Received: 6 July 2023 | Revised: 16 July 2023 | Accepted: 7 August 2023

https://doi.org/10.1016/j.rpth.2023.102195

RESEARCH LETTER

Citrullination of α2-antiplasmin is unlikely to contribute to enhanced plasmin generation in COVID-19 pathophysiology

COVID-19 is caused by the SARS-CoV-2 [1]. Severe COVID-19 is characterized by immune cell activation, leading to inflammation and the formation of neutrophil extracellular traps (NETs) [2–4]. COVID-19 is also associated with an imbalance in coagulation and fibrinolytic activity [5]. Exuberant fibrinogen production secondary to the inflammatory response, coupled with elevated thrombin generation, provokes fibrin deposition. However, despite paradoxically increased plasmin generation potential in plasma from patients with COVID-19 [6,7], fibrinolytic pathways are unable to counter this process [8]. Patients with severe COVID-19 have a high risk of developing venous thromboembolism [9], and patients with COVID-19 with venous thromboembolism have an increased risk of death [10].

In response to inflammation, the enzyme peptidylarginine deiminase 4 (PAD4) present in neutrophil granulocytes becomes activated, leading to histone citrullination, chromatin decondensation, and NET formation and release. During these events, exposed PAD4 may citrullinate extracellular and plasma proteins, particularly those present at high concentrations that have a relatively long circulating half-life ($t_{1/2}$) (eg, fibrinogen, antithrombin, and α2-antiplasmin [AP]) [11,12]. PAD4 can also citrullinate proteins with short $t_{1/2}$ and low plasma concentration (eg, tissue factor pathway inhibitor) *in vitro* [13]. Citrullination of coagulation and fibrinolytic proteins has been observed in inflammatory diseases [11,12]. For example, citrullinated fibrinogen ($t_{1/2}$ 3-5 days) has been detected in rheumatoid arthritis and is thought to incite the production of anti-citrullinated protein antibodies [14]. Importantly, citrullination can alter the functional activity of these proteins. Citrullination of AP ($t_{1/2}$ 2.6 days), the canonical inhibitor of the fibrinolytic enzyme plasmin, abolishes its activity [15]. Given these observations, we hypothesized that during COVID-19, extensive neutrophil activation and release of PAD4 promotes plasma protein citrullination and that the generation of citrullinated AP (CitAP) causes increased plasmin generation potential [6,7].

We tested this hypothesis by characterizing plasma samples from 28 patients with COVID-19 (11 males, 17 females, mean age 56 [range, 28-76], 19 of whom had diabetes and/or hypertension, 45% males and 12% females had a fatal outcome). Race/ethnicity of patients with COVID-19 were as follows: 24 White (17 non-Hispanic and 7 Hispanic), 2 American Indian, 1 Black, and 1 Native Hawaiian. These patients were compared with 20 age- and gender-matched healthy controls (18 White [17 non-Hispanic and 1 Hispanic], 1 Asian, and 1 unknown). Neutrophil activation is accompanied by secretion of myeloperoxidase, and as expected, plasma myeloperoxidase was significantly elevated in patients with COVID-19 vs controls (Figure 1A). We confirmed that patients with COVID-19 have abnormal plasmin generation potential [16] (Figure 1B–F), including a prolonged lag time (Figure 1C), time to peak (Figure 1D), elevated velocity index (Figure 1E), and plasmin peak (Figure 1F). Myeloperoxidase levels correlated with the plasmin generation lag time (Figure 1G) and time to peak (Figure 1H), and these correlations were driven by measurements in patients with COVID-19. These changes suggest that patients with COVID-19 may have quantitative or qualitative changes in fibrinolytic proteins. However, patients with COVID-19 have normal or slightly reduced plasminogen [17,18], and AP activity is in the normal range [19,20], although some studies have reported higher [21] or lower [22] AP activity in patients with COVID-19. We used immunoblotting and densitometry to confirm that AP antigen was not different in our patient cohort (Figure 1I, J).

To verify that citrullination reduces AP function, we used PAD4 (Cat# 10500, Cayman Chemical) to citrullinate plasma-purified AP. To detect CitAP, we first tested a biotinylated phenylglyoxal probe that labels citrulline residues [23]; however, we detected considerable nonspecific binding of this probe to negative (noncitrullinated) controls (data not shown). Therefore, we used an alternate method to detect CitAP with an Anti-Citrulline (Modified) Detection Kit (Cat# 17-347B, MilliporeSigma). This method involves first modifying citrulline residues with 2,3-butanedione and antipyrine in the presence of $FeCl_3$ under acidic conditions [24]. We then separated the proteins with sodium dodecyl sulphate-polyacrylamide gel electrophoresis , transferred them to polyvinylidene fluoride membranes, and probed the membranes with an antibody that detects the modified citrulline. We visualized the signal with enhanced chemiluminescence (Figure 2A, upper panel). This strategy successfully detected citrullinated protein and did not recognize noncitrullinated protein. We reprobed the blots with anti-AP antibody and IRDye 800CW-labeled secondary antibody (Cat# 926-32211, Licor) to visualize total AP (Figure 2A, lower panel). We then spiked normal pooled plasma with exogenous AP or CitAP (final amounts indicated) and measured the effects on plasmin generation potential. In contrast, the addition of AP to normal pooled plasma reduced plasmin generation in a concentration-dependent manner, and the addition of even high

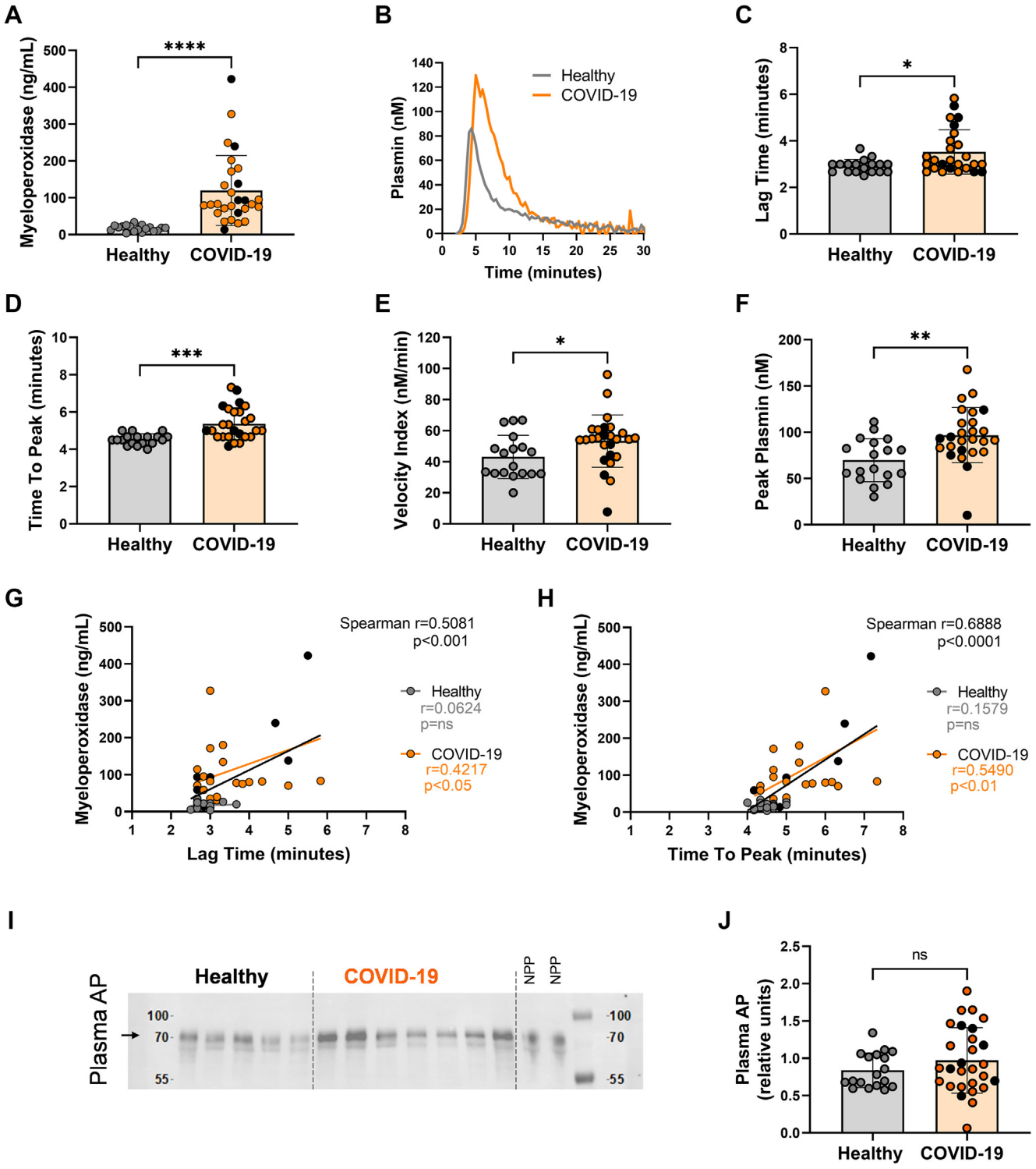

FIGURE 1 Patients with COVID-19 have increased plasmin generation and normal α2-antiplasmin (AP) levels. (A) Myeloperoxidase measured in plasma from healthy donors and patients with COVID-19 by ELISA (Cat# DY3173, R&D Systems). All samples were analyzed in duplicate and averaged. (B) Representative plasmin generation curves. Diluted plasma was mixed with tissue factor, tissue plasminogen activator, and phospholipids or with α2-macroglobulin-plasmin complex, and reactions were triggered by dispensing fluorogenic substrate and $CaCl_2$. Quantitative parameters included (C) lag time, (D) time to peak, (E) velocity index, and (F) peak plasmin calculated from the plasmin generation curves. All samples were analyzed in duplicate. Spearman correlations for myeloperoxidase and plasmin generation (G) lag time and (H) time to peak. (I) Representative immunoblot of plasma AP. Samples were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene membrane. Plasma AP levels in healthy donors and patients with COVID-19 were detected by anti-AP antibody (1 μg/mL, Cat# 13228-1-AP, Proteintech) and IRDye 800CW-labeled secondary antibody (Cat# 926-32211, Licor). (J) Total plasma AP was quantified by densitometry using a ChemiDoc MP Imaging System and Image Lab 6.1.0 software (Bio-Rad). Each blot contained normal pooled plasma (NPP) in duplicate, and the mean plasma AP value of NPP was used to calculate the relative values of the plasma AP of the donors. Mann–Whitney U-test, $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$, ns, not significant. Each dot represents a separate individual; black dots represent deceased patients.

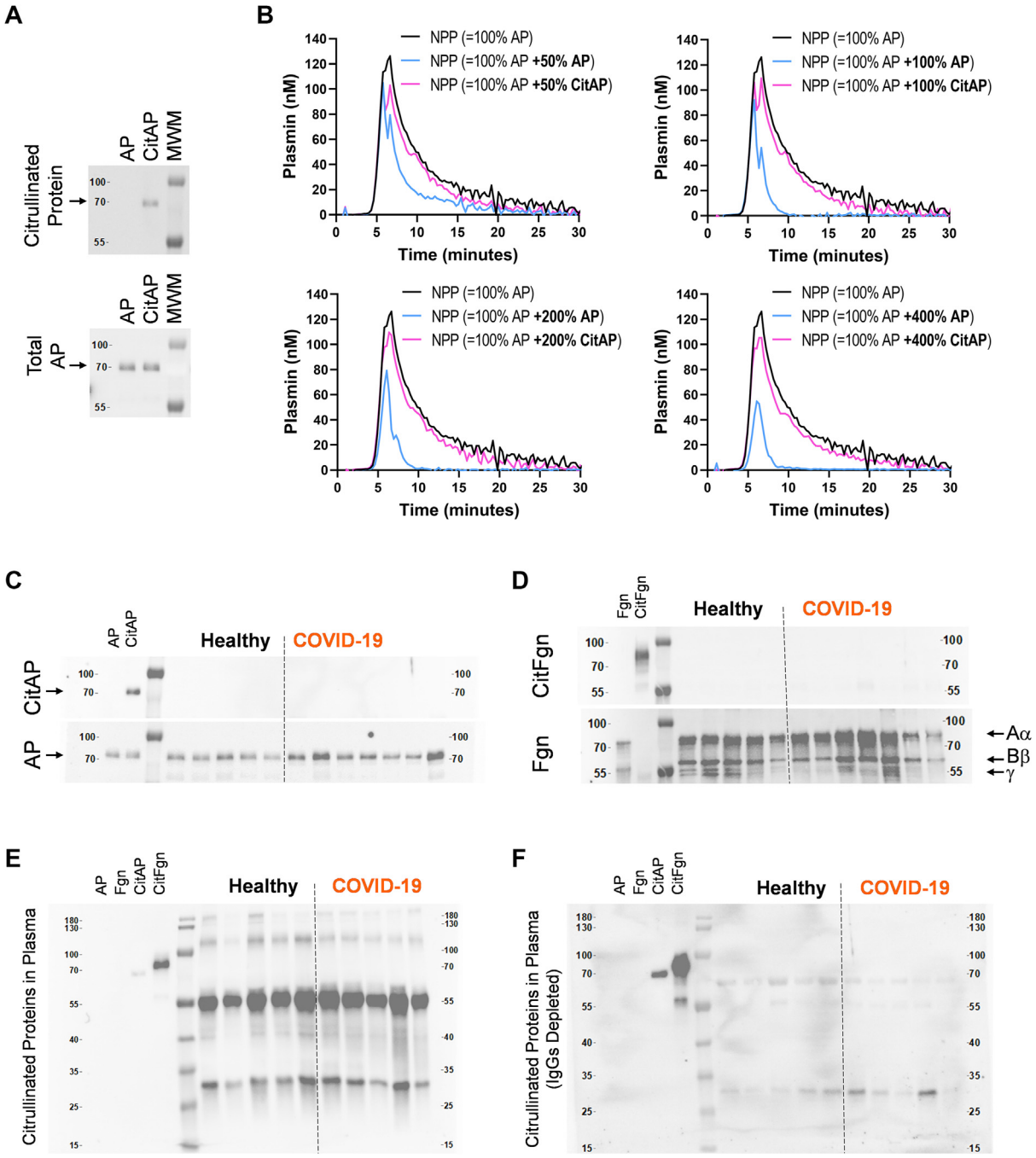

FIGURE 2 Patients with COVID-19 do not have detectable citrullinated antiplasmin (CitAP) or citrullinated fibrinogen (CitFgn). (A) Representative immunoblots of α2-antiplasmin (AP) and CitAP were probed for citrullinated proteins (upper panel) and total AP (lower panel). Citrullinated protein was detected using the Anti-Citrulline (Modified) Detection Kit (Cat# 17-347B, MilliporeSigma), and the same blot was probed for total AP as in Figure 1. (B) Normal pooled plasma (NPP) was estimated to contain 0.7 μg of endogenous AP, and 50%, 100%, 200%, or 400% (0.35, 0.7, 1.4, and 2.8 μg, respectively) of additional AP or CitAP was spiked into NPP before initiating plasmin generation reactions. As a control, NPP was spiked with a buffer containing no AP or CitAP. All samples were analyzed in quadruplicate. (C) Representative immunoblots of CitAP and AP were purified from plasma samples via immunoprecipitation (Cat# 26149, Thermo Fisher Scientific) and detected as described above. (D) Representative immunoblots of CitFgn and Fgn purified from plasma samples via immunoprecipitation and detected as described above, but using primary anti-Fgn antibody (1 μg/mL, Cat# A0080, Dako-Agilent); fibrinogen runs as Aα, Bβ, and γ chains. (E) Representative immunoblot of total citrullinated proteins in plasma samples detected as described above. The secondary antibody (goat anti-human IgG) used in the kit recognizes the human plasma antibody heavy chain (~55 kDa) and light chain (~30 kDa). (F) Representative immunoblot of citrullinated proteins in plasma samples where the IgGs were depleted using protein A/G columns. For immunoblots and plasma spiking experiments, AP and CitAP and/or Fgn and CitFgn were incubated with or without peptidylarginine deiminase 4 (Cat# 10500, Cayman Chemical, 1:10 ratio of peptidylarginine deiminase 4:protein) in 100 mM Tris-HCl pH 7.5, 5 mM $CaCl_2$ for 2.5 hours at 37 °C; 20 ng of (Cit)AP and 100 ng of (Cit)Fgn were loaded as controls on the blots.

amounts of CitAP (up to 400% of normal levels, constituting 80% of total AP) did not (Figure 2B). These results confirmed [15,25] that CitAP is unable to inhibit plasmin.

To detect CitAP in the plasma of patients with COVID-19, we immunoprecipitated total AP using anti-AP antibodies; this step enabled us to specifically identify AP and CitAP within the complex plasma milieu where multiple proteins may undergo citrullination. We confirmed that this method captures and detects immunoprecipitated CitAP. We also immunoprecipitated fibrinogen (Fgn) as a control. We then subjected the proteins to the Anti-Citrulline (Modified) Detection Kit (Cat# 17-347B, MilliporeSigma) protocol to identify citrullinated proteins and reprobed the membranes with anti-AP or anti-Fgn antibodies to quantify the total AP or Fgn, respectively. However, we were unable to detect CitAP or CitFgn in the samples purified from the plasma of patients with COVID-19 or controls (Figure 2C, D, upper panels). To assess if patients with COVID-19 have elevated total citrullinated plasma proteins, we also analyzed unfractionated plasma samples for citrullinated proteins as described above. Since the secondary antibody in the Anti-Citrulline (Modified) Detection Kit (Cat# 17-347B, MilliporeSigma) binds to human immunoglobulins, the detected proteins at ~55 and ~30 kDa are likely endogenous IgG heavy and light chains, respectively (Figure 2E). However, when quantifying either total protein in the lane or just the bands above and below these bands, there was no difference in citrullinated proteins between controls and patients with COVID-19 (data not shown). We also depleted immunoglobulins from the samples using protein A/G columns to overcome antibody interference, but there was still no difference in citrullinated proteins between controls and patients with COVID-19 (Figure 2F).

It is possible that plasma protein citrullination occurs in COVID-19, but these proteins are consumed faster [25] or incorporated into the fibrin network [26]. However, our *in vitro* experiments show normal plasmin generation even when up to 80% of available AP is citrullinated. Thus, the inability to detect even small amounts of citrullinated proteins in these plasma samples with abnormal plasmin generation and fibrin formation (Bouck et al. [6] and Figure 1) suggests that the abnormal function cannot be explained by the presence of CitAP or CitFgn. It is also possible that a minor fraction of CitAP with citrullination of specific arginine residues may have reduced activity but is difficult to detect with immunoblotting; targeted mass spectrometry may be required to detect extremely low-level modifications. Interestingly, Ordonez et al. [12] showed that although patients with rheumatoid arthritis have increased levels of citrullinated antithrombin, the citrullination impacted only an insignificant portion of antithrombin, and there was no association between the citrullinated antithrombin and the risk of thrombosis.

Although neutrophil-derived PAD4 citrullinates histones and is essential for NETosis, PAD4 may not have a major role in citrullinating circulating proteins [27]. In particular, protein citrullination depends not only on exposure to PAD enzymes but also on the local environment (eg, high calcium), which may not be achieved on a scale or for sufficient time to citrullinate substantive amounts of circulating proteins [28]. Collectively, our data show that despite extensive neutrophil activation and NETosis, Fgn, and AP are not widely citrullinated in plasma from patients with COVID-19, suggesting this modification is unlikely to contribute to abnormal fibrin formation or plasmin generation in COVID-19.

ACKNOWLEDGMENTS

The authors thank Dr Amritangshu Chakravarty for technical assistance.

FUNDING

This study was supported by funding from the National Institutes of Health (HL160046, HL163019, and HL126974).

INFORMED PATIENT CONSENT

Written informed consent in accordance with the Declaration of Helsinki was obtained from each donor. The study protocols were approved by the University of Utah (IRB00051506 and IRB00102638).

AUTHOR CONTRIBUTIONS

K.K. performed experiments, interpreted the data, and wrote the manuscript. R.A.C. provided samples and analyzed data. R.T. contributed to the study design. M.J.F. and A.S.W. contributed to the study design and data interpretation. All authors reviewed and edited the manuscript and approved the final version.

RELATIONSHIP DISCLOSURE

The authors have no competing financial interests to declare.

Kadri Kangro[1]
Robert A. Campbell[2]
Ronak Tilvawala[3]
Matthew J. Flick[1]
Alisa S. Wolberg[1]

[1]*Department of Pathology and Laboratory Medicine, UNC Blood Research Center, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA*
[2]*Department of Internal Medicine, University of Utah, Salt Lake City, Utah, USA*
[3]*Department of Molecular Biosciences, University of Kansas, Lawrence, Kansas, USA*

Handling Editor: Dr Henri Spronk

Correspondence
Alisa S. Wolberg, Department of Pathology and Laboratory Medicine, University of North Carolina at Chapel Hill, 8018A Mary Ellen Jones Building, CB #7035, Chapel Hill, NC 27599-7035, USA.
Email: alisa_wolberg@med.unc.edu

TWITTER

Robert A. Campbell @racampbell04
Matthew J. Flick @fib390_396A
Alisa S. Wolberg @aswolberg

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