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Use Of The Human Serum Proteome To Assess The Progression Of Fracture Healing

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Abstract:

Introduction: Serum protein markers may potentially be used to predict fracture healing. However, only a limited number of studies in either animals or humans have been performed (Reviewed in 1). The overall goal of this preliminary prospective observational study was to define the potential efficacy of a serum protein-based diagnostic for predicting the progression or failure of fracture healing in closed humeral diaphyseal fractures through observation of the serum proteome over a six-month healing period.

Methods: Patient Group: Patients were enrolled under an approved sIRB overseen by the Major Extremity Trauma Research Consortium. We assessed nine patients (seven that healed and two non-unions). Primary Inclusion criteria: 1) Skeletally mature, ages 18-70 (inclusive), 2) Diagnosis of extra-articular fractures of the proximal humeral metaphysis (AO/OTA 11 type A2.1, A2.2 and/or A2.3) with diaphysis involvement or diagnosis of isolated closed extra-articular fractures of the humeral shaft (AO/OTA 12), 3) Treated by closed non-operative immobilization in a functional brace, and 4) Approached for consent within three weeks of injury. Primary Exclusion criteria: 1) Humeral shaft fractures that extend into the articular surface (i.e., AO/OTA 13), 2) Pathologic fractures, 3) Additional bone injuries or injury that involved a trauma activation with entry of more than two AIS codes, and 4) Pregnant or lactating women. Time Course of Study: The first study visit was at the first follow up visit at 1-3 weeks after injury and thereafter at 4-6, 7-9, 12-16 and 26-32 weeks. At the regularly scheduled visit at 7-9 weeks, progression to union was assessed. Nonunion was defined as gross motion at the fracture site and mRUST score of less than six at this visit. If assessed as a nonunion the treating physician discussed the option of waiting until next examination to further assess healing or consider surgical treatment to try to improve healing. If the decision is to have surgical treatment, the last blood draw was at the time of surgery. Proteomic Assay: Total plasma was prepared using a standardized protocol

across all participating sites. Samples were stored at -80°C until they were shipped on dry ice to the primary study site where they were organized and freeze thawed one time to generate 200 microliter aliquots. Samples were then transferred to Somalogic (Boulder, Colorado) who provided an aptamer based proteomic profiling as a commercial service using the SomaScan Discovery Assay that screened ~7000 proteins per sample. **Statistical and Bioinformatics Approaches:** In this analysis, the first time point (1-3 weeks post injury) was used as the reference for statistical comparisons, to which each subsequent time was compared. A paired t-test was used to compare the first visit against each subsequent visit (temporal change), and 2-sample t-test to compare the two outcome groups (union vs. nonunion). Only proteins showing a fold change (FC) at 1.25 or greater and a $p < 0.01$ were considered to be differentially expressed (DE). The protein groups showing differences by time and between the union and nonunion groups were then used for ontology analysis using either DAVID (2) or Metascape (3).

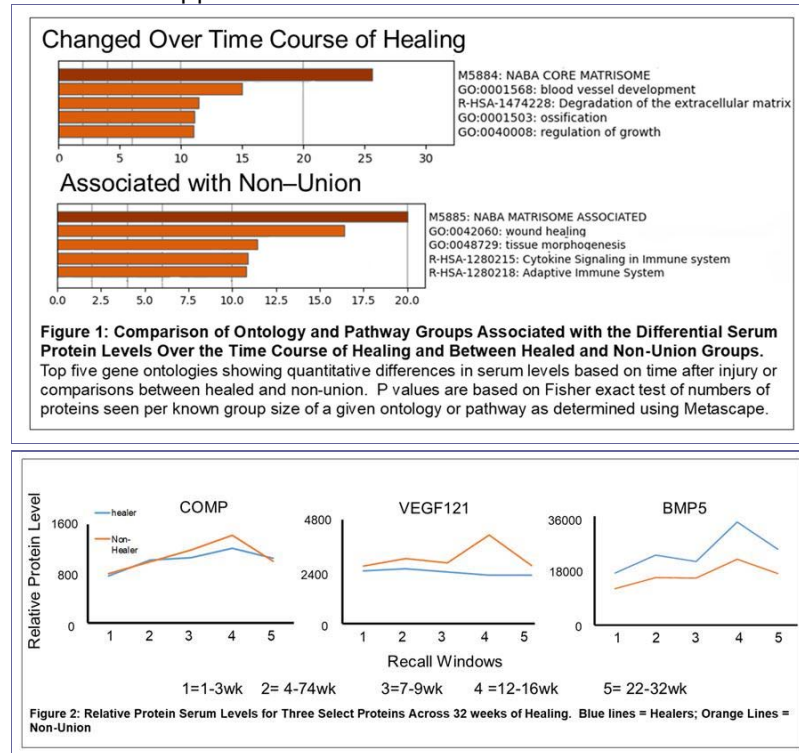


Results: 739 proteins were identified that met our identification criteria that were different by time. 576 showed differences between the nonunion and union groups. 100 proteins overlapped between the two groups. Figure 1 shows a comparison between the top five ontology groups associated with the temporal DE proteins seen in serum of the patient groups that healed normally (Top panel) compared to those patients that developed nonunion (Bottom panel). A total, 121 of the 739 proteins were associated with matrisome (extracellular matrix production). Some notable proteins included Col10A1, periostin, and Col9A1. The second most prominent group seen in normal healing were associated with blood vessel development of which 61 proteins were observed. The proteins associated with the matrisome also were the most prevalent type of protein that passed screening for DE between the nonunion and union groups. Other interesting groups that are of note seen only in the non-healing protein group included immune cytokines and proteins associated with the adaptive immunity. We next show the individual graphs of three representative proteins (Figure 2) that showed both changes in expression over time and were different between the union and nonunion groups. One protein COMP-1 (Cartilage oligomeric protein) is shown as an example of a protein that changed over time but did not show a difference in its expression between healers and non-unions. Two other proteins VEGF 121 (Vascular Endothelia Growth Factor A121) and BMP5 (Bone Morphogenetic Protein 5) are shown as examples of proteins with different patterns of serum profiles between the healer and non-union groups. The selection of these three proteins is shown to demonstrate the potential efficacy of the further development of the approach, since examination of individual proteins are both informative mechanistically to the underlying biology of healing and non-union and of their potential use diagnostically to identify non unions.

Discussion: In this first analysis, our goal was to show both the feasibility and efficacy of using a serum proteomic approach to observe the biological progression of fracture healing to predict nonunion. While none of the protein groups had significance with a False Discovery Rate (FDR) < 0.05 , which is the basis for acceptance for this type of large data analysis, we did observe biological relevancy of the protein groups that were identified based on our criteria for identifying the DE, to the known biology of fracture healing. In order to assess how many patients we would need to reach acceptable levels of statistical significance, we carried out a simulation study for power assessment of our current data using varying sample sizes, proportions of DE proteins, FC differences and varying FDR values. As an example using 10% DE proteins (~760), while controlling FDR at 5%, we found that FDRs will be controlled on average at 0.054, 0.043, 0.035 for $N=25$, 30, and 35, respectively. These assessments suggest that our analysis is easily scalable to identify individual proteins having both prognostic and diagnostic value.

Significance/Clinical Relevance: Even though the current study was less statistically robust, the ontology and pathway analysis of the DE proteins was useful to both successfully identify biological processes associated with the progression of healing and identify biological mechanism(s) that are associated with failed healing. As an example, the current data, *for the first time*, shows the actual temporal progression of the biological processes of human fracture healing. It places the initiation of an observable endochondral process between 4-6 week after injury while placing peak of cartilage formation during endochondral bone formation in the humerus in a window between 7 and 12 weeks.

From a mechanistic point of view, it will provide further clarity on why healing fails, identify what adjunct therapies may have useful efficacy, and when adjunct therapies might show greatest efficacy in their clinical application



Author Disclosure Information:

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Additional Questions (Complete):

* **Abstract Description:** Clinical

* **Abstract Collaboration:** 9

Abstract Collaboration Details: : Major Extermity Trauma Research Consortium

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* **Abstract references:** : 1. Chitwood et al. Biomarkers 2021, 26(8) 703. 2. Huang da, W. et al. Nucleic Acids Res.35 (Web Server issue). 3. Zhou, Y. et al. Nat Commun. 2019, 3;10(1):1523.

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Category for Review (Complete): Clinical Research - Omics, Biomarkers Genetics ; Bone - Injury and Healing ; Cartilage and Synovium - Growth, Development and Aging

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