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BRIEF REPORT

A novel factor IXa–specific enzyme-linked immunosorbent assay detects factor IXa in human plasma

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Abstract

Background: Factor (F)IXa activity has been detected in human plasma and may impact thrombotic risk. Current FIXa activity assays are complex and cumbersome.

Objectives: To develop a reproducible enzyme-linked immunosorbent assay (ELISA) using a novel monoclonal antibody that detects total FIXa in human plasma.

Methods: A monoclonal antibody was raised against the new N-terminus exposed upon activation of FIX to FIXa by cleavage after R226. This antibody is specific for FIXa protease and does not recognize FIX zymogen or FIXα. The antibody was used to develop a FIXa-specific ELISA capable of quantifying total FIXa (free FIXa and FIXa-antithrombin complex) in human plasma. Total FIXa quantified using the ELISA was compared to that of FIXa-antithrombin quantified using modifications of a previously described ELISA.

Results: The FIXa-specific ELISA was reproducible and quantified total FIXa in human plasma. Total FIXa levels correlated with FIXa-antithrombin levels.

Conclusion: A monoclonal antibody was developed that specifically detects human FIXa protease. A FIXa-specific ELISA using the new antibody is capable of reproducibly measuring total FIXa in human plasma (both free FIXa and FIXa-antithrombin). This assay should facilitate the evaluation of total FIXa levels in a variety of clinical circumstances.

KEYWORDS
antithrombin, coagulation, COVID-19, ELISA, factor IXa, human, monoclonal antibody, protease, zymogen

Essentials

- Coagulation factor (F)IXa plasma levels may impact the risk of thrombosis.
- A new monoclonal antibody was developed that specifically detects FIXa.
- An assay to measure total FIXa levels in human plasma was developed using new antibodies.
- The new FIXa assay should be a useful tool for measuring FIXa levels.

1 | INTRODUCTION

Coagulation factor (F)IX is essential for normal hemostasis. It circulates in plasma as an inactive zymogen and is activated to FIXa protease by FVIIa–tissue factor (TF) and FXIa. Plasma FIXa is primarily inhibited by antithrombin (AT) [1]. However, the basal rate of FIXa inhibition by AT is 40-fold slower than for the homologous protease FXa [2,3]. Isolated FIXa is poorly reactive with both substrates and inhibitors [4,5], reflecting the "zymogen-like" structure of the protease [6] and making it uniquely suited to persist in circulation. Consistent with these observations, the plasma half-life of FIXa activity is remarkably prolonged [7].

Elevation of plasma FIX activity is a risk factor for deep vein thrombosis [8–10]. FX activation by FIXa in the intrinsic Xase (FIXa-FVIIIa) complex is the rate-limiting step for thrombin generation [11–13]. Likewise, the *in vivo* thrombogenicity of infused bovine FIXa in a rabbit model is 10- to 100-fold more potent than FXa or thrombin [14]. Elevated levels of FIXa complexed with AT (FIXa-AT) have been demonstrated in patients with atrial fibrillation [15] and COVID-19 [16] but generally remain understudied. Elevated FIXa activity has been demonstrated in premenopausal women using hormonal contraception and may contribute to that hypercoagulable state [17]. Thus, total plasma FIXa may represent an essential biomarker in the activation of coagulation.

Existing FIX activity assays [18,19] do not distinguish between the zymogen and the FIXa protease. FIX activation peptide radioimmunoassays reflect FIX that has been activated [20,21], but the polyclonal antibody reagents are no longer available. FIXa activity can be measured using a modified thrombin generation assay; however, the assay is technically demanding and unsuitable in plasma not specifically collected for that purpose [17]. FIXa-AT can be measured in an enzyme-linked immunosorbent assay (ELISA) [22,23], but the assay does not detect free FIXa. Described here is a novel monoclonal antibody-based ELISA that quantifies total FIXa in human plasma, including both free FIXa and FIXa-AT complex.

2 | METHODS

Recombinant FIXaα and FIXα were prepared as described previously [24]. As described, cleavage of FIX to FIXaα or FIXα is not quantitative [7]. N-terminal HPC4-tagged TF (amino acids 3-244) was expressed in *Escherichia coli*, purified with modifications of previous methods [24,25], and relipidated [26] prior to use. Human FIXaβ, AT, and FXIa were purchased from Prolytix. Recombinant mouse FIX was purchased from Sino Biological (#50362-M08H) and activated to FIXa using FXIa. Activation was verified via sodium dodecyl-sulfate polyacrylamide gel electrophoresis (data not shown). Gly-Pro-Arg-Pro was purchased from EMD Millipore (#03-34-0001).

Patient samples in this study were collected at the beginning of the COVID-19 pandemic (May to December 2020) under an institutional review board-approved protocol at the University of Wisconsin-Madison. The cohort consisted of 16 inpatients positive for COVID-19 with blood collected in citrate for clinical studies; the residual specimens were frozen following collection. The earliest available plasma specimen from each of the patients with COVID-19 was analyzed. The median age of patients with COVID-19 was 70 years, with 69% being men. Clinical variables (Supplementary Table) were determined retrospectively by computer review of electronic medical records.

For healthy controls, we obtained 50 normal plasma samples in citrate from George King Bio-Medical. The median age of controls was 40 years, and 50% were men. Because of the source of these plasmas, analogous data to those in the Supplementary Table for patients with COVID-19 was not available, but presumably, the values were within the normal range. FIX immune-depleted (ID) plasma (Prolytix) and pooled normal plasma (PNP) (George King Bio-Medical) were used as controls.

Murine monoclonal antibodies were raised in collaboration with Green Mountain Antibodies and are available commercially from the company. An antibody specific for activated human FIXa (unreactive with FIX), designated 16E11, was raised against the new N-terminus exposed upon activation of FIX to FIXa by cleavage after R226, using the peptide VVGGEDAKPGQFPWQC. 16E11 does not bind to mouse FIXa (Supplementary Figure S1A). An antibody specific for the N-terminus of FIX/FIXa designated 13B10, was raised against the N-terminal human FIX/FIXa peptide YNSGKL(Gla)(Gla)FVQGNLGGC. 13B10 also binds to mouse FIX (Sino Biological) and mouse FIXa but with lower affinity than for the corresponding human proteins (Supplementary Figure S1B).

Initial antibody specificity was determined via Western blot analysis. BioRad 4% to 20% TGX minigels were run under reducing conditions and transferred to polyvinylidene fluoride membranes. The membranes were blocked with Tris-buffered saline (10 mM Tris, 150 mM NaCl, pH7.4) + 0.05% Tween 20 (TBST) containing 0.25% gelatin, probed with 1 μg/mL monoclonal antibody, and incubated with peroxidase-conjugated donkey anti-mouse immunoglobulin G (IgG) (Jackson ImmunoResearch Laboratories, Inc).

Calcium dependence of antibody binding to FIX/FIXa was determined via ELISA using 96-well plates (Costar, #3590) coated with FIX or FIXa in Tris-buffered saline (TBS) overnight at 4 °C. Plates were washed well with TBST between each step. Coated wells were blocked with 3% bovine serum albumin (BSA) in TBS for 1 hour at room temperature, washed, and incubated with 0.5 μg/mL of either 13B10 or 16E11 in 1% BSA/TBS ± 5 mM $CaCl_2$ for 1 hour at 37 °C. Wells were then washed and incubated with peroxidase-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc) in 1% BSA/TBS ± 5mM $CaCl_2$ for 1 hour at 37 °C. Detection was done using KPL SureBlue peroxidase substrate (SeraCare), followed by quenching with KPL Stop Solution (SeraCare) and measuring absorbance at 450 nm.

The activation of FX by the intrinsic Xase was determined ±25 μg/mL antibody as described previously [27]. The antibody was incubated with FIXa for 2 minutes prior to assay. Monoclonal GMA-001, anti-FIX gla domain inhibitory antibody (Green Mountain Antibodies) [28], was used as a positive control, while mouse IgG (EMD Millipore) was used as a negative control.

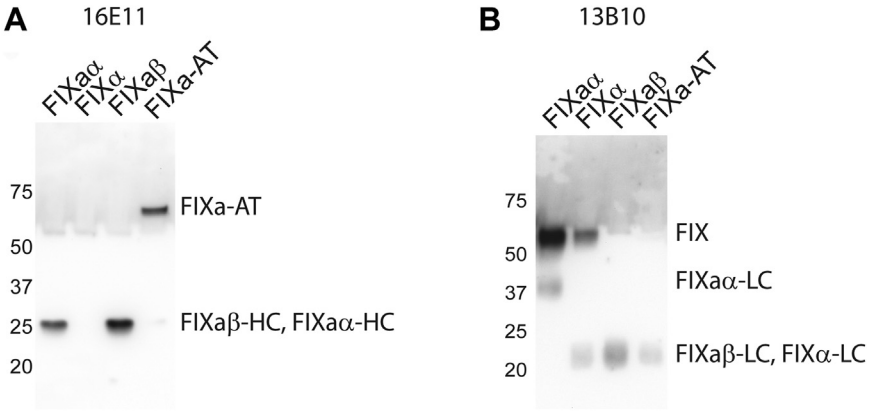

FIGURE 1 Western blot analysis of purified proteins under reducing conditions. (A) Monoclonal antibody 16E11, raised against a peptide with the sequence of the new N-terminus that is generated when factor (F)IX is cleaved after R226, detected the heavy chains (HC) of FIXaβ and FIXaα and activated FIX (FIXa) complexed to antithrombin (AT) (FIXa-AT), but did not detect FIXα. (B) Monoclonal antibody 13B10, raised against the N-terminus of FIX, detected FIX and the light chains (LC) of FIXaβ, FIXaα, and FIXα. Cleavage of FIX to FIXaα or FIXα was not quantitative; thus, the parental FIX remained in these preparations [7]. Sodium dodecyl-sulfate polyacrylamide gel electrophoresis and Western blotting were performed as described in Methods.

A FIXa-specific ELISA that detects free FIXa protease and FIXa-AT equally but does not detect FIX was constructed using 13B10 as the capture antibody and biotinylated-16E11 as the detection antibody. 16E11 was biotinylated using a Lightning Link Rapid Biotin Kit (Abcam, #ab201795) and run over a Zeba Dye and Biotin binding column (Thermo Fisher Scientific, #A44300). The wells of a 96-well plate (Costar, #3590) were coated with 100 µL of 5 µg/mL 13B10 in TBS overnight at 4 °C. Wells were washed well between each step with TBST. Coated wells were blocked with 3% BSA/TBS for 1 hour at room temperature, then washed and incubated with either human FIXa standards (Prolytix) or sample plasma diluted in Plasma Sample Diluent (Immunochemistry Technologies, #696) and incubated for 1 hour at 37 °C. Next, wells were washed and incubated with biotinylated-16E11 in 1% BSA/TBS for 1 hour at 37 °C and then washed and incubated with Pierce Streptavidin Poly-HRP (Thermo Fisher Scientific, #21140) in 1% BSA/TBS for 30 minutes at 37 °C. Finally, FIXa was detected by incubation with KPL SureBlue peroxidase substrate, followed by quenching with KPL Stop Solution and measuring absorbance at 450 nm. The limit of detection was 16 pM FIXa. Plasma quantitation was done by fitting the FIXa standard curve to a 5-parameter logistics model, interpolating unknowns, and accounting for dilutions.

FIXa-AT levels were measured with modifications of the previously described ELISA [22,23]. Our capture antibody was 13B10, while our detection antibody was biotinylated-SαhATIII (PAHAT-S, Prolytix) labeled with biotin using a Lightning Link Rapid Biotin Kit (Abcam #ab201795), as described above.

Activation of endogenous plasma FIX was measured using the FIXa ELISA or FIXa-AT ELISA. PNP or FIX ID citrated plasma was recalcified by addition of 12.5 mM $CaCl_2$ and activated with either 5 nM TF or 1.8 nM FXIa at 37 °C in the presence of 10 mM Gly-Pro-Arg-Pro (peptide inhibits fibrin polymerization). Aliquots were removed at the indicated time points, quenched with EDTA, and assayed. Heparin (0.5 mg/mL) was added to EDTA-quenched samples when indicated.

2.1 | Statistical analysis

Statistical analysis was performed using GraphPad Prism 10.0. *P* values were determined using Mann–Whitney U-test. The association between FIXa and FIXa-AT was summarized using Spearman's rank correlation coefficient (r_s) and a linear trend fit using simple linear regression.

3 | RESULTS AND DISCUSSION

Sodium dodecyl-sulfate polyacrylamide gel electrophoresis and Western blotting demonstrate that 16E11 detected the 28 kD heavy chain of FIXaβ and FIXaα, but not the 39 kDa heavy chain of FIXα; it also detected FIXa complexed with AT (Figure 1), confirming specificity for FIXa cleaved after R226 [29]. 13B10 detected intact FIX, the 18 kD light chain of FIXaβ and FIXα, and the 29 kDa light chain of FIXaα, confirming specificity for the N-terminus of the molecule. The calcium dependence of the antibodies was examined in an ELISA with FIX and FIXa coating the plate as described in Methods. The binding affinity of 16E11 to FIXa was unaffected by the presence of added calcium (Figure 2A). 13B10 binding affinity for FIX and FIXa was highest in the absence of calcium, with dramatically decreased binding observed in the presence of 5 mM $CaCl_2$ (Figure 2B). Neither 13B10 nor 16E11 inhibited FIXa activity in the intrinsic Xase assay up to a concentration of 25 µg/mL (data not shown). Under the same conditions, the inhibitory monoclonal antibody, GMA-001, inhibited 95% of the intrinsic Xase activity.

A sandwich ELISA to quantify plasma FIXa was developed using the new antibodies. 13B10 was used as the capture antibody, while biotinylated 16E11 was the detection antibody. Titration curves from the FIXa ELISA illustrate the specificity of the assay for FIXa. In a purified system, free FIXaβ and FIXa-AT were detected equally, while FIX was not detected (Figure 3A). The ELISA also detected FIXaα but not FIXα (Figure 3B). The process for generating FIXaα from FIX yields

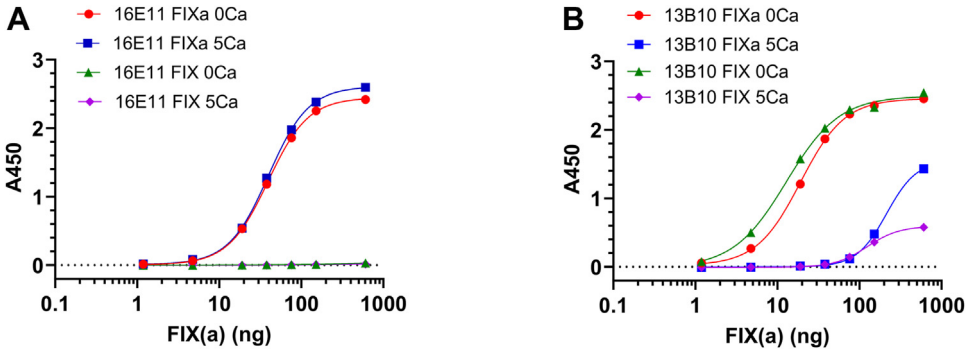

FIGURE 2 Calcium dependence of monoclonal antibodies 16E11 and 13B10. (A) Monoclonal 16E11 bound factor (F)IXa equally ± 5mM $CaCl_2$ but did not bind FIX. (B) Monoclonal 13B10 bound both FIXa and FIX optimally in the absence of $CaCl_2$. Addition of 5 mM $CaCl_2$ dramatically decreased binding. Binding was determined as described in Methods. The graphs are representative curves.

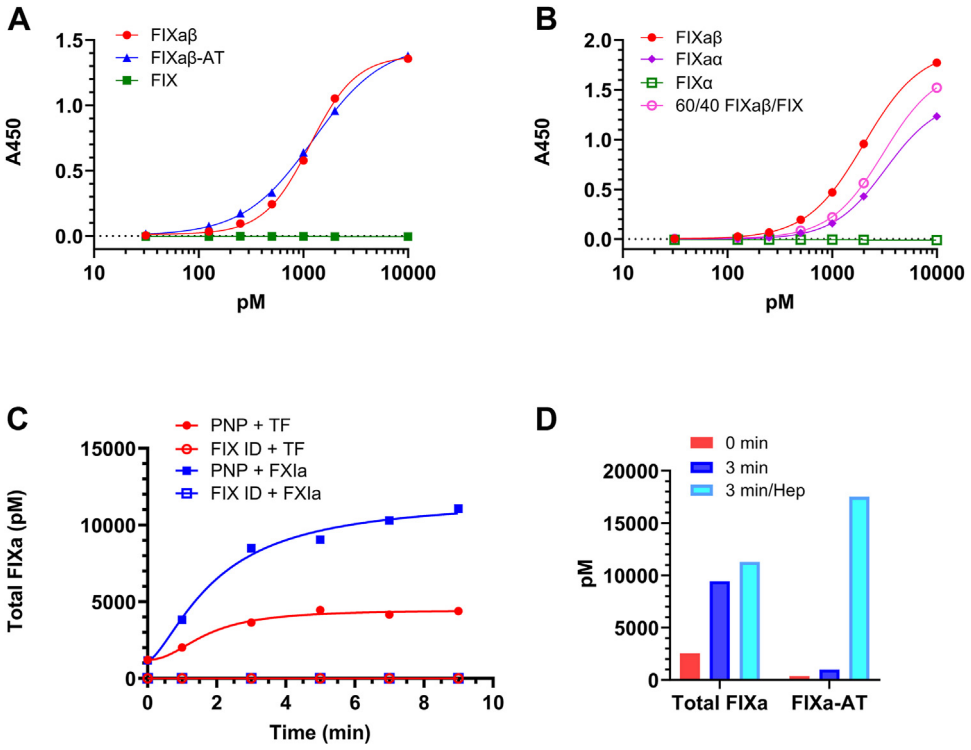

FIGURE 3 Factor (F)IXa enzyme-linked immunosorbent assay (ELISA) titrations and plasma activation. The specificity of the FIXa ELISA for various FIX forms was examined using purified proteins. (A) The FIXa-specific ELISA detected both free FIXaβ and FIXaβ-antithrombin (AT) complexes but not FIX. (B) The FIXa-specific ELISA detected FIXaβ and FIXaα, but not FIXα. As explained in the Results, the FIXaα preparation contained approximately 40% unactivated FIX (by Western blot analysis), so a 60% FIXaβ/40% FIX mixture was included to reflect how an approximately equivalent number of FIXa heavy chains within the FIXaα population would react in the assay. (C) The FIXa-specific ELISA detected the time-dependent generation of FIXa in human plasma. Citrated pooled normal plasma (PNP) or FIX immune-depleted (ID) plasma was recalcified and activated with either tissue factor (TF) or FXIa as described in Methods. The FIXa ELISA was used to measure the increase in plasma FIXa in activated PNP over time. FIX ID plasma did not have measurable FIXa, even after activation. (D) Addition of heparin (Hep) resulted in an increase in FIXa-AT. The FIXa-specific ELISA or the FIXa-AT ELISA was used to measure the increase in plasma FIXa in PNP after activation with FXIa for 3 minutes at 37 °C. Hep was added after quenching, as indicated (light blue). The graphs are representative curves.

a preparation containing 60% FIXaα and 40% unactivated FIX by Western blot analysis [24], so a mixture of 60% FIXaβ/40% FIX was used to approximate the quantity of catalytically competent FIXaα in the preparation (Figure 3B). This report refers to FIXa as the catalytically active forms of FIX (FIXaβ and FIXaα), recognizing that FIXaβ is the predominant form in normal plasma [30,31]. The intra-assay coefficient of variation obtained in 5 separate assays of PNP was 2.2%. The interassay coefficient of variation in 11 separate assays was 20%. FIX ID plasma gave no detectable signal in the assay, as expected. Recovery of FIXa added into FIX ID plasma was excellent, with

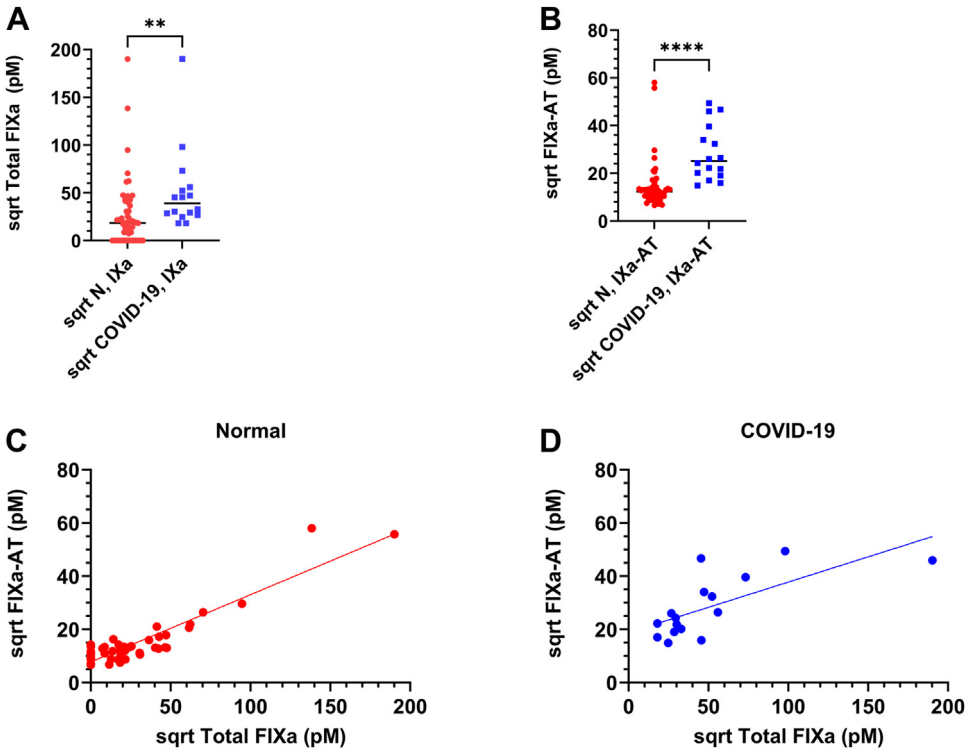

FIGURE 4 Factor (F)IXa vs FIXa-antithrombin (AT) in normal and COVID-19 plasmas. (A) Total FIXa was determined in plasma from normal, healthy patients and patients with COVID-19, as described in the Methods. The square root (sqrt) of total FIXa was compared in normal vs COVID-19 plasma. (Normal FIXa was significantly different from COVID-19 FIXa, $^{**}P = .0013$.) (B) FIXa-AT was determined in plasma from normal, healthy patients and patients with COVID-19, as described in the Methods. The sqrt of FIXa-AT was compared in normal vs COVID-19 plasma. (Normal FIXa was significantly different from COVID-19 FIXa, $^{****}P < .0001$.) Correlations between individual total FIXa and FIXa-AT levels were determined using GraphPad Prism. (C) Normal plasma ($N = 50$), $r_s = 0.6566$, $P < .0001$. (D) COVID-19 plasma ($N = 16$), $r_s = 0.7206$, $P = .0023$.

an average recovery of 99% ($N = 10$). The FIXa-specific ELISA was able to detect a time-dependent increase in plasma FIXa due to activation of endogenous FIX by TF or FXIa in PNP (Figure 3C). No FIXa was observed in FIX ID plasma ± TF or FXIa.

AT is the primary plasma inhibitor of FIXa and activated FIXa in plasma complexes rapidly and more quantitatively with AT in the presence of heparin than in its absence [1,32]. Figure 3D illustrates a dramatic increase in FIXa-AT complexes when heparin is added to plasma following 3 minutes of activation with FXIa (light blue bar, right side of graph) compared to the same reaction without the addition of heparin (dark blue bar, right side of graph). In contrast, there was little difference in total FIXa when heparin was added following activation (left side of the graph), indicating that free FIXa is detected by this assay and that the addition of heparin drives free FIXa into a complex with AT, as expected [32].

The FIXa-specific ELISA was used to determine total FIXa in normal, healthy, adult plasma and adult COVID-19 patient plasma, which would be expected to have elevated FIXa levels [16]. FIXa was measurable in plasma from 72% of the healthy adults using this FIXa-specific ELISA, with a median level of 342 pM (IQR, 0-1554) ($N = 50$). In contrast to the healthy adults, 100% of the patients with COVID-19 had measurable FIXa in their plasma, with a median level of 1570 pM (IQR, 792-2831) ($N = 16$). The FIXa-AT ELISA was used to determine the FIXa-AT level in the same plasma samples. The median level of FIXa-AT in healthy adults was 154 pM (IQR, 109-198) ($N = 50$), while it was 634 pM (IQR, 395-1262) in patients with COVID-19 ($N = 16$) (Figure 4B). Thus, patients with COVID-19 demonstrated an approximately 4-fold elevation in total FIXa ($P = .0013$) and FIXa-AT levels ($P < .0001$) compared to healthy controls. Total FIXa correlated with FIXa-AT in normal, healthy, plasma ($r_s = 0.6566$; $P < .0001$) and COVID-19 plasma ($r_s = 0.7206$; $P = .0023$) (Figure 4C, D). The use of square roots yielded a more optimum normal distribution of data points.

FIXa is slowly inhibited by AT (relative to other proteases) [2,3] and demonstrates a prolonged plasma half-life [7], suggesting that total FIXa levels, rather than FIXa-AT levels, might be an important measure in certain situations. For example, acquired AT deficiency can occur in cases of increased consumption due to extreme inflammation, such as in COVID-19 [33–35]. The power of our novel FIXa-specific ELISA is that it measures free FIXa as well as FIXa-AT to yield total FIXa, making it an ideal tool to study FIXa levels in health and disease.

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ETHICS STATEMENT

Healthy individuals and patients provided informed consent. The study's protocols were approved by the Institutional Review Board of the University of Wisconsin-Madison (University of Wisconsin-Madison BioBank Institutional Review Board 2016-0934). The University of Wisconsin-Madison BioBank provided the patient samples, and the Clinical Research Data Service searched the database for the patient information.

AUTHOR CONTRIBUTIONS

T.M.M., B.S.S., K.T.K., and J.P.S. contributed to conception and design of the study. T.M.M. performed the experiments. T.M.M. and M.R.L. analyzed the data. All authors wrote the manuscript.

RELATIONSHIP DISCLOSURE

B.S.S. is employee of Morgridge Institute for Research. M.R.L. serves on a protocol review committee for cancer-related studies and reports the following grants or contracts: American College of Radiology—algorithms for accurate adaptive audit methods (July 1, 2020, to June 30, 2022); National Institutes of Health (NIH)—biochemical and molecular newborn screening for familial hypercholesterolemia (May 1, 2021, to April 30, 2023); NIH—Institute for Clinical and Translational Research–Biostatistics (commenced on September 21, 2017; ongoing) provides statistical support to clinicians and investigators; and NIH/National Cancer Institute—UW Comprehensive Cancer Center Support (commenced on January 1, 2016; ongoing) provides statistical support for cancer researchers. J.P.S. reports funding from National Institutes of Health National Heart, Lung, and Blood Institute (R01 HL149855 as PI for In Vivo Regulation of Factor IXa by Protein S in Hemophilia and Systemic Hypercoagulability; R01HL149364 as co-PI for TFPI, Protein S, FXIIa, and Plasma FIXa in Hormone-Induced Hypercoagulability; and R01 HL152066 as coinvestigator for Comprehensive Characterization of Missense Mutants in Factor IX); grant or contract from Bayer AG; payment for expert testimony from Genentech/Paul, Weiss, Rifkind, and Wharton & Garrison LLP; and support for attending the 2023 International Society on Thrombosis and Haemostasis Congress from University of Wisconsin Comprehensive Bleeding Disorders Program. T.M.M. is an employee of Morgridge Institute for Research and reports support for attending meetings and/or travel from Morgridge Institute for Research. K.T.K. has no competing interests to disclose.

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SUPPLEMENTARY MATERIAL

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