Host derived biomarkers of inflammation, apoptosis, and endothelial activation are associated with clinical outcomes in patients with bacteremia and sepsis regardless of microbial etiology

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LETTER TO THE EDITOR

Sepsis, defined as the systemic inflammatory response syndrome (SIRS) in the setting of suspected infection, is directly responsible for at least 200,000 deaths per year in the United States, accounting for more attributable deaths than breast cancer, colon cancer, leukemia, lymphoma, and ovarian cancer combined. By consensus, sepsis is defined as infection combined with the systemic inflammatory response syndrome (SIRS). The pathophysiology of sepsis is complex, and the relative contributions of host-mediated responses and the specific causative pathogen to poor patient outcomes are unclear. In a patient population with clearly defined bloodstream infections, we sought to determine whether acute bloodstream infection with different pathogens was associated with different concentrations of host biomarkers reflective of different physiologic processes.

From the standpoint of the host response, there are substantial differences between the degree of acute inflammation induced by specific bacterial species in pre-clinical animal model systems with controlled inocula of pathogen. In pediatric populations with hematologic malignancy, differences were observed in inflammatory cytokine production in patients with gram-positive cocci when compared to gram-negative bacilli infections. In adults, higher levels of pro-inflammatory cytokines IL-6, IL-8, sFAS, sVAM-1, and sTNFR-1 have been reported in human leukocytes stimulated with LPS when compared to heat-killed S. aureus, a finding mirrored in the elevations of the serum of patients with unspecified “infections” from gram-positive pathogens.

The in vitro differences are contrasted by in vivo findings from a different group who found no difference in IL-6 between patients with sepsis from pure gram-positive infection when compared to pure gram-negative infections. No differences were found at the level of mRNA expression of neutrophils from patients with gram-positive versus gram-negative infections. In terms of clinical outcomes using administrative databases, authors have reported either no difference in outcome or a roughly 6% crude mortality difference with respect to the inciting pathogen.

In experimental systems, the acute hyperinflammatory response has traditionally been thought to be followed by a subsequent compensatory anti-inflammatory response syndrome (CARS) where patients are thought to be at risk from pathogens of normally low virulence. This two-phase response has been brought into question recently as markers associated with both inflammation and an anti-inflammatory response (e.g. IL-10) are elevated simultaneously in both routine clinical care and experimental systems. We are aware of no data examining the host response to pathogens of normally low virulence.

Immunologic signals are thought to mediate organ dysfunction by contributing to disruption of endothelial integrity. Soluble TNF receptor (sTNFR-1) is considered to play an anti-inflammatory role by binding TNF and preventing activation of the membrane form of TNFR on target cells. sTNFR-1 has been associated

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with outcomes in sepsis but has not been investigated in the context of different pathogens. The Ang-1-Ang-2 axis has emerged as a strong candidate biomarker for measurement endothelial physiology. Ang-1 is present at stable, detectable levels in the blood of healthy volunteers and serves to maintain endothelial integrity. Ang-2 is not normally present in the circulation of healthy individuals but acute inflammation induces the release of Ang-2 from endothelial cells. Ang-2 competes with Ang-1 for binding to the tyrosine kinase receptor, Tie-2, promoting microvascular leak in preclinical mouse models. The response of the angiopoietin system to different classes of endovascular bacterial infection has not been investigated to date.

Septic patients also have an increase in cellular apoptosis, primarily occurring in the spleen and lymphatic organs. Signaling via the canonical death receptor FAS (CD95) receptor is a key event in the one of the major pathways that induces apoptosis. Gram-negative bacteria contain a conserved Type III secretion system that targets this pathway, which is absent in gram-positive bacteria. It is unknown if there are differences in FAS mediated responses with respect to different pathogens but sFAS has been associated with the development of sepsis after trauma.

Since there is complexity in the host response to severe infection, biomarker panels have been proposed as a method to capture composite information. If different responses could be observed early between bacterial class, differential patterns of biomarker concentrations could be used to stratify patients for novel interventions that targeted various elements of the host response. Efforts to understand the different host response to various pathogens are complementary to current efforts to improve the rapidity of microbiologic identification using molecular techniques. For example, if low virulence organisms were associated with an altered ratio of inflammatory to anti-inflammatory markers, it would suggest that immunostimulatory strategies should be pursued.

The goals of our pilot study were to determine: 1) whether sepsis caused by specific classes of bacterial pathogens is associated with differences in concentrations of biomarkers reflective of the host response, and 2) whether bloodstream infection with different pathogens is associated with variations in clinical outcomes in sepsis.

Subjects: The Harborview SIRS cohort prospectively enrolled subjects meeting at least 2 SIRS criteria within 24 hours of enrollment who were admitted to the ICU and clinically suspected to have infection from December 2006-December 2010, as previously described. Exclusion criteria included known immunosuppression, HIV infection, current diagnosis of cancer, major trauma, or intracranial hemorrhage. For all subjects, plasma samples were obtained within 24 hours of enrollment. The cohort was initially restricted to Caucasians to minimize the potential for confounding due to underlying genetic differences. Of this larger prospective cohort, we retrospectively identified a subset of 100 patients who had a defined bloodstream infection within 24 hours of when the plasma sample was obtained. Bloodstream infection was defined according to Centers for Disease Control and Prevention (CDC) criteria. In concordance with CDC criteria, we excluded patients with microbiologic growth of an alternate pathogen at any other site, and each infection was classified as “gram-positive cocci,” “gram-negative bacilli” or “low virulence” on the basis of the pathogen’s reported ability to cause clinical sepsis in immunocompetent hosts. We applied strict criteria to the low virulence class that included at least two positive cultures and an absence of an alternative diagnosis at any other site, given the fact that many of the organisms in this class (e.g., coagulase negative staphylococci) are common colonizers of the skin. See supplementary Table 1 for full details of the organisms identified. APACHE III scores were calculated using data from the first 24 hours after admission.

Protein measurement: Frozen plasma samples were thawed no more than twice and concentrations of IL-6, IL-8, G-CSF, sTNFR-1, Ang-1, Ang-2, sVCAM-1 and sFAS were measured using a multiplex immunoassay (Meso Scale Discovery, Rockville, MD). To fit the samples to a standard curve, samples were diluted. The dynamic ranges of the assays were as follows: IL-6, IL-8, sTNFR-1, G-CSF (0.08–2500 pg/mL); Ang-1 (3–100,000 pg/mL); Ang-2 (0.5 to 10,000 pg/mL); and sVCAM-1 (0.05 to 1000ng/mL). In 9 patients, there were insufficient sample volume for testing of IL-6, IL-6, G-CSF, or sTNFR-1. Samples above or below the range of the assay were assigned the upper or lower limit of the assay, respectively.

Statistical analysis

We used the Mann-Whitney U test for comparison between the groups “alive at 28 days” and “dead at 28 days.” The data were not normally distributed, so we transformed biomarker concentrations using log-2 transformation in order to apply linear models. We generated a Spearman correlation matrix in order to determine the degree of correlation between each biomarker when compared to each other biomarker. Weak correlation was defined as a Spearman correlation of less than 0.5.

Our initial analysis utilized unadjusted logistic regression to test for associations with biomarkers and 28-day
health evaluation (APACHE) III score into our model. We then incorporated the Acute Physiology and Chronic Health Evaluation (APACHE) III definition, adjusting for potential confounders with respect to mortality.

We also tested for unadjusted associations with each class of bacteria with respect to 28-day mortality or hypotension, using gram-positive cocci as the reference. As above, we then proceeded to use multivariable logistic regression models to adjust for covariates and acute illness. We tested for differences between biomarker concentrations in patients with bloodstream infection using bacterial class as a categorical variable using a Mann Whitney U test, considering p < 0.05 to represent statistical significance.

Statistical analysis was performed using R 3.0.0 and graphics were generated in R unless otherwise specified. The dot plots graphs for each biomarker were generated using Prism 6 (Graphpad Software).

The demographics of the patients who survived to 28 days did not differ statistically from those who died within 28 days (Table 1). The population was composed primarily of patients admitted to the medicine service (83%), and had a median age of 53.5 years. Roughly half of all patients experienced hypotension within 72 hours. The mean APACHE III score was 53.7 (+/- 27.6) in patients who survived to 28 days and 80.2 +/- 38.7 in patients who died. In our population, 17% of all patients in the cohort died within 28 days. Because of low sample volume, we were unable to perform biomarker analysis on nine samples.

**Correlations among pro-inflammatory biomarkers**

We evaluated the correlation of each biomarker to each other biomarker using a Spearman matrix (Supplementary Table 2). In general, biomarkers were weakly correlated across pre-established categories and more strongly correlated within categories. Notable exceptions included the following: weak correlation (Spearman correlation <0.5) within the category of “endothelial homeostasis” (Ang-1, Ang-2, and sVCAM-1); strong correlation (Spearman correlation >0.5) between sFAS and sTNFR-1 (0.68); and sFAS and sVCAM-1 (0.56).

**Biomarker association with 28 day mortality**

There were differences in the mean of biomarker concentrations between patients who survived and patients who did not, as shown in Table 2. In unadjusted logistic regression analysis (Table 3), all biomarkers were associated with 28-day mortality with a p value < 0.05 (with the exception of Ang-1 and G-CSF). The ratio of Ang-2/Ang-1 was associated with 28-day mortality with an odds ratio of 1.26 (1.00-1.61). All biomarkers were associated with the development of hypotension in unadjusted analysis (data not shown).

Since tobacco use, age, chronic renal insufficiency and diabetes could influence the development of hypotension and or the expression of biomarkers, we then adjusted

| Table 1. Demographics of HMS SIRS study populations. |
|----------------------------------------------------|
| **Characteristic** | **Alive at 28 days (N = 83)** | **Dead at 28 days (N = 17)** |
| Patient Age, mean ± SD | 51.9 ± 16.94 | 64.0 ± 13.1 |
| Male patients, no. (%) | 55 (66) | 13 (76) |
| Caucasian, no. (%) | 83 (83) | 17 (17) |
| Source of ICU admit, no (%) | | |
| Medical | 59 (71) | 15 (88) |
| Surgical | 24 (29) | 2 (12) |
| APACHE III score, mean ± SD | 53.9 ± 27.6 | 80.2 ± 38.7 |
| Comorbidities, no. (%) | | |
| Diabetes | 24 (29) | 5 (29) |
| Cirrhosis | 4 (5) | 3 (18) |
| Chronic Renal Insufficiency | 3 (4) | 2 (12) |
| Tobacco Use | 45 (54) | 13 (76) |
| Hypotension within 72 hours (%) | 42 (47) | 9 (53) |
| Acute Lung Injury, no (%) | 22 (27) | 7 (43) |
| AKIN ≥ Stage I, no (%) | 50 (60) | 13 (75) |

**Table 2. Comparison of plasma biomarkers and 28-day mortality.**

| Biomarkers (pg/mL) | Subjects (N) | Alive at 28 Days, Median (IQR) | Dead at 28 Days, Median (IQR) | P Value |
|--------------------|--------------|-------------------------------|-------------------------------|---------|
| **Inflammatory:** |
| IL-6 | 90 | 142 (74, 295) | 260 (104, 757) | 0.036 |
| IL-8 | 90 | 12 (7, 23) | 31 (13, 79) | 0.005 |
| G-CSF | 90 | 29 (19, 56) | 35 (18, 127) | 0.145 |
| sTNFR-1 | 90 | 9962 (6322, 16971) | 18197 (11302, 31017) | 0.003 |
| **Endothelial Homeostasis:** |
| Ang-1 | 98 | 5719 (2642, 10123) | 2504 (1417, 5760) | 0.183 |
| Ang-2 | 98 | 15177 (8707, 35769) | 42063 (17094, 76983) | 0.021 |
| Ang-2/Ang-1 | 98 | 3 (1, 12) | 15.6 (4.0, 60.4) | 0.039 |
| sVCAM-1 | 99 | 647 (447, 811) | 819 (553, 1249) | 0.006 |
| sFAS | 99 | 11584 (8398, 16006) | 819 (553, 1249) | 0.000 |

Notes. Definition of Abbreviations: IQR = Interquartile Range; IL-6 = Interleukin-6; IL-8 = Interleukin-8; G-CSF = Granulocyte colony stimulating factor; sTNFR-1 = Soluble Tumor Necrosis Factor Receptor-1; Ang-1 = Angiopoietin-1; Ang-2 = Angiopoietin-2; sVCAM-1 = Soluble Vascular Adhesion Molecule-1.

*P value for Mann-Whitney U Test
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sFAS remained independently associated with mortality APACHEIII in a logistic model, and we determined that and APACHEIII did not survive to 28 days.

For hypotension, we identified a population of patients with high sFAS and APACHEIII <50 with poor outcomes (quadrant IV of Fig. 2). The median sFAS concentration in our population was 12168 pg/mL. Only one patient with sFAS measurement slightly below the median (10884 pg/mL) did not survive to 28 days.

**Table 3. Multivariate analysis of plasma biomarkers association with clinical outcomes.**

| Biomarkers (pg/mL) | Unadjusted OR (95%CI) for 28 day mortality | Adjusted OR with comorbidities for development of hypotension | Adjusted OR with APACHE III and comorbidities for 28 day mortality |
|-------------------|------------------------------------------|----------------------------------------------------------|-------------------------------------------------------------|
| Inflammation:     |                                          |                                                          |                                                             |
| IL-6              | 90 1.35 (1.02, 1.79)**                   | 1.22 (0.93, 1.61)**                                      | 1.09 (0.85, 1.42)**                                        |
| IL-8              | 90 1.41 (1.04, 1.91)**                   | 1.32 (0.92, 1.90)**                                      | 1.27 (0.92, 1.75)**                                        |
| G-CSF             | 90 1.18 (0.95, 1.47)                     | 0.76 (0.55, 1.05)**                                      | 0.96 (0.77, 1.19)**                                        |
| sTNFR-1           | 90 1.93 (1.26, 2.97)**                   | 1.03 (0.84, 1.26)**                                      | 1.33 (0.82, 2.15)**                                        |
| Endothelial Homeostasis: |                                        |                                                          |                                                             |
| Ang-1             | 98 0.81 (0.58, 1.13)                     | 0.93 (0.59, 1.45)**                                      | 0.91 (0.57, 1.44)**                                        |
| Ang-2             | 98 1.59 (1.07, 2.35)**                   | 1.24 (0.74, 2.08)**                                      | 1.23 (0.73, 2.10)**                                        |
| Ang-2/Ang-1       | 98 1.26 (1.00, 1.61)**                   | 1.11 (0.79, 1.55)**                                      | 1.11 (0.80, 1.52)**                                        |
| sVCAM-1           | 99 3.09 (1.48, 6.46)**                   | 2.46 (1.09, 5.52)**                                      | 1.79 (0.69, 4.61)**                                        |
| Apoptosis:        |                                          |                                                          |                                                             |
| sFAS              | 99 4.56 (2.16, 9.64)**                   | 3.15 (1.23, 8.03)**                                      | 2.89 (1.10, 7.56)**                                        |

Notes:
1Adjusted for Age, Gender, Chronic Renal Insufficiency, Smoking status, Diabetes Mellitus.
2Hypotension was defined as systolic blood pressure less than 90mmHg with 500cc fluid or receipt of a vasopressor.
p-values: **< 0.05  ***< 0.01

We classified patients with bloodstream infections into “gram-positive cocci,” “gram-negative bacilli,” and “low virulence organisms.” “Gram-positive cocci” infections were largely Staphylococcus aureus, whereas “gram-negative bacilli” infections were composed of enteric pathogens (mostly Escherichia coli). Low virulence organisms were predominantly coagulase negative Staphylococci (Table S1). Using “gram-positive” infections as the reference, no single organism class was uniquely associated with 28-day mortality or development of septic shock. Adjustment for either covariates or severity of illness (APACHEIII) did not change the association results (Table 4).

**Bacterial class and biomarker concentrations**

We then examined whether bacterial class was associated with different levels of host biomarker expression. There was no statistically appreciable difference between biomarker concentrations with respect to sVCAM-1, sTNFR-1, IL-6, IL-8, or sFAS (Fig. 1). However, individuals with “gram-negative bacilli” infections had higher circulating plasma concentrations of Ang-2/Ang-1 when compared to individuals with “gram-positive cocci” (p = 0.0184) or “low virulence” infections (p = 0.0088). Individuals with “gram-negative bacilli” infections had higher concentrations of G-CSF when compared to individuals with “low virulence” infections (p = 0.0096).

In our population of patients with sepsis caused by bacterial bloodstream infection, elevated circulating levels of sFAS were strongly associated with 28-day mortality. Bacterial class, however, was not associated with the clinical outcome of hypotension or 28-day mortality or substantial difference in the pattern of putative biomarker concentrations.

Our report is consistent with prior reports that also found no evidence for differences in clinical outcome by pathogen.43 We are the first group to demonstrate that..

**Table 4. Multivariate analysis of organism type association with clinical outcomes.**

| Gram - (reference) | Unadjusted OR (95%CI) for 28-day mortality | Adjusted OR (95%CI) for 28-day mortality | Adjusted OR (95%CI) for hypotension | Adjusted OR (95%CI) for Apache III |
|-------------------|------------------------------------------|----------------------------------------|-----------------------------------|-----------------------------------|
| Gram -            | 0.68 (0.18, 2.49)                        | 0.54 (0.14, 2.09)                      | 0.57 (0.15, 2.17)                 | 0.43 (0.11, 1.62)                 |
| Low Virulence     | 0.47 (0.13, 1.70)                        | 0.53 (0.10, 2.83)                      | 0.59 (0.11, 3.3)                  | 0.63 (0.09, 4.33)                 |
| p-values for tests above |                                        |                                        |                                   |                                   |
| Gram -            | 0.56                                     | 0.39                                   | 0.44                             | 0.27                             |
| Low Virulence     | 0.25                                     | 0.43                                   | 0.52                             | 0.59                             |

Notes:
1Adjusted for Age, Gender, Smoking status, and Diabetes Mellitus.
2Hypotension was defined as systolic blood pressure less than 90mmHg with 500cc fluid or receipt of a vasopressor.
p-values: **< 0.05  ***< 0.01
in a restricted cohort of patients with bacteremia, the host biomarker profile does not vary substantially by the infectious etiology.

The lack of association of bacterial class with mortality is similar to a study by Abe et al. of a Japanese population with bloodstream infection. Our findings contrast with those of Abe et al. with respect to IL-6, as their group identified higher IL-6 levels in patients with gram-negative bacteremia when compared to gram-positive cocci infections. This difference could be due to patient populations, given that their population was entirely of Japanese descent whereas our cohort was uniformly Caucasian. Also, there are potential differences in the underlying methodology. The population in the Abe et al. study were drawn from a larger inpatient population that had IL-6 levels measured as part of routine clinical care (and was identified retrospectively), rather than enrolled as part of a prospective observational cohort of patients with suspected sepsis.

In summary, our data demonstrate that candidate biomarkers reflective of the host response to sepsis were associated with mortality in bacteremic patients whereas the type of bacterial class was not associated with mortality or the development of shock. Host biomarkers have been associated with clinical outcomes previously.\textsuperscript{9–12, 19, 25, 46}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Scatter Plots of Circulating Plasma Biomarker Concentrations According to Bacterial Class IL-6, IL-8, sTNFR-1, granulocyte-colony stimulating factor, soluble FAS, soluble vascular cell adhesion molecule-1, and Ang2/Ang1 were measured in the plasma of 100 subjects meeting CDC criteria for bloodstream infection and sepsis within 24 h of admission to the ICU. The graphs depict individual subjects (symbols) and a horizontal line representing the mean with vertical lines representing the standard error measures for each biomarker. *p < 0.05, ** p < 0.01.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{sFAS Discriminates a Subset of Patients with Low APACHEIII who Subsequently Died of Sepsis. The measured values for soluble FAS and APACHEIII score were calculated for individual patients with sepsis from defined bloodstream infection. The values were plotted for each individual patient on an X and Y axis. The population was divided into four quadrants with the APACHEIII divided into populations above and below the clinically meaningful value of 50. Survivors are depicted with circles and nonsurvivors with triangles.}
\end{figure}
The association with host-derived sFAS remained following adjustment for covariates, acuity of illness (APACHEIII score) or a combination of acuity of illness and covariates (Table 3). Only one patient with sFAS slightly below the median did not survive to 28 days (Fig. 2). These data suggest that circulating sFAS in patients with bacteremic sepsis provides may provide clinically informative insight into a process not currently captured by standard information or the APACHEIII score. Importantly, increased Ang-2/Ang-1 ratios were commonly observed in this population along with elevations in biomarkers reflective of inflammation (IL-6, IL-8). Elevations in all of these markers were associated with 28-day mortality in unadjusted analysis, but the association did not remain after incorporating clinical information into our model (Table 3). The latter finding is most likely due to the relatively low number of mortality events (only 17 deaths) in this cohort.

The strengths of our study include prospective enrollment, a rigorous definition of bloodstream infection, and early measurement of candidate biomarkers that coincides with the onset of clinical deterioration. Some of the limitations are that our population is derived from a single center with a uniformly Caucasian population, and there were a relatively low number of total outcomes (only 17), so our findings require replication in larger, more heterogeneous cohorts. Furthermore, we can only speculate as to the causal basis for the association with an increase in observed mortality. Our hypothesis is that the source of sFAS is primarily lymphocytes based upon autopsy studies of septic patients that have demonstrated an increased in immunohistochemical markers of apoptosis in the spleen. Lymphocyte apoptosis could affect the host ability to respond to secondary infections, consistent with our observation that 41% (7/17) of patients died after seven days of hospitalization. Alternatively, elevations in sFAS could represent a proxy for susceptibility to other forms of end-organ damage. A previous investigation using this cohort determined that polymorphisms in sFAS were associated with development of acute lung injury.

This study is the first to document an association with mortality and sFAS levels in severe infection and to demonstrate that sFAS concentration was associated with 28-day mortality even after adjustment for available clinical information. Another group did not find an association with sFAS and 28-day mortality in patients with bacteremia. We suspect that key differences in patient populations account for this discrepancy. Specifically, our population was drawn prospectively from patients experiencing physiologic disturbance leading to ICU admission, whereas Huttunen et al. selected their study population from a mixed inpatient group on the basis of bacterial growth in blood cultures drawn during routine clinical care. Furthermore, our population had sampling of plasma for biomarkers within 24 hours of the bloodstream cultures, such that the biological information captured by the measurement of biomarkers was at or before the time of bloodstream infection.

sFAS levels below the median early in the development of sepsis were strongly associated with protection from mortality at 28-days. Elevations in the plasma concentration of the endothelial markers sVCAM-1 and Ang-2/Ang-1, the anti-inflammatory signal sTNFR-1, and the proinflammatory signals G-CSF, IL-6, IL-8 were also associated with mortality. We have recently demonstrated similar associations in a broader population of patients with SIRS. Because the host response in our population was relatively uniform irrespective of pathogen (with the exception of Ang-2/Ang-1 and G-CSF), our findings support the importance of endothelial dysfunction and/or apoptosis in the pathophysiology of sepsis irrespective of pathogen. Our findings support the general concept that host response to infection rather than the causative pathogen per se determines patient outcome in clinical sepsis.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Author contributions

WOH, CM, JH, MMW, and WCL designed the study. CM and SHB performed and analyzed the experiments. BLP and RK performed statistical analysis. WOH, MMH and WCL interpreted the results and drafted the manuscript. All authors reviewed, revised and approved the manuscript for submission.

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