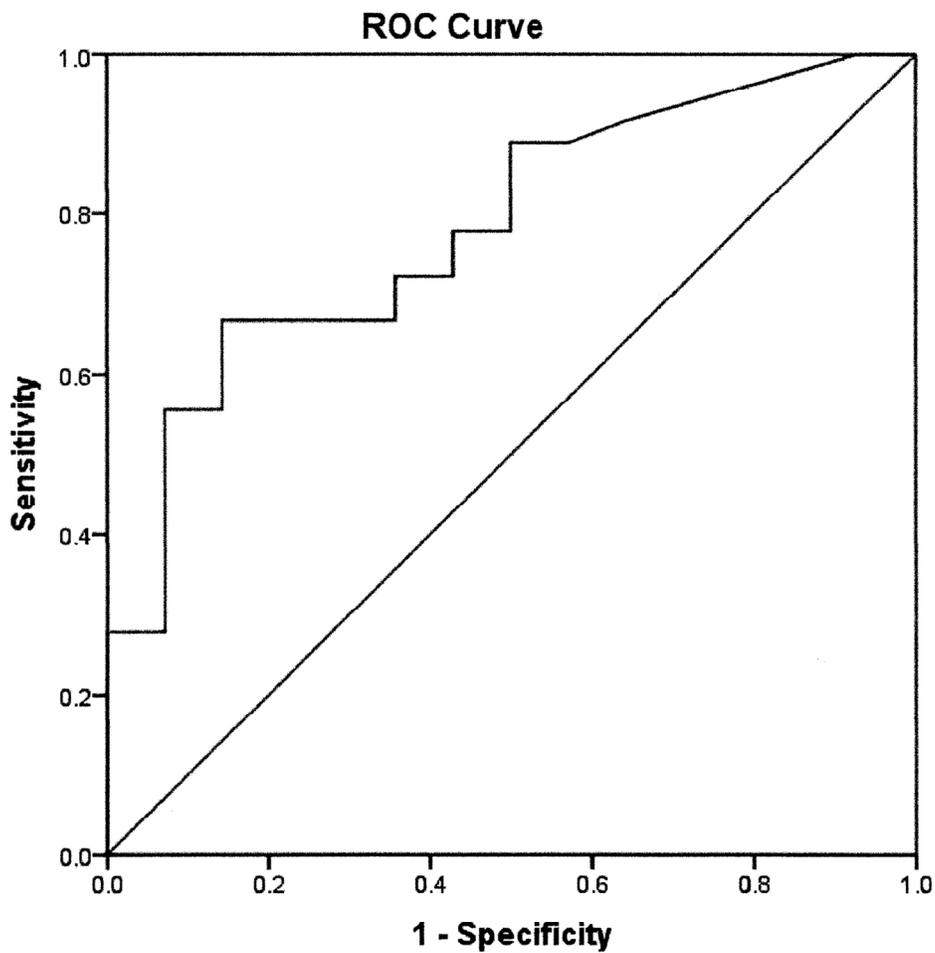
Accept



Diagonal segments are produced by ties.

Fig 4. ROC curve for HNE (all wounds).
131x135mm (300 x 300 DPI)

Acc



Diagonal segments are produced by ties.

Fig 5: ROC curve for HNE (wounds with highest reliability of non-healing determination).
132x138mm (300 x 300 DPI)

Acc