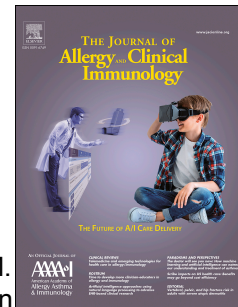


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Characterization of the Cytokine Storm Reflects Hyperinflammatory Endothelial Dysfunction in COVID-19

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DISCLOSURE STATEMENTS

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Rodgers, Nicoletta Bivi, Brian J. Nickoloff, Anabela Cardoso, Robert J. Konrad, Stephanie de

Bono, Richard E. Higgs, Robert J. Benschop, and Ajay Nirula are all employees of Eli Lilly and

Company. Justin Stebbing is editor-in-chief of Oncogene. JS has sat on several scientific

advisory boards, including Benevolent AI, and consults with Lansdowne partners, Vitruvian, and

Eli Lilly and Company, and sits on the Board of Directors for BB Biotech Healthcare Trust. Mario Corbellino and Giacomo Casalini report no conflicts of interest.

ABSTRACT

Background: Physicians treating COVID-19 patients increasingly believe that the hyperinflammatory acute stage of COVID-19 results in a cytokine storm. The circulating biomarkers seen across the spectrum of COVID-19 have not been characterized compared to healthy controls, but such analyses are likely to yield insights into the pursuit of interventions that adequately reduce the burden of these cytokine storms.

Objective: To identify and characterize the host inflammatory response to SARS-CoV-2 infection, we assessed levels of proteins related to immune responses and cardiovascular disease, in patients stratified as mild, moderate, and severe, versus matched healthy controls.

Methods: Blood samples from adult patients hospitalized with COVID-19 were analyzed using high-throughput and ultrasensitive proteomic platforms and compared with age- and sex-matched healthy controls to provide insights into differential regulation of 185 markers.

Results: Results indicate a dominant hyperinflammatory milieu in the circulation and vascular endothelial damage markers within COVID-19 patients, and strong biomarker association with patient response as measured by Ordinal scale. As patients progress, we observe statistically significant dysregulation of IFN γ , IL-1RA, IL-6, IL-10, IL-19, MCP-1, -2, -3, CXCL9, CXCL10, CXCL5, ENRAGE and PARP-1. Furthermore, in a limited series of patients who were sampled frequently confirming reliability and reproducibility of our assays, we demonstrate that intervention with baricitinib attenuates these circulating biomarkers associated with the cytokine storm.

Conclusion: These wide-ranging circulating biomarkers show an association with increased disease severity and may help stratify patients and selection of therapeutic options. They also provide insights into mechanisms of SARS-CoV-2 pathogenesis and the host response.

Key Messages:

- o COVID-19 patients experience an increasing chemokine/cytokine burden in circulation with increasing severity that suggests dysregulation of neutrophil, NK cell, and macrophage activity along with a concomitant increase in markers of endothelial inflammation, some of which correlate with changes in Ordinal scale.
- o COVID-19 patients experience a reduction in plasma biomarkers related to antigen presentation with increasing severity of illness.
- o Treatment with baricitinib results in corrective changes in some of these biomarkers to healthy control levels over time.

Capsule Summary: This detailed analysis of plasma biomarkers in COVID-19 patients provides definitive understanding of the cytokine storm and changes in vascular endothelial markers that may inform us of the changes in the Ordinal scale for COVID-19.

Key Words: COVID-19, Biomarkers, Cardiovascular, Inflammation, Ordinal Scale, Baricitinib

Abbreviations:

Abbreviation	Word
COVID-19	Coronavirus disease 2019
GDF-2	Growth/differentiation factor 2
ICU	Intensive care unit
IFN	Interferon
IL	Interleukin
JAK	Janus kinase

MCP	Monocyte chemoattractant protein
MMP	Matrix metalloproteinase
PEA	Proximity extension array
PTX3	Pentraxin-related protein 3
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SCF	Stem cell factor
STAT	Signal transducer and activator of transcription
tSNE	T-distributed stochastic neighbor embedding

INTRODUCTION

The COVID-19 pandemic created an overwhelming need to define host-derived molecular mediators of disease severity evident in hospitalized patients. One approach to dissect protective and pathological immune responses to COVID-19 infection is to measure plasma biomarkers correlated to various stages of COVID-19 and determine which pro-inflammatory mediators are modulated in response to therapies improving patient outcomes(1).

RESULTS & DISCUSSION

Proteomic profiling of peripheral blood samples from COVID-19 patients

We utilized multiplex methodology from Olink Proteomics to assess 184 analytes, spanning a broad range of cytokines and chemokines involved in inflammation (INF I) and cardiovascular-linked processes (CVD II), and, based on the inflammatory responses described in SARS-CoV-2-infected lung epithelium, we also measured IL-19 levels using a highly sensitive and specific assay as previously described(4, 6, 7). Analysis of the differential protein regulation patterns within severity classes of COVID-19 cases compared to HC revealed strong dysregulation of IFN γ , IL-6, IL-10, MCP-3, CXCL9, CXCL10, CXCL11, ENRAGE, PARP-1, and IL-1RA (Fig. 1A, B-bottom, C). These changes were significant and ranged from increases of 21-fold for IFN γ , 18-fold for IL-6, 12-fold for MCP-3, to 9-fold for CXCL10, to more than 3 fold for MCP-1 and -2, when comparing the average observed concentration in critical patients to HC (Fig. 1A; 2A,B-left). We observed a 2-fold increase in IL-19 levels and, along with the aforementioned changes in IL-10, MCP-1, -2, and -3, is consistent with the important role for inflammatory monocytes/macrophages in the immunopathogenesis of COVID-19-infected patients(8).

Common clinical observations derived from biomarker profiling of COVID-19 patients

Our analysis is consistent with a more prominent increase in IFN γ and IL-10 levels in COVID-19 patients as seen in the moderate/severe and critical patients who are more likely to suffer from adult respiratory distress syndrome(9). While interferon signaling is certainly implicated with the

dramatic increase in IFN γ , additional insights into type I interferon family members was out of scope of this manuscript due to the lack of reliable ultrasensitive assays for these analytes. Furthermore, we observed PTX3, Gal9, and CD8A upregulation in all patients regardless of severity (Fig. 1A, B-bottom; 2C-left). The stark enhancement of inflammatory analytes observed from mild patients to moderate/severe patients points toward enhanced activation of innate immune responses. Markers that are reduced and shown to the right of the heatmap in blue, tend to be involved in antigen presentation and neutrophil-mediated immune surveillance, such as FLT3L, TRANCE, CCL17, and CXCL5 (Fig. 1A, B-bottom)(10). Chemokines, such as CXCL5 and CXCL8 (IL-8), are important for neutrophil recruitment and accumulation (10). Steady-state development of plasmacytoid dendritic cells (pDCs) and conventional dendritic cells (cDCs) requires the ligand for FMS-like tyrosine kinase 3 receptor (FLT3L)(11). This decrease in dendritic cells and neutrophils may enhance the unbridled viral expansion and escape from standard immune surveillance in these patients leading to a potentially worse outcome over time.

COVID-19 patient clustering by severity classification

Using both tSNE analysis and hierarchical clustering to reflect the separation of the plasma samples from these patients in 2 dimensions, we observed 2 distinct sample groups segregating based on severity of disease (data not shown). This indicates that inflammatory proteins observed in the plasma of COVID-19 patients could be predictive of disease state and potentially clinical outcomes. Pathway mapping of the most prominent biomarkers that are altered in severe COVID-19 patients (Supplementary Table 3) suggests changes in cytokines correlated with pneumonia such as IFN γ and IL-10, along with chemokines such as CXCL10, MCP-3, CCL20, CXCL9, IL-8, ENRAGE, and MCP-2(8, 12, 13).

We observed strong positive correlation ($Cor > 0.7$) between reductions in the Six-Point Ordinal Scale and numerous inflammatory proteins in patients treated with Baricitinib, including MCP-3 (IL-18R1, PTX3, CTSL1, TRAILR2, Gal9, (Fig. 1A, B-top; Supplementary Table 4)(14, 15).

147 Importantly, many analytes not only possessed some meaningful positive correlation with known
148 clinical inflammatory markers, such as LDH, CRP, and Ferritin, but we specifically observed that
149 MCP-3, CTSL1, and PTX3 were more meaningfully correlated to changes in the Ordinal Scale
150 than these widely used readouts (Supplementary Table 4). We observed several analytes
151 inversely correlated to the same magnitude ($\text{Cor} < -0.7$) with both LDH, CRP, and Ferritin and the
152 Ordinal Scale: MMP12, GIF, GDF-2, BOC, SCF, and CD6 (Fig. 1A, B-top, Supplementary Table
153 4).

154 In addition to these identified immunological changes, there is a growing consensus on the
155 thromboembolic risk seen in COVID-19 patients. Severe endothelial injury, vascular thrombosis,
156 and intussusceptive angiogenesis characterizes the lung damage observed, and the incidence
157 of venous thromboembolism among severe and critically ill COVID-19 patients is higher
158 compared to that reported in other studies including patients with other disease conditions(16,
159 17). Recent recommendations suggest that all hospitalized COVID-19 patients should receive
160 thromboprophylaxis or full therapeutic-intensity anticoagulation if such an indication is
161 present(18). Therefore, our emphasis on the CVD II Olink panel offers some insight into
162 potential disruptions in the vascular endothelial markers, such as IL-6, PTX3, IL-1RA, CTSL1,
163 IL-18, and RAGE. These changes suggest potential viral-mediated changes in endothelial
164 function that may provide clues to the increased incidence of thromboembolic risks in COVID-19
165 patients. Cardiovascular risk studies focused on biomarker analysis across large cohorts have
166 identified a causal relationship between elevated levels of interleukin-6, interleukin-18, and
167 CD40 ligand correlations to plaque rupture or thrombotic events(19). Notably, in
168 moderate/severe and critical COVID-19 patients these plasma markers are elevated relative to
169 mild patients and HC, which is consistent with thromboembolic risk in these patients potentially
170 attributed to vast changes in the underlying endothelium of the most at-risk patients(20).
171 Changes in PARP-1 were most pronounced in the severe, and critical patients and this marker
172 have been implicated in cardiovascular disease(21). It is also well known that PARP-1 activity

correlates with viral infection and, conceivably, this marker reflects the added tissue viral burden in such patients; however, in this limited set, PARP-1 levels did not correlate with change in the Ordinal Scale or viral load (Fig. 1B). Furthermore, it has been reported that GDF-2 (BMP-9) plays a protective role against inflammation-mediated damage on endothelial and cardiac tissues. Likewise, we observed downregulation of GDF-2 in the plasma of COVID-19 patients (Fig. 1B). In GDF-2^{-/-} mice subjected to transverse aortic constriction, loss of GDF-2 activity promoted cardiac fibrosis and impairs heart function(22). Therefore, reductions in GDF-2 potentially may result in endothelial and cardiac damage in an inflammatory condition, and therapeutic restoration of factors such as GDF-2, such as observed with Baricitinib-treated patients, can help stabilize cardiac function in these at-risk patients (Fig. 2D).

Biomarker profiles in COVID-19 patients treated with Baricitinib

Changes in cytokines and other biomarkers in a series of 4 patients treated with Baricitinib offers insights in the pleiotropic nature of this JAK1/JAK2 inhibitor. The biomarkers that were most robustly decreased longitudinally in the Baricitinib-treated cohort compared to baseline include IL-6, MCP-3, IL-10, CXCL10, and IFN γ (Fig. 1-middle; Fig. 2-right). These were followed by cytokines such as PTX3, IL-1RA, MCP-2, CCL19, and IL-18R1 along with sPDL1 and CCL23. One sees a clear time-dependent decrease in PTX3 levels. PTX3 behaves as an acute phase response protein, as the low blood levels of PTX3, in normal conditions, increase rapidly and dramatically during endotoxic shock, sepsis, and other inflammatory and infectious conditions, correlating with the severity of the disease(23, 24). In recent work conducted in COVID-19 PBMCs using single cell RNASeq, it was reported that a reconfiguration of peripheral immune cell phenotype leads to a heterogeneous interferon-stimulated gene signature, HLA class II downregulation, and appearance of a neutrophil population with acute respiratory failure requiring mechanical ventilation(7). Importantly, they hypothesize that peripheral monocytes and lymphocytes do not express substantial amounts of pro-inflammatory cytokines which may imply

that the robust increases we observe in plasma biomarkers originate from the tissues that are affected by SARS-CoV-2 infection-related inflammation(25).

In conclusion, we identified a subset of plasma biomarkers in COVID-19 patients enhancing our understanding of the cytokine storm, along with changes in vascular endothelial markers that may better inform us of the therapeutic response, as observed by change in Ordinal Scale, in COVID-19 patients. Most notably, in addition to these increases, one observes a decrease in neutrophil and pDC-mediated immune surveillance that may facilitate SARS-CoV-2 viral expansion in tissues. Importantly, a reversal in hyperinflammatory endothelial biomarker levels, some of which correlate with improvements in the Ordinal scale, was observed in a limited subset (4 patients) treated with Baricitinib.

For detailed methods, please see the Methods section in this article's Online Repository at www.jacionline.org."

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AUTHOR CONTRIBUTIONS

J.T.S. and V.K. conceived the study. J.T.S., S.M.E., and G.H.R. conducted the laboratory experiments. J.T.S., V.K., C-Y.C., S.M.E., G.C., and J.S. designed and conducted the main analyses and interpreted the results. V.K. wrote the first draft of the manuscript. G.H.R., N.B., B.J.N., R.J.K., R.E.H., G.C., and M.C. also contributed to the analysis. J.T.S., V.K., C-Y.C., S.M.E., and J.S. wrote the manuscript, with contributions and review by all other authors.

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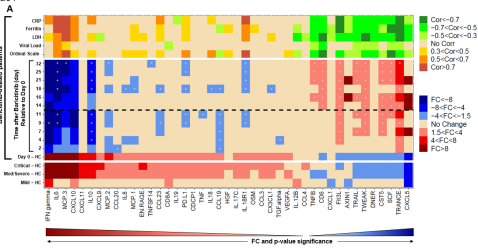
FIGURE LEGENDS

Figure 1. A, B, C) Inflammatory panel (a) or the Cardiovascular panel II (b) fold change in baseline protein levels, COVID-19 patients vs. HC (n=20) (bottom). Baseline COVID-19 patient samples were classified based on patients receiving treatment for ≤ 2 days (n=25). Heatmaps of fold change relative to baseline (Day 0) are shown for moderate/severe patients treated with Baricitinib (n=4) (middle). Red or blue represents significantly up- or down-regulated markers, respectively, with 1.5X-4X in light color, 4X-8X in medium color and $>8X$ in dark color. Analytes are ordered along the x-axis by p-value significance ($p < 0.05$) based on their positive and negative fold change ($>1.5X$) relative to healthy controls. A "+" sign designates that Baricitinib-treated patients were within 1.5X of HC. Correlations of biomarkers and clinical assessments such as Ordinal Scale, nasopharyngeal viral load (the most sensitive viral target gene, N gene, is shown), and inflammation status (CRP, Ferritin, LDH) are shown for each biomarker across all longitudinal timepoints for COVID-19 patients treated with Baricitinib (top). Orange or green represents significantly positive or negative correlated markers, respectively, with $0.3 < \text{Cor} < 0.5$ in light color, $0.5 < \text{Cor} < 0.7$ in medium color, and $\text{Cor} > 0.7$ in dark color. Dashed line represents the last day a patient received Baricitinib treatment. (c) Volcano plots showing the Inflammatory panel and IL-19 (top) or Cardiovascular panel II (bottom) biomarkers at baseline for mild (left), moderate/severe (middle), and critical (right) patients compared to HC. The y-axis is the $-\log_{10}$ of the p-value with higher numbers reflecting greater significance and the x-axis represents the $\log_2$ fold change, wherein color designation of blue or red represents decreased or increased presence of these markers relative to HC, respectively.

Figure 2: A) IL-6, B) MCP-3, C) PTX3, and D) GDF-2, levels in HC versus mild, moderate/severe or critical patients (left), correlation of analyte levels to Ordinal Scale for 4 patients (A, B, C, D) over the course of treatment with Baricitinib (middle), and time-dependent changes in 4 patients treated with Baricitinib (right). Dotted line identifies the levels seen in

326 matched healthy controls (n=20). Dashed bar represents longest course of Baricitinib treatment
327 administered to a patient in this study (11 days). *p<0.05.

A
Γ



B

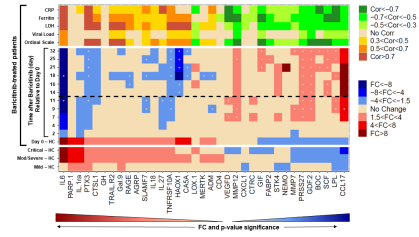


Figure 2

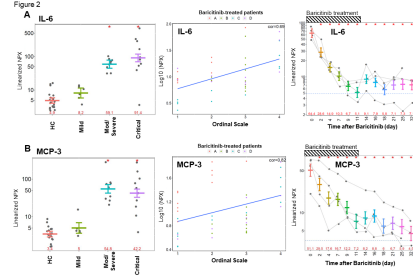


Figure 2

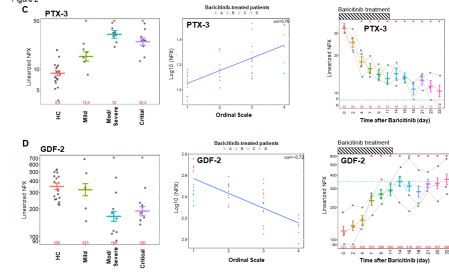


Figure 1C

