

Utility of Plasma Protein Biomarkers and Mid-Infrared Spectroscopy for Diagnosing Fracture-Related Infections: A Pilot Study

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## Abstract

**Objectives:** To compare a large panel of plasma protein inflammatory biomarkers and mid-infrared (MIR) spectral patterns between patients with confirmed fracture related infections (FRIs) and controls without infection.

**Design:** Prospective case-control.

**Setting:** Academic, level 1 trauma center.

**Patients:** Thirteen patients meeting confirmatory FRI criteria were matched to 13 controls based on age, time after surgery, and fracture region.

**Intervention:** Plasma levels of 49 proteins were measured using enzyme-linked immunosorbent assay (ELISA) techniques. Fourier transform infrared (FTIR) spectroscopy of dried films was used to obtain MIR spectra of plasma samples.

**Main Outcome Measurements:** Plasma protein levels and MIR spectra of samples.

**Results:** Multivariate analysis-based predictive model developed utilizing ELISA-based biomarkers had sensitivity, specificity, and accuracy of  $69.2\pm 0.0\%$ ,  $99.9\pm 1.0\%$ , and  $84.5\pm 0.6\%$ , respectively, with PDGF-AB/BB, CRP, and MIG selected as the minimum number of variables

explaining group differences ( $P<0.05$ ). Sensitivity, specificity, and accuracy of the predictive model based on MIR spectra were  $69.9\pm 6.2\%$ ,  $71.9\pm 5.9\%$ , and  $70.9\pm 4.8\%$ , respectively, with six wavenumbers as explanatory variables ( $P<0.05$ ).

**Conclusions:** This pilot study demonstrates the feasibility of using a select panel of plasma proteins and FTIR spectroscopy to diagnose FRI. The preliminary data suggest that measurement of these select proteins and MIR spectra may be potential clinical tools to detect FRI. Further investigation of these biomarkers in a larger cohort of patients is warranted.

**Running Title:** Biomarkers for Fracture Related Infection Diagnosis

**Level of Evidence:** Diagnostic Level III. See Instructions for Authors for a complete description of levels of evidence.

**Key Words:** fracture, infection, biomarker, infrared spectroscopy

## INTRODUCTION

The incidence of fracture-related infection (FRI) is commonly reported as 5-10%<sup>1</sup> with treatment costs exceeding \$23,000 per infection. Despite the socio-economic impact, the ability to diagnose FRIs remains challenging.<sup>2</sup> Diagnostic work-up for infection is largely based upon history and physical exam, white blood cell (WBC) count, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and imaging. Unfortunately, these diagnostic tools are of limited utility.<sup>3,4</sup> Quantitative histology and culture from biopsied samples can be useful, but they are

invasive, dependent on sample quality, and results are delayed due to need for sample processing.<sup>5</sup>

Recent studies have shown that WBC, ESR, and CRP are not sufficiently accurate predictors of FRI.<sup>6,7</sup> Few studies have evaluated other inflammatory biomarkers, such as interleukin-6 (IL-6).<sup>8-10</sup> Measurements of inflammatory biomarkers commonly utilizes enzyme-linked immunosorbent assay (ELISA) methodology, and large panels that are disease-specific may limit their clinical applicability due to cost and need for specialized laboratory access. Fourier transform infrared spectroscopy (FTIR) of biological fluids is a simple, cost effective methodology that is a clinically accessible tool previously used in diagnosing a variety of disease processes in animals and humans.<sup>11-18</sup> FTIR spectroscopy produces a signal that is reflective of the sum of all mid-infrared (MIR)-active molecular bonds in a sample.<sup>19</sup> The unique spectrum of a sample can then be used as a “fingerprint” to discriminate between different samples. The purpose of this pilot study was to evaluate a large panel of protein inflammatory biomarkers and MIR spectra from plasma samples to discriminate between patients with confirmed FRIs and controls without infection. Specifically, the aims were to 1) identify potential plasma proteins that might predict presence of FRI and 2) investigate whether MIR spectral patterns of plasma samples can identify FRI.

## **METHODS**

### *Patients*

This diagnostic, level III<sup>20</sup> study was performed at a single level-one trauma center over nine months. Inclusion and exclusion criteria (see **Table, Supplemental Digital Content 1, <http://links.lww.com/JOT/B718>**) were the same for both the confirmed FRI and control groups. The confirmed FRI group had an additional inclusion criterion of a clinically suspected and

subsequently confirmed FRI. No patient had rheumatologic disease or other known chronic inflammatory condition. Patients who had received antibiotic treatment leading up to their FRI diagnosis were not excluded. All FRI confirmed patients were enrolled prior to surgical intervention for their infection. ESR, CRP, and WBC, as well as three intraoperative cultures and gram stains, were obtained as part of the standard of care for the FRI patients. Patients in the control cohort were identified and matched to the FRI patients based on age ( $\pm 15$  years), time after surgery ( $\pm 2$  weeks), and fracture region. Fracture regions were categorized as upper extremity long bones (humerus, radius/ulna, clavicle), lower extremity long bones (femur and tibia), and other lower extremity bones (e.g., patella, ankle, tarsal bones). Controls were patients undergoing fracture care follow-up who had to be infection-free for a minimum of six months after enrollment based on routine clinic follow-up, chart review, or phone calls. Informed consent was obtained from all participants prior to enrollment (IRB#1905884760).

#### *Blood Collection and Processing*

Blood samples were obtained from the FRI cohort preoperatively on the day of surgical intervention to address infection. Blood samples were obtained from the control cohort during routine fracture care follow-up visits. Approximately 5ml of peripheral venous blood was collected in an EDTA purple top tube (BD Vacutainer®, Becton, USA). The tube was inverted 4-5 times allowing the blood to mix with the anticoagulant before it was centrifuged at 1500g for 10 minutes. Plasma was then extracted, aliquoted into 500 $\mu$ L tubes, and stored at  $-80^{\circ}\text{C}$  until batch analysis.

#### *Plasma Protein Measurement*

The Human Cytokine/Chemokine/Growth Factor Panel A 48 Plex (EMD Millipore Corporation, Burlington, MA) was used for protein multiplex ELISA utilizing the Luminex

xMAP technology (Luminex, IL) according to the manufacturer's protocol. The panel contains the proteins listed in Table, **Supplemental Digital Content 2**, <http://links.lww.com/JOT/B719> and was selected because it contains a substantial number of proteins that have been associated with the inflammatory response to infection.<sup>21</sup> Additionally, CRP levels were measured on all samples using the hospital laboratory (Beckman Coulter, CA). Samples were thawed at room temperature (22°C) and then spun at 10,000g for 10 minutes. All samples were run in duplicate. For samples with biomarker concentrations that were undetectable, ½\*lowest detectable value was used for analysis.<sup>22</sup> Samples exceeding maximal detectable values were diluted to fall within the assay range, re-measured, and corrected for dilution.

#### *Infrared Spectroscopy*

Thawed plasma samples were diluted with potassium thiocyanate (KSCN, SigmaUltra, Sigma-Aldrich Inc., MO) as an internal control in a 2:1 ratio. Using a previously described technique, three 8µL replicates of each sample were applied on a 96-welled silicon microplate (Silicon Specialists, Inc.) and allowed to dry at room temperature.<sup>23,24</sup> Each microplate was placed in the multi-sampler (HTS-XT, Bruker Scientific, USA) attachment of an FTIR spectrometer (INVENIO S, Bruker Scientific, USA). Mid-IR (MIR) absorbance spectra in the wavenumber range of 400 to 4,000cm<sup>-1</sup> were recorded using the OPUS software (version 6.5, Bruker Optics, Germany). For each sample, 512 interferograms were signal averaged and Fourier transformed to produce a nominal resolution of 4cm<sup>-1</sup> for the resulting spectrum.<sup>23,25-27</sup>

#### *Statistical Analysis*

Bivariate and multivariate analyses of the protein and spectral data were performed to obtain complementary information from each analytical method.<sup>28,29</sup> Bivariate analyses were performed using two-sided matched *t*-tests. Although change/paired data are typically normally

distributed, plasma protein results can be skewed, so non-parametric signed rank tests were also performed to verify the results of the paired *t*-tests (similar findings, results not shown). The adjusted *P* value for multiple comparison was based on the Bonferroni correction, and statistical significance was set a  $P < 0.001$ . Given the exploratory nature of this study, results with unadjusted *P* value  $< 0.05$  were still evaluated for their relative potential as predictive biomarkers. To analyze the ability of plasma proteins to accurately predict a sample's group (FRI versus control), logistic regression models were performed, and Receiver Operator Characteristics (ROC) curves were generated. The optimal cut-point values for each protein were determined using the Youden Index. A cumulative index, ranging from 0 to 4 for any proteins with  $P < 0.05$  difference, was also calculated by summing the number of proteins that were above separate Youden values, and ROC analyses were performed on each category in order to determine if this can give a more accurate prediction. Bivariate analyses were performed using SAS software (v9.4 SAS Institute, USA).

For multivariate analysis, both data sets (proteins and MIR spectra) were imported into the MATLAB<sup>®</sup> software (MathWorks-R2015b (8.6.0.267246), USA). In-house written scripts were utilized for processing. Initially, spectral data were smoothed using the Savitsky-Golay filter<sup>30</sup> (2<sup>nd</sup> degree polynomial functions and 11-point smoothing window).<sup>31</sup> Standard normal variate transform (SNV)<sup>32</sup> and normalization to the KSCN peak were further used. Verification of whether an observation was an outlier or not relied on the values of the two statistics,  $T^2$  and  $Q$ , for which both the null hypothesis was tested at a 95% confidence level.<sup>33</sup> The average of the three replicates for each sample was used for analysis. Statistical significance based on permutation tests for the multivariate analysis was set at  $P < 0.05$ .<sup>34-36</sup>

To allow comparison of the utility of ELISA-based proteins and sample spectral patterns as predictors of FRI, both data sets were used to build multivariate classification models to discriminate between FRI and control samples, with subsequent validation. Partial least squares discriminant analysis (PLS-DA) was used for classification to address the low number of patient samples in the training set compared to the number of measured variables for both data sets.<sup>37,38</sup> In order to improve classification accuracy and to identify a minimum set of proteins and spectral wavenumbers out of the whole set of variables, PLS-DA classification algorithm was coupled with covariance selection (CovSel).<sup>39</sup> The CovSel-PLS-DA model was built and validated through a repeated double-cross-validation (rDCV) procedure with 13 segments in the outer loop and 12 in the inner loop using 50 repetitions.<sup>35,40</sup> For each cancellation group in the outer loop, predictions were carried out on a model built on the remaining samples. The best subset of original variables as inputs and optimal number of latent variables were selected as those leading to the minimum classification error in the inner-loop cross-validation procedure. Data were auto-scaled prior to the analysis. The selected variables were integrated in a mid-level data fusion approach.<sup>41</sup> The predictors were autoscaled, and all data were further block-scaled to allow equal contributions. For each comparison the accuracy, sensitivity, and specificity of the predictive model, as well as the area under the curve (AUC) of the ROC curve, were reported as measures of the model's performance.<sup>42</sup> The steps of multivariate analysis are summarized in Figure, see **Supplemental Digital Content 3**, <http://links.lww.com/JOT/B720>.

## RESULTS

Eighty-two patients were screened of which 13 FRIS matched with controls met the inclusion criteria. **Table, Supplemental Digital Content 4**, <http://links.lww.com/JOT/B721>

summarizes patient clinical information for both groups. There was a statistically significant difference in time post-operative at which samples were obtained ( $P=0.045$ ), however, the mean difference of a week is unlikely clinically significant. Seven patients in the FRI group had received antibiotics within two weeks of their blood sampling.

Bivariate analysis of measured proteins did not reveal statistically significant differences (all  $P>0.001$ ) (Table, **Supplemental Digital Content 2**, <http://links.lww.com/JOT/B718> and **Figure 1**). Exploratory analysis based on unadjusted  $P$  value ( $P<0.05$ ), identified four proteins (i.e., IL-6, platelet-derived growth factor AB/BB (PDGF-AB/BB), vascular endothelial growth factor-A (VEGF-A), and CRP). These four were carried forward into ROC curve analyses. Cut-points optimizing the ROC analyses were 7.8, 10,443, 77.5 pg/mL for IL-6, PDGF-AB/BB, and VEGF-A, respectively. Cut point was 2.8mg/dL for CRP. Individual AUCs for these cut points ranged from 0.654-0.731 showing weak to acceptable performance (see **Table, Supplemental Digital Content 5**, <http://links.lww.com/JOT/B722>). Examined cumulatively, having all four of these biomarkers below their respective cut-points had a specificity of 100% (see **Table, Supplemental Digital Content 6**, <http://links.lww.com/JOT/B723>). Alternatively, having at least one of these markers above the cut-point may also be diagnostically useful (sensitivity of 84.6%).

Multivariate analysis using plasma protein levels as predictors resulted in PDGF-AB/BB, CRP, and MIG (Monokine induced by gamma interferon, also known as CXCL9) being retained to build the classification model. The overall classification accuracy on the external loop samples was  $84.5\pm 0.6\%$ , with  $69.2\pm 0.0\%$  sensitivity and  $99.9\pm 1.0\%$  specificity (**Figure 2A**). The AUC for this model was  $0.826\pm 0.018$ , demonstrating an excellent discriminant ability. The visual interpretation of the results is presented in **Figure 3**.

Analysis of the spectral data, during the model-building phase, resulted in six latent variables as optimal complexity (**Figure 4A**). The overall classification accuracy on the external loop samples was  $70.9\pm 4.8\%$ , with  $69.9\pm 6.2\%$  sensitivity and  $71.9\pm 5.9\%$  specificity (**Figure 2B**). The AUC for this model was  $0.761\pm 0.041$  indicating an acceptable discriminant ability. Permutation test confirmed that the observed figures of merit were all  $P < 0.05$ . The visual interpretation of the results is presented in **Figure 4B**. The scores plot indicated that FRI patients have predominantly negative values, whereas the majority of controls have positive scores. Comparison with the weight values suggests that FRI patients are characterized by higher absorbance at  $1624.3$  and  $1188.2\text{cm}^{-1}$  and lower absorbance at  $610.6$ ,  $1592.9$ ,  $1648.6$  and  $3288.7\text{cm}^{-1}$  (**Figure 4C**).

Lastly, the predictive variables from the previous two steps were autoscaled, and the blocks of data (proteins and MIR spectra) were further block-scaled to allow equal contributions. The model consistently included four variables (i.e., PDGF-AB/BB, CRP, MIG, and  $610.6\text{cm}^{-1}$ ) that contributed significantly to the model (**Figure 5**) and provided an overall classification accuracy on the external loop samples of  $75.2\pm 4.5\%$ , with  $61.5\pm 6.3\%$  sensitivity and  $88.9\pm 6.6\%$  specificity (**Figure 2C**). The area under the curve was  $0.795\pm 0.054$ , indicating near excellent discriminant ability.

## DISCUSSION

This is the first study demonstrating differences between FRI and control patients based on measured values of CRP, PDGF-AB/BB, and MIG. Exploratory bivariate analysis of the 49 measured proteins also demonstrated the potential for VEGF-A, IL6, and CRP. These results confirm that CRP may be useful in the diagnostic work up of FRI. Platelet-derived growth

factors are released from platelets and, upon binding with specific receptors, are proponents of inflammatory responses.<sup>43</sup> MIG is an interferon-induced chemokine with involvement in the innate immune response and has been investigated with regards to its bactericidal effects in various infections.<sup>44</sup> The specific role of PDGF-AB/BB and MIG in FRI warrants further investigation. Although IL-6, and VEGF-A were not identified as predictive markers based on multivariate analysis, they showed potential in the exploratory bivariate analysis. IL-6 and VEGF-A have been previously reported as elevated in patients with FRI and periprosthetic joint infection,<sup>8-10,21</sup> and VEGF-A has been utilized as a therapeutic target for anti-inflammatory therapy.<sup>45</sup> Therefore, the lack of statistically significant differences in this study is likely due to type II error. It is noteworthy that the results of this study are associative and do not imply any causality of these markers with the local or systemic response to FRI.

In this study, we used complementary approaches of bivariate and multivariate analytical methods to explore biomarker differences from a large panel of inflammatory proteins and MIR spectral signal in plasma from FRI patients and controls. Both analytical approaches have identified PDGF-AB/BB and CRP as candidate biomarkers with discriminatory abilities. The multivariate method showed MIG combined with PDGF-AB/BB and CRP to be the minimum number of non-redundant variables that contributed to the final predictive model. On the other hand, only exploratory bivariate analysis (based on unadjusted *P* value) identified IL-6 and VEGF-A to be potential biomarkers that may be different between groups. Such discrepancy in results of bivariate and multivariate analytical approaches has been previously identified in other metabolomics studies where high number of variables with small sample sizes are encountered.<sup>28,29</sup> However, as a pilot study, the number of patients in this study is in-line with recommendations for the discovery phase of diagnostic or prognostic biomarkers.<sup>46</sup> The

combination of these two analytical methods provides complementary results that reduces loss of information encountered from either approach.<sup>28</sup> Therefore, the results of each analytical approach also requires individual interpretation rather than an attempt to validate the results of one method against the other. Lack of significance for MIG in the exploratory bivariate approach may be due to an existing covariance of this plasma protein with PDGF-AB/BB and CRP that is identifiable through the multivariate approach. On the other hand, lack of IL-6 and VEGF-A being selected in the multivariate analysis may be due to less correlation/covariance between these and other selected biomarkers. However, this does not imply that these two biomarkers do not have significant differences between the two groups, but that the combination of PDGF-AB/BB, CRP, MIG variables was the minimum number of non-redundant variables that was able to best demonstrate the group differences in the multivariate approach.

There is limited literature on novel methods for diagnosis of FRIs.<sup>4</sup> Historically, the standard inflammatory markers used to aide in the diagnosis of FRI have been peripheral WBC, CRP, and ESR. A systematic review of diagnostic accuracy of these “classic” plasma inflammatory markers determined that they are insufficient.<sup>7</sup> In that review, sensitivity and specificity based on CRP ranged from 60-100% and 34-86%, respectively. The ability to predict FRI based on PDGF-AB/BB, CRP, MIG in this study was comparable in sensitivity to previous reports based on CRP alone but significantly improved for specificity and accuracy. The predictive model based on MIR spectra alone showed similar sensitivity to that of select protein biomarkers. However, despite having an acceptable discriminatory ability, the specificity and accuracy were lower than those based on protein biomarkers. Another interesting finding in this study was that combining the selected protein biomarkers and spectral variables improved the discriminatory ability of the final predictive model compared to spectral data alone, but it did not

surpass the performance of the model based on protein biomarkers alone. These results suggest that, in this cohort of patients, the predictive model based on this select panel of protein biomarkers is the more accurate and specific discriminatory tool, with similar sensitivity compared to spectral fingerprint alone. These results must be interpreted with caution, as the sample size for development of the models are small and cannot be generalized to larger populations. However, the comparable results based on MIR spectral data demonstrate the potential ability of this FTIR spectroscopy method to be used as a surrogate for this protein panel as a potential point of care diagnostic screening tool. Advantages of using FTIR spectroscopy compared to ELISA-based biomarkers include lack of need for adjuvants and cost effectiveness (~5% the cost of ELISA methods). The spectroscopy technique used here relies on spectral patterns of the sample as a “fingerprint” to identify differences between samples rather than identification of unique molecules responsible for the observed changes. Future studies looking to identify the molecules responsible for the observed spectral differences will require “omics” techniques (e.g., metabolomics, proteomics) such as mass spectroscopy.

The main limitations of this pilot study include small sample size and the exploratory nature of the bivariate analysis. It is important to point out that the bivariate analysis with adjusted *P* value for multiple comparisons did not reveal any of the measured proteins to be different between the two groups. The multivariate analytical approach, however, is more effective in handling the problem of small sample size (i.e., number of patients) compared to the large number of measured variables (i.e., spectral data and protein measurements), particularly with the repeated double cross validation scheme with the use of permutation tests.<sup>34-36,47</sup> However, the predictive models based on this pilot study require validation in larger populations. Several potential confounders may have also resulted in elevated inflammatory biomarkers in the

FRI group despite strict inclusion criteria and matching for age, time after initial surgery, and fracture region. It is well known that inflammatory biomarkers, such as CRP, increase with surgery with a subsequent time-dependent return to normal post-operatively.<sup>48,49</sup> The temporal change following surgery of other protein biomarkers investigated in the present study is unknown. Additionally, acute inflammatory phases during trauma, fracture healing, and the number of broken can affect systemic measures of inflammation.<sup>50-52</sup> These factors may have affected our results as cases were not matched for these variables. Finally, seven of the FRI patients had received antibiotics, which may have blunted measured protein levels. Therefore, we recommend caution in interpretation of these results before larger confirmatory studies have been completed.

In conclusion, this is a first step towards evaluation of plasma protein profiling and MIR spectral pattern analysis as potential methods to diagnose FRI. Preliminary results of this pilot study are promising for acceptable to good diagnostic accuracy and predictive ability based on multivariate analytical methods. Developing validated diagnostic biomarkers can prove beneficial for clinical use, as well as a trial enrichment strategy in interventional studies.<sup>53</sup> Validation and testing of plasma protein profiling and MIR spectral pattern analysis requires future studies with increased sample size.

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### **Figure Legends**

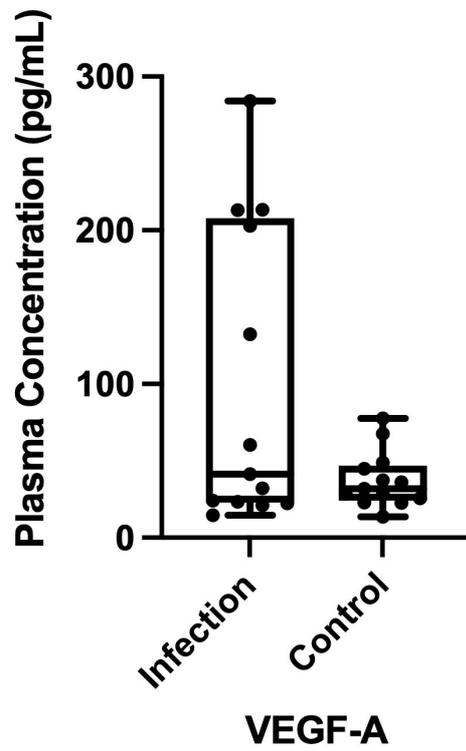
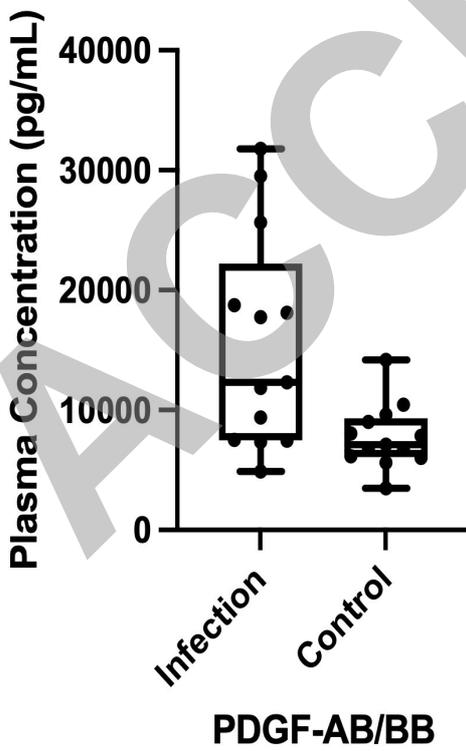
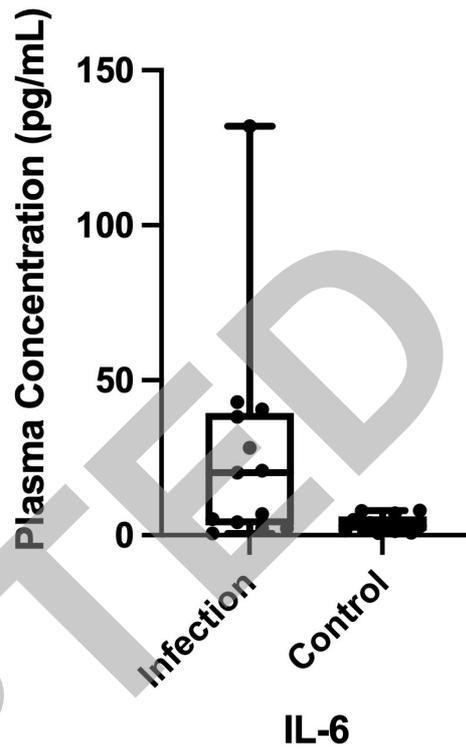
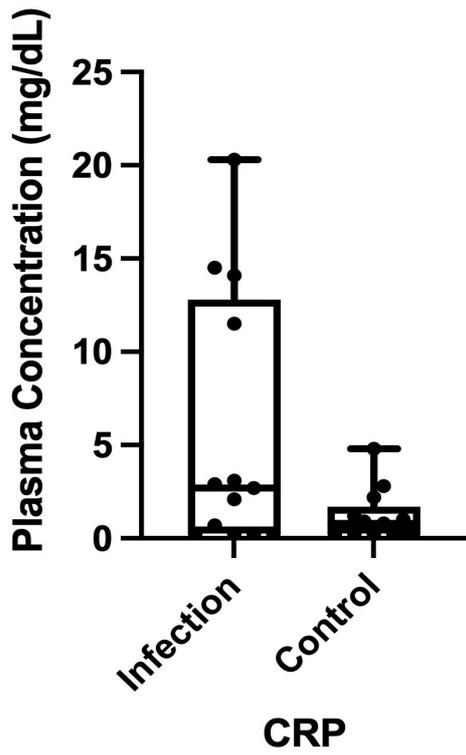
**Figure 1: Box and Whisker Plots for CRP, IL-6, PDGF-AB/BB, and VEGF-A.** CRP, C-reactive protein; IL-6, Interleukin 6; PDGF-AB/BB, platelet derived growth factor AB/BB; VEGF, vascular endothelial growth factor.

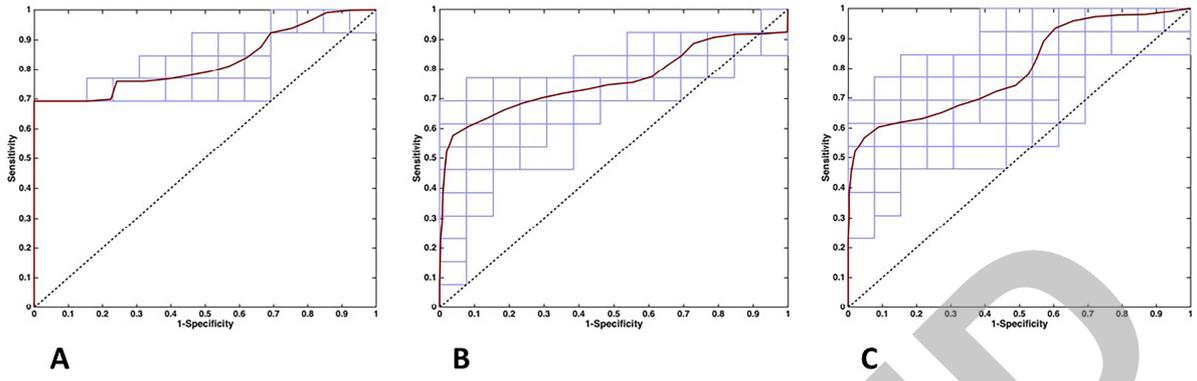
**Figure 2: Receiver operator characteristic (ROC) curves for A) ELISA-based biomarkers, B) FTIR spectral data, and C) Data fusion of ELISA-based biomarker and spectral data variables.** ROC curves calculated based on predictions from the repeated (x50) double-cross-validation (rDCV) outer loops are reported (both the ones corresponding to the individual DCV runs, blue lines, and their average, thick dark red line). X axis is 1-specificity (false positive rate), and Y axis is sensitivity (false negative rate).

**Figure 3: Results of CovSel-PLS-DA modeling on ELISA-based protein biomarker data.** A) Double-cross-validated projections of the outer loop samples onto the only canonical variate of the classification model showing the difference in the values of the scores (bars indicate mean and whiskers the corresponding 95% confidence intervals) between FRI and control patients' samples and B) Weights of the selected markers for the construction of that projection direction (canonical variate). FRI, fracture related infection group; Ctrl, control group; PDGF-AB/BB, platelet derived growth factor AB/BB; CRP, C-reactive protein; MIG, Monokine induced by gamma interferon.

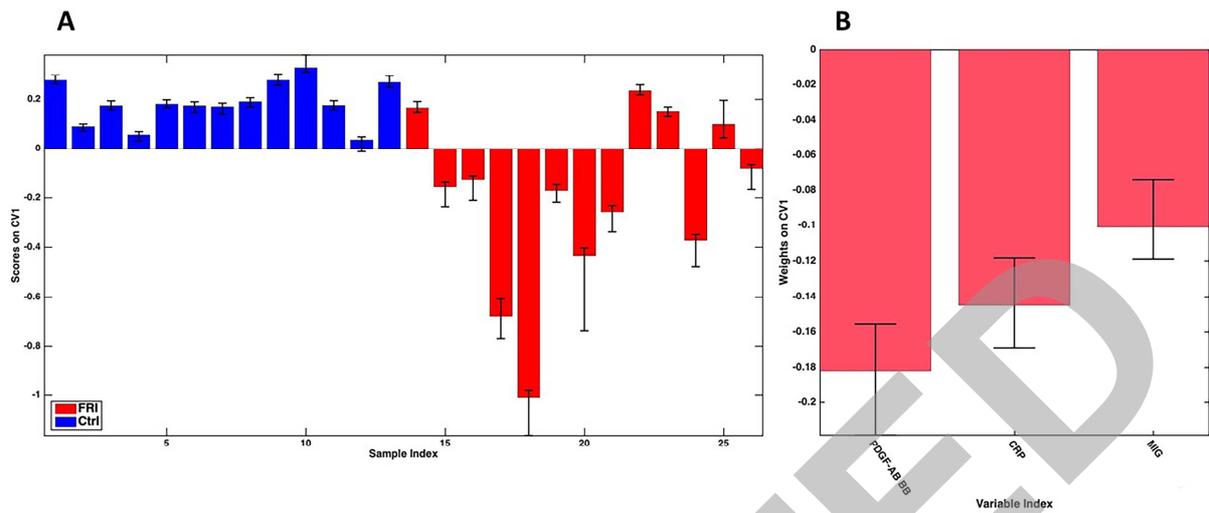
**Figure 4: Results of CovSel-PLS-DA modeling on IR spectroscopic data.** A) Identification of the six variables (wavenumbers) selected as relevant by the CovSel algorithm (3288.7, 1648.6, 1624.3, 1592.9, 1188.2 and 610.6 cm<sup>-1</sup>; red vertical bars) over the mean IR spectrum of the samples. B) double-cross-validated projections of the outer loop samples onto the only canonical variate of the classification model showing the difference in the values of the scores (bars indicate mean and whiskers the corresponding 95% confidence intervals) between FRI and control patient samples and C) Weights of the selected markers for the construction of that projection direction (canonical variate). FRI, fracture related infection group; Ctrl, control group.

**Figure 5: Results of PLS-DA data fusion of selected ELISA-based biomarkers and FTIR spectral variables.** A) Double-cross-validated projections of the outer loop samples onto the only canonical variate of the classification model showing the difference in the values of the scores (bars indicate mean and whiskers the corresponding 95% confidence intervals) between FRI and control patients' samples and B) Weights of the selected markers for the construction of that projection direction (canonical variate). FRI, fracture related infection group; Ctrl, control group; CV, coefficient variable; PDGF-AB/BB, platelet derived growth factor AB/BB; CRP, C-reactive protein; MIG, Monokine induced by gamma interferon.

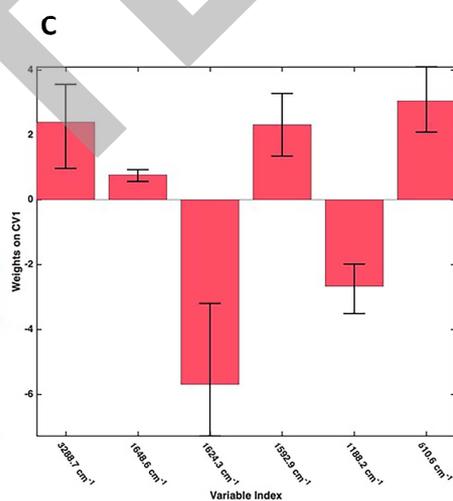
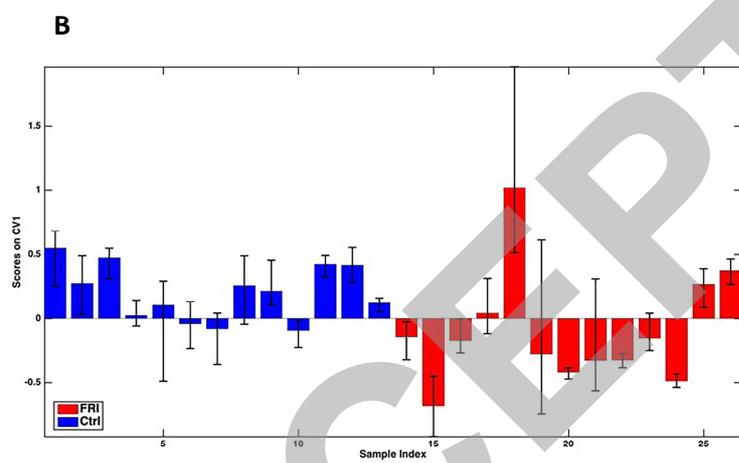
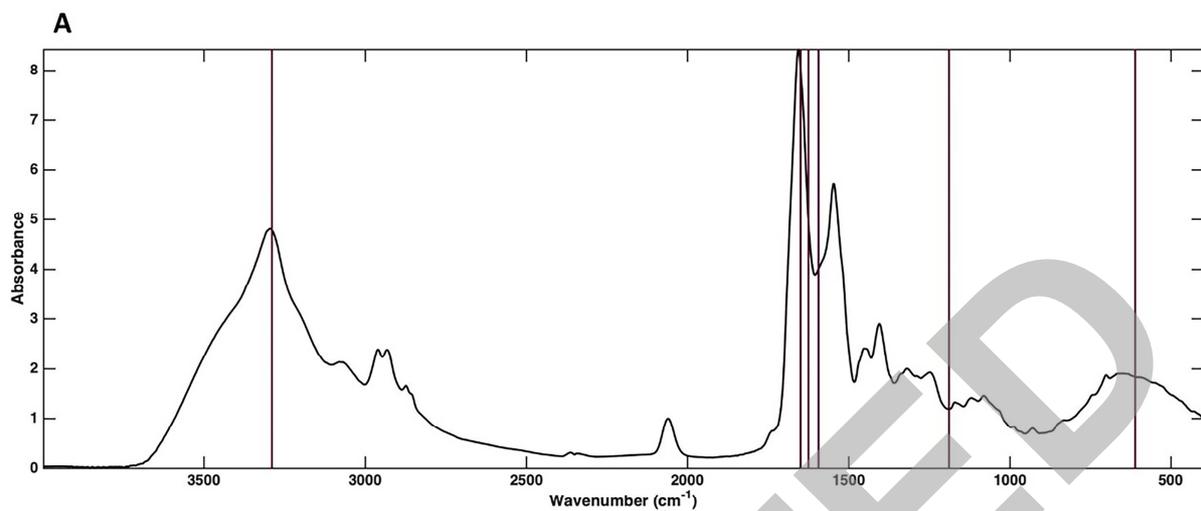


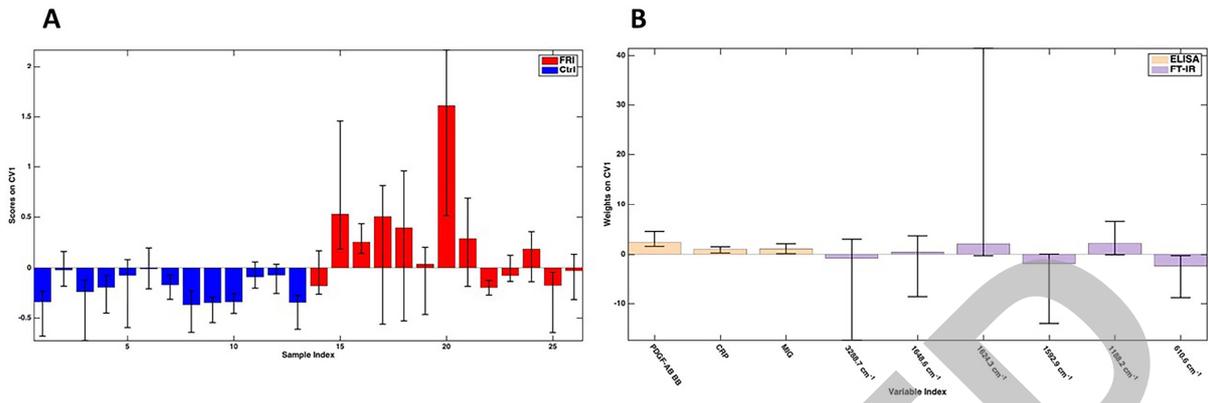


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