



Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.



Increased histone-DNA complexes and endothelial-dependent thrombin generation in severe COVID-19

Beth A. Bouchard^{a,*}, Christos Colovos^b, Michael A. Lawson^b, Zachary T. Osborn^b,
Adrian M. Sackheim^b, Kara J. Mould^c, William J. Janssen^c, Mitchell J. Cohen^d,
Devdoot Majumdar^b, Kalev Freeman^b

^a Department of Biochemistry, University of Vermont, Burlington, VT, USA

^b Department of Surgery, University of Vermont, Burlington, VT, USA

^c Department of Medicine, National Jewish Health, Denver, CO, USA

^d Department of Surgery, University of Colorado School of Medicine, Aurora, CO, USA

ARTICLE INFO

Keywords:

COVID-19
Endothelial cells
Fibrin
Histones
SARS-CoV-2
Thrombin

ABSTRACT

Coagulopathy in severe COVID-19 is common but poorly understood. The purpose of this study was to determine how SARS-CoV-2 infection impacts histone levels, fibrin structure, and endogenous thrombin potential in the presence and absence of endothelial cells. We studied individuals with SARS-CoV-2 infection and acute respiratory distress syndrome at the time of initiation of mechanical ventilation compared to healthy controls. Circulating histone-DNA complexes were elevated in the plasma of COVID-19 patients relative to healthy controls (n=6, each group). Using calibrated automated thrombography, thrombin generation was altered in COVID-19 patient plasma samples. Despite having increased endogenous thrombin potential, patient plasma samples exhibited prolonged lag times and times to peak thrombin in the presence of added tissue factor and PCPS. Strikingly different results were observed when endothelial cells were used in place of tissue factor and PCPS. While healthy control plasma samples did not generate measurable thrombin after 60 min, plasma samples from COVID-19+ patients formed thrombin (mean lag time ~20 min). Consistent with the observed alterations in thrombin generation, clots from COVID-19 subjects exhibited a denser fibrin network, thinner fibers and lower fibrin resolvability. Elevated histones, aberrant fibrin formation, and increased endothelial-dependent thrombin generation may contribute to coagulopathy in COVID-19.

1. Introduction

The emergence of the severe acute respiratory syndrome novel corona virus 2 (SARS-CoV-2) resulted in a global pandemic that afflicted over 200 million people globally and over 40 million in the United States alone as of October 2021. The associated disease, coronavirus disease 2019 (COVID-19), accounts for over 4.8 million deaths worldwide and over 700,000 in the United States [1]. A consistent finding amongst patients with SARS-CoV-2 is derangements in coagulation markers and increased incidence of thrombotic complications [2–6]. Derangement in coagulation parameters was associated with a coagulation phenotype similar to disseminated intravascular coagulopathy and associated with death [5,7]. Findings of micro and macro vascular thromboses resulting

in organ failure are observed in autopsy studies. Emerging evidence of the benefits provided by therapeutic anticoagulation further supports the idea that coagulopathy contributes to SARS-CoV-2 disease progression.

Cellular release of histones, major mediators of death in sepsis and neutrophil extracellular trap (NET)¹ formation, have been implicated in COVID-19 coagulopathy [8–12]. Histones increase plasma thrombin generation directly through interactions with coagulation proteins [13,14], platelets, neutrophils, and endothelial cells [15,16]. The purpose of this study was to determine how SARS-CoV-2 infection impacts histone levels, fibrin structure, and thrombin generation in the presence and absence of endothelial cells.

* Corresponding author at: University of Vermont, 89 Beaumont Avenue, Given C440, Burlington, VT 05405-0068, USA.

E-mail address: beth.bouchard@uvm.edu (B.A. Bouchard).

¹ NET, neutrophil extracellular trap; HBS, 20 mM Hepes, 0.15 M NaCl, pH 7.4; DMEM, Dulbecco's Modified Eagle Medium; PCPS, synthetic vesicles consisting of 80% phosphatidylcholine and 20% phosphatidylserine

<https://doi.org/10.1016/j.vph.2021.106950>

Received 10 August 2021; Received in revised form 29 October 2021; Accepted 30 November 2021

Available online 3 December 2021

1537-1891/© 2021 Elsevier Inc. All rights reserved.

Table 1
COVID-19 patient characteristics.

Patient	Survival (day 28)	Age (years)	Gender	Race/Ethnicity	BMI*	Diabetes	HTN [†]	Smoker
1	Y	53	M	White	30.8	Y	Y	Never
2	Y	54	M	Hispanic or Latino	30.4	N	N	Never
3	Y	57	F	White	28.6	Y	Y	Never
4	Y	70	M	Hispanic or Latino	32.0	N	Y	Never
5	N	62	M	Black	24.8	N	Y	Current
6	N	63	F	Black	27.5	N	Y	Former

Patient	Anticoagulation	Steroids	Convalescent plasma	RDV [‡]	CMV (days) [§]	CMV (total days)
1	Lovenox	N	N	N	2	17
2	Lovenox	Y	N	N	6	19
3	Lovenox	Y	Y	Y	7	22
4	Lovenox	Y	Y	Y	4	7
5	Heparin	N	N	N	8	15
6	Lovenox	N	N	Y	4	24

Patient	Days since admit	Days since + COVID test	Arterial pH	APACHE score [¶]	SAPS**	LIPS ^{††}
1	4	7	7.52	16	35	5.5
2	6	6	7.48	10	37	6.5
3	13	17	7.41	16	41	3.5
4	4	4	7.44	15	43	4.5
5	8	8	7.31	17	51	7
6	4	4	7.45	11	37	5.5

* Body mass index, kg/m².[†] Hypertension.[‡] Remdesivir.[§] Continuous mandatory ventilation.[¶] Acute physiologic assessment and chronic health evaluation

** Simplified acute physiology score

^{††} Lung injury prediction score

2. Materials and methods

2.1. Study participants

We recruited hospitalized ICU patients (n=6) with acute SARS-CoV-2 infections confirmed by RT-PCR at St. Joseph's Hospital in Denver, CO. All patients required mechanical ventilation due to the severity of lung infection. At the time of blood draw, patients were receiving lovenox (5/6) or heparin (1/6). All participants provided written consent using a protocol approved by National Jewish Health IRB. Healthy donors (n=6) were recruited and provided written consented using a protocol approved by the University of Vermont Committee on Human Research. All work with human subjects was carried out in accordance with the Declaration of Helsinki and our respective institutional guidelines.

2.2. Sample acquisition

Plasma from patients and healthy donors was prepared from citrated (3.2%) whole blood obtained at the onset of mechanical ventilation from a central line or the antecubital vein using standard methods. All plasma samples were stored at -80 °C in small aliquots.

2.3. Measurement of histone levels

Cell-free histone levels were assessed in plasma from healthy controls and COVID-19 patients using a photometric enzyme immunoassay (Cell Death Detection ELISAPLUS kit, Roche, Indianapolis, IN), which measures histone-associated DNA fragments, according to the manufacturer's instructions.

2.4. In situ fibrin polymerization

Fibrin clot formation was assessed as previously described [17]. Plasma was diluted 1:1 with 20 mM Hepes, 0.15 M NaCl, pH 7.4 (HBS) and incubated on glass chamber slides with AlexaFluor488-labeled fibrinogen (220 nM) (ThermoFisher Scientific, Waltham, MA), relipidated tissue factor₁₋₂₄₂ (8.7 pM) (a gift from Dr. R. Lunblad, Baxter Healthcare Corp.), and CaCl₂, with HistoChoice (MilliporeSigma, Burlington, MA) and an anti-photobleaching agent (Agilent Technologies, Santa Clara, California). Clots were visualized by confocal microscopy using a Nikon A1R Confocal Microscope (Nikon Instruments Inc., Melville NY) with a 60X/1.5 oil immersion objective. For each clot, a three-dimensional Z-stack image series consisting of 40 images at 0.25 μm steps through each sample was obtained. All plasma clots were formed and imaged in duplicate. Fiber resolvability was quantified by standard deviation mapping of the images using custom in-house software as described previously [17]. Analysis of fiber resolvability, as determined by standard deviation mapping, provides a reliable assessment of the clot architecture that is unaffected by the overall brightness of the fluorescence [18].

2.5. Endothelial cell culture

Human endothelial cells (EA.hy926; ATCC® CRL-2922™) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 5 μg/mL gentamycin (complete medium) at 37 °C, 5% CO₂. Prior to use, the complete medium was removed and the cells incubated with DMEM for 1 hr. at 37 °C, 5% CO₂. The cells were released from the tissue culture wells with trypsin and subjected to centrifugation (170 x g, 7 min). Cell pellets were washed one time by resuspension in HBS followed by centrifugation. The final cell pellets

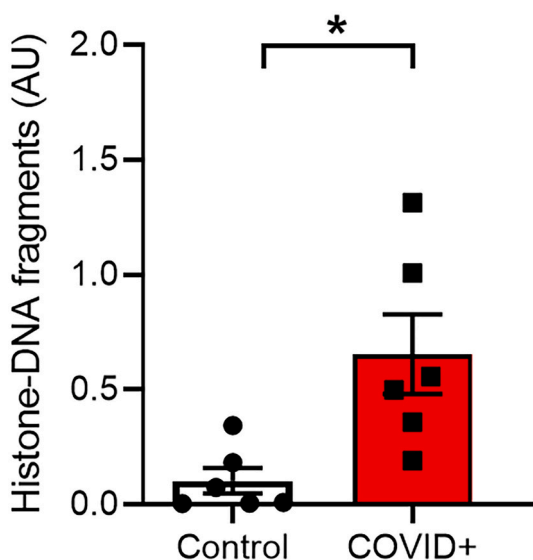


Fig. 1. Plasma from COVID-19 patients contains increased levels of cell-free histones. Histone-DNA fragments were measured in plasma from healthy controls (n=6) and COVID-19 patients (n=6) using a commercially available assay (*p=0.0126).

were resuspended in HBS and adjusted to a final concentration of 1×10^7 /mL.

2.6. Thrombin generation

Thrombin generation was assessed by modified calibrated automated thrombography [19]. Plasma was thawed at 37 °C in the presence of corn trypsin inhibitor (0.1 mg/mL final concentration). Plasma was incubated with the thrombin substrate Z-Gly-Gly-Arg 7-amido-4-methylcoumarin hydrochloride (0.42 mM) (Bachem AG, Switzerland) and CaCl_2 (15 mM) (3 min, 37 °C), and the reactions initiated by the addition of relipidated tissue factor₁₋₂₄₂ (6.5 pM) and synthetic vesicles consisting of 80% phosphatidylcholine and 20% phosphatidylserine (PCPS) (20 μM), or EA.hy926 cells (2×10^5). Fluorescence was measured (ex = 370 nm/em = 460 nm) for 1 hour with a Cytation 3 imaging reader (BioTek, Winooski, VT). Changes in fluorescence were converted to thrombin concentrations using a calibration curve created from sequential dilutions of human thrombin. If no change in fluorescence

was noted after 60 min, the lag time for the sample was defined as >60 min.

2.7. Statistical analysis

Data are expressed as mean ± standard error of the mean. Unpaired, two-tailed t-tests were performed to compare the mean histone levels and measures of thrombin generation between controls and patients. For comparison of clot resolvability, the Kolmogorov-Smirnov test was used to compare cumulative distributions with 95% confidence. A p-value less than 0.05 was considered significant.

3. Results

3.1. COVID-19 patient characteristics

We enrolled patients diagnosed with COVID-19 (n=6 patients) and healthy controls (n=6). The patient characteristics are described in Table 1.

3.2. Cell-free histone levels are elevated in patients with COVID-19

Using an assay that detects cell-free, histone-associated DNA fragments, it was observed that histones were significantly elevated in COVID-19 patient plasma relative to plasma from healthy controls (Fig. 1).

3.3. Altered fibrin clot structure as a consequence of infection with SARS-CoV-2

Fibrin clot structure was also assessed using AlexaFluor488-labeled fibrinogen. Comparison of the clot structures by confocal microscopy demonstrated that fibrin clots formed using plasma from COVID-19 patients were markedly different from those formed using healthy control plasma. Control clots consistently demonstrated a robust fibrin network with high fiber resolvability (Fig. 2). In contrast, clots made from COVID-19 patient samples were densely packed with extremely thin, almost hair-like fibers that were significantly less resolvable ($p < 0.05$) suggesting that thrombin generation in the COVID-19 plasma was altered [20].

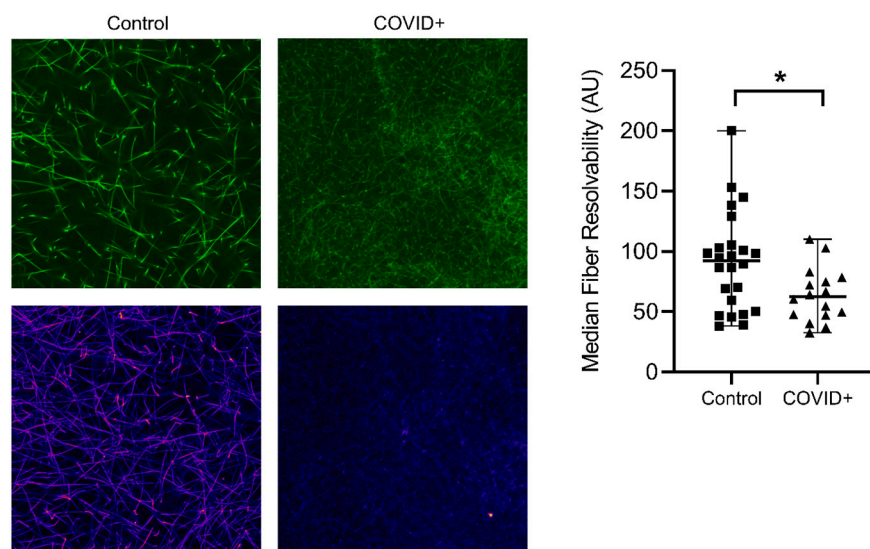


Fig. 2. Fibrin clot structure is altered in plasma from COVID-19 patients. Representative confocal microscopy images (top) and corresponding standard deviation maps (bottom) of fluorescently labeled fibrinogen polymerized in the presence of control and COVID+ patient plasma samples. Clots were imaged on a Nikon A1R Confocal microscope (60X/1.5 oil immersion). Each image is a representative cross-section from a three-dimensional Z-stack (0.25 μm steps) of 40 images captured from each clot. For each clot, median fiber resolvability was quantified by standard deviation mapping using custom in-house software [17]. *p<0.05 was considered significant.

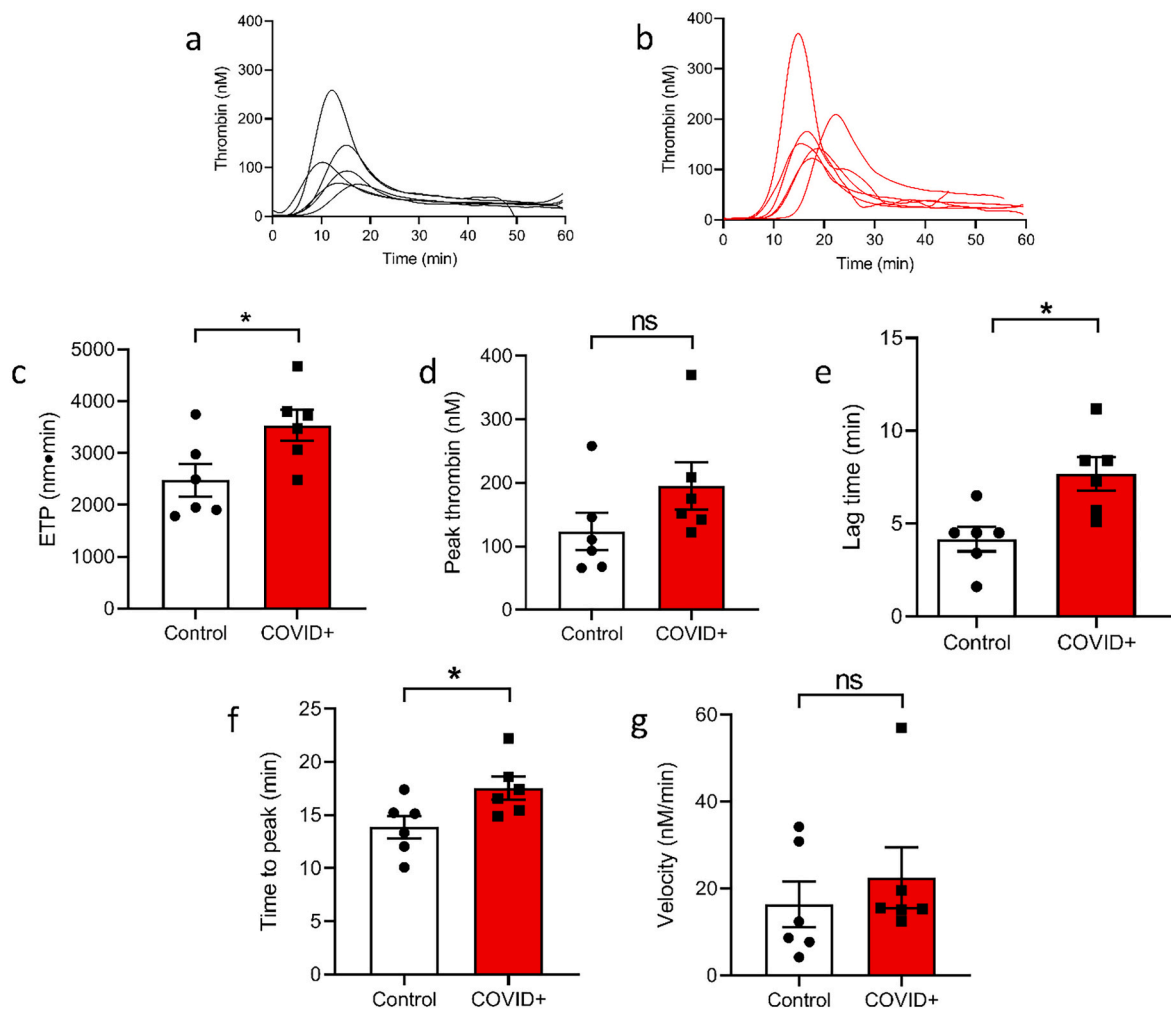


Fig. 3. Thrombin generation is altered in plasma from COVID-19 patients. Calibrated automated thrombography was used to measure thrombin generation using plasma from healthy controls (n=6) or COVID-19 patients (n=6) in the presence of tissue factor (6.5 pM) and PCPS (20 μM). The mean of duplicate thrombin generation curves for each healthy control (a) and COVID-19 patient (b) is shown. Endogenous thrombin potential (c), peak thrombin (d), lag time (e), time to peak thrombin (f), and rate of thrombin generation (velocity) (g) of the controls and patients under these conditions were compared. *p<0.05 was considered significant. ETP = endogenous thrombin potential; ns = not significant

3.4. Thrombin generation is dysregulated in the plasma from patients with severe COVID-19

Thrombin generation in healthy control plasma and plasma from COVID-19 patients were compared by calibrated automated thrombography in the presence of added tissue factor and PCPS (Figs. 3a & 3b) or cultured EA.hy926 cells (Figs. 4a & 4b). In the presence of tissue factor and PCPS, the endogenous thrombin potential (ETP) (Fig. 3c) of plasma from COVID-19 patients (n=6) was significantly higher than plasma from healthy controls (n=6) although peak levels of thrombin (Fig. 3d) were not different. Despite the apparent hypercoagulability of the COVID-19 patient plasma, significantly longer lag times (Fig. 3e) and times to peak thrombin (Fig. 3f) were observed in plasma from patients as compared to healthy controls, while the rates of thrombin generation (Velocity) (Fig. 3g) were not different. Strikingly different results were observed when EA.hy926 cells were used in place of added tissue factor and PCPS (Fig. 4). Under these conditions, none of the healthy control plasma samples generated thrombin (lag time >60 min) (Fig. 4a & 4e). Unexpectedly, and in marked contrast to the results obtained with control plasma, four of the COVID-19 patient plasma samples generated thrombin (mean lag time ~20 min) (Fig. 4b & e) albeit at levels lower than that observed in the presence of added tissue factor and PCPS. Consistent with this observation, the mean endogenous thrombin

potential (Fig. 4c) and peak levels of thrombin (Fig. 4d) were significantly higher in patient vs healthy control plasma, while the times to peak thrombin (Fig. 4f) were not different. Rates of thrombin generation were unable to be calculated in the absence of added tissue factor and PCPS.

4. Discussion

These combined observations suggest that thrombin generation by plasma from patients with severe COVID-19 infections is dysregulated, and that high levels of histones or another molecule(s) effect(s) blood coagulation in the presence of endothelial cells. Histones act as damage-associated molecular pattern molecules following their release from cells by NETs, cell apoptosis, or cell necrosis [21]. Circulating levels of nucleosomes and histones are significantly elevated and correlate with the severity or poor outcome of several pathophysiological processes such as acute bacterial infection, sepsis, autoimmune diseases, cerebral stroke, trauma, cancer [21], and acute pulmonary embolism [22]. Here, we show increased histone-associated DNA fragments in SARS-CoV-2 infection resulting in severe COVID-19. This is consistent with prior reports of elevated NETs in COVID-19, as evidenced by increased cell-free DNA, myeloperoxidase-DNA complexes, and citrullinated-histone H3 [23–25]. Similar observations were recently made in two

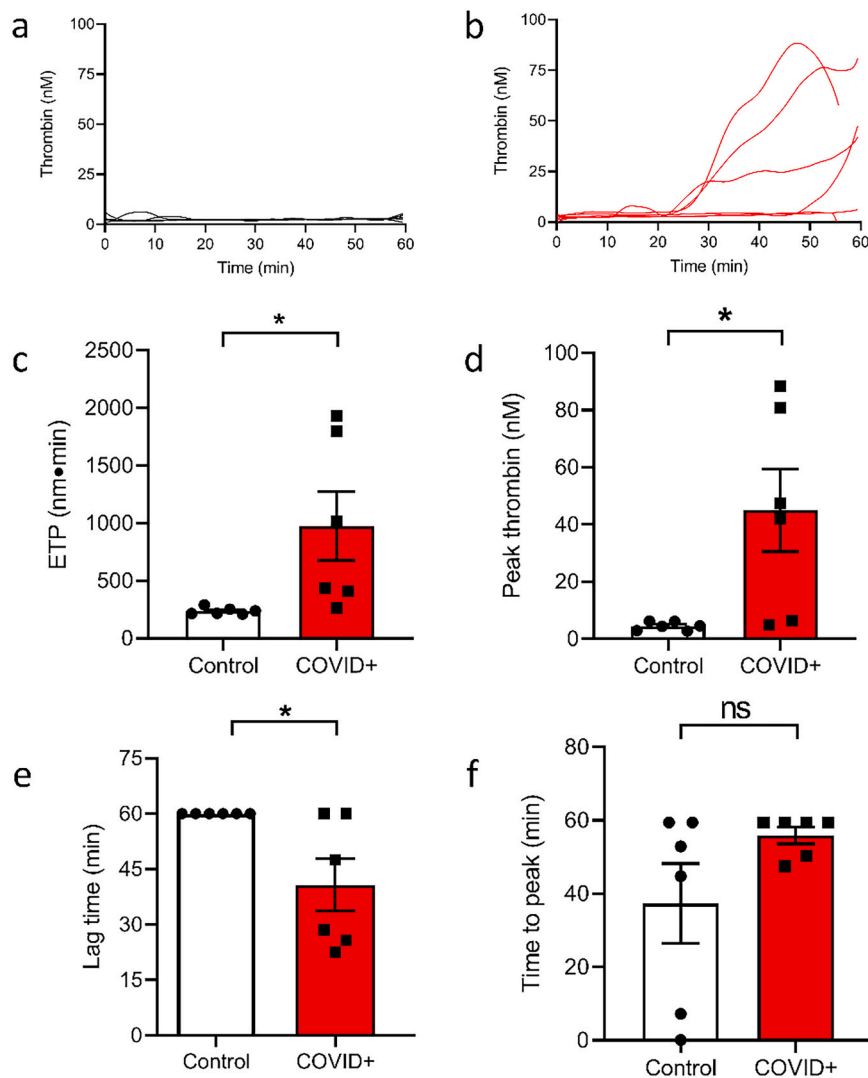


Fig. 4. Plasma from COVID-19 patients is hypercoagulable in the presence of cultured endothelial cells. Calibrated automated thrombography was used to measure thrombin generation using plasma from healthy controls (n=6) or COVID-19 patients (n=6) in the presence of EA.hy926 cells. The mean of duplicate thrombin generation curves for each healthy control (a) and COVID-19 patient (b) is shown. Endogenous thrombin potential (c), peak thrombin (d), lag time (e), and time to peak thrombin (f) of the controls and patients under these conditions were compared. For the plasma samples that did not clot in the presence of cells over the duration of the experiment (60 min), the lag times were set to 60 min. ETP = endogenous thrombin potential; ns = not significant.

independent cohorts of COVID-19 positive patients with a quantitative nucleosome immunoassay that measured cell-free H3.1 nucleosomes [26]. These investigators demonstrated that nucleosomes were highly elevated in plasma of COVID-19 patients with a severe course of the disease relative to healthy controls and that both the histone 3.1 variant and citrullinated nucleosomes increase with disease severity. In another study by Guéant and colleagues, circulating histone-DNA levels and other markers of neutrophil activation were increased in patients with COVID-19 [27]. Histone-DNA was associated with markers of disease severity including intensive care admission, body temperature, lung damage, markers of cardiovascular outcomes, renal failure, and increased interleukin-6, interleukin-8 and C-X-C motif chemokine receptor 2.

Extracellular histones enhance plasma thrombin generation by reducing thrombomodulin-dependent protein C activation [14]. Additionally, isolated histone proteins bind to prothrombin via fragment 1 and fragment 2 (non-catalytic portions), and reduce the need for factor Xa in clotting [13]. Indeed, the overall higher amounts of thrombin generated by COVID-19 patient plasma is consistent with a more procoagulant state. However, unexpectedly, the mean lag time and time to peak thrombin were significantly reduced as compared to control patients suggesting that thrombin generation in COVID-19 patients is dysregulated. Consistent with our observations are very recent studies by Bouck and colleagues [28] who also demonstrated that compared with healthy donors, patients with SARS-CoV-2 infection had increased

thrombin generation potential but a prolonged lag time. Additional experiments demonstrated increased endogenous plasmin potential and delayed plasmin formation. These perturbations led to increased fibrin formation.

The fibrin clots formed *in situ* by plasma from patients with severe COVID-19 are denser with thinner fibers and lower fiber resolvability as compared to clots formed from the plasma of healthy controls. Patients with thromboembolic diseases also form structurally abnormal clots that are resistant to fibrinolysis [29]. Similar observations have been made in patients with severe trauma [17], which is also characterized by a disseminated intravascular coagulation-like phenotype [30]. As patients with acute thromboembolic disease [22] and trauma [13] also have elevated circulating histone levels, these structurally- and functionally-altered clots may be the result of covalent (via factor XIIIa crosslinking) and noncovalent interactions of histones with fibrin [31] in addition to dysregulated thrombin formation. Additional studies are needed to examine the interactions of histones with fibrin.

Histones can also promote thrombotic events by inducing endothelial cells to release pro-inflammatory cytokines [15], increase cell surface adhesion molecules [16], and express tissue factor [15]. When cultured endothelial cells were used in place of added tissue factor and PCPS, we observed considerable thrombin generation by COVID-19 patient plasma suggesting that histones or (an)other molecule(s) present in COVID-19 plasma induce(s) an inflammatory and procoagulant endothelial cell phenotype. This notion is supported by a recent

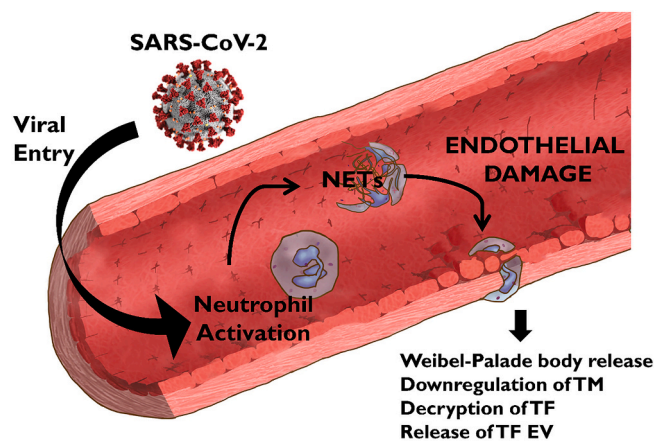


Fig. 5. Hypothetical model of vascular injury and coagulopathy after SARS-CoV-2 infection. SARS-CoV-2 activates neutrophils resulting in the release of histone-containing NETs [23–25]. We hypothesize that circulating histones promote coagulopathy through their interactions with endothelial cells (Weibel-Palade body exocytosis [34], thrombomodulin (TM) downregulation [14], and sphingomyelinase-mediated decryption of tissue factor (TF) [15,33] and procoagulant extracellular vesicle (EV) release [33]) resulting in dysregulated thrombin formation and abnormal fibrin clot formation.

observation by Nagashima and colleagues [32] who demonstrated that post-mortem lung samples from patients with COVID-19 have higher endothelial cell expression of interleukin-6, tumor necrosis factor- α , intracellular adhesion molecule-1 and caspase-1, a marker of pyroptosis, an inflammatory form of programmed cell death, as compared to H1N1 and control groups, and proposed that this endothelial cell dysfunction leads to systemic thrombosis. In addition, Rao and colleagues recently demonstrated that SARS-CoV-2 infection rapidly induces the activation and translocation of acid sphingomyelinase [33] to the outer leaflet of monocyte-derived macrophage membranes leading to decryption of tissue factor and release of tissue factor positive extracellular vesicles.

5. Conclusion

While the impact of this study is limited by its small sample size, its results confirm that circulating histones are elevated in severe COVID-19 and that thrombin generation using a plasma-based assay is dysregulated. This study extends these observations by demonstrating a role for endothelial cells in COVID-19 coagulopathy and that fibrin clot structure is altered. Based on these collective observations, we hypothesize that histones in COVID-19 patient plasma activate endothelial cells leading to dysregulated thrombin generation by release of von Willebrand factor and P-selectin from Weibel-Palade bodies [34], activation and translocation of sphingomyelinase resulting in tissue factor decryption [15,33] and procoagulant microparticle release [33], and/or decrease of thrombomodulin expression [14] (Fig. 5). Histones may also mediate the formation of structurally abnormal clots directly or as a result of altered thrombin formation. Neutralization of histones with sulfated polyanions such as heparin may mitigate these coagulopathic responses and reduce morbidity and mortality due to thrombosis. Additional studies with a larger patient cohort and access to larger plasma volumes are warranted to test these hypotheses.

Funding

This research was supported by grants (R01GM123010 to K.F., UM1HL120877 to K.F., R35HL140039 to W.J.) from the National Institutes of Health (NIH). It was also funded, in part, by the NIH Agreement 1OT2HL156812. The views and conclusions contained in this document are those of the authors and should not be interpreted as representing the official policies, either expressed or implied, of the NIH.

Author statement

Beth A. Bouchard designed and performed experiments, analyzed experiments, interpreted data, and wrote the manuscript; Christos Colovos interpreted data and wrote the manuscript; Michael A. Lawson designed and performed experiments, and analyzed and interpreted data; Zachary T. Osborn designed and performed experiments and analyzed data; Adrian M. Sackheim performed experiments and analyzed data; Kara J. Mould provided patient samples and clinical data and edited the manuscript; William J. Janssen provided patient samples and clinical data and edited the manuscript; Mitchell J. Cohen provided patient samples; Devdoot Majumdar interpreted data; Kalev Freeman conceived of the research, designed experiments, interpreted data and wrote the manuscript.

All authors read and approved the final version prior to submission.

This article is the authors' original work, hasn't received prior publication, and isn't under consideration for publication elsewhere.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

The authors would like to thank Nicholas Grubinger for quantification of fibrin clot fiber resolvability, Dr. Roger Lunblad (Baxter Healthcare Corp.) for the generous donation of recombinant tissue factor₁₋₂₄₂ and Dr. Ralph Budd's laboratory (University of Vermont) for the use of the Cytation 3 imaging reader.

References

- [1] E. Dong, H. Du, L. Gardner, An interactive web-based dashboard to track COVID-19 in real time, *Lancet Infect. Dis.* 20 (5) (2020) 533–534.
- [2] F. Zhou, T. Yu, R. Du, G. Fan, Y. Liu, Z. Liu, J. Xiang, Y. Wang, B. Song, X. Gu, L. Guan, Y. Wei, H. Li, X. Wu, J. Xu, S. Tu, Y. Zhang, H. Chen, B. Cao, Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study, *Lancet* 395 (10229) (2020) 1054–1062.
- [3] W.J. Guan, Z.Y. Ni, Y. Hu, W.H. Liang, C.Q. Ou, J.X. He, L. Liu, H. Shan, C.L. Lei, D. S.C. Hui, B. Du, L.J. Li, G. Zeng, K.Y. Yuen, R.C. Chen, C.L. Tang, T. Wang, P. Y. Chen, J. Xiang, S.Y. Li, J.L. Wang, Z.J. Liang, Y.X. Peng, L. Wei, Y. Liu, Y.H. Hu, P. Peng, J.M. Wang, J.Y. Liu, Z. Chen, G. Li, Z.J. Zheng, S.Q. Qiu, J. Luo, C.J. Ye, S. Y. Zhu, N.S. Zhong, C. China medical treatment expert group for, clinical characteristics of coronavirus Disease 2019 in China, *N. Engl. J. Med.* 382 (18) (2020) 1708–1720.
- [4] C. Huang, Y. Wang, X. Li, L. Ren, J. Zhao, Y. Hu, L. Zhang, G. Fan, J. Xu, X. Gu, Z. Cheng, T. Yu, J. Xia, Y. Wei, W. Wu, X. Xie, W. Yin, H. Li, M. Liu, Y. Xiao, H. Gao, L. Guo, J. Xie, G. Wang, R. Jiang, Z. Gao, Q. Jin, J. Wang, B. Cao, Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China, *Lancet* 395 (10223) (2020) 497–506.
- [5] N. Tang, D. Li, X. Wang, Z. Sun, Abnormal coagulation parameters are associated with poor prognosis in patients with novel coronavirus pneumonia, *J. Thromb. Haemost.* 18 (4) (2020) 844–847.
- [6] Q.A. Zhou, J. Kato-Weinstein, Y. Li, Y. Deng, R. Granet, L. Garner, C. Liu, D. Polshakov, C. Gessner, S. Watkins, Potential Therapeutic Agents and Associated Bioassay Data for COVID-19 and Related Human Coronavirus Infections, *ACS Pharmacol Transl Sci* 3 (5) (2020) 813–834.
- [7] F. Zhou, T. Yu, R. Du, G. Fan, Y. Liu, Z. Liu, J. Xiang, Y. Wang, B. Song, X. Gu, L. Guan, Y. Wei, H. Li, X. Wu, J. Xu, S. Tu, Y. Zhang, H. Chen, B. Cao, Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study, *Lancet* 395 (10229) (2020) 1054–1062.
- [8] Y. Zuo, S. Yalavarthi, H. Shi, K. Gockman, M. Zuo, J.A. Madison, C. Blair, A. Weber, B.J. Barnes, M. Egeblad, R.J. Woods, Y. Kanthi, J.S. Knight, Neutrophil extracellular traps in COVID-19, *JCI Insight* 5 (11) (2020).
- [9] B.J. Barnes, J.M. Adrover, A. Baxter-Stoltzfus, A. Borczuk, J. Cools-Lartigue, J. M. Crawford, J. Dassler-Plenker, P. Guerci, C. Huynh, J.S. Knight, M. Loda, M. R. Looney, F. McAllister, R. Rayes, S. Renaud, S. Rousseau, S. Salvatore, R. E. Schwartz, J.D. Spicer, C.C. Yost, A. Weber, Y. Zuo, M. Egeblad, Targeting potential drivers of COVID-19: Neutrophil extracellular traps, *J. Exp. Med.* 217 (6) (2020).
- [10] E. Gremese, G. Ferraccioli, The pathogenesis of microthrombi in COVID-19 cannot be controlled by DOAC: NETosis should be the target, *J. Intern. Med.* 289 (3) (2021) 420–421.
- [11] S. Gupta, V. Sahni, The intriguing commonality of NETosis between COVID-19 & Periodontal disease, *Med. Hypotheses* 144 (2020) 109968.

- [12] S.A. Sawadogo, B. Dighero-Kemp, D.D. Ouedraogo, L. Hensley, J. Sakande, How NETosis could drive "Post-COVID-19 syndrome" among survivors, *Immunol. Lett.* 228 (2020) 35–37.
- [13] S.T. Abrams, D. Su, Y. Sahraoui, Z. Lin, Z. Cheng, K. Nesbitt, Y. Alhamdi, M. Harrasser, M. Du, J.H. Foley, D. Lillicrap, G. Wang, C.H. Toh, Assembly of alternative prothrombinase by extracellular histones initiates and disseminates intravascular coagulation, *Blood* 137 (1) (2021) 103–114.
- [14] C.T. Ammollo, F. Semeraro, J. Xu, N.L. Esmon, C.T. Esmon, Extracellular histones increase plasma thrombin generation by impairing thrombomodulin-dependent protein C activation, *J. Thromb. Haemost.* 9 (9) (2011) 1795–1803.
- [15] J. Xu, X. Zhang, R. Pelayo, M. Monestier, C.T. Ammollo, F. Semeraro, F.B. Taylor, N.L. Esmon, F. Lupu, C.T. Esmon, Extracellular histones are major mediators of death in sepsis, *Nat. Med.* 15 (11) (2009) 1318–1321.
- [16] B. McDonald, R.P. Davis, S.-J. Kim, M. Tse, C.T. Esmon, E. Kolaczowska, C. N. Jenne, Platelets and neutrophil extracellular traps collaborate to promote intravascular coagulation during sepsis in mice, *Blood* 129 (10) (2017) 1357–1367.
- [17] N. Dow, J.R. Coleman, H. Moore, Z.T. Osborn, A.M. Sackheim, G. Hennig, S. Butenas, M.T. Nelson, E.E. Moore, K. Freeman, Dense and dangerous: The tissue plasminogen activator-resistant fibrinolysis shutdown phenotype is due to abnormal fibrin polymerization, *J. Trauma Acute Care Surg.* 88 (2) (2020) 258–265.
- [18] K.P. Quinn, I. Georgakoudi, Rapid quantification of pixel-wise fiber orientation data in micrographs, *Biomed Opt* 18 (4) (2013), 046003.
- [19] S. Butenas, A. Undas, M.T. Gissel, K. Szuldrzynski, K. Zmudka, K.G. Mann, Factor XIa and tissue factor activity in patients with coronary artery disease, *Thromb. Haemost.* 99 (1) (2008) 142–149.
- [20] A.S. Wolberg, Thrombin generation and fibrin clot structure, *Blood Rev.* 21 (3) (2007) 131–142.
- [21] R. Chen, R. Kang, X.-G. Fan, D. Tang, Release and activity of histone in diseases, *Cell Death Dis.* 5 (8) (2014), e1370.
- [22] M. Ząbczyk, J. Natowska, A. Janion-Sadowska, A. Metzger-Gumiela, M. Polak, K. Plens, M. Janion, G. Skonieczny, K. Mizia-Stec, A. Undas, Prothrombotic fibrin clot properties associated with NETs formation characterize acute pulmonary embolism patients with higher mortality risk, *Sci. Rep.* 10 (2020) 11433.
- [23] F.P. Veras, M.C. Pontelli, C.M. Silva, J.E. Toller-Kawahisa, M. de Lima, D. C. Nascimento, A.H. Schneider, D. Caetite, L.A. Tavares, I.M. Paiva, R. Rosales, D. Colon, R. Martins, I.A. Castro, G.M. Almeida, M.I.F. Lopes, M.N. Benatti, L. P. Bonjorno, M.C. Giannini, R. Luppino-Assad, S.L. Almeida, F. Vilar, R. Santana, V. R. Bollela, M. Auxiliadora-Martins, M. Borges, C.H. Miranda, A. Pazin-Filho, L.L. P. da Silva, L.D. Cunha, D.S. Zamboni, F. Dal-Pizzol, L.O. Leiria, L. Siyuan, S. Batah, A. Fabro, T. Mauad, M. Dolhnikoff, A. Duarte-Neto, P. Saldiva, T.M. Cunha, J. C. Alves-Filho, E. Arruda, P. Louzada-Junior, R.D. Oliveira, F.Q. Cunha, SARS-CoV-2-triggered neutrophil extracellular traps mediate COVID-19 pathology, *J. Exp. Med.* 217 (12) (2020).
- [24] M.H. Busch, S. Timmermans, M. Nagy, M. Visser, J. Huckriede, J.P. Aendekerk, F. de Vries, J. Potjewijd, B. Jallah, R. Ysermans, A.M.L. Oude Lashof, P. H. Breedveld, M.C.G. van de Poll, I.C.C. van de Horst, B.C.T. van Bussel, R. Theunissen, H.M.H. Spronk, J. Damoiseaux, H. Ten Cate, G.A.F. Nicolaes, C. P. Reutelingsperger, P. van Paassen, Neutrophils and Contact Activation of Coagulation as Potential Drivers of COVID-19, *Circulation* 142 (18) (2020) 1787–1790.
- [25] E.A. Middleton, X.Y. He, F. Denorme, R.A. Campbell, D. Ng, S.P. Salvatore, M. Mostyka, A. Baxter-Stoltzfus, A.C. Borczuk, M. Loda, M.J. Cody, B.K. Manne, I. Portier, E.S. Harris, A.C. Petrey, E.J. Beswick, A.F. Caulin, A. Iovino, L. M. Abegglen, A.S. Weyrich, M.T. Rondina, M. Egeblad, J.D. Schiffman, C.C. Yost, Neutrophil extracellular traps contribute to immunothrombosis in COVID-19 acute respiratory distress syndrome, *Blood* 136 (10) (2020) 1169–1179.
- [26] E. Cavalier, J. Guiot, K. Lechner, A. Dutsch, M. Eccleston, M. Herzog, T. Bygott, A. Schomburg, T. Kelly, S. Holdenrieder, Circulating Nucleosomes as Potential Markers to Monitor COVID-19 Disease Progression, *Front. Mol. Biosci.* 8 (2021), 600881.
- [27] J.-L. Guéant, R.-M. Guéant-Rodriguez, J. Fromonot, A. Oussalah, H. Louis, C. Chery, M. Gette, S. Gleye, J. Callet, J. Raso, F. Blanchecotte, P. Lacolley, R. Gueiu, V. Regnault, Elastase and exacerbation of neutrophil innate immunity are involved in multi-visceral manifestations of COVID-19, *Allergy* 76 (6) (2021) 1846–1858.
- [28] E.G. Bouck, F. Denorme, L.A. Holle, E.A. Middelton, A.M. Blair, B. de Laat, J. D. Schiffman, C. Con Yost, M.T. Rondina, A.S. Wolberg, R.A. Campbell, COVID-19 and Sepsis Are Associated With Different Abnormalities in Plasma Procoagulant and Fibrinolytic Activity, *Arterioscler. Thromb. Vasc. Biol.* 41 (1) (2021) 401–414.
- [29] A. Undas, R.A.S. Ariens, Fibrin clot structure and function: a role in the pathophysiology of arterial and venous thromboembolic diseases, *Arterioscler. Thromb. Vasc. Biol.* 31 (12) (2011) e88–e99.
- [30] L.Z. Kornblith, H.B. Moore, M.J. Cohen, Trauma-induced coagulopathy: The past, present, and future, *J. Thromb. Haemost.* (2019), <https://doi.org/10.1111/jth.14450> [Epub ahead of print].
- [31] M. Locke, C. Longstaff, Extracellular Histones Inhibit Fibrinolysis through Noncovalent and Covalent Interactions with Fibrin, *Thromb. Haemost.* 121 (4) (2021) 464–476.
- [32] S. Nagashima, M. Castilho Mendes, A.P. Camargo Martins, N.H. Borges, T. M. Godoy, A.F.R. Dos Santos Miggiolaro, F. da Silva Dezidério, C. Machado-Souza, L. de Noronha, Endothelial dysfunction and thrombosis in patients with COVID-19 - Brief Report, *Arterioscler. Thromb. Vasc. Biol.* 40 (10) (2020) 2404–2407.
- [33] J. Wang, U.R. Pendurthi, G. Yi, L.V.M. Rao, SARS-CoV-2 infection induces the activation of tissue factor-mediate coagulation via activation of acid sphingomyelinase, *Blood* 138 (4) (2021).
- [34] A. Michels, S. Albáñez, J. Mewburn, K. Nesbitt, T.J. Gould, P.C. Liaw, P.D. James, L.L. Swystun, D. Lillicrap, Histones link inflammation and thrombosis through the induction of Weibel-Palade body exocytosis, *J. Thromb. Haemost.* 14 (11) (2016) 2274–2286.