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A Bioinformatic Approach to Utilize a Patient’s Antibody-Secreting Cells against *Staphylococcus aureus* to Detect Challenging Musculoskeletal Infections

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**ABSTRACT**

Noninvasive diagnostics for *Staphylococcus aureus* musculoskeletal infections (MSKI) remain challenging. Abs from newly activated, pathogen-specific plasmablasts in human blood, which emerge during an ongoing infection, can be used for diagnosing and tracking treatment response in diabetic foot infections. Using multianalyte immunoassays on medium enriched for newly synthesized Abs (MENSA) from Ab-secreting cells, we assessed anti–*S. aureus* IgG responses in 101 MSKI patients (63 culture-confirmed *S. aureus*, 38 *S. aureus*–negative) and 52 healthy controls. MENSA IgG levels were assessed for their ability to identify the presence and type of *S. aureus* MSKI using machine learning and multivariate receiver operating characteristic curves. Eleven *S. aureus*–infected patients were presented with prosthetic joint infections, 15 with fracture-related infections, 5 with native joint septic arthritis, 15 with diabetic foot infections, and 17 with suspected orthopedic infections in the soft tissue. Anti–*S. aureus* MENSA IgG levels in patients with non-*S. aureus* infections and healthy controls were 4-fold (**p = 0.0002) and 8-fold (**p < 0.0001) lower, respectively, compared with those with culture-confirmed *S. aureus* infections. Comparison of MENSA IgG responses among *S. aureus* culture–positive patients revealed Ags predictive of active MSKI (IsdB, SCIN, Gmd) and Ags predictive of MSKI type (IsdB, IsdH, Amd, Hla). When combined, IsdB, IsdH, Gmd, Amd, SCIN, and Hla were highly discriminatory of *S. aureus* MSKI (area under the ROC curve = 0.89 [95% confidence interval 0.82–0.93, p < 0.01]). Collectively, these results demonstrate the feasibility of a bioinformatic approach to use a patient’s active immune proteome against *S. aureus* to diagnose challenging MSKI. *ImmunoHorizons*, 2020, 4: 339–351.
INTRODUCTION

Musculoskeletal infections (MSKI) continue to be a significant source of complications and increased cost in orthopedic surgery. This socioeconomic burden of MSKI, especially after surgery, will only increase in the United States because of the aging population that requires increased care for age-related conditions such as arthritis and geriatric fractures (1–3). Despite recent advances in surgical procedures, improved quality of implants, and novel antimicrobial therapies, the infection rates in following total joint replacement (TJR) arthroplasties and trauma surgery have remained mostly unchanged over the last 50 y (3). There is an urgent need to control MSKI post-TJR, as these arthroplasties are projected to increase by ~400% by 2030 in the United States (4), and importantly, treatment failure rates after MSKI remains high (5–7).

Most MSKI in TJR arthroplasties are caused by Staphylococcus aureus, frequently by difficult-to-treat methicillin-resistant S. aureus and emerging strains with pan-resistance (8–11). This commensal pathogen is implicated in several types of orthopedic infections, including prosthetic joint infections (PJI), fracture-related infections (FRI), native joint septic arthritis (SEP), and diabetic foot infections (DFI) (12–20). The ability of S. aureus to cause the aforementioned MSKI could be attributed to the numerous immune evasion mechanisms it deploys to invade the bone niche successfully. These mechanisms include biofilm formation, osteocytic-canalicular invasion of cortical bone, bone marrow abscess formation, and intracellular invasion of non-professional phagocytes (21–27).

Diagnosis of deep-seated MSKI has been challenging, as patients typically present with nonspecific signs such as fever, pain, swelling, and erythema. The current diagnostic paradigm for these infections involves the analysis of nonspecific WBC counts and inflammatory markers such as erythrocyte sedimentation rate and C-reactive protein (CRP) (28). Unfortunately, these standard-of-care tests are neither pathogen specific nor anatomically specific. In the clinic, S. aureus MSKI are primarily diagnosed by direct observation of the pathogen, either by an invasive culture or by PCR of the wound swab. Identification of S. aureus infections via wound cultures has major limitations, including 1) limited utility for difficult-to-sample infections like PJI, 2) inability to distinguish the commensal from infecting pathogen that is responsible for MSKI, and 3) high false-negative rates particularly after the initiation of antibiotic therapy.

Noninvasive, blood-based approaches, describing anti–S. aureus humoral immune response, are an attractive alternative for diagnosing MSKI. Several groups, including ours, have described serum-based S. aureus–specific Abs that are produced during colonization and infection (29–35). Recently, we developed a multiplex immunoassay that has shown promise for the diagnosing and tracking of S. aureus DFI (36). We demonstrated that anti–S. aureus Abs secreted from a population of newly activated, pathogen-specific plasmablasts (Ab-secreting cells [ASCs]), which emerge into the bloodstream during an active infection, can be used for the diagnosis of ongoing S. aureus DFI in patients. More importantly, the secreted medium enriched for newly synthesized Abs (MENSA) produced by ASCs measures the host immune response at that moment and can be used to track the success and failure of antibiotic therapy in DFI patients (36).

In this study, we expanded the utility of our MENSA-based immunoassay to various S. aureus MSKI. We hypothesized that MENSA-based IgG responses could accurately diagnose S. aureus as the infecting pathogen in various S. aureus MSKI. Because different MSKI (PJI versus FRI versus SEP versus DFI) will generate unique humoral immune responses, we also hypothesized that our ASC-based immunoassay could reliably identify and differentiate these ongoing S. aureus MSKI. To test these hypotheses with a clinical proof-of-concept study, using bioinformatic approaches, we evaluated anti–S. aureus IgG responses in 101 MSKI patients (with S. aureus and non–S. aureus infections) to assess the sensitivity and specificity of Ag-specific MENSA for the presence and type of S. aureus MSKI. To our knowledge, this is the first study to demonstrate the utility of ASCs to identify the presence and type of ongoing S. aureus MSKI. As such, it represents a significant step toward a personalized medicine blood-based diagnostic for challenging S. aureus MSKI.

MATERIALS AND METHODS

Ethics statement

This was a study approved by the University of Rochester Medical Center’s Research Subjects Review Board (RSRB#00046924, RSRB#00057719). Patient enrollment was performed after obtaining written, informed consent. The principal investigator or study coordinators conducted all enrollment, consenting, and sample procurement activities. All study personnel involved in patient recruitment were approved by our institutional review board, with current Collaborative Institutional Training Initiative certification.

Patient enrollment

One hundred and one patients presenting to the emergency department or orthopedics department of our tertiary care center were enrolled in the current study from February of 2014 to January of 2018. This included patients with one of the following MSKI: 1) PJI, 2) FRI, 3) SEP, 4) DFI, or 5) suspected orthopedic infections in the soft tissue (SSTI). Patients with immunodeficiencies were excluded, as were minors. Both types I and II patients with diabetes were permitted to be enrolled. All patients received bedside or intraoperative wound cultures, as well as clinical laboratories, including blood glucose, hemoglobin A1c (HbA1c), body mass index, WBC, erythrocyte sedimentation rate, and CRP. Sixty-three out of the 101 patients were culture-confirmed to have S. aureus MSKI. Fifty-two individuals with no active orthopedic or nonorthopedic infections were also recruited as a control population. These individuals are preprimary patients scheduled for elective total knee or hip replacement arthroplasties. The aforementioned clinical parameters were obtained for 26 of the 52 control patients.
**Whole-blood processing**

Whole blood (6–10 ml) was obtained from the study subjects after informed consent and was taken to sample each patient after hospital admission and prior to or <24 h after the initiation of antibiotic therapy. Whole blood was processed immediately to isolate PBMCs using the Ficoll-Paque lymphocyte preparation method as described previously (25, 36). Harvested PBMCs were washed extensively using sterile PBS to remove serum Igs and cultured at a density of 10⁶ cells per milliliter in RPMI-1640 supplemented with an antibiotics-antimycotics solution, glutamine, and 20% FBS at 37°C in 5% CO₂ for 72 h. The resulting culture supernatant MENSA was harvested, aliquoted, and maintained at −80°C for long term storage (25, 36).

**Luminex-based multiplex immunoassays**

Anti-\(S. \text{aureus}\) IgG levels were determined using a custom multianalyte Luminox immunoassay, the development of which has been previously described (35, 36). Briefly, avidin-coated magnetic LumAvidin microspheres with unique spectral signatures were coupled to individual recombinant \(S. \text{aureus}\) Ags belonging to distinct functional classes as described previously (35, 36). These include 1) iron-scavenging proteins/iron-regulated surface determinant proteins (IsdA, IsdB, and IsdH); 2) cell wall enzymes autolysin (Atl), glucosaminidase (Gmd), and aminidase (Amid); 3) immune evasion proteins chemotaxis inhibitory protein of \(S. \text{aureus}\) (CHIPS), staphylococcal inhibitory protein (SCIN), and staphylococcal protein A (Spa); 4) secreted toxins \(\alpha\)-hemolysin (Hla), phenol soluble modulins (PSM), and leukocidin LukF-PV; and 5) cell attachment proteins clumping factor B (ClfB), fibrinogen binding protein A (FnBPA), serine-aspartate repeat protein C (SdrC), and bone-sialoprotein binding protein (Bsbp, also known as SdrE).

For the detection of \(S. \text{aureus}\) Ag-reactive IgG, 1000 magnetic beads per analyte per well were mixed, sonicated, and incubated with 100 \(\mu\)l of MENSA (in duplicates) for 2 h. After washing, the samples were incubated for 1 h with a secondary detection reagent, PE-conjugated goat anti-human IgG (SouthernBiotech), and run on a Luminex Flow Cytometer (Bio-Plex 200; Bio-Rad Laboratories, Life Sciences Research). The fluorescent intensity of the beads (to determine specificity) and PE (to determine level of bound Ab) (100 beads per analyte per well) were acquired for analysis. As described previously (36), the generated data from the multiplex immunoassay was accepted for downstream analyses only if the coefficient of variation (the ratio of SD to the mean) among the replicates was <20%. Consistent with our prior study (36), the cumulative lower limit of detection for all Ags was calculated using the formula lower limit of detection = median fluorescent intensity (MFI) of assay buffer + 2 × SEM of assay buffer MFI.

**Data analyses**

Patient characteristics were compared using Fisher exact test or the Wilcoxon rank-sum test for variations in parameters among the infected patients and healthy controls. Comparisons of the 16-array anti-\(S. \text{aureus}\) Ab measurements across patients with \(S. \text{aureus}\) infections, non-\(S. \text{aureus}\) infections, and healthy individuals were performed using nonparametric multivariate ANOVA (MANOVA). Similarly, MANOVA tests were also performed to compare the different classes of MSKI within \(S. \text{aureus}\)-infected patients. MENSA IgG levels for each Ag were assessed for their predictive ability in discriminating the presence and type of \(S. \text{aureus}\) MSKI using receiver operating characteristic (ROC) curve analysis, with overall prediction accuracy summarized by the area under the ROC curve (AUC). ROC analyses were also conducted for a combination of Ags using best subsets selection with multivariate logistic regression models to identify clusters of Abs that best diagnosed and discriminated \(S. \text{aureus}\) MSKI infections. Additionally, each individual Ab’s contribution to discriminatory ability in combinations of Ags was quantified by its average AUC across all combinations with one, two, and three other Abs, which is an average of 120 + 560 + 1820 = 2500 AUC’s for each of the 16 Ags. Nonparametric estimates for the AUC for each predictor, along with 95% CIs and p values for testing each AUC’s significance, were also computed. Furthermore, binary random forest classifiers were trained for each pair of cohorts (healthy controls, \(S. \text{aureus}\), and non-\(S. \text{aureus}\)) using a leave-one-out cross-validation scheme to assess the discriminatory importance of Abs based on the corresponding mean decrease in the Gini coefficient (mean ± SD), averaged over all cross-validation folds. The AUC curve of these models was computed as an overall measure of their discriminatory power. All analyses were performed using GraphPad Prism version 8.4.1, SAS version 9.4, and R version 3.6.2. A p value <0.05 was considered significant.

**RESULTS**

**Patient demographics and clinical outcomes**

Patients were recruited from those entering the University of Rochester Medical Center’s Emergency Department with suspected orthopedic infections who were subsequently transferred to the Department of Orthopedics for care. A total of 101 patients with five distinctive types of infection, 1) PJIs (n = 19), 2) FRI (n = 19), 3) SEP (n = 34), DFIs (n = 34), and SSTI (n = 22), were enrolled in the study. Results from culture-based diagnostic procedures identified 63 patients who were infected by \(S. \text{aureus}\) and 38 patients whose infections were caused by one or more other species. In addition, 52 uninfected healthy individuals were recruited as controls. Patient demographics and clinical information are described in Table I. Statistical analyses revealed that the uninfected and infected cohorts are well matched with the exception of age and HbA1c status.

**Anti-\(S. \text{aureus}\) IgG responses in MENSA can diagnose patients with \(S. \text{aureus}\) MSKI**

 Newly secreted anti-\(S. \text{aureus}\) Ab levels specific for 16 different \(S. \text{aureus}\) Ags from ASCs were analyzed using our custom multiplex immunoassay (Fig. 1). Immunoanalyses were performed on 136 out of the 153 enrolled subjects, as 17 were excluded because of missing/unreliable clinical data or sample mismanagement. MENSA IgG levels were undetectable in uninfected control subjects (Fig. 1A). Similarly, most patients with non-\(S. \text{aureus}\) infections had low Ab levels (Fig. 1B). However, 9 out of 30 (30%)
had detectable levels against ≥5 Ags (primarily IsdA, IsdB, Amd, Gmd, Hla, IsaA, and Spa), suggesting the presence of \textit{S. aureus} cross-reactive Abs or misdiagnosis via culture. Interestingly, 12 non–\textit{S. aureus} DFI patients (DFU012, DFU013, DFU015, DFU016, DFU017, DFU023, DFU024, ASCI17, ASCO31, ASCI04, ASCI07, ASCI10) with reported chronic polymicrobial infections had higher IgG levels compared with other patients with non–\textit{S. aureus} infections (Fig. 1B, \textit{**} \textit{p} = 0.0011, MANOVA). In sharp contrast, the IgG titers in MENSA from culture-confirmed \textit{S. aureus}–infected patients were significantly higher (Fig. 1C) for one and, typically, several Ags (**** \textit{p} < 0.00001 versus controls, \textit{****} \textit{p} < 0.0001 versus non–\textit{S. aureus} infections, MANOVA). Overall, anti–\textit{S. aureus} MENSA IgG levels in patients with non–\textit{S. aureus} infections and healthy controls were 4.38-fold (** \textit{p} = 0.0002, ANOVA) and 8.35-fold (**** \textit{p} < 0.0001, ANOVA) lower, respectively compared with those with culture-confirmed \textit{S. aureus} infections.

To formally examine the potential of a single \textit{S. aureus} Ag for diagnosing an ongoing infection in MENSA, receiver operator AUC characteristics were performed (Fig. 2). Interestingly, a direct comparison of the immunodominant Ag IsdB-specific IgG levels between the control subjects and patients with culture-confirmed \textit{S. aureus} infections yielded the highest ROC curve with an AUC of 0.857 (**** \textit{p} < 0.0001, Fig. 2A, 2B). Intriguingly, a control patient ASCO28 had high anti-IsdB MENSA IgG levels. Examination of the potential of each Ag to identify subjects with ongoing infections (Fig. 2C) compared with control subjects yielded highly significant separation of the two groups and AUC values ranged between 0.6182 (FnBPA) and 0.8472 (Hla). Similarly, a direct comparison of the \textit{S. aureus}–infected population and the patients with non–\textit{S. aureus} infections yielded comparable results with some notable differences (Fig. 2D). For example, IsdB with an AUC of 0.6637 (\textit{p} = 0.011) was not the most discerning Ag in this comparison. Conversely, SCIN, an Ag associated with early infections and unique to \textit{S. aureus}, was the most discriminatory yielding an AUC of 0.8623 (**** \textit{p} < 0.0001, Fig. 2C) of the several Ags that can discriminate these two groups reasonably well (AUC > 0.8).

Finally, when comparing patients with non–\textit{S. aureus} infections to control subjects, none of the Ags achieved significance in its ability to identify ongoing infections (Fig. 2E).

**Anti–\textit{S. aureus} IgG responses in MENSA can detect various classes of \textit{S. aureus} MSKI**

An implicit hypothesis in this examination of the humoral immunity against ongoing \textit{S. aureus} MSKI has been that the antigen specificity will be unique for various MSKI classes due to differences in the host microenvironment niche at the infection site. To test this hypothesis, we examined immunodominant Ags in each of the five classes of infections (PJI, FRI, SEP, DFI, and SSTI) included in our enrolled population. In the illustrated heat map in Fig. 3A, the \textit{S. aureus}–infected patients have been grouped by the site of their infections, and log-transformed MENSA IgG titers of each Ag has been color-coded in a concentration-dependent manner with very high concentrations indicated in red and very low in blue (Fig. 3A).

This analysis produced three remarkable findings. First, \textit{S. aureus} infections in each of the five sites were significantly distinguishable from the control population by nonparametric MANOVA tests (Fig. 3B), so the ability to identify ongoing \textit{S. aureus} infections is not limited to any subset of these infection groups. Second, \textit{S. aureus} infections were distinguishable from non–\textit{S. aureus} infections for the PJI, DFI, and SSTI groups, but not from the FRI group, and the SEP group was equivocal due to only five patients. Third, patients with non–\textit{S. aureus} infections were not resolvable from the control population except for the predominantly polymicrobial DFI group (\textit{**} \textit{p} = 0.0072), which had higher IgG MENSA levels compared with other non–\textit{S. aureus} infections (Fig. 1B).

Next, we examined if MENSA IgG responses against \textit{S. aureus} Ags are diagnostic for each class of \textit{S. aureus} infection using ROC analysis and the AUC values against uninfected controls or patients with non–\textit{S. aureus} infections (Fig. 3C). Two Ags were conspicuously effective at detecting all five infection groups: Gmd and SCIN (AUC > 0.75 for each Ag alone). Iron-scavenging proteins (IsdA, IsdB, and IsdH) were strong distinguishers of

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**TABLE I. Patient clinical and demographic characteristics**

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<th></th>
<th>Healthy Controls</th>
<th>\textit{S. aureus} Infection</th>
<th>Non–\textit{S. aureus} Infection</th>
<th>Total</th>
<th>\textit{p} Value</th>
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<td>63</td>
<td>38</td>
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<td><strong>Age (years)</strong></td>
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<td>10</td>
<td>4</td>
<td>17</td>
<td></td>
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<td></td>
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<tr>
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<td>34</td>
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<td>SSTI</td>
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<td>5</td>
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<td><strong>Clinical laboratories</strong></td>
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<td>HbA1C (%)</td>
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<td>WBC count (×1000)</td>
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<td>Body mass index (kg/m²)</td>
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<td>31.2 ± 6.6</td>
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S. aureus–infected patients from control subjects but were not quite so discerning against patients with infections caused by non–S. aureus pathogens. Interestingly, the IsdB Ag was highly discriminatory of DFI infections. Additionally, Hla was highly discriminatory of SEP and DFI S. aureus infections when compared against control subjects and non–S. aureus infections. Moreover, IgG responses against SdrC, CHIPS, LukF-PV, and Amd were intriguing because they were more discerning only in certain classes of S. aureus infections. Among the 16 Ags, the responses against six Ags (FnBPA, ClfB, IsaA, Spa, PSM, and Bsbp) were poor overall at discerning most infection classes with the possible exception of DFI.

Next, we examined if IgG responses against specific Ags can be associated with certain classes of infection. To this end, we assessed Ag-specific MENSAs IgG response in pairwise combination of S. aureus infections (PJI versus FRI versus SEP versus DFI versus SSTI). One example illustrates a significant difference in the host response against IsdH between patients with PJI and those with SEP infections (**p < 0.01, Fig. 4A). Similarly, pronounced differences were observed in anti-IsdB responses between DFI patients and those with SSTI (****p < 0.0001, Fig. 4B). The response against all 16 Ags and the 10 pairwise combinations of infection classes are presented as AUC values from pairwise ROC analyses (Fig. 4C). Responses by patients experiencing PJI, SSTI, and FRI were not readily differentiable from each other, but SEP (by Amd) and DFI (by IsdB) infections were distinguishable from the other groups and possibly from each other (by Amd). In conclusion, Ags IsdB, Amd, Hla, and IsdH were highly discriminatory of the various classes of S. aureus MSKI.

The diagnostic prediction can be improved by increasing cross-functional antigenic diversity in Ag combinations

We previously reported that increasing cross-functional antigenic diversity can markedly improve our immunoassay’s ability to diagnose an ongoing S. aureus DFI infection (36). In the current

**FIGURE 1. Anti–S. aureus Ab IgG levels in MENSAs in patients with orthopedic infections.**

The MFIs for each of the 16 Ags were determined using multiplex Luminex immunoassay on MENSAs samples. The primary anti–S. aureus IgG levels in MENSAs in (A) healthy controls (n = 45), (B) patients with non–S. aureus infections (n = 30), and (C) S. aureus–infected individuals (n = 61) are depicted in this study. Primary IgG data were log-transformed, and heatmaps illustrating the expression levels between the groups are illustrated in this study. Although highly variable, the global MENSAs IgG levels in patients with S. aureus orthopedic infections were significantly higher (****p = 0.00001, MANOVA) than those measured in healthy controls and patients with non–S. aureus infections (****p = 0.00001, MANOVA).

https://doi.org/10.4049/immunohorizons.2000024
study, we examined this intriguing observation for various *S. aureus* MSKI. Multivariate ROC analyses on MENSA IgG levels using combinations of recombinant *S. aureus* Ags chosen within or across distinct functional classes (iron-scavenging proteins, cell wall enzymes, immune evasion proteins, secreted toxins, and adhesins) was performed. The best combinations of two and three Ags for distinguishing *S. aureus*–infected patients from control subjects (Fig. 5A) are presented as AUC values from ROC analyses. Host IgG responses against the three secreted toxins Hla, LukF-PV, and PSM yielded the best AUC among Ags within a functional group, which was still significantly lower than two-Ag cross-functional combination of Hla + SCIN (*p* = 0.029). The two-or-three Ag combinations that had Ags from different functional categories yielded improved AUC values of greater than 0.8 when comparing patients with *S. aureus* infections and controls. The same observation was made for the discrimination of patients suffering *S. aureus* infections and those with infections caused by other pathogens (Fig. 5B), although expectedly, the Ag combinations were different. Additionally, each of the 16 Ags’ discriminatory ability in combinations of Ags was computed to yield the average AUC for *S. aureus* infections versus controls and *S. aureus* versus non-*S. aureus* infections (Fig. 5C). Interestingly, Ags Hla, IsdH, Gmd, SCIN, and IsdA yielded high average AUCs, >0.8.

**Ags IsdB, IsdH, Gmd, Amd, SCIN, and Hla are the best discriminators of *S. aureus* MSKI**

We next examined how many, among the 16 Ags, are absolutely essential for identifying the presence and type of ongoing *S. aureus* MSKI in a patient. This information is crucial for eventually developing and validating a robust clinical diagnostic assay for *S. aureus* MSKI. To probe for essential discriminatory Ags, we used a combination of 1) objective machine learning approach and 2) subjective manual approach with defined AUC discrimination criteria.
In the objective approach, the MENSA IgG responses were trained using random forest classifiers for each pair of cohorts (healthy controls, S. aureus, and non–S. aureus infections), and the resulting discriminatory importance of Ags was rank-ordered from low to high using the Gini coefficient output (Fig. 6A–C). Interestingly, Ags Hla, IsdB, IsdA, SCIN, and SdrC were the top five discriminators when comparing S. aureus–infected patients against controls (Fig. 6A). Ags SCIN, Gmd, IsdH, IsdA, and Amd
were the top discriminators when comparing *S. aureus* against non-*S. aureus*–infected patients (Fig. 6B). ROC curve analyses on these random forest models yielded high AUCs of 0.875 (**p < 0.001, Fig. 6D) and 0.756 (**p < 0.001, Fig. 6E), respectively, for these cohorts. Expectedly, non-*S. aureus* infections were not resolvable from the control population with a low AUC (Fig. 6F). In the subjective approach, we defined the following criteria to narrow down to most informative discriminatory Ags in *S. aureus* MENSA IgG responses: 1) Ags that yielded diagnostic AUC of >0.8 when identifying *S. aureus* MSKI (Fig. 2), 2) Ags that produced an AUC of >0.8 for functional class diagnosis (Fig. 3), and finally, 3) Ags with predictive AUC of >0.8 while differentiating two or more classes of MSKI (Fig. 4).

Combining the above approaches resulted in six best discriminatory Ags belonging to various functional classes: 1) iron-scavenging proteins (IsdB, IsdH), 2) cell wall enzymes (Amd, Gmd), 3) immune evasion protein (SCIN), and 4) secreted toxin (Hla). Remarkably, the six-Ag combination yielded highly significant separation of *S. aureus* infections versus healthy with a predictive AUC of 0.8889 (**p < 0.001, Fig. 7A, 7C) and *S. aureus* versus non-*S. aureus* infections with an AUC of 0.8645 (**p < 0.001, Fig. 7B, 7D).

**DISCUSSION**

Reliable, fast, and minimally invasive diagnosis of *S. aureus* MSKI is a critical first step to administering optimal treatment, and the absence of such a test is a major healthcare problem. The work presented is a step toward achieving such a diagnostic test of high

FIGURE 4. Anti–*S. aureus* IgG responses in MENSA can differentiate various classes of orthopedic infections. Primary IgG data illustrating MENSA Ab levels’ predictive ability to discriminate (A) PJI versus SEP and (B) DFI versus SSTI. *S. aureus* infections are illustrated in this study. (C) AUC heatmap comparisons illustrating pairwise discrimination of PJI versus FRI versus SEP versus DFI versus SSTI *S. aureus* infections are depicted. Asterisks indicate Ag-specific discrimination among the various classes. \*p < 0.05, \**p < 0.01, \***p < 0.001. \****p < 0.0001.
FIGURE 5. The diagnostic prediction can be markedly improved by increasing cross-functional antigenic diversity in Ag combinations.

The graphs illustrate AUCs from combinations of Ags with similar and different functions for (A) S. aureus infections versus healthy controls (n = 106) and (B) S. aureus versus non-S. aureus infections (n = 91). Note that Ag combinations across functions improve diagnostic predictive power. (C) Each Ag’s discriminatory ability as measured by its average AUC across all combinations with one, two, and three other Ags (average of 120 + 560 + 1820 = 2500 AUC’s for each of the 16 Ags) was computed as a combined mean AUC for diagnosis. Subsequently, the mean AUCs are rank-ordered and displaced for S. aureus infections versus healthy controls and S. aureus versus non-S. aureus infections. ****p < 0.0001.

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clinical value. The current study has two significant findings: 1) MENSA-based IgG responses can reliably be used to identify various classes of *S. aureus* MSKI with excellent sensitivity and specificity, and 2) antigenic specificity during humoral immune responses will be unique for various MSKI due to differences in host microenvironment niche. A key advantage of Ab-based diagnosis in MENSA is that we can identify the infecting pathogen. Using a combinatorial approach, this study identified six functionally distinct Ags (IsdB, IsdH, Gmd, Amd, SCIN, and Hla) that are highly specific, discriminatory, and can be used as diagnostic biomarkers for *S. aureus* infections in orthopedic indications.

IsdB and IsdH are necessary for iron acquisition (37, 38), and we previously demonstrated that higher anti-IsdB Ab levels in *S. aureus*–induced PJI patients are prognostic of morbid outcomes (35). In the current study, IsdB levels were highly discriminatory of DFI infections, which indicates antigenic specificity to a host niche. Additionally, change in anti-IsdH IgG levels in MENSA overtime was predictive of healing status in DFI patients undergoing antibiotic foot salvage therapy (36). Interestingly, two of the six Ags are the subunits of the cell wall autolysin AtlA (Amd and Gmd), enzymes essential for bacterial cell division (39).

In contrast to the Isd proteins, serum anti-Gmd levels were protective against *S. aureus* infections in patients undergoing orthopedic surgeries (40, 41). A recent study demonstrated that anti-Gmd Abs identified in synovial fluid is highly sensitive and specific for diagnosing chronic *S. aureus* osteomyelitis (42). Our laboratory has explored these proteins as potential targets for passive immunotherapy against *S. aureus* osteomyelitis (40, 41, 43, 44). Another discriminatory protein SCIN is a secreted immuno-evasion protein that is present in 90% of clinical strains of *S. aureus*. It is involved in the establishment of biofilms and blocking of host immune response by binding to the human complement system (45, 46). Finally, the multifunctional pore-forming cytotoxin Hla, which contributes to superficial and invasive *S. aureus* infections (47, 48), was a highly discriminatory Ag of *S. aureus* MSKI. Recently, neutralizing mAbs against Hla were shown to prevent *S. aureus* biofilm formation on orthopedic implants in a murine hematogenous PJI model (49). Complementary to MENSA, serum anti-SCIN and anti-Hla IgG responses were also highly discriminatory of *S. aureus* osteomyelitis patients compared with healthy controls (35). Surprisingly, with the exception of SdrC, MENSA IgG responses against most bacterial adhesion proteins were not useful biomarkers.

**FIGURE 6.** Machine learning classification of infections identifies discriminatory Ags of *S. aureus* MSKI.

Leave-one-out cross-validated random forest classification models were fit on MENSA IgG levels for (A) *S. aureus* infections versus healthy controls (n = 106), (B) *S. aureus* versus non-*S. aureus* infections (n = 91), and (C) non-*S. aureus* infections versus healthy controls (n = 75). The resulting mean decrease in Gini coefficient (mean ± SD across all cross-validation runs), which is a measure of each feature’s importance in the model, was used to order the Ags from most important to least important in (A)–(C). ROC curves based on the random forest model leave-one-out cross-validation predictions were computed and the resulting AUC values are displayed for (D) *S. aureus* infections versus healthy controls (AUC = 0.875 [95% CI 0.8033–0.9447, ***p < 0.001]), (E) *S. aureus* versus non-*S. aureus* infections (AUC = 0.756 [95% CI 0.6370–0.8741, ***p < 0.001]), and (F) non-*S. aureus* infections versus healthy controls (AUC = 0.696 [95% CI 0.5634–0.8282, **p = 0.005]).

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of \textit{S. aureus} MSKI. However, these proteins were shown to have diagnostic and prognostic roles in staphylococcal septicemia (50).

Serologic detection of anti-\textit{S. aureus} Abs against acute \textit{S. aureus} infections such as bacteremia and skin infections are being pursued by other investigators (51–54). A recent study involving 42 children with invasive \textit{S. aureus} infections demonstrated that anti-LukAB Abs could be a useful diagnostic predictor (55), although leukocidin was not a biomarker for MSKI. These studies further highlight the differences in the microenvironment pressures that contribute to differential \textit{S. aureus} humoral immune response. Other nonspecific diagnostic biomarkers such as IL-6 and synovial leukocyte esterase have shown some promise in improved sensitivity and specificity, although there remains room for improvement (56, 57). Interestingly, nonspecific biomarkers such as IL-8 and CCL2 are also being evaluated as prognostic tools for identifying patients with the highest risk for bacteremia-associated mortality (58).

The current study has its limitations. First, the sample size of the enrolled populations is modest, especially in the SEP group (7 patients), limiting the power of our conclusions pertaining to that group. Second, the primary diagnostic tool (wound culture) that we needed to use as a “standard” is not very reliable and prone to high false negatives of up to 15% (59–61). There were few patients in our non-\textit{S. aureus}–infected group with modest anti-\textit{S. aureus} MENSA IgG responses. These patients most likely were misdiagnosed by culture or possibly had polyclonal infections that include coagulase-negative Staphylococci. In the future, we will need to use criteria for identification of the infecting pathogen (e.g., PCR), although that too has similar potential shortcomings (62). A third limitation is patient follow-up. Only a single blood draw was performed at the time of hospital admission and there was no

\[ \text{Log-transformed MENSA IgG levels for the six best discriminatory Ags are represented with heatmaps for (A) \textit{S. aureus} infections versus healthy controls and (B) \textit{S. aureus} versus Non-\textit{S. aureus} infections. The combined diagnostic predictive values of the six best Ags were computed using multivariate logistic regression for (C) \textit{S. aureus} versus healthy controls yielding the equation} \]

\[1.02 \times 10^{-3} \times \text{IsdB} + 8.30 \times 10^{-4} \times \text{IsdH} + 1.02 \times 10^{-3} \times \text{GMD} - 1.27 \times 10^{-4} \times \text{AMD} + 2.39 \times 10^{-3} \times \text{SCIN} + 1.37 \times 10^{-2} \times \text{Hla} \]

\[ \text{to yield a highly discriminatory AUC of 0.89 (95% CI 0.83–0.95, ***p < 0.001). (D) Similar calculations were performed for \textit{S. aureus} versus non-\textit{S. aureus}, yielding the regression equation} \]

\[2.41 \times 10^{-4} \times \text{IsdB} + 3.23 \times 10^{-3} \times \text{IsdH} + 7.28 \times 10^{-3} \times \text{GMD} - 1.39 \times 10^{-4} \times \text{AMD} + 8.02 \times 10^{-4} \times \text{SCIN} - 6.16 \times 10^{-4} \times \text{Hla}, \]

\[\text{which also yielded a high AUC of 0.86 (95% CI 0.78–0.94, ***p < 0.001).} \]
provision for follow-up to track therapy, as demonstrated in our previous study with DFI patients (36). A primary future goal is to monitor MENSA IgG responses over time to track the success of therapeutic interventions in patients with orthopedic infections. Reducing the complexity of ASC preparation from whole blood, shortening the time needed to produce pathogen-specific MENSA, and expanding this analytical approach to detect/track non-S. aureus polymicrobial infections will enable widespread adoption of this MENSA-based diagnostic immunoassay in the clinic.

DISCLOSURES

E.M.S. is a founder of Telephus Medical LLC (San Diego, CA). J.L.D. and F.E.-H.L. are cofounders of MicroB-plex, Inc. (Atlanta, GA) and work there part-time. The other authors have no financial conflicts of interest.

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