Kinetic Analysis of Biomarkers in a Cohort of US Patients With Ebola Virus Disease

Anita K. McElroy,1,2 Jessica R. Harmon,1,2 Timothy D. Flietstra,1 Shelley Campbell,1 Aneesh K. Mehta,1,4 Colleen S. Kraft,1,4 Marshall G. Lyon,1 Jay B. Varkey,1 Bruce S. Ribner,1 Christopher J. Kratochvil,1 Peter C. Iwen,1 Philip W. Smith,1 Rafi Ahmed,2 Stuart T. Nichol,1 and Christina F. Spiropoulou1

1Viral Special Pathogens Branch, US Centers for Disease Control and Prevention, 2Division of Pediatric Infectious Disease, 3Emory Vaccine Center, 4Division of Infectious Diseases, and 5Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, Georgia, 6Office of the Vice Chancellor for Research, University of Nebraska Medical Center, 7Department of Pathology and Microbiology, and 8Department of Internal Medicine, Division of Infectious Diseases, University of Nebraska Medical Center College of Medicine, Omaha

Background. Ebola virus (EBOV) infection causes a severe and often fatal disease. Despite the fact that more than 30,000 individuals have acquired Ebola virus disease (EVD), the medical and scientific community still does not have a clear understanding of the mechanisms by which EBOV causes such severe disease.

Methods. In this study, 54 biomarkers in plasma samples serially collected from 7 patients with EVD were analyzed in an attempt to define the kinetics of inflammatory modulators. Two clinical disease groups were defined (moderate and severe) based on the need for clinical support. Biomarkers were evaluated for correlation with viremia and clinical disease in an effort to identify pathways that could be useful targets of therapeutic intervention.

Results. Patients with severe disease had higher viremia than those with moderate disease. Several biomarkers of immune activation and control were significantly elevated in patients with moderate disease. A series of pro-inflammatory cytokines and chemokines were significantly elevated in patients with severe disease.

Conclusions. Biomarkers that were associated with severe EVD were pro-inflammatory and indicative of endothelial or coagulation cascade dysfunction, as has been seen historically in patients with fatal outcomes. In contrast, biomarkers that were associated with moderate EVD were suggestive of a strong interferon response and control of both innate and adaptive responses. Therefore, clinical interventions that modulate the phenotype and magnitude of immune activation may be beneficial in treating EVD.

Keywords. Ebola; biomarkers; cytokines; immunity; EVD.

Ebola virus (EBOV) infection in humans causes a severe and often fatal disease. The current understanding of the pathophysiology of EBOV disease (EVD) is limited because few published studies have evaluated human samples [1–6]. These studies have used residual clinical samples that were collected as part of EVD outbreak responses, often with only 1 or 2 samples available from each patient. Collectively, the prior studies reported increased levels of both pro- and anti-inflammatory cytokines in patients with EVD, indicating marked dysregulation of innate immune responses, most notably in patients who succumbed to disease. Prior studies also reported an association between lymphocyte apoptosis and fatal outcome, as well as decreased numbers of total and activated T cells in fatal cases.

Minimal healthcare infrastructure and infectious risk have limited the collection of serial blood samples from patients with EVD in Africa. In contrast, patients who acquired EVD during the 2014 West African outbreak and were repatriated to the United States or Europe for care had access to a more resource-rich level of care. Although these patients had access to more services than patients in West Africa, their clinical care was still limited by the infectious nature of EBOV [7]. Most of these patients had a central line placed for fluid and medication administration, and this allowed for regular, frequently daily, blood sampling to monitor hemato logic or blood chemistry parameters. As a consequence of regular blood sampling for advanced care, residual banked samples were available for analysis.

In this study, the expression kinetics of inflammation, coagulation, endothelial function, and lymphocyte function biomarkers were examined to identify pathways associated with severe and moderate EVD. Our goal was to provide clinicians and scientific investigators insight into EVD pathophysiology for the design of targeted therapeutics.

METHODS

Patient Enrollment
Any patient admitted with EVD to Emory University Hospital, Atlanta, Georgia, or University of Nebraska Medical Center, Omaha, was eligible, and all eligible patients were included. Written informed consent to participate in local institutional review board–approved protocols (Emory University [IRB00076700], University of Nebraska Medical Center [716-14-EP], and US

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Correspondence: C. F. Spiropoulou, 1600 Clifton Rd, MS G14, Atlanta, GA 30333 (ccs8@cdc.gov).
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Centers for Disease Control and Prevention [CDC; 6643, 6774, and 1652]) was obtained from all surviving patients with EVD and from healthy control donors. Patients were randomly assigned an EVD number; in the case of EVD2, 5, 9, and 15, the number remains the same as used in a prior publication [8]. Patients were followed to time of discharge.

Biosafety
Work with infectious specimens was performed in CDC’s biosafety level 4 laboratories. Plasma samples were γ-irradiated with $5 \times 10^6$ rads prior to processing for quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR), enzyme-linked immunosorbent assay (ELISA), and biomarker analyses.

ELISA
EBOV nucleoprotein-specific ELISA was performed as previously described [8]. Data were analyzed using Excel (Microsoft Corp, Redmond, Washington) and Prism (GraphPad Software Inc, La Jolla, California) software.

Virus-Specific qRT-PCR
RNA was isolated from plasma using MagMax Total RNA Isolation Kit (Life Technologies, Grand Island, New York). qRT-PCR of RNA was conducted using established primer/probe sets that target the EBOV nucleoprotein gene [9]. A standard curve, expressed as tissue culture infective dose 50 (TCID$_{50}$/mL), was generated from a passage 3 stock of EBOV isolated from patient EVD2. This stock was inoculated into human plasma and γ-irradiated with $5 \times 10^6$ rads to control for the effects of irradiation on the qRT-PCR reaction. The standard curve was used to convert raw $C_t$ values to relative TCID$_{50}$/mL.

Biomarker Assays
Assays were obtained from Affymetrix (Santa Clara, California) and performed according to manufacturer’s instructions; a 34-plex assay for B-cell activating factor, fractalkine, granulocyte colony stimulating factor, granulocyte monocyte colony stimulating factor, granzyme B, interferon (IFN)-α, IFN-β, IFN-γ, IFN-γ-induced protein 10, interleukin (IL) 1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-29, IL-1 receptor antagonist (IL-1RA), monocyte colony stimulating factor (MCSF), monocyte chemoattractant protein (MCP)-1, MCP-2, plasminogen activator inhibitor–1, platelet endothelial cell adhesion molecule (PECAM)-1, tumor necrosis factor (TNF)-α, TNF-related apoptosis-inducing ligand, thrombopoietin, soluble CD40 ligand, sE-selectin, soluble Fas cell surface death receptor ligand (sFas-L), sIL-2 receptor, sL-selectin, sP-selectin, tissue plasminogen activator, and MCP-3; a 7-plex assay for apoptosis antigen-Fas (APO-Fas), melanoma growth stimulation activity alpha (Gro-alpha) Gro-α, IL-1α, macrophage inflammatory protein (MIP)-1α, MIP-1β, TNF Receptor-I (TNFR-I), and TNFR-II; a 3-plex assay for D-dimer, soluble intracellular adhesion molecule (sICAM)-1, and soluble vascular cell adhesion molecule (sVCAM)-1; and a single-plex assay for regulated on activation normal T-cell–expressed and secreted (RANTES). Assays were obtained from Millipore (Billerica, Maryland) and performed according to manufacturer’s instructions: a 2-plex assay for tissue factor (TF) and thrombomodulin; a 3-plex assay for fibrinogen, platelet factor-4, and von Willebrand factor (vWF); and single-plex assays for ferritin, complement factor H, anti-thrombin-III, and a disintegrin and metalloproteinase with a thrombospondin type 1 motif 13. If samples had values outside of the standard curve range, additional dilutions were made to obtain accurate values for all analytes. Data were collected on a Luminex 200. Samples from 10 healthy human donors were analyzed in each assay since many of these assays do not have an established normal range.

Statistical Analysis
For each patient, 2 acute illness phase cutoffs were defined: the last day of fever and the last day of measurable viremia. For each of the
54 biomarkers, a T test was conducted in order to compare values measured during the acute phase in patients with severe EVD against the values of moderately ill patients. The false discovery rate method [10] was used to determine the statistical significance of each biomarker with an overall $\alpha = 0.05$, taking into account the multiple tests run. For sensitivity analysis, individual patients (EVD 2, 5, 10, 11, or 15) were removed from the dataset, and the analysis was repeated to discover if this impacted the significance of specific biomarkers. Only associations that remained significant after the sensitivity analysis were included. The correlation of each biomarker with level of viremia was analyzed using the nonparametric Spearman coefficient of rank correlation.

RESULTS

Clinical Diversity

Seven EVD patients treated at Emory University Hospital or University of Nebraska Medical Center in 2014 were included in this study. The patients were aged 29–59 years; 5 were male and 2 were female. Their clinical histories have been reported [11–15]. Table 1 provides a brief description and clinical classification for all patients. Severity of disease was defined by the degree of clinical support required; the 2 patients who required extracorporeal support with dialysis and ventilation were classified as having severe disease, and the 5 who did not were classified as having moderate disease. It is not known if extracorporeal support could have influenced the

### Table 2. Statistically Significant Associations Between Biomarkers and Disease Severity During the Acute Phase of Ebola Virus Disease

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Disease Association</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>APO-Fas</td>
<td>Moderate</td>
<td>.0001880</td>
</tr>
<tr>
<td>Interferon-β</td>
<td>Moderate</td>
<td>.0045833</td>
</tr>
<tr>
<td>IL-29</td>
<td>Moderate</td>
<td>.000235</td>
</tr>
<tr>
<td>IL-5</td>
<td>Moderate</td>
<td>.000058</td>
</tr>
<tr>
<td>sFas-L</td>
<td>Moderate</td>
<td>.154270</td>
</tr>
<tr>
<td>TNFR-II</td>
<td>Moderate</td>
<td>.004500</td>
</tr>
<tr>
<td>D-dimer*</td>
<td>Severe</td>
<td>.000182</td>
</tr>
<tr>
<td>Fractalkine</td>
<td>Severe</td>
<td>.000733</td>
</tr>
<tr>
<td>Granzyme B*</td>
<td>Severe</td>
<td>.0001579</td>
</tr>
<tr>
<td>IL-10</td>
<td>Severe</td>
<td>.000791</td>
</tr>
<tr>
<td>IL-1RA*</td>
<td>Severe</td>
<td>.0010439</td>
</tr>
<tr>
<td>IL-8</td>
<td>Severe</td>
<td>.0056186</td>
</tr>
<tr>
<td>MCP-1*</td>
<td>Severe</td>
<td>.000063</td>
</tr>
<tr>
<td>MCP-2*</td>
<td>Severe</td>
<td>.0033463</td>
</tr>
<tr>
<td>Monocyte colony stimulating factor</td>
<td>Severe</td>
<td>.004575</td>
</tr>
<tr>
<td>Macrophage inflammatory protein-1β*</td>
<td>Severe</td>
<td>.000180</td>
</tr>
<tr>
<td>Platelet endothelial cell adhesion molecule-1*</td>
<td>Severe</td>
<td>$6.8 \times 10^{-11}$</td>
</tr>
<tr>
<td>P-selectin</td>
<td>Severe</td>
<td>.0000122</td>
</tr>
<tr>
<td>Soluble intracellular adhesion molecule*</td>
<td>Severe</td>
<td>$9.99 \times 10^{-9}$</td>
</tr>
<tr>
<td>Thrombomodulin*</td>
<td>Severe</td>
<td>.000812</td>
</tr>
<tr>
<td>Tissue factor</td>
<td>Severe</td>
<td>.0008097</td>
</tr>
<tr>
<td>TNFR-1*</td>
<td>Severe</td>
<td>.0055870</td>
</tr>
<tr>
<td>von Willebrand factor*</td>
<td>Severe</td>
<td>.000527</td>
</tr>
<tr>
<td>IL-6</td>
<td>Severe</td>
<td>N/A*</td>
</tr>
</tbody>
</table>

For each patient, the acute phase of disease was determined as the time prior to the cessation of viremia. The 2 patients classified as having severe disease were grouped together, and the 5 remaining patients formed the moderate disease group. The groups were compared with one another to identify biomarkers associated with disease severity. Asterisk (*) indicates a biomarker that also correlated with viremia.

Abbreviations: APO-Fas, apoptosis antigen-Fas; IL, interleukin; MCP, monocyte chemoattractant protein; N/A, not applicable; sFas-L, soluble Fas ligand; TNFR, tumor necrosis factor receptor.

The lack of significant amounts of IL-6 in patients with moderate disease prevented a statistical comparison, but levels were clearly elevated in patients with severe disease.
levels of the biomarkers independent of the disease process; however, the 2 patients with severe disease also had the highest detected viremia. The patients received a variety of experimental therapeutics that could have influenced their clinical course or immune response. Neither platelet count nor aspartate aminotransferase levels correlated with disease severity. Six patients survived EBOV infection and 1 succumbed to disease; EVD9, who was classified as severe but survived, would have succumbed to disease without medical intervention.

Viremia
All surviving patients had detectable viremia that declined over time (Figure 1A). Since each patient was received for care at different time points in the disease course, only later time points were available for analysis for some patients. Because of this, peak viremia is only known for EVD9, in whom EBOV peaked at more than $10^7$ relative TCID$_{50}$/mL. Notably, the viremia of the patient who died (EVD12) increased in serial samples late in the disease course at the time when viremia in all surviving patients was declining. This phenomenon has been historically reported in patients with fatal outcomes [16].

Humoral Responses
All patients had detectable IgM (Figure 1B) and IgG (Figure 1C) antibody responses against EBOV. ZMapp treatment (EVD2, EVD5, and EVD12) would not have affected these results, but convalescent plasma administration may have. This is clearly seen in EVD15, who had detectable anti-EBOV IgM after administration of convalescent plasma and prior to developing anti-EBOV IgM titers. Convalescent plasma administration appeared to have no detectable effect on ELISA titer for EVD9, but no conclusions can be made regarding plasma administration to EVD10 or EVD11 since plasma was administered prior to the first available sample. Previous studies have shown that patients often succumb to EVD without developing detectable virus-specific antibody responses [3, 16]. EVD12, the patient who died, received convalescent whole blood and plasma, so it is unclear if IgG titers in samples from EVD12 are the donor’s or the recipient’s antibody response. However, the presence of detectable IgM suggests that EVD12 may have developed EBOV-specific IgM prior to death, since convalescent plasma would not have significant amounts of EBOV-specific IgM.

Biomarker Measurements
Fifty-four biomarkers were selected based on their roles in innate immunity, adaptive immunity, endothelial function, or coagulation/fibrinolysis. For all biomarkers tested, at least 1 EVD patient had levels outside of the reference range, which was based on samples collected from 10 healthy humans. Convalescent plasma administration did not appear to alter biomarker levels in the recipients based on examination of before and after data from EVD9, the patient for whom there was the most kinetic data. The kinetic data for all biomarkers that are not otherwise discussed below are contained in the Supplementary Material.

To determine which biomarkers were associated with moderate vs severe EVD, biomarker values from samples collected during the acute phase (while patients had detectable viremia) from the 2 patients with severe disease were compared with those from

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**Figure 2.** Biomarkers that were associated with moderate Ebola virus disease (EVD). Expression of each biomarker was assayed in all patient plasma samples as described. Each patient’s data are indicated by the specified color and symbol. The x-axis shows days after onset of self-reported symptoms. The gray area represents the reference values of each biomarker as measured in samples from 10 healthy humans. The dotted line is the limit of the assay. For any sample that had a biomarker level that was below the limit of detection or undetectable, a non-zero value below the limit of detection was assigned to allow visualization of the data on the graph. This applies to apoptosis antigen-Fas (APO-Fas) and soluble Fas ligand (s-FasL). Abbreviations: IFN, interferon; IL, interleukin; TNFR, tumor necrosis factor receptor.
the 5 patients with moderate disease. Six biomarkers had a statistically significant association with moderate disease and 17 with severe disease (Table 2). Patients with severe disease had higher viremia than those with moderate disease (P = .0003). Therefore, an additional analysis was performed to determine which biomarkers correlated with viremia. Sixteen biomarkers correlated with viremia (Supplementary Table 1) and, not surprisingly, 11 of those were also associated with severe disease (Table 2).

**Biomarkers Associated With Moderate Disease**

APO-Fas, IFN-β, IL-29, IL-5, sFas-L, and TNFR-II were elevated in patients with moderate disease (Figure 2). EVD5 had the highest levels of APO-Fas, IFN-β, IL-29, and IL-5; in sensitivity analysis, the association of these markers with moderate disease was still observed if EVD5 was excluded from the dataset. The physiologic significance of the association between APO-Fas and moderate disease is unclear, since most EVD patients had APO-Fas levels that fell within the reference range. Elevated levels of IFN-β in moderately ill patients were consistent with the observation that administering IFN-β to EBOV-infected macaques prolonged survival [17]. IL-29 (also known as IFN-λ) has never been examined in the context of EVD, and higher levels in patients with moderate disease could simply reflect its antiviral function similar to IFN-β. Elevated IL-5 in patients with moderate disease could indicate skewing of the adaptive response toward a Th2 phenotype, although this was not statistically significant for the main Th2 cytokine, IL-4 (Supplementary data). Elevated sFas-L and TNFR-II in patients with moderate EVD is suggestive of timely and appropriate control of the innate and adaptive immune responses.

**Biomarkers Associated With Severe Disease**

Severe EVD was associated with biomarkers of innate immune function, coagulation, endothelial function, lymphocyte activity, and inflammation. IL-6, IL-8, IL-10, MCSF, MCP-1, MCP-2, IL-1RA, MIP-1β, and TNFR-I were elevated in patients with severe disease, indicating innate immune dysregulation (Figure 3), as previously described [1]. IL-6 differences between moderate
and severe disease were not statistically significant due to the small numbers of samples in the moderate group that had detectable levels. However, it is clear that there is a direct association between severity and IL-6 (Figure 3). Biomarkers suggesting coagulation pathway imbalance (D-dimer, vWF, TF, and thrombomodulin; Figure 4A) and biomarkers indicating endothelial dysfunction (P-selectin, PECAM-1, and sICAM; Figure 4B) were associated with severe EVD. Fractalkine, a marker of lymphocyte homing, and granzyme B, a marker of lymphocyte cytotoxicity, were also associated with severe EVD (Figure 4C).

**DISCUSSION**

**Endothelial Function and EVD**

Previous studies have suggested endothelial dysfunction in EVD: third-spacing in patients [12, 18], detection of viral antigen in endothelial cells in fatal cases [19], and association of multiple markers of endothelial dysfunction with disease and sometimes fatal outcome [20]. Selectins are upregulated on the surface of activated leukocytes (L-selectin), activated endothelium (P-selectin or E-selectin), or activated platelets (P-selectin) and subsequently shed into the endovascular space [21]. The levels of L-selectin in all patients studied were
essentially normal, while P-selectin and E-selectin were elevated in all patients, indicating endothelial and platelet activation during EVD (Figure 4B and Supplementary data). Activated endothelia upregulate expression of ICAM and VCAM on the surface and shed soluble forms into plasma. sICAM levels were elevated in all patients, while sVCAM levels were within normal range in most patients. PECAM-1, which maintains tight junctions between endothelial cells [22], was markedly elevated in all patients. Furthermore, P-selectin, sICAM, and PECAM-1 correlated with disease severity in a statistically significant manner and remained elevated throughout early convalescence, supporting the role of endothelial activation in the EBOV disease process and highlighting the need to consider targeted endothelial therapies for EVD treatment. Many existing therapeutic strategies could be evaluated for efficacy against EVD [23], particularly statins, which downregulate ICAM and P-selectin, both of which are associated with severe EVD.

Given the overlap between viremia and outcome [16], it is difficult to derive conclusions about biomarkers associated with both severe EVD and viremia. Fractalkine, IL-10, IL-8, MCSF, IL-6, P-selectin, and TF correlated only with severe EVD, and not viremia. The last 2 could be related to endothelial activation and subsequent release of endothelium-derived procoagulant molecules, further highlighting the need for therapeutics that target the endothelium.

Coagulation and EVD
EVD was originally called Ebola hemorrhagic fever because of the hemorrhagic manifestations observed during the first 2 outbreaks in 1976 [24, 25]. However, most studies from the current outbreak reported hemorrhagic manifestations (any form of bleeding) in fewer than 5% of patients [26–28]. Despite the lack of frank bleeding, there is an intimate relationship between coagulation and inflammation. An activated endothelium is both proinflammatory and procoagulant. The patients in this study have clear evidence of endothelial activation, which could trigger some aspects of the coagulation and fibrinolysis pathways. This battle to balance coagulation and fibrinolysis is clearly exemplified by the simultaneous upregulation of biomarkers of coagulation, anticoagulation, and fibrinolysis observed in these 7 patients; however, only thrombomodulin, TF, vWF, and D-dimer were associated with severe disease. All of these proteins are released from activated or damaged endothelial cells, and increased expression could reflect the endothelial dysfunction seen in EVD patients. Elevations in D-dimer have been associated with fatal EVD and were also associated with severe EVD in this cohort. One study on TF levels in human plasma during EVD reported no association with fatal outcomes [20], as had been suggested by nonhuman primate data [29]. However, in this study, the patients with severe disease had higher TF levels and both exhibited clinical coagulopathy (EVD9, oozing from venipuncture sites, and EVD12, elevated PT and INR) during their clinical course.

Cellular Immunity and EVD
In a previous study of human immune responses to EVD, lymphocyte markers of activation and cytotoxicity were measured; expression of CX3CR1 (the fractalkine receptor) and intracellular granzyme B on CD8 cells indicated that these cells were homing to sites of inflammation and functionally cytotoxic [8]. Consistent with those data, both free plasma fractalkine and granzyme B were elevated in all patients studied, and both biomarkers correlated with disease severity. These markers of T-cell activity may reflect the higher viremia in the severely affected patients, indicating more T-cell–stimulating antigen. Consistent with this, granzyme B correlated with both viremia and disease severity. Additionally, these effector CD8 T cells may cause direct tissue damage and contribute to the disease process.

Immune Modulation and EVD
The human immune response to a viral infection generally allows for clearance of the virus via antibody-mediated neutralization and destruction of infected cells via cell-mediated immunity. However, this response can be destructive to the host if it continues unchecked. A dysregulated innate immune signaling system, with prolonged elevation of various cytokines, has been noted in patients with fatal EVD, suggesting, at least in part, a potentially harmful failure to downregulate the immune response in fatal cases. The results of the present study show that immune activity associated with moderate EVD includes antiviral interferons (IFN-β and IL-29/IFN-λ) and timely and appropriate contraction of the adaptive immune response (sFas-L and TNFR-II). It is possible that immune control mediated via sFas-L contributes to the moderate disease phenotype by preventing immunopathology. Supporting this hypothesis, administering sFas-L improved clinical outcome in herpes simplex virus–mediated keratitis, presumably by activating apoptosis of inflammatory cells [30]. The finding that TNFR-II is released in soluble form by T-regulatory cells and can control IL-6–mediated inflammation [31] adds further credence to the idea of immune control playing a critical role in controlling EBOV clinical severity. Additionally, in collagen-induced arthritis, TNFR-II was associated with T-regulatory activity, while TNFR-I was proinflammatory [32]. These data complement our findings of elevated TNFR-I in patients with severe disease and an association of TNFR-II with moderate disease and begin to distinguish between beneficial vs harmful host immune responses.

The findings in this study suggest a number of potential targets for clinical intervention. General downregulation of immune signaling pathways via administration of prednisone, specific administration of anti-IL-6R antibody [33] to block proinflammatory signaling by IL-6, or downregulation of the adaptive response by inhibition of lymphocyte proliferation using anti-IL-2R antibody [34] are possible ways to inhibit an overreactive immune response. Additionally, activating IFN signaling by direct IFN administration could augment other therapeutic modalities. Not only will using the correct therapies
important, but also the timing of administration will be critical. Immune inhibition, if initiated at an inappropriate time, could be detrimental to the host. Our biomarker data argue for expanded evaluation of potential immunomodulatory therapies for EVD treatment using available animal models.

The limitations of our study include the small sample size; late medical attention received by some patients, preventing our ability to see the entire time course of viremia; and different forms of experimental therapeutics received by the patients. Despite these limitations, this extensive kinetic biomarker study can be useful for determining which molecular and cellular pathways are significant in the pathophysiology of the disease. The results from this study illuminate the beneficial vs harmful host immune responses in EVD. The next step is to generate testable hypotheses about these physiologic processes and evaluate them using relevant animal models.

### Supplementary Data

**Supplementary materials** are available at [http://cid.oxfordjournals.org](http://cid.oxfordjournals.org). Consisting of data provided by the author to benefit the reader, the posted materials are not copyrighted and are the sole responsibility of the author, so questions or comments should be addressed to the author.

### Notes

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**Disclaimer.** The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention (CDC).

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