No evidence of hemoglobin damage by SARS-CoV-2 infection


Haematologica 2020 [Epub ahead of print]


Publisher's Disclaimer.
E-publishing ahead of print is increasingly important for the rapid dissemination of science. Haematologica is, therefore, E-publishing PDF files of an early version of manuscripts that have completed a regular peer review and have been accepted for publication. E-publishing of this PDF file has been approved by the authors. After having E-published Ahead of Print, manuscripts will then undergo technical and English editing, typesetting, proof correction and be presented for the authors' final approval; the final version of the manuscript will then appear in print on a regular issue of the journal. All legal disclaimers that apply to the journal also pertain to this production process.
No evidence of hemoglobin damage by SARS-CoV-2 infection

Anthony W. DeMartino1*, Jason J. Rose1,2,3§, Matthew B. Amdahl1, Matthew R. Dent1, Faraaz A. Shah2,4, William Bain2, Bryan J. McVerry2, Georgios D. Kitsios2, Jesús Tejero1,2,3,5, and Mark T. Gladwin1,2,3

1 Heart, Lung, Blood, and Vascular Medicine Institute, University of Pittsburgh, Pittsburgh, PA, USA, 2 Division of Pulmonary, Allergy and Critical Care Medicine, University of Pittsburgh, Pittsburgh, PA 15261, United States; 3 Department of Bioengineering, University of Pittsburgh, Pittsburgh, PA 15260, United States; 4 VA Pittsburgh Healthcare System, Pittsburgh, Pennsylvania 15240 5 Department of Pharmacology and Chemical Biology, University of Pittsburgh, Pittsburgh, Pennsylvania 15261, United States

*These authors contributed equally

§Correspondence to:

Jason J. Rose, Pittsburgh Heart, Lung, Blood, and Vascular Medicine Institute, University of Pittsburgh, Pittsburgh, PA 15261; Division of Pulmonary, Allergy, and Critical Care Medicine, University of Pittsburgh, Pittsburgh, PA 15261; E-mail: rosejj@upmc.edu

Key Points
- Comparing clinical data from 21 critically ill COVID-19-patients to 21 ARDS controls, there is no evidence of hemolytic anemia in COVID-19.
- SARS-CoV-2 does not have a direct effect on red blood cells by iron removal or heme degradation; the hemoglobin-oxygen dissociation curve is normal.

Abstract
SARS-CoV-2 disease (COVID-19) has affected over 22 million patients worldwide as of August 2020. As the medical community seeks better understanding of the underlying pathophysiology of COVID-19, several theories have been proposed. One widely shared theory suggests that SARS-CoV-2 proteins directly interact with human hemoglobin (Hb) and facilitate removal of iron from the heme prosthetic group, leading to the loss of functional hemoglobin and accumulation of iron. Herein, we refute this theory. We compared clinical data from 21 critically ill COVID-19 patients to 21 non-COVID-19 ARDS patient controls, generating hemoglobin-oxygen dissociation curves from venous blood gases. This curve generated from the COVID-19 cohort matched the idealized oxygen-hemoglobin dissociation curve well (Pearson correlation, $R^2 = 0.97$, $P<0.0001$; CV(RMSD) = 7.3%). We further analyzed hemoglobin, total bilirubin, lactate dehydrogenase, iron, ferritin, and haptoglobin levels. For all analyzed parameters, patients with COVID-19 had similar levels compared to patients with ARDS without COVID-19. These results indicate that patients with COVID-19 do not exhibit any hemolytic anemia or a shift in the normal hemoglobin-oxygen dissociation curve. We therefore conclude that COVID-19 does not impact oxygen delivery through a mechanism involving red cell hemolysis and subsequent removal of iron from the heme prosthetic group in hemoglobin.

Introduction
The severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) pandemic has progressed around the globe with over 22 million cases at the time of this writing. In the early stages of the SARS-CoV-2 disease (COVID-19) pandemic, there were reports of unique clinical phenotypes in affected patients. One study of the initial experiences of critically ill patients in Italy described an atypical form of viral pneumonia-induced acute respiratory distress syndrome (ARDS) with
normal lung compliance and low ventilation-to-perfusion ratio. Initial reports had suggested very high mortality rate (>80%) for patients with respiratory failure from SARS-CoV-2 disease (COVID-19), compared to pre-COVID-19 ARDS mortality rates in the range of 30-40%. A clinical syndrome of “silent” or “happy” hypoxemia has been widely observed, with patients exhibiting minimal dyspnea or signs of neurocognitive dysfunction despite severe hypoxemia detected via pulse oximetry. A number of hospitalized COVID-19 patients appear to have significant hemostatic activation, with 25 - 31% prevalence of venous thromboembolism observed in some cohorts. More recent clinical studies have shown that the mechanical ventilation requirements in COVID-19 patients are similar to populations of patients enrolled in ARDS clinical trials without COVID-19. Further observational studies have reported mortality rates for COVID-19 in the range of 25-32%, similar to the mortality rates for non-COVID-19 ARDS. While the exact pathophysiology of COVID-19 remains a topic of active investigation, growing evidence suggests that respiratory failure in COVID-19 patients behaves similarly to respiratory failure in patients with severe viral pneumonia that triggers ARDS.

The uncertainty at the outset of this pandemic as well as some persistently unique features of the disease (e.g., increased thromboembolic risks), have led to a large number of proposed hypotheses regarding the pathophysiological mechanisms of SARS-CoV-2. One widely proposed theory holds that viral proteins directly interact with human hemoglobin (Hb) and facilitate removal of iron from the protein’s heme prosthetic group, resulting in the loss of functional hemoglobin and the toxic accumulation of iron. This theory originated from a manuscript by Liu and Li posted in the preprint server ChemRxiv (over 1.89 million manuscript views as of August 2020). This work by Liu and Li which has not been peer-reviewed and continues to be cited, uses in silico approaches to model interactions between several viral proteins and hemoglobin. In brief, the manuscript suggests that viral proteins ORF1ab, ORF10, and ORF3a are derived from infected plasma cells and work in concert to remove heme from the beta chain of hemoglobin, strip iron from heme, and sequester the resulting iron-free protoporphyrin IX (PPIX). The authors speculate that this coordinated "attack" of hemoglobin occurs in the plasma following immune hemolysis, resulting in the release of toxic amounts of iron, diminished functional hemoglobin levels, and disrupted heme metabolism. Finally, Liu and Li further purport that the consequences of such hemoglobin degradation account for some of the irregular clinical characteristics reported early in the pandemic (Figure 1). While this work has received significant attention from scientists, physicians, the press, and the general public, the study advances a theory of viral interaction with hemoglobin that is inconsistent with well-characterized mechanisms of physiological heme degradation, and, critically, advances a pathophysiological mechanism inconsistent with the clinical presentation of COVID-19 patients. Herein, we compare clinical laboratory data from confirmed COVID-19 patients admitted to the intensive care unit (ICU) at University of Pittsburgh Medical Center (UPMC) to patients with ARDS but without COVID-19. These empirical data challenge the theory that SARS CoV-2 proteins directly remove iron from human hemoglobin as a pathophysiological mechanism of COVID-19.

Methods
We reviewed laboratory data from 21 patients with known COVID-19 (PCR positive for SARS-CoV-2) admitted to the ICU at UPMC and compared with 21 patients with non-COVID19 ARDS from different etiologies, who had been enrolled in the Acute Lung Injury Registry (ALIR) and Biospecimen Repository at UPMC. Patient data were de-identified. This study was approved by the University of Pittsburgh Institutional Review Board.

We recorded clinically available venous blood gas values of partial pressure of carbon dioxide (PvCO2), partial pressure of oxygen (PvO2), pH, and venous oxygen saturation of hemoglobin
We recorded initial hemoglobin and total bilirubin levels. Values for iron, ferritin, haptoglobin, and LDH levels (whenever available during the inpatient admission) were collected as well.

Hemoglobin and total bilirubin levels between COVID-19 and non-COVID-19 patients were compared using a Student’s t-test. For iron, ferritin and LDH, Mann-Whitney testing was used. Values for SvO2 versus PvO2 were plotted to determine a line of best fit. The deviation of the PvO2 by COVID-19 and ARDS cohorts from the theoretical-standard-predicted-PvO2 (described by JW Severinghaus) was evaluated using a Pearson correlation and a coefficient of variation of the root-mean-square deviation or CV(RMSD). All data variance is described as standard error of mean, except for age, which is described as standard deviation.

**Results**

Oxygen-hemoglobin dissociation curves were generated using COVID-19 patient data and non-COVID-19 ARDS patient data and compared against the theoretical standard curve generated by Severinghaus. The average age of the COVID-19 cohort was 62 ± 9 versus 47 ± 17 for the ARDS cohort. The distribution of sex was 12 males and 9 females in the COVID-19 cohort and 10 males and 11 females in the non-COVID-19 ARDS cohort. The oxygen-hemoglobin dissociation curve generated from patients with COVID-19 was similar to the curve generated from patients with ARDS without COVID-19. The fitting generated from COVID-19 patient data matched the ideal oxygen-hemoglobin dissociation curve well (Pearson correlation, $R^2 = 0.97$, $P<0.0001$; CV(RMSD) = 7.3%). The fitting generated from non-COVID-19 ARDS patients also matches the ideal oxygen-hemoglobin dissociation curve reasonably well (Pearson correlation, $R^2 = 0.92$, $P<0.0001$; CV(RMSD) = 9.4%, Figure 2). This comparison of oxygen-hemoglobin dissociation curves suggests that hemoglobin oxygen affinity is not altered in patients with COVID-19 admitted to the ICU.

Patients with COVID-19 had similar total hemoglobin, total bilirubin, ferritin, iron and LDH levels compared to patients with ARDS without COVID-19 (none were significantly different, Figure 2). Few patients had haptoglobin levels available. In those COVID-19 patients that did, the average haptoglobin level was similar to that of ARDS patients (mean 205 mg/dL, n=2, vs. 283 ± 19, n=6). There was no laboratory evidence of on-going red blood cell hemolysis or degradation of hemoglobin.

**Discussion**

Liu and Li generated computational results obtained by sequence analysis, molecular modeling, and docking approaches to propose a novel model of viral degradation of hemoglobin-derived heme. However, the authors employed methodologies and docking simulations that have been heavily criticized, as thoroughly discussed in a recent report by Read. It is important to note that Liu and Li do not present any experimental support for their theories, and though they have revised the initial manuscript and vastly changed their calculated parameters (as of version 9), their new calculations are speculative at best, as Read has recently addressed in an addendum to his manuscript (version 2). Moreover, the hypotheses originally put forth by Liu and Li remain unchanged. Our work thus focuses on the unique mechanism of SARS-CoV-2 proposed by Liu and Li: direct virus triggered hemoglobin degradation. We highlight that this hypothesis is not supported by existing evidence, known pathologies of coronaviruses, Li and Liu’s own docking calculations, or the clinical data presented herein.

Most nonstructural viral proteins of coronaviruses are not found in large amounts in plasma but rather localize in infected cells where they play important roles in RNA replication. Thus, these
proteins are unlikely to access appreciable amounts of hemoglobin. There is no evidence suggesting that the virus enters erythrocytes, where heme concentrations are 15-20 mM, and these highly specialized cells lack the requisite cellular machinery needed to produce viral proteins.20 As a result, intraerythrocytic hemoglobin is likely protected from exposure to viral proteins. Liu and Li13 posit that the interaction between viral proteins and hemoglobin may occur in the plasma after immune hemolysis, but significant hemolysis has not been documented in COVID-19 patients,8,21,22 nor do we observe hemolysis in our patients. Any elevation in lactate dehydrogenase levels seen in COVID-19 patients appears to derive from hepatocellular injury and not intravascular hemolysis. Liu and Li13 suggest that the virus could infect plasma cells via the ACE2 receptor and induce secretion of viral proteins from infected plasma cells. However, there is no evidence of SARS-CoV-2 infecting plasma cells.23,24 Further, secretion of viral proteins from any infected cells is extremely rare and does not occur in any viruses related to SARS-CoV-2.25

The manuscript's flawed18 and experimentally unverified docking models lead the authors to suggest that a non-structural viral protein, ORF10, binds to heme and releases heme-derived iron. In this putative mechanism, ORF1ab and ORF3a bind the hemoglobin protein and cause conformational changes that expose the heme to ORF10, which subsequently breaks down the cofactor into iron and PPIX; the latter is then theoretically captured by ORF1ab. This model would represent an entirely novel mechanism of hemoglobin degradation, as hemoglobin has not been documented to undergo large conformational shifts as a result of protein-protein interactions.26 Further, the removal of iron from PPIX by ORF10, a protein of only 38 amino acids, is unlikely considering that heme degradation is catalyzed by significantly larger and more complex, well-characterized heme-oxygenase proteins.16 As the authors’ hypothesis represents a completely novel and unexpected model of heme degradation, careful in vitro and in vivo studies would be required to confirm such a mechanism. It is worth noting that we do not observe a statistical difference in iron, bilirubin, or ferritin levels between COVID-19 patients and ARDS control-patients (Figure 2), indicating that heme breakdown is not occurring above typical catabolic levels in the COVID-19 cohort.

We further assert that the clinical syndrome observed in COVID-19 patients is not consistent with Liu and Li’s13 model of heme degradation. The clinical evidence presented here does not suggest hemolysis, hemoglobin degradation, removal of iron from the heme molecule, nor altered oxygen affinity of hemoglobin (Figure 2). The oxygen dissociation curve calculated from real-life patient data fits quite well with the idealized standard curve in both the COVID and ARDS cohorts (R² = 0.97, R² = 0.92, respectively). There is minimal deviation from the idealized curve (CV(RMSD) of 7.3 and 9.4%, respectively). This finding is in agreement with another recent study that uses hemoglobin isolated from the red blood cells of COVID-19 patients demonstrating normal hemoglobin-oxygen dissociation properties ex vivo.27

The clinical data presented here suggests no significant hemolysis or abnormal hemoglobin-oxygen dissociation characteristics. As aforementioned, newer clinical reports of critically ill COVID-19 patients suggest both mortality rates and mechanical ventilation requirements similar to other forms of ARDS.2,7-10 However, there has been no evidence to suggest a unique hemoglobin-specific mechanism such as large-scale hemoglobin degradation. In addition to the work here, there has been no other evidence of significant anemia or iron overload.8,21,22 While the finding of elevated transaminase levels has been widely described, this condition exists without hyperbilirubinemia, which would be a signal of excessive hemolysis and release of hemoglobin into the plasma.26,28 Increased risk of thromboembolism is observed in COVID-19 patients5,6, however, acute infections are known to increase the risk of thromboembolism29 and thromboembolism is not generally driven by a hemoglobin-based toxicity. Neither “silent
hypoxia", nor patients who maintain a normal work of breathing before rapid onset of ARDS suggest an undetected hemoglobin-based toxicity. Any removal of iron and/or heme from hemoglobin would not have an effect on pulse oximetry measurements, as the resulting heme-free hemoglobin does not absorb light in the wavelength range used by these detectors and thus would not interfere with such measurements. Hemoglobin desaturation correlates well with decreases in partial pressure of oxygen in patients with COVID-19 respiratory failure: in our cohort, we did not observe gross abnormalities in the partial pressure of oxygen versus hemoglobin oxygen saturation.

The world community is rapidly working to understand the pathophysiology of COVID-19 in an effort to better prevent the spread of the disease, manage patients, and ultimately develop definitive therapies. There is no suggestion that patients with COVID-19 exhibit a hemolytic anemia or a shift in the normal hemoglobin-oxygen dissociation curve. Thus, COVID-19 does not impact oxygen delivery through a mechanism involving red cell hemolysis and removal of iron from the heme prosthetic group in hemoglobin.

Author Contributions
AWD, JJR, MBA, MRD, FAS, WB, BJM, GDK, JT, and MTG all contributed to designing and performing the research. JJR, FAS, BJM, GDK all contributed to data collection. JJR, AWD, JT, MTG all contributed to data analysis, statistical analysis. AWD, JJR, MBA, MRD, FAS, BJM, GDK, JT, and MTG all contributed to writing the manuscript. AWD and JJR generated the figures.

Disclosed Conflict of Interest
MTG and JJR are co-inventors on a provisional patent describing sodium nitrite as a therapeutic for pneumonia-related lung injury. MTG, JT, and JJR are shareholders in Globin Solutions, Inc, which has a license to technology using nitrite in cardiovascular diseases. JJR and JT are officers and directors of Globin Solutions. MTG is a director and advisor of Globin Solutions. GDK has received research funding from Karius, Inc. BJM has received research funding from Bayer Pharmaceuticals, Inc.

Funding
This work is funded by 2 P01 HL114453-06 to BJM and 1 K08 HL136857 to JJR.

References


Figure Legends

Figure 1 Description of pathophysiology of SARS-CoV-2 infection via the lungs (open arrows) resulting in hypoxemia (left) vs direct ‘attack’ by COVID-19 viral proteins on red blood cell (RBC) hemoglobin (Hb) as proposed by Liu and Li (right, hashed arrows/gray boxes). Hypothesized pathways crossed out by red X’s and highlighted in gray boxes are not supported by available clinical evidence, in vitro/in vivo studies, our results, nor mechanisms of viral interaction with RBCs. See text for details.

Figure 2 Summary of clinical data of COVID-19 and non-COVID-19 ARDS patient cohorts. Oxygen-hemoglobin dissociation curve (left): standard curve as described by JW Severinghaus (black squares, gray line), values of oxygenation saturation plotted versus PvO2 for patients admitted to ICU with COVID-19 (blue diamonds) and ICU patients with ARDS but without COVID-19 (red circles). Individual replicates (i.e., same PvO2) are displayed. Corresponding lab values of hemoglobin, total bilirubin, ferritin, iron, and LDH were similar between ICU patients with COVID-19 and those with ARDS without COVID-19 (right, no significant differences: P=0.76, P=0.28, P=0.82, P=0.52, P=0.94, respectively). Few patients had haptoglobin data, but available COVID-19 values were similar to ARDS (mean 202 versus 283, respectively). Error bars represent standard error of mean.