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Protein C Pathway, Inflammation, and Pump Thrombosis in Patients With Left Ventricular Assist Devices

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Abstract

Use of left ventricular assist devices (LVADs) for management of advanced heart failure is becoming increasingly common; however, device associated thrombosis remains an important cause of mortality in this patient population. We hypothesize that inflammation in LVAD implanted patients dysregulates the protein C pathway, creating a hypercoagulable state leading to thrombosis. Plasma samples from 22 patients implanted with the Thoratec HeartMate II LVAD were analyzed by commercial ELISAs. Retrospective sample selection included those collected 1-3 months prior to and within 1 month after a thrombotic or bleeding event. Unrelated to warfarin dosing, total protein S and free protein S ($p = 0.033$) levels were 20% lower in patients with LVAD-thrombosis than in patients with LVAD-bleeding. Levels of protein C, soluble endothelial cell protein C receptor, and soluble thrombomodulin were similar in both groups before and after the event. Compared to normal, C-reactive protein levels were 25-fold elevated in LVAD-thrombosis patients but only 9-fold elevated in LVAD-bleeding patients. This study suggests that protein S, influenced by the inflammatory state, is a gatekeeper for the function of protein C in patients with LVAD-associated thrombosis.

Keywords

heart failure, thrombosis, protein C

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Background

Heart failure is a serious health concern that continues to increase in prevalence. Currently in the United States, a 40-year-old adult has a 1 in 5 lifetime risk of developing heart failure.[1] American Heart Association Heart Disease and Stroke Statistics from 2018 demonstrate that approximately 1 in 9 Americans will die with heart failure and that heart failure is the number one reason for hospitalization, costing the health-care system more than 30 billion dollars annually.[1,2] While there are pharmacological therapies that can help patients with milder forms of heart failure, patients with advanced heart failure require more intensive therapies such as implantation of a left ventricular assist device (LVAD) or heart transplant. Since transplantable hearts are limited in availability and not all patients are good candidates for heart transplant, VADs are an important option to enhance the quality of life and increase the lifespan of patients with end stage heart failure.[3] The number of patients receiving left ventricular assist devices increased from 98 in 2006 to 2423 in 2014 with steady improvements in in-hospital mortality rate, hospital length of stay, and survival.[2]

Although there has been considerable progress in LVAD technology since it first was implemented in 1966 by Dr. Michael E. DeBakey,[4] device associated complications including infection, pump thrombosis, and gastrointestinal bleeding remain common causes of morbidity and mortality.[5-7] Bleeding is the most common adverse event after LVAD implantation due to arteriovenous malformations, hepatic dysfunction, and other causes. This bleeding risk is complicated by the required antiplatelet and anticoagulant therapies.[8]

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Hemodynamic alterations following LVAD implantation can be a trigger for blood clot formation via platelet activation and enhanced release of von Willebrand factor.[9] Activation of either the extrinsic (tissue factor expression with vascular injury) or intrinsic (foreign surface) pathways of the coagulation system leads to thrombin generation, fibrin formation, and stabilization of a platelet plug. In LVAD patients, alteration of these hemostatic pathways leads to overproduction of thrombin and subsequent arterial and venous thrombosis, arteriovenous malformations, neurologic events, and pump thrombosis.[10]

Antithrombin and protein C are 2 important physiologic control mechanisms that regulate thrombin generation. Protein C binds to endothelial protein C receptor (EPCR). When thrombin is generated, the thrombin-thrombomodulin complex converts protein C to activated protein C (APC). With its cofactor protein S, APC inhibits the activated forms of factors V and VIII, effectively decreasing thrombin generation by the coagulation system.[11] Protein S also exhibits a direct anticoagulant effect by inhibiting the factor Va-Xa complex, reducing the generation of thrombin.[12] Deficiency or dysfunction of protein C or protein S are directly correlated to the risk of developing a thrombotic event.[13]

Increased interest in the protein C pathway for LVAD patient quality of care stems from the relationship between protein S and inflammation. Protein S circulates either free or bound to complement C4b binding protein (C4bBP); only free (unbound) protein S is functionally active.[14,15] C4bBP is an acute phase reactant that is upregulated with inflammation, and patients with implanted VADs are in a heightened state of inflammation.[16]

We hypothesize that in LVAD patients with thrombosis, C4BP levels are upregulated, causing a decrease in the active free form of protein S, thereby decreasing the efficacy of the protein C pathway to control thrombin generation and clot formation. To test the hypothesis that LVAD-associated thrombosis can be linked to a dysregulation of the protein C pathway, we evaluated the components of the protein C pathway in patients with implanted LVADs, correlating the findings to clinical outcomes.

Methods

Patients

This study was approved by the Institutional Review Board of Loyola University Chicago, Health Sciences Division, Maywood, IL. All patients and healthy volunteers provided written informed consent prior to participation in the study.

Twenty-two (22) patients (16 males, 6 females) who were implanted with the HeartMate II continuous flow LVAD (Thoratec Corp., Pleasanton, CA) were recruited over a 1-year period from one medical center. All patients were invited to enroll. All patients received warfarin targeted to an INR of 2.0 to 3.0 and 325 mg aspirin daily per institution protocol.

Blood samples were collected during routine post-operative clinic visits following implantation until transplant, withdrawal from the study, explantation of the LVAD device, or death. Plasma samples were stored frozen (-80° C) until analysis.

Clinical information including INR values, laboratory parameters, hospitalizations, procedures (including pump exchange), and adverse clinical events were collected from the electronic medical record, an internal REDCap database, and the Interagency Registry for Mechanically Assisted Circulatory Support (INTERMACS) registry.

For the analysis reported herein, stored samples were selected from the enrolled patients who had an LVAD-related event. LVAD-associated thrombosis ($n = 18$ events) was defined according to INTERMACS criteria as a cerebrovascular accident (stroke or transient ischemic attack diagnosed by a neurologist), lactate dehydrogenase (LDH) or plasma free hemoglobin 2X above the upper limit of normal, or pump thrombosis. Pump thrombosis was diagnosed based on changes in LVAD parameters consistent with clot formation in the LVAD inflow or outflow cannula, clot seen on imaging, or surgical pump exchange due to pump thrombosis. LVAD-associated bleeding ($n = 8$ events) was defined according to INTERMACS criteria as an acute anemia (decrease in hemoglobin $\geq$2 g/dL) or gastrointestinal bleeding (GIB) events, which were defined by endoscopic evidence of active or recent bleeding (visualization of a source of bleeding or the presence of blood in the GI tract), hemoccult positive stool, or hemolysis.

Some patients experienced multiple LVAD associated adverse events during the study period (for example, both a bleeding and thrombosis event, multiple thrombosis events, or multiple bleeding events). In these cases, study samples were taken at the time of each event and analyzed individually.

Study Controls

For a control group, a single citrated blood sample was collected from healthy individuals ($n = 20$). Samples were processed in the same manner as patient samples. Results are represented as the group mean value labeled as normal human plasma (NHP).

Analysis

From stored samples, patient plasma samples were selected as those nearest the time following the thrombotic or bleeding event (3 weeks on average; range 3 days to 5 weeks), as well as a sample taken 1-3 months prior to the event on the same patient.

All samples were analyzed with commercially available ELISA kits for total protein S (Diagnostica Stago, Parsippany, NJ), free protein S (Stago), protein C (Stago), soluble thrombomodulin (sTM; R&D Systems, Minneapolis, MN), soluble endothelial cell protein C receptor (sEPCR; Stago), and C-reactive protein (CRP; eBioscience, San Diego, CA). Results are given as % of normal or gravimetric quantity according to the calibrators in each assay kit.

Table 1. Protein C Pathway Components in Patients With an Implanted LVAD Before and After LVAD-Associated Thrombotic and Bleeding Events.

	Pre-Bleeding	Bleeding	Pre-Thrombosis	Thrombosis
CRP (mg/L)	8.0*	9.9	27.5*	25.4
Total S (%)	47.5	55.6	40.0	34.3
Free S (%)	50.3	47.3	42.9**	34.7**
Protein C (%)	51.5	56.8	57.5	50.5
sEPCR (ng/mL)	444	496	513	437
sTM (ng/mL)	3.3	3.3	3.5	2.9

Results are given as median levels; n = 18 thrombotic events; n = 8 bleeding events.
*$p = 0.003$.
**$p = 0.033$.

Statistical Analysis

Normally distributed data was compared using the t-test (SigmaPlot; Chicago, IL), and data that was not normally distributed was compared using the rank sum test (SigmaPlot). Comparison of patient values to normal controls was determined using an unpaired t-test (GraphPad Prism; LaJolla, CA). For patients with multiple events, each event was treated individually.

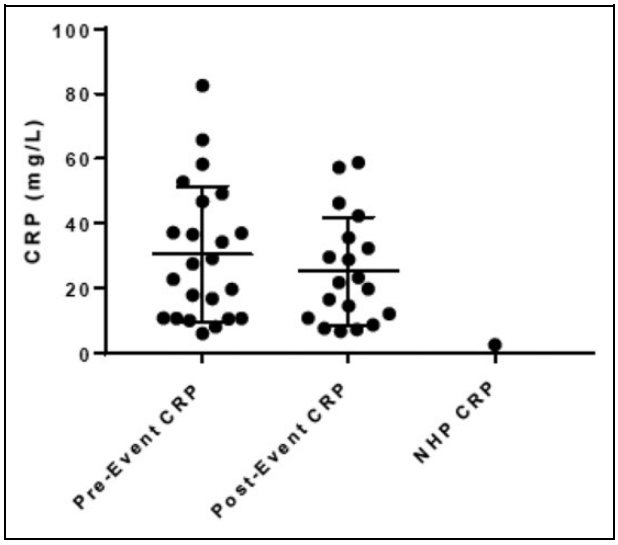

Figure 1. C-reactive protein (CRP) levels in LVAD implanted patients before and after LVAD associated thrombotic events. Elevated CRP levels in this patient population before the thrombotic event (mean 32.7 mg/L *vs* 2.5 mg/L in normal healthy individuals [NHP], $p < 0.0001$) did not alter following a thrombotic event. The mean CRP level in LVAD patients before and after an LVAD associated bleeding event was approximately 9 mg/L.

Results

The characteristics of the protein C pathway parameters in the LVAD implanted patients of this study are summarized in Table 1. Comparing the pre-thrombosis group to both pre-bleeding and bleeding groups (*i.e.*, patients at risk of thrombosis compared to all non-thrombotic LVAD patients), the group that developed thrombosis was characterized as having a higher CRP level ($p = 0.003$) and lower total protein S and free protein S levels. Among patients that developed thrombotic complications, protein C, protein S, and sEPCR were lower in the days following the thrombotic complication when compared to pre-thrombosis levels in the same patient. The decrease in free protein S level from pre-thrombosis to time of thrombosis was significant ($p = 0.033$).

Patients who experienced LVAD-associated thrombosis exhibited high CRP levels, a biomarker of inflammation. Figure 1 details the CRP levels for the LVAD patients before and at the time of a thrombotic event. All patients with LVAD-associated thrombosis had higher than normal CRP (>5.0 mg/L; $p \leq 0.0001$). Patients in the thrombosis group had approximately 3-fold higher CRP levels than patients in the bleeding group (median CRP level 27.5 mg/L compared to 8.0 mg/L; $p = 0.003$). CRP levels did not alter comparing before thrombosis to time of thrombosis (interquartile range (IR) pre: 10.7–46.8 mg/L *vs* IR post: 10.8–35.6 mg/L). Conversely, CRP levels in patients either before or after an LVAD-bleeding event rarely had values higher than 10.0 mg/L; however, compared to healthy controls the LVAD-bleeding group had elevated CRP levels ($p = 0.001$).

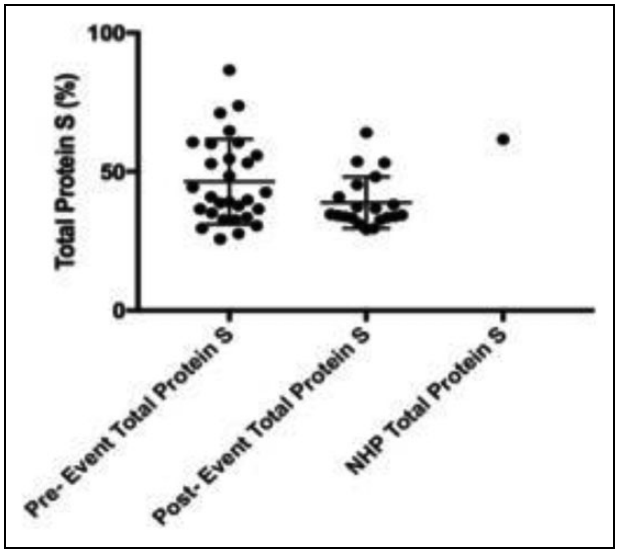

Figure 2. Total protein S levels in LVAD implanted patients before and after LVAD associated thrombotic events. Total protein S levels were lower than normal in this patient population before the thrombotic event (mean 48.5% *vs* 70.4% in normal healthy individuals [NHP], $p < 0.05$) and slightly decreased following a thrombotic event to 40.3%.

Components of the protein C pathway measured in patients who developed LVAD-associated thrombotic complications are detailed in Figures 2–6. The healthy normal mean value is included as a reference. The mean levels pre-LVAD-thrombosis for total protein S (Figure 2), free protein S (Figure 3), protein C (Figure 4), and sEPCR (Figure 5) were lower

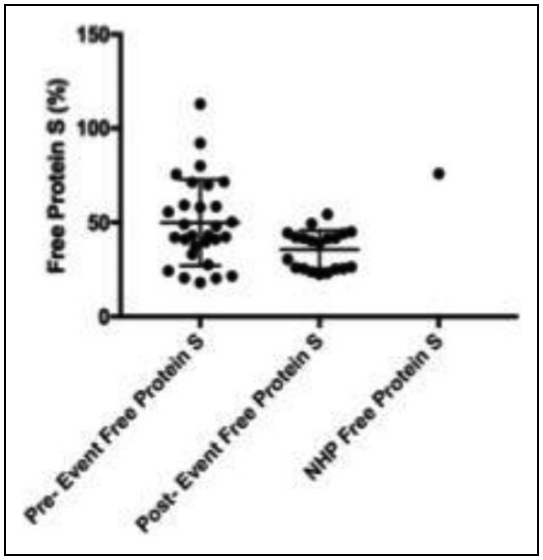

Figure 3. Free protein S levels in LVAD implanted patients before and after LVAD associated thrombotic events. Free protein S levels were lower than normal in this patient population before the thrombotic event (mean 50.3% *vs* 82.5% in normal healthy individuals [NHP], $p < 0.05$) and markedly decreased following a thrombotic event to 37.7% ($p = 0.033$).

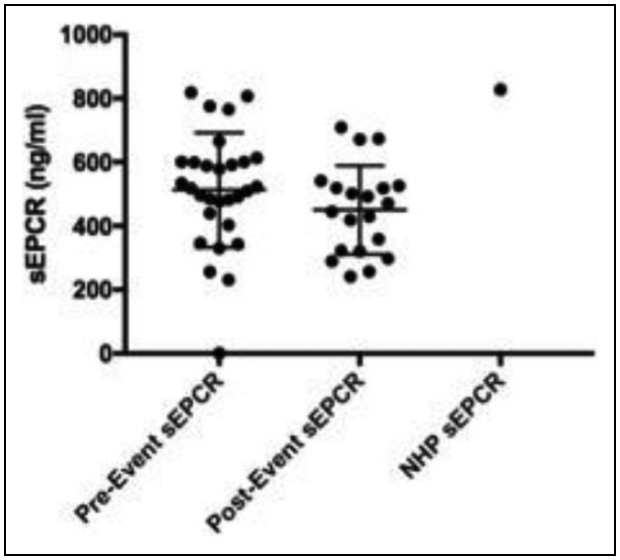

Figure 5. Soluble EPCR levels in in LVAD implanted patients before and after LVAD associated thrombotic events. Soluble EPCR levels were lower than normal in this patient population before the thrombotic event (mean 513 ng/mL *vs* 831 ng/mL in normal healthy individuals [NHP], $p < 0.05$) and slightly decreased following a thrombotic event to 437 ng/mL.

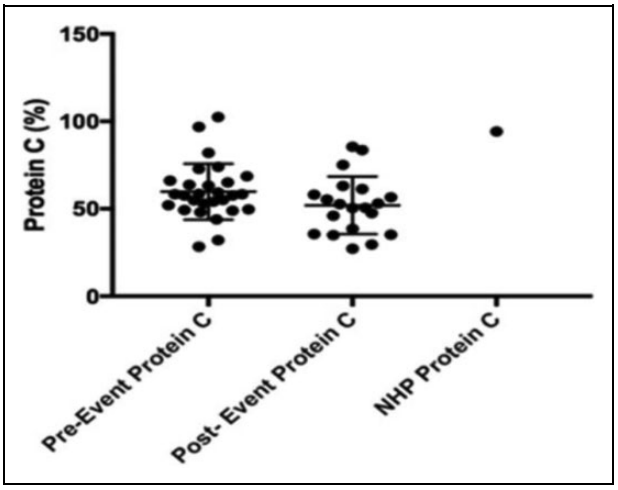

Figure 4. Protein C levels in LVAD implanted patients before and after LVAD associated thrombotic events. Protein C levels were lower than normal in this patient population before the thrombotic event (mean 57.5% *vs* 99.6% in normal healthy individuals [NHP], $p < 0.05$) and slightly decreased following a thrombotic event to 50.5%.

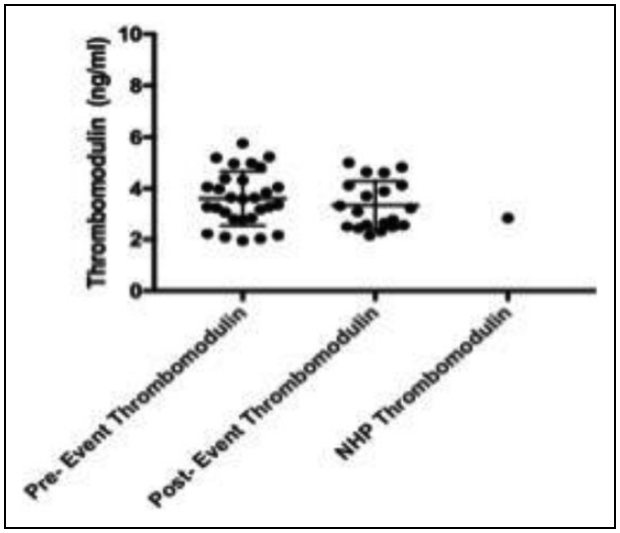

Figure 6. Soluble thrombomodulin levels in LVAD implanted patients before and after LVAD associated thrombotic events. Soluble thrombomodulin levels were at normal levels in this patient population before the thrombotic event (mean 3.5 ng/mL *vs* 3.3 ng/mL in normal healthy individuals [NHP]) and did not alter following a thrombotic event.

than the healthy normal mean value, with the free protein S and total protein S values lower than the protein C value.

Comparing before and at time of thrombosis samples, levels for protein C (IR) pre: 50.0–65.4% *vs* IR post: 37.1–59.7%) and sEPCR (IR pre: 370–600 ng/mL *vs* IR post: 321–533 ng/mL) decreased but not significantly. Free protein S levels were <25% of normal in one-third of the patients who developed thrombosis. In contrast, only one patient with a bleeding episode had a low free protein S level of 20% of normal. Comparing before thrombosis to time of thrombosis, both total protein S (IR pre: 33.9–55.5% *vs* IR post: 33.5–44.5%) and free protein S (IR pre: 37.9-59.0% *vs* IR post: 25.4–44.2%) decreased. Free protein S demonstrated a significant 20% decrease in its level when thrombosis occurred ($p = 0.033$).

The level of soluble thrombomodulin (Figure 6) was normal and did not change in response to thrombotic events (IR pre: 2.8–4.4 ng/mL *vs* IR post: 2.5–4.1 ng/mL).

The therapeutic use of warfarin, which decreases the levels of the vitamin K-dependent protein C and protein S,[17] confounds this investigation. To circumvent this issue, data for the pre-event groups was evaluated in terms of warfarin use. In the pre-thrombosis group, the individual patient results revealed a high frequency of patients at the normal to high normal level for total protein S (38% of pre-thrombosis patients; Figure 2), free protein S (27% of pre-thrombosis patients; Figure 3), and protein C (14% of pre-thrombosis patients; Figure 4). A therapeutic warfarin effect (in a non-LVAD patient) would produce an expected decrease to approximately 20-40% of plasma circulating levels of the vitamin K-dependent proteins.[18] The impact of LVAD support on protein C and protein S has not been described in the contemporary literature. The high plasma levels observed in the LVAD patients of this study, all warfarin-treated per institutional protocol, suggest that the anticoagulation goal of the warfarin therapy may not have been fully achieved in all patients.

INR values were similar at a mean of 2.2 for both the pre-bleeding and the pre-thrombosis samples. However, there was an approximately 15% lower level of free protein S and total protein S in the pre-thrombosis group (40% of normal plasma circulating level) compared to the bleeding groups (50-60% of normal plasma circulating level) which is not fully accounted for by warfarin therapy. Values for protein S were further decreased in the post-thrombosis samples, including the observation of no highly elevated free protein S or total protein S values, coincident with increased dosing of warfarin to a mean INR of 3.3.

Discussion

The protein C pathway produces an endogenous anticoagulant or control function that protects against excess thrombin generation and thrombosis.[11,13] Protein S, an essential component of this pathway, exists both free (functional) and bound to C4b binding protein (C4bBP). C4bBP is upregulated in circulating concentration with enhanced states of inflammation.[14] Protein S, unlike C4bBP, is not an acute phase protein, but it binds with high affinity to available C4bBP. The impact of LVAD support on protein C and protein S has not been described in the contemporary literature.

It has been reported that LVAD implanted patients have a heightened state of inflammation,[16] which has been confirmed in our study. Our study is the first to show, however, that patients with LVAD-associated thrombosis have a higher degree of inflammatory upregulation than the general LVAD population. Patients without an LVAD-associated thrombotic event had CRP levels at the high end of the reference range, but the majority of patients with LVAD-associated thrombosis had CRP levels 3 to 4 fold higher than other LVAD patients. These CRP levels were well into the diagnostic range for acute disease/tissue damage. The FDA Guidance document states the following for interpretation of CRP levels: healthy is <1 mg/L; normal is 1-5 mg/L; >10 mg/L is diagnostic for disease (tissue damage) with an acute range of 20-500 mg/L.[19]

Knowing that upregulated inflammation causes an increase in the amount of C4bBP and that protein S bound to C4bBP effectively reduces its anticoagulant function,[14,15] we aimed to determine if a dysfunction of the protein C pathway could be an unreported mechanism of LVAD-associated thrombosis. Our study demonstrated that patients with higher CRP were more likely to experience thrombosis, and that these patients had lower total protein S and free protein S levels—a finding that does not appear to be related to warfarin therapy as detailed in the results section. There was no evidence that warfarin therapy was excessive causing a severe deficiency of protein C or protein S. We believe that the mechanism of inflammation causing a reduction of protein S, combined with the underlying reduction in functional protein S from warfarin therapy, creates an environment of hypercoagulability from an excess of thrombin generation not controlled by the protein C pathway. The data from the selected study samples revealed that in about one-third of these LVAD patients another potential reason for thrombosis was being out of the therapeutic range for warfarin.

Our data did not demonstrate an alteration of the ratio in the circulating levels of total *vs* free protein S in association with increased CRP/enhanced inflammation that we anticipated. This may be explained by the specificity of the assays used in the study or that activity of these factors was not evaluated in the immunoassays. It is, however, more likely related to the suggested physiological control of maintaining a near-constant functional level of free protein S.[20] A further level of complexity is yet added when considering that protein S and other components of the protein C pathway are involved in biological functions that are both protein C pathway dependent[12] and independent.[21-23]

The protein C, soluble EPCR, and soluble thrombomodulin levels were the same before and after the thrombotic event, providing more strength to our data interpretation that protein S, associated with an elevated inflammatory state, played a role in the formation of pump thrombus through dysregulation of the protein C pathway.

One final potential confounding issue for this study is that coagulation factors are consumed during the thrombotic process. Studies in patients with active venous thromboembolism (VTE) have demonstrated this phenomenon, but interestingly protein C and protein S do not necessarily follow the same pattern as fibrinogen, for example. Minuk and colleagues demonstrated that protein C and protein S can be normal in many patients during the first 24 hours of symptomatic VTE prior to anticoagulation.[24] As the LVAD-associated thrombosis most likely produces a lighter burden of thrombosis than an active VTE, the consumption of protein C and protein S due to thrombosis in the LVAD patients of our study may not be an applicable confounding problem.

In conclusion, this study provides the first evidence that in patients with an implanted LVAD who experience pump thrombosis there exists an exaggerated inflammatory state leading to low levels of functional protein S. LVAD management protocols include the use of warfarin to inhibit clot formation. While INR is used universally as a marker of

anticoagulation efficacy, it is an imperfect marker of overall hemostatic status as it does not reveal the functional status of protein S. Although this study suggests that the protein C pathway is relevant to the health status of LVAD patients, it was limited by its retrospective nature and small size. If our findings are validated in larger studies, monitoring the inflammatory state, monitoring protein S activity levels, potentially using tests of thrombin generation, and addition of anti-inflammatory therapy, may be used to reinforce tight INR control to obtain successful LVAD patient clinical outcomes.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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