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Metalloproteinase Expression is Associated with Traumatic Wound Failure

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Background. **Matrix metalloproteinases (MMPs) are crucial in the inflammatory and remodeling phases of wound healing. We previously reported the correlation between pro-inflammatory cytokines and timing of successful combat-wound closure. We now extend our studies to investigate the correlation between wound-remodeling MMP expression and wound healing.**

Methods. **Thirty-eight wounds in 25 patients with traumatic extremity combat wounds were prospectively studied. Surgical debridement with vacuum-assisted closure (VAC) device application was repeated every 48 to 72h until surgical wound closure. Wound effluent and patient serum were collected at each wound debridement and analyzed for five matrix metalloproteinases using the Luminex multiplex system; Millipore Corp, Billerica, MA. The primary outcome was wound healing within 30 d of definitive wound closure. Impairment was defined as delayed wound closure (> 21 d from injury) or wound dehiscence. MMP expression was compared between impaired and normal healing wounds.**

Results. **Elevated levels of serum MMP-2 and MMP-7 and reduced levels of effluent MMP3 were seen in impaired wounds ($n = 9$) compared with wounds that healed ($n = 29$; $P < 0.001$). Receiver operating characteristic (ROC) curve analysis yielded area-under-the-curve (AUC) of 0.744, 0.783, and 0.805, respectively.**

Conclusions. **Impaired wound healing is characterized by pro-inflammatory MMP-2 and MMP-7. Serum and effluent concentrations of MMP-2, MMP-3, and MMP-7 can effectively predict the outcome of traumatic war wounds and can potentially provide decision-supportive, objective evidence for the timing of wound closure.** Published by Elsevier Inc.

Key Words: **Luminex; MMP-2; MMP-3; MMP-7; effluent; multiplex; dehiscence; acute wound; VAC.**

INTRODUCTION

Advances in body armor have contributed to increased survival of some of our most severely injured combat casualties [1]. However, this survivability often comes at a devastating cost. Blasts from improvised explosive devices (IEDs) and other high-energy weaponry cause excessive extremity trauma that often renders limbs unsalvageable or requires extensive surgical care [1–4]. The resulting large wound beds are often difficult to manage due to location, extent of tissue damage, and the frequent additional complications of contaminating debris and recalcitrant bacteria [5–8].

Negative-pressure vacuum-assisted closure device (VAC) usage, in combination with serial debridements of devitalized tissue and high-pressure wound irrigation, has greatly reduced the morbidity of such traumatic extremity wounds [9, 10]. Although these methods have been accepted as the standard of care in many institutions, timing of surgical closure of a wound remains a subjective process in which the surgeon relies on criteria such as the patient's overall condition, wound location, wound bed gross appearance, and local perfusion. In spite of this quality care and success rate, some wounds still result in dehiscence. Conversely, unnecessary surgical debridements occur on wounds that could have been closed earlier than

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the surgeon had surmised, which results in additional patient risk and expense [8]. The study herein investigates the association of matrix metalloproteinases (MMPs) and the timing of successful surgical closure of acute traumatic wounds.

Acute wounds typically heal by a complex and interdependent sequence of events that can be divided into four phases: initiation (clotting), inflammation, proliferation, and maturation [11]. Progression through each phase from initial injury to wound healing and resolution is highly dependent on the molecular environment at the wound site [12]. MMPs play crucial roles in the inflammation, proliferation, and maturation phases by performing several functions related to inflammatory signaling and wound remodeling [13–15]. MMPs, which are proteins that incorporate a Zn^{2+} or Ca^{2+} ion in their enzymatic active sites [14], can be generally classified into five classes based on their primary substrate: collagenases, gelatinases, stromelysins, matrilysins, and membrane-type matrix metalloproteinases. However, recent research asserts that many MMPs have overlapping functions [16]. A thorough literature search has revealed that the major MMPs involved in wound remodeling are MMPs 1, 2, 3, 7, 8, 9, 10, 12, and 13, although, with ongoing research, it is likely that all MMPs play essential roles in wound healing. [13, 14, 16–20].

Collagenases (MMP-1, MMP-8, and MMP-13) mainly cleave type I, II, and III fibrillar collagen present in the extracellular matrix (ECM). This allows for properly-oriented keratinocyte migration and ultimately sets the stage for later wound remodeling [13, 14, 21]. Similarly, gelatinases (MMP-2 and MMP-9) have been shown to participate in wound remodeling by cleaving type IV, V, VII, and X collagen, elastin, and other basement-membrane proteins [13–15]. More recently, MMP-2 and MMP-9 have been implicated in inflammatory cell recruitment as well as in the establishment of chemotactic gradients that direct immune cell migration [14]. Similar to the actions of gelatinases, stromelysins (MMP-3, MMP-10, and MMP-11) have been shown to degrade elastin and collagens IV, V, and X [13, 14]. MMP-3 has also been shown to possess regulatory functions in chemokine signaling as well as a critical role in both reepithelialization and wound contraction[14]. Matrilysin (MMP-7) shares many of the functions of MMP-3 such as processing elastin and wound bed reepithelialization, but also demonstrates the inflammatory functions of MMP-2 and MMP-9 by enhancing neutrophil migration across epithelial layers via chemokine processing [14]. Membrane-type matrix metalloproteinases (MMPs 14–17) are present on cell membranes and appear to aid in binding other MMPs to cell surfaces for local activation and wound reepithelialization [13].

These highly regulated and interdependent processes exist during "normal" wound healing, defined herein as wounds that are surgically closed within 21 d post-injury with no dehiscence within the following 30 d. We have observed that wounds exhibiting delayed healing (surgical closure>21 d post-injury) or subsequent dehiscence are the result of an inflammatory dysregulation of the wound-bed molecular environment [7]. This state of excessive or prolonged inflammation seems to 'stall' progression through the wound healing phases, thus resulting in impaired wound healing. Elevated levels of the proteases MMP-2 and MMP-3 and low levels of their respective inhibitors have been measured in wound effluent of chronic pressure ulcers treated with VAC [17]. Such elevated MMP expression and disproportionate expression of their inhibitors has been proposed as a cause of wound chronicity [15, 22–24].

By analyzing concentrations of MMP-2, MMP-3, MMP-7, MMP-9, and MMP-13 in traumatic extremity wound effluent throughout the treatment process, this study sought to parallel these studies in acute wounds. Additionally, our goal was to build a systemic picture by monitoring serum levels of these representative MMPs. We hypothesized that MMP expression, as an objective marker of healing, is indicative of timing for successful surgical wound closure and avoidance of dehiscence.

MATERIALS AND METHODS

Study Methodology

This serial, observational study with prospective data collection was conducted in accordance with the ethical standards of the committee on human experimentation as approved by the institutional review board of the National Naval Medical Center (NNMC) and the Naval Medical Research Center (NMRC). Study participants were recruited from wounded U.S. service members evacuated to the National Capital Area from Iraq and Afghanistan between January 5, 2007 and May 30, 2008, and treated at the National Naval Medical Center (Bethesda, MD). Informed consent was obtained for all participating patients. Inclusion criteria for this study were defined as all service men and women who sustained penetrating injuries to one or more extremities. Up to three wounds per patient were studied. Patients with confounding comorbid conditions, such as immune disorders, connective tissue disorders, or any conditions requiring immunosuppressive agents, were excluded. One patient was excluded from data analysis due to death prior to wound closure. One patient declined the study. Ten eligible patients were not entered as a third party could not be contacted for consent prior to the first surgical procedure at our facility. Although a third party was contacted by telephone, two eligible patients were not entered into the study as third-party consent was only accepted if the party was physically present. Recorded demographic variables included age, gender, date, body mass index, nicotine use, injury severity score (ISS), concomitant traumatic brain injury, location and mechanism of injury, wound size, associated major vascular injury to the affected limb, type of wound closure, number of wound debridements (Table 1), and Acute Physiology and Chronic Health Evaluation II (APACHE-II) scores (data not shown). Surgical debridement, pulse lavage, and VAC application were repeated every 48 to 72 h until surgical wound

TABLE 1

Patient (Wound) Demographics

	Wounds $n = 38$	Normal healing $n = 29$ (76.3%)	Impaired healing $n = 9$ (23.7%)	P value
Age (y)*		23±5	21±2	0.05
Gender - no. (%)				NA
Male	38	29 (100)	9 (100)	
Female	0			
Body mass index*		25.5±3.7	22.0±3.7	0.02
Tobacco use - no. (%)	11	8 (27.6)	3 (33.3)	1.0
Injury severity score (ISS) *		18±10	39±7	<0.001
Traumatic brain injury - no. (%)	17	14 (48.3)	3 (33.3)	0.48
Mechanism of injury - no. (%)				0.28
GSW	2	2 (6.9)	0 (0)	
Blast	35	27 (93.1)	8 (88.9)	
Crush	1	0 (0)	1 (11.1)	
Evacuation time (d) to NNMC*		4.6±1.9	7.0±3.9	0.10
Wound location - no. (%)				0.22
Upper extremity	12	11 (37.9)	1 (11.1)	
Lower extremity	26	18 (62.1)	8 (88.9)	
Traumatic amputation - no. (%)	15	11 (37.9)	4 (44.4)	1.0
Size of wound (cm^3)*		158±239	583±673	0.10
Associated vascular injury - no. (%)	7	2 (6.9)	5 (55.6)	0.004
Wound closure method - no. (%)				
Suture	29	24 (87.8)	5 (55.6)†	0.17
Skin graft	9	5 (17.2)	4 (44.4)‡	
Number of wound debridements*		3.0±1.3	7.6 ± .6	0.02

*Mean±SD.

†Three wounds dehisced, two wounds delayed.

‡One wound dehisced, three wounds delayed.

closure or coverage, according to current institutional standards of practice. Timing of closure was at the discretion of the attending surgeon.

Sample Collection

Peripheral venous blood (8 mL) was drawn prior to each surgical debridement and collected in a Red-Top Serum BD Vacutainer (Becton Dickinson, Franklin Lakes, NJ). Wound effluent samples (≥30 mL) were collected from the VAC canister (without gel pack; Kinetic Concepts, Inc., San Antonio, TX) 2 h following the first surgical debridement and over a 12-h period prior to each subsequent wound debridement. All serum samples were immediately separated using a centrifuge (Thermo-Electron Corp., Waltham MA) at 2500×*g* for 10 min. Serum supernatants and effluent samples were transferred to individually labeled Cryo-Loc polypropylene tubes (Lake Charles Manufacturing, Lake Charles, LA), flash-frozen in liquid nitrogen, and stored at -80°C until analysis at the National Naval Medical Center (Silver Spring, MD).

Matrix Metalloproteinase Analysis

Serum and wound effluent proteins of interest were quantitated using a Luminex 100 IS xMAP Bead Array Platform (Millipore Corp, Billerica, MA). Five matrix metalloproteinases (MMP-2, MMP-3, MMP-7, MMP-9, and MMP-13) were quantitated using R & D Systems Fluorokine MAP Human Base Kit, MMP panel (R&D Systems, Minneapolis, MN; cat. no. LMP 000) according to the manufacturer's instructions and as previously described.[8] Briefly, serum and wound effluent samples were diluted based on initial optimization (3-fold for MMP-7 and MMP-13; 150-fold for MMP-2, MMP-3, and MMP-9; optimization data not shown), and incubated with MMP-specific monoclonal antibodies covalently linked to uniquely fluorescent microparticle beads. Subsequently, biotinylated monoclonal antibodies specific for the bead-linked antibody:MMP complexes were introduced; this secondary complex was then detected by streptavidin-phycoerythrin. Individual Human MMP Fluorokine MAP microparticle/biotin kits were used (LMP902, LMP513, LMP907, LMP911, and LMP511; R and D Systems) for MMP-2, 3, 7, 9, and 13, respectively).

Wound Closure and Follow-up

All wounds were examined once daily following surgical wound closure or coverage until suture removal. All patients were followed clinically for 30 d. The primary clinical outcome measure was successful wound healing after definitive closure or coverage with skin graft. Impaired wound healing included delayed wound closure or wound dehiscence after closure or coverage. Delayed wound closure was defined as definitive closure occurring ≥21 d after injury, or two standard deviations outside of the mean normal wound closure time period of 10 d. Dehiscence was defined as spontaneous partial or complete wound disruption after primary closure or >90% skin graft loss. Wounds that progressed to healing at 30 d without requiring a return to the operating room were considered healed.

Statistical Analysis

A Kruskal-Wallis test was performed to detect MMP differences between all time points followed by a post-hoc Mann-Whitney U-test for each time point. Similarities between groups were evaluated *versus* known risk factors using one-way analysis of variance with a *post-hoc* Tukey-Kramer method, or by contingency analysis as appropriate. Associations between categorical variables were studied with the χ^2 test. Each wound was considered independently for statistical analysis of MMP expression and healing outcome. Receiver operating characteristic (ROC) curves were generated by plotting sensitivity *versus* 1-specificity, and area-under-the-curve (AUC) values were calculated to assess the predictive value of MMPs as biomarkers of

healing. Statistical analysis was performed using SPSS (SPSS 16.0, SPSS Inc., Chicago, IL). A two-tailed P value < 0.05 was considered statistically significant. Data is represented as mean ± standard error (SEM) unless otherwise specified.

RESULTS

Patient and Wound Characteristics

Thirty-eight extremity war wounds in 25 patients were investigated. Nine (23.7%) wounds on five (20%) patients demonstrated impaired healing. These included five (13.1%) delayed wound closures in three patients and four (10.5%) wound dehiscences in two patients. The decision to delay wound closure was secondary to clinical concerns of ongoing wound infection ($n = 3$) or severe systemic illness ($n = 2$).

Risk Factors for Impaired Wound Healing

Significant clinical determinants for impaired wound healing included elevated ISS ($P < 0.001$), associated vascular injury ($P = 0.004$), and BMI prior to injury ($P = 0.02$) (Table 1). Number of debridements was significantly higher in impaired healing wounds by definition. The P value of 0.02 confirms that our delineation between impaired and normal healing wounds was appropriate. Age, tobacco use, traumatic brain injury, mechanism of injury, evacuation time, wound location, traumatic amputation, wound size, and closure method were not associated with wound healing ($P \geq 0.05$).

Serum Levels of MMP-2 and MMP-7 are Associated with Impaired Wound Healing

Figure 1 shows the progression of serum MMP-2 and MMP-7 levels after acute traumatic injury for the first three serial debridements and at time of surgical closure by suture or skin graft. MMP-2 and MMP-7 levels, sampled at each surgical debridement, were statistically higher in patients who demonstrated impaired wound healing compared with patients with normally healing wounds ($P < 0.001$). All other MMPs measured in patient serum did not significantly differ between the groups. Additionally, serum MMP-2 did not differ significantly when grouped by vascular injury or ISS, whereas serum MMP-7 did.

Wound VAC Effluent MMP-3 Expression is Associated with Impaired Wound Healing

Figure 1 also shows the progression of MMP-3 levels in the wound bed for the first three debridements and final washout prior to closure. Patients with impaired wound healing expressed statistically lower wound VAC effluent MMP-3 levels throughout the debridement and healing process compared with patients with wounds that healed normally ($P < 0.001$). Effluent MMP-3 did not differ significantly when grouped by vascular injury or ISS. Although not statistically significant, effluent measurements of MMP-7, MMP-9, and MMP 13 demonstrate a measurable difference in expression in impaired healing compared with normal healing wounds. MMP-7 tends to become elevated by time of closure. MMP-9 remained suppressed throughout treatment, and MMP-13, although seemingly corrected by the time of surgical closure, exhibits decreased expression. The difference in average effluent values for MMP-2 was negligible under the assay conditions (data not shown).

Serum and Effluent MMP Biomarkers Objectively Predict Wound Healing

Receiver operating characteristics for individual serum and effluent biomarkers were analyzed. Serum MMP-2 and MMP-7 and effluent MMP-3 were statistically predictive of wound healing outcome throughout the initial three and final debridements prior to wound closure ($P < 0.001$). These three MMPs are strongly correlated with wound healing ($AUC_{MMP2} = 0.744$, $AUC_{MMP7} = 0.783$, $AUC_{MMP3} = 0.805$) (Table 2; Fig. 2).

DISCUSSION

Evidence-based, decision-supportive tools for application in the early stages of traumatic wound healing are lacking. It has been hypothesized that acute wound failures are a consequence of an exaggerated inflammatory response [25]. Key regulators of tissue infiltration are zinc- or calcium-dependent matrix metalloproteinases (MMPs). MMPs serve a pro-inflammatory function by establishing chemotactic gradients for cellular recruitment and by breaking down components of the extracellular matrix to enhance migration. In wound healing, MMPs also fulfill a dual role in tissue remodeling in that they not only have proteolytic activity to degrade components of connective tissue and basement membrane, but also function to polymerize some of these same components in a carefully orchestrated fashion. Because of this, and the established contribution of MMP dysregulation to persistent chronic wounds [15, 22–24], expression of MMPs following traumatic extremity injury is a reasonable marker of such an exaggerated inflammatory response that would potentiate acute wound failure.

Of the five classes of matrix metalloproteinases, we selected two gelatinases (MMP-2 and MMP-9), a stromelysin (MMP-3), a matrilysin (MMP-7), and a collagenase (MMP-13) for investigation and found that the 'pro-inflammatory' MMPs, 2 and 7, were increased over multiple debridements, and that the 'wound

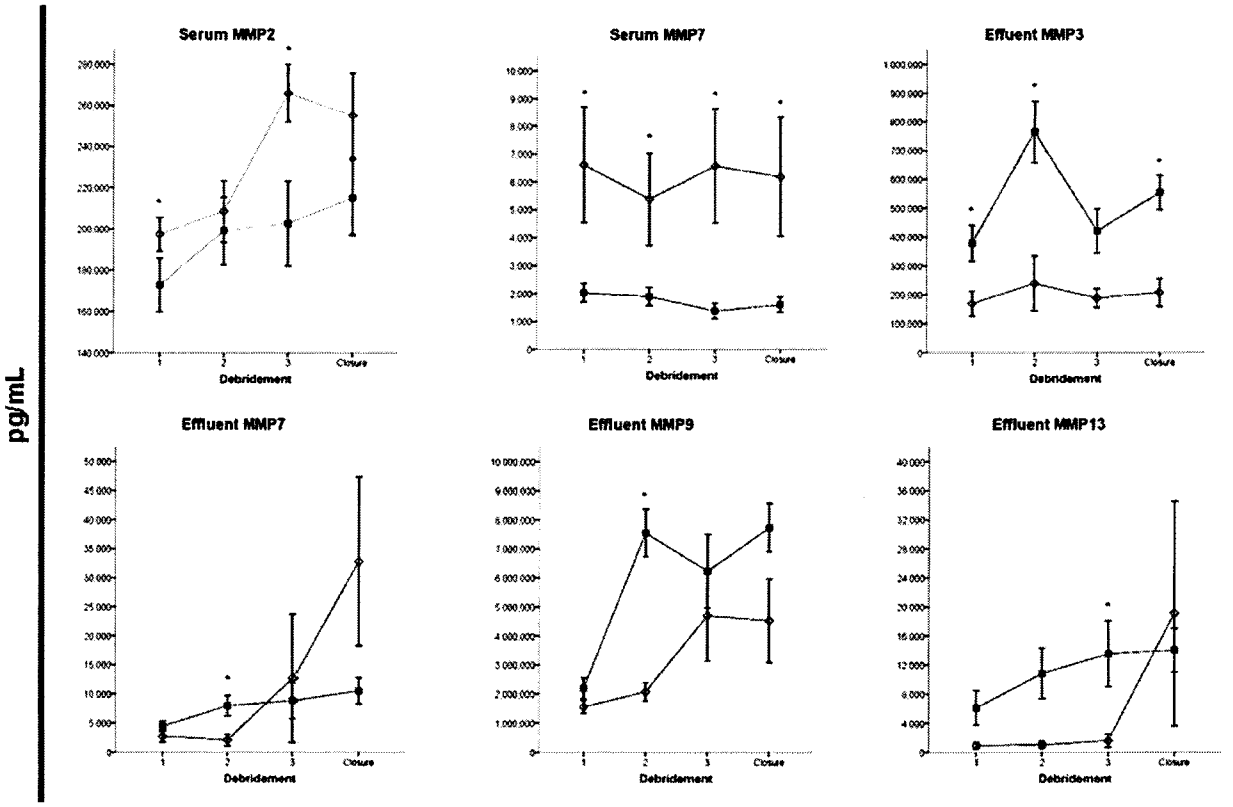

FIG. 1. Serum and effluent matrix metalloproteinase expression at each wound debridement for normal (filled square) and impaired (open diamond) wound healing. Data is depicted in pg of protein per mL of serum or effluent sample as mean ± SEM. *$P < 0.05$ from normal healing patients.

resolution' MMP, MMP-3, was decreased in the impaired healing group with statistical significance (Fig.1).

The average serum level of MMP-2 increased from hospital admission to surgical wound closure for both normal healing and impaired healing wounds in our patient population. However, patients with impaired healing wounds expressed significantly higher serum MMP-2 levels at the first and third serial debridements as opposed to patients who had normal healing wounds (Fig. 1). Additionally, when considering all values over the first three debridements and at time of surgical closure, elevated MMP-2 expression is a significant predictor of wound healing as evidenced by the ROC analysis (Table 2; Fig. 2). Whether this was a systemic response or a serum representation of the local wound environment is not known at this time. Elevated serum MMP-2 levels are consistent with an increase in both collagen and basement membrane cleaving activity as well as immune cell recruitment. While these actions are important for the proper progression of wounds through the stages of healing, excessive extracellular matrix cleavage and inflammatory cell recruitment brought on by too high of a concentration of MMP-2 clearly has deleterious effects on wound healing by contributing to an exaggerated inflammatory response [15, 22–24].

Similar to the results of serum MMP-2, serum MMP-7 levels were significantly higher in impaired healing wounds compared with normal healing wounds throughout the serial debridements (Fig. 1). However, unlike the expression of MMP-2, both normal and impaired wounds maintained a relatively constant expression of MMP-7 throughout the healing process.

TABLE 2

Receiver Operating Characteristics for Individual Serum and Effluent Biomarkers

MMP	(AUC)*	95% CI†	*P* value
Effluent MMP-3	0.805	0.718–0.892	<0.001
Serum MMP-7	0.783	0.683–0.882	<0.001
Serum MMP-2	0.744	0.653–0.836	<0.001
Effluent MMP-9	0.661	0.554–0.768	0.01
Serum MMP-9	0.655	0.543–0.767	0.014
Effluent MMP-13	0.644	0.534–0.754	0.022
Effluent MMP-7	0.632	0.497–0.768	0.035

*Area-under-the-curve.
†Confidence interval.

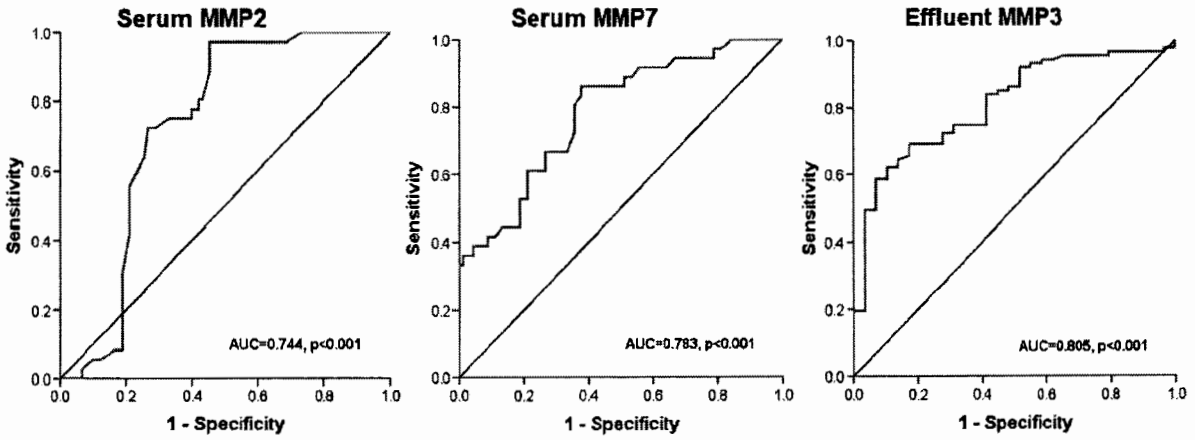

FIG. 2. Area-under-the-curve graphs for serum MMP-2, serum MMP-7, and VAC effluent MMP-3. (Color version of figure is available online.)

Since the role of MMP-7 is in the processing of ECM, re-epithelialization, and neutrophil migration, it is likely that excessive levels of this protease contributed to an exaggerated inflammatory response and subsequent impairment of wound healing similar to that of excessive MMP-2 expression.

Conversely, the average effluent MMP-3 expression for normal healing wounds was higher overall, and significantly so at the first, second, and final debridements. Since MMP-3 performs degradation of the ECM as well as wound re-epithelialization and contraction, elevated expression of this MMP signifies wound remodeling and resolution. Wounds that exhibited impaired healing were associated with suppressed MMP-3 levels relative to normal healing. A paucity of MMP-3 is consistent with insufficient signaling required to enter the wound proliferation and maturation phases of normal wound healing.

We acknowledge that there are limitations to the biological interpretation of this study. The multiplex assays measured pro-, mature, and tissue inhibitors of metalloproteinases (TIMP)-bound MMPs. Measuring the total MMP levels in this manner might not have reflected MMP functionality. Additionally, although elevated serum MMP-7 was associated with impaired wound healing, our analysis revealed a statistical difference between the wounds with or without vascular injury. Though vascular injury was also associated with wound outcome, it remains unclear whether serum MMP-7 is associated solely with wound outcome, or whether it may be a surrogate for vascular injury alone. Despite this, the real correlation of MMP expression to wound healing illustrated by ROC curve analysis presents the potential of these biomarkers as functional, decision-supportive tools for trauma surgeons. The influence of wound bacterial colonization on MMP expression in our patient population warrants further investigation as well.

Our research efforts continue to be aimed at understanding the biology as well as the physiology of wound healing. The interplay between microbial colonization, host response, and wound outcome remains a focus of our investigations. Knowledge of the concordance of MMP level and healing outcome would affect current wound care protocol by warranting additional surgical care for wounds that have not progressed along a healing trajectory or by encouraging earlier wound closures when they have. Although we focused on prediction of wound outcome based on biomarker expression, there is expanding, wound-healing research utilizing dressings (bandages, foams, modified gelatin microspheres, etc.) impregnated with MMPs, or their inhibitors, to alter local concentrations or protease functionality in order to subsequently encourage healing and prevent acute wounds from becoming chronic. Potentially, our study may be applicable as an indicator to begin or end such interventions.

We feel that with the advent of point-of-care devices for measuring MMP expression [26, 27], the lessons learned here will have broad implications for both civilian and combat casualty care as objective markers of wound failure are acutely needed.

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