Received: 21 May 2019 | Revised: 11 November 2019 | Accepted: 14 November 2019

DOI: 10.1002/rth2.12291

BRIEF REPORT

Serum amyloid A4 is a procoagulant apolipoprotein that it is elevated in venous thrombosis patients

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Funding information
Support was provided, in part, by a grant RO1-HL133728 from the National Institutes of Health.

Handling Editor: Cihan Ay

Abstract

Background: Serum amyloid A4 (SAA4) is an apolipoprotein that is in the SAA family and it is constitutively translated. Previously, acute-phase SAA1 and SAA2 levels were associated with venous thromboembolism (VTE).

Objective: We investigated the association of plasma SAA4 with VTE and the role of SAA4 in coagulation.

Patients and Methods: The association of SAA4 with VTE in a case-control study of adult VTE subjects (N = 113 each group) and the effects of recombinant SAA4 on plasma blood coagulation assays and prothrombin activation initiated by factor Xa were evaluated.

Results: Plasma SAA4 levels in VTE subjects were higher vs. controls (48.1 vs. 38.4 μg/mL; $P < .001$). Elevated plasma SAA4 level (above the 90th percentile of controls) was associated with increased VTE occurrence (odds ratio, 3.8; 95% confidence interval, 1.8-8.0). This association remained significant after the adjustment for acute-phase SAA level, suggesting that SAA4 associated with VTE is independent of acute-phase SAA. Two isoforms of SAA4, that is, glycosylated and nonglycosylated SAA4 isoforms, were each higher in VTE patients. When recombinant SAA4 was added to plasma, it shortened factor Xa-1-stage clotting times, showing that it enhances clotting in plasma. In reaction mixtures containing purified factors Xa and Va and prothrombin, recombinant SAA4 increased prothrombin activation, showing that it enhances prothrombinase activity.

Conclusion: Elevated plasma constitutive SAA4 levels were linked to VTE in adults, and SAA4 can enhance thrombin generation in plasma. Our data highlight a previously unknown procoagulant activity of SAA4 that appears to be related to risk of venous thrombotic events.

KEYWORDS

factor Xa, prothrombinase, serum amyloid A, serum amyloid A4, venous thrombosis

Fernández and Deguchi are equally contributed to this study.

Essentials

- Plasma SAA4 levels were higher in patients with venous thrombosis.
- Elevated plasma constitutive SAA4 levels were linked to VTE independent of acute-phase SAA.
- Recombinant SAA4 was procoagulant in plasma factor Xa-1-stage clotting assays.
- Recombinant SAA4 promoted thrombin generation in purified prothrombinase assays.

1 | INTRODUCTION

Venous thrombosis and arterial cardiovascular disease were traditionally regarded as separate diseases. However, several studies have shown that patients with venous thromboembolism (VTE) have an increased risk of subsequent arterial disease.[1–6] An association between VTE and risk factors for atherosclerotic vascular diseases is emerging and may help identifying new risk factors for VTE.[7] This is the case of the association between lipoprotein levels and VTE that could be explained by common factors that are a consequence of lipid properties on the hemostasis balance and inflammation.[8] These factors include changes in levels of C-reactive protein and acute-phase serum amyloid A (SAA).[9] Recent studies show that acute-phase SAA can directly bind to fibrin and affect coagulation by promoting amyloid formation in fibrin.[10]

The SAA gene family has 4 members. SAAs can be divided into 2 subgroups, comprising acute-phase SAA1 and SAA2 associated with HDL and inflammation in the first subgroup[11] and SAA4 in the second subgroup. SAA3 is expressed in mice but not in humans. SAA1 and SAA2 are acute-phase apolipoproteins that may rise >100-fold in acute inflammation.[12–14] Constitutive SAA4, where "constitutive" denotes its level is not subject to remarkable alterations,[15,16] is linked to a certain group of HDL particles (HDL_3).[17] More recent studies have identified a minor portion of SAA4 that is in the LDL and VLDL subfractions.[18,19] SAA4 comprises >90% of the total serum SAA proteins[17] in the absence of inflammatory pathologies with a serum concentration of 42 to 86 μg/mL, which is high in comparison with basal acute-phase SAA (SAA1 plus SAA2) levels (average, 1-2 μg/mL).[9,12,13] SAA4 expression is implicated during inflammation including atherosclerosis; notably, SAA4 lacks expression in normal arteries but is expressed in carotid lesions.[20] Thus, SAA4 is a histologic and serologic biomarker for atheromatous lesions.[21,22] However, the specific physiological role of SAA4 has not been well defined.[12,13]

Previously, we reported that increased levels of acute-phase SAA is associated with the risk of VTE.[9] However, constitutive SAA4, which is present in plasma at much higher concentrations than acute-phase SAA, has not yet been investigated for its association with VTE. The absence of any specific studies of SAA4 related to hemostasis led us to measure SAA4 plasma levels in a cohort of VTE subjects and to assay recombinant purified SAA4 in coagulation assays. This study suggests an association between high levels of plasma SAA4 and increased risk of VTE independent of acute-phase SAA and suggests that the procoagulant properties of SAA4 might be involved in the pathology for development of VTE.

2 | MATERIALS AND METHODS

2.1 | Materials

Human factor Va (FVa), factor Xa (FXa), Gla-domainless (DG)-factor Xa, and biotinylated factor Xa were purchased from Hematologic Technologies Inc (Essex Junction, VT, USA). Prothrombin and chromogenic substrate Pefachrome TH were from Enzyme Research Laboratories (South Bend, IN, USA). Recombinant SAA4 was from Novoprotein (Summit, NJ, USA). Normal human pooled plasma for clotting assays and immunoblots was purchased from George King Bio-Medical Inc (Overland Park, KS,USA). All other chemicals and reagents used were of the highest purity available.

2.2 | VTE patient and control groups

The Scripps Venous Thrombosis Registry is a case-control study of risk factors for VTE.

Plasma samples were drawn from 113 VTE patients and 113 age- and sex-matched subjects.[8,23] Patients with objectively documented deep venous thrombosis with or without pulmonary embolism were recruited from the Scripps Anticoagulation Service and the community. Inclusion criteria for this study included age at thrombosis <55 years, >3 months since diagnosis of acute thrombosis, a life expectancy of at least 3 years, and no lipid-lowering medications or cancer. Age-matched (± 2 years) healthy controls were recruited through the General Clinical Research Center's normal blood donation service. Donor controls were from the community, but most were employees or former employees of Scripps. The protocol was approved by the Institutional Review Board, and subjects provided written informed consent. Participants in the blood donation service had normal complete blood count and negative HIV and hepatitis B and C testing. Clinical characteristics and the frequency of identified risk factors are shown in Table 1.

2.3 | ELISA measurements of total SAA4 in plasma

ELISA measurements of total SAA4 were performed using a commercial kit from Aviscera Bioscience Inc (Santa Clara, CA, USA).

TABLE 1 Clinical profile of study groups in VTE populations

	VTE populations		
	Scripps registry		Control vs VTE
	VTE	Control	*P* value
Total, N	113	113	
Race, Caucasian, N (%)	106 (93.8)	105 (92.9)	
Gender, female, N (%)	64 (56.6)	64 (56.6)	
Years at blood sampling from the VTE event, N			
<1 (%)	57 (50.4)	…	
1-3 (%)	27 (23.9)	…	
>3 (%)	29 (25.7)	…	
Pulmonary embolism, N (%)	36 (31.9)	…	
Factor V Leiden, N (%)	26 (23.0)	6 (5.3)	.0002
Prothrombin G20210A, N (%)	9 (8.0)	4 (3.5)	.15
Warfarin use, N (%)	89 (78.8)	0	
Hormone use (female only), N (%)	31 (48.4)	32 (50.0)	.86
Age at blood drawing (SD), years old	45.0 (9.8)	45.2 (9.3)	.98
Age at event, y (SD)		40.1 (10.1)	…
BMI, mean, (SD)	29.3 (6.6)	27.2 (5.5)	.0068
VLDL particles (IQR), nmol/L	45.7 (27.2-63.6)	49.9 (26.9-66.6)	.53
LDL particles, nmol/L (IQR)	909 (719-1136)	1006 (795-1263)	.02
HDL particles, μmol/L (IQR)	26.3 (23.2-29.3)	25.2 (21.4-27.6)	.005
SAA(1 + 2), μg/mL (IQR)	3.7 (6.5-75)	2.1 (3.2-79)	<.0001
CRP, μg/mL (IQR)	2.2 (1.0-5.1)	1.7(0.86-4.4)	.24

Note: The number and percentage, median values for each parameter, and the difference between VTE and control (*P* values) were shown.

Abbreviations: BMI, body mass index; CRP, C reactive protein; HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein; SAA, serum amyloid A; VTE, venous thromboembolism; IQR, Interquartile range; SD, standard deviation; N, number; y, year.

2.4 | Immunoblotting of SAA4 isomers in plasma

Plasma was diluted 1/50 in Tris-buffered saline buffer, and 5 μL of diluted plasma was incubated with 1 μL of 100 mmol/L Iodoacetamide and 10 μL of BioRad SDS Lammeli buffer for 5 minutes at room temperature (nonreducing conditions). The incubation mixture was loaded on an 18% Criterion BioRad Tris/Glycine gel, and the gel was blotted on low-fluorescence polyvinylidene difluoride membranes using a semidry BioRad blotting system. For detection of SAA4 bands an AVIVA rabbit anti-peptide antibody (0.5 μg/mL) was used as primary antibody and goat anti-rabbit IRDdye 800 fluorescence labeled antibodies as the secondary antibody. For quantification of the bands, the Li-Cor software Image Studio version 4.0 (Lincoln, NE, USA) was used. The blots were normalized against an internal control (George King Bio-Medical pooled plasma) for interassay variations. A standard curve from a 2-fold serial dilution series over 12 dilutions of a pooled normal plasma was made to calculate the linear dynamic range of detection for the SAA4 antibody. The SAA4 concentration of each sample was calculated from the intensities of the 2 SAA4 bands at 14 and 19 kDa.

2.5 | Clotting assays

For FXa 1-stage clotting assays, citrated plasma was incubated with FXa (34 nmol/L) and varying concentrations of rat SAA4. Clotting was initiated by adding 30 mM $CaCl_2$.

2.6 | Prothrombin activation assays

Prothrombin activation by FXa/FVa or Gla-domainless-FXa/FVa were assayed in the presence of various concentrations of rat SAA4. Prothrombin (0.76 μmol/L final) activation was also assayed in the absence of FVa and phospholipid by adding purified FXa (0.125 nmol/L final) plus or minus varying concentrations of SAA4 preparations using a 120-minute incubation.

2.7 | Statistical analysis

Statistical analysis, including media and interquartile values, Mann–Whitney test, and 2-tailed Spearman correlation test, were

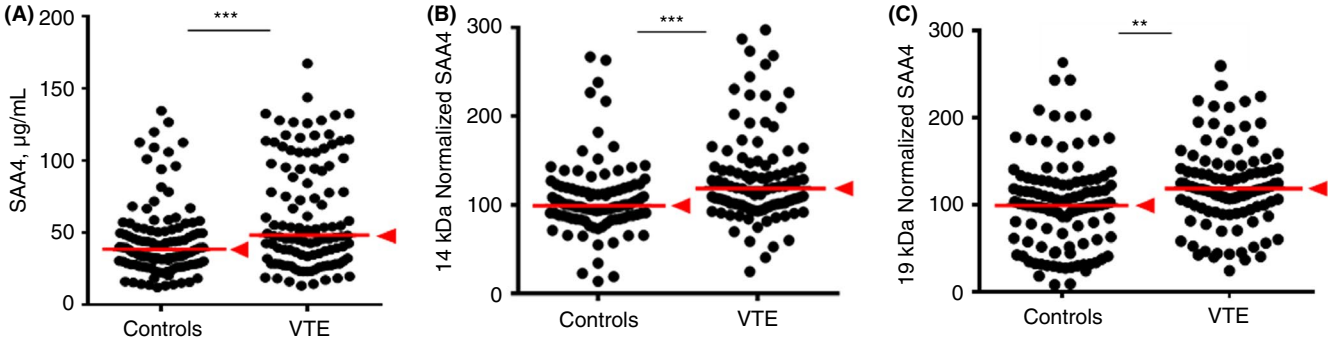

FIGURE 1 Plasma SAA4 antigen levels are higher in VTE patients than controls. The Scripps Venous Thrombosis Registry is a case-control study of risk factors for VTE (113 VTE patients and 113 age-matched controls).[8,23] The plasma samples used for SAA4 measurement were collected between 2002 and 2003 and stored at −80°C as aliquots. The samples, which had never been previously thawed, were analyzed for SAA4 antigen in 2018. (A) Total SAA4 antigen was quantified for Scripps VTE registry subjects using an ELISA kit from Aviscera Biosciences. The results are expressed in micrograms per milliliter of SAA4 from the standard curve supplied by the kit. The median of total plasma SAA4 level was higher in VTE cases than in controls [48.1 µg/mL (interquartile range [IQR], 32.2-94.9) vs. 38.4 µg/mL (IQR, 28.2-51.8;, $P < .001$). (B and C) SAA4 antigen values for the 2 SAA4 isoforms, 14-kDa and 19-kDa bands, were quantified using a quantitative immunoblotting. The antigen values of the 14-kDa monomer band (B) and the 19-kDa glycosylated band (C) are expressed in percentage of normalized values obtained from a standard curve using dilutions of normal pooled plasma (George King Bio-Medical) where 100% was defined as the median value for controls for each band. The median of plasma 14 kDa and 19 kDa SAA4 levels were higher in VTE cases than in controls (120.2%; [IQR, 99.7-155.2] vs. 100%; [IQR, 88.1-119.3], $P < .001$ for 14-kDa SAA4; and 119.4% [IQR, 95.8-149.8] vs. 100% [IQR, 59.2-95.8], $P < .01$ for 19-kDa SAA4]. The median values are indicated by a red arrowhead and red line. The *P* values were calculated for the difference in median values between patients with VTE and controls using the Mann-Whitney test using Prism 6.0. $^{***}P < .001$; $^{**}P < .01$. SAA4, serum amyloid A4; VTE, venous thromboembolism

performed using Prism 6.0 software (Graph Pad Software Inc, La Jolla, CA, USA).

3 | RESULTS AND DISCUSSION

Here, we discovered that the median plasma SAA4 level determined by SAA4 ELISA was higher in VTE cases than in matched controls (48.1 vs. 38.4 µg/mL; $P < .001$; Figure 1A). Elevated plasma SAA4 levels, defined as above the 90th percentile of controls, were associated with VTE (odds ratio [OR], 3.8; 95% confidence interval, 1.8-8.0; Table 2). Besides 90th percentile analysis, OR for the 75th and 67th percentiles were calculated; these OR values differed from 90th percentile values but remained statistically significant (OR, 3.8, 2.5, and 1.9 for 90th, 75th, and 67th percentiles, respectively; Table 2). Known risk factors for VTE, ie, factor V Leiden and prothrombin nt G20210A, were not associated with SAA4 levels. Since SAA4 is carried by lipoproteins,[17–19] the association of SAA4 levels with lipoprotein particle levels, that is, with factors that may reflect metabolism of lipoproteins (hormone use and obesity) was analyzed (Table 3). Among them, HDL particles levels were correlated with SAA4. However, the association of elevated plasma SAA4 levels above the 90th, 75th and 67th percentile of controls with VTE occurrence remained significant after adjustment for HDL particle levels (Table 2, Model II). When the association of SAA4 with acute SAA levels (SAA1 + SAA2) and another acute inflammatory marker, C-reactive protein (CRP), were evaluated, plasma SAA4 levels positively correlated with acute-phase SAA and CRP levels ($r = .28$, $P < .0001$; and $r = .15$, $P = .023$, respectively). However, when plasma levels of acute-phase SAA and CRP were included as covariates, the association of elevated plasma SAA4 with VTE occurrence remained significant (Table 2, Models III-V). These findings suggest that elevated plasma SAA4 is indeed associated with increased occurrence of VTE independent of these other parameters.

Total plasma SAA4 levels determined by ELISA is composed of 2 isoforms because SAA4 in plasma is partially glycosylated at Asn76 resulting in a 19-kDa glycosylated monomer in addition to the 14-kDa nonglycosylated monomer.[13] Hence, quantitative immunoblotting was used to measure each isoform in VTE cases and controls. Plasma median levels of both the 14-kDa isoform (Figure 1B) and the 19-kDa SAA4 isoform (Figure 1C) were elevated in VTE patients compared to controls (120.2% vs. 100%, $P < .001$; and 119.4% vs. 100%, $P < .01$, respectively).

In recent years, studies have identified minor abundance plasma lipids and apolipoproteins that may influence hemostasis or cardiovascular events.[24,25] We previously found that acute-phase SAA measured by ELISA, that is, SAA1 and SAA2, was elevated in VTE patients.[9] Here, we studied constitutive SAA4, the other member of the human SAA family, which contains 112 residues and shares substantial homology with mature SAA1, which contains 104 residues. Previously, nothing was known about SAA4's association with blood coagulation.

To assess its procoagulant activity, recombinant 14-kDa monomer SAA4 made in E. coli was studied in various coagulation assays. Recombinant SAA4 dose-dependently shortened the FXa-1-stage clotting time from 320 seconds to 55 seconds (Figure 2A). When SAA4 was added to purified prothrombinase assays, consisting of purified FXa, FVa, prothrombin, and Ca^{++}, SAA4 promoted

TABLE 2 Odds ratio for VTE based on elevated plasma SAA4 level above 90th, 75th, and 67th percentile of controls without and with adjustments

Percentile of SAA4	Odds ratio (95% CI)		
	90th	75th	67th
Cutoff values	**80.0 μg/mL**	**52.7 μg/mL**	**48.0 μg/mL**
Model and adjustments			
I. None	3.8 (1.8-8.0)	2.5 (1.4-4.4)	1.9 (1.1-3.3)
II. HDL particles	4.6 (2.1-10)	3.2 (1.7-5.8)	2.4 (1.3-4.2)
III. Acute-phase SAA	3.7 (1.7-7.8)	2.4 (1.4-4.3)	1.8 (1.1-3.2)
IV. Acute-phase SAA, CRP	3.9 (1.8-8.2)	2.5 (1.4-4.5)	1.9 (1.1-3.4)
V. HDL particles + acute phase SAA(1 + 2) + CRP	4.7 (2.1-10)	3.2 (1.7-5.9)	2.3 (1.3-4.2)

Note: Logistic regression was used to evaluate the association between elevated plasma SAA4 levels and VTE occurrence using STATA (StataCorp LLC, College Station, TX, USA) and odds ratio (95% confidence interval) for VTE based on elevated plasma SAA4 levels of VTE patients above the 90th percentile of controls are shown. The value for 90th, 75th, and 67th percentile of plasma SAA4 in normal subjects served as the reference group. Models II to V were adjusted by variables indicated in the table. All the variables were used as continuous variables. Acute-phase SAA (SAA1 and SAA2) were measured by ELISA (BioSource International, Inc, Camarillo, CA, USA),[9] which detects acute-phase SAA. CRP values were obtained using CRP high-sensitivity ELISA (Calbiotech, Inc, El Cajon, CA, USA).

prothrombin activation (Figure 2B). In this prothrombinase assay system, acute-phase recombinant SAA1, SAA4's homolog, did not promote prothrombinase activity (data not shown). The Gla domain of FXa was necessary for the SAA4's procoagulant effect (Figure 1B). These data showing the procoagulant activity of SAA4 provides biological plausibility for a potential causal prothrombotic role for SAA4. SAA4 may act as a template for the formation of the prothrombinase complex in certain pathologies where SAA4 is deposited, as in the atheroma plaque where significant amounts of SAA4 are found.[19] These findings also raise the possibility that SAA4 procoagulant activity might contribute to normal hemostasis.

One limitation of this study is the modest number of VTE patients (113 patients). Replication studies and further analyses are needed to confirm our discovery and to determine whether SAA4 levels might be of value in predicting VTE. The timing of a blood draw in relation to the clinical event can raise an issue of validity of conclusions, as all blood samples came long after the clinical event. Some plasmas were obtained >5 years after the clinical presentation. However, subgrouping of the Scripps patients with VTE was made based on time since the clinical event (ie, <1 year, 1-3 years, >3 years), and the SAA4 median values were not different between subgroups (Table 3). The use of warfarin at the time of blood collection might be a potential issue, as the Scripps VTE registry included some warfarin users among VTE cases. However, there was no difference in plasma levels of SAA4 between warfarin users and nonusers, suggesting that warfarin use did not affect the association of elevated SAA4

TABLE 3 Associations and correlations of plasma SAA4 levels with other parameters

	Association between covariates and SAA4 levels	
	Median (IQR), μg/mL	***P* value**
Gender		
Male	36.1 (27.4-51.4)	.0008
Female	48.0 (33.4-73.4)	
Years at blood sampling from the VTE event		
<1	50.3 (36.6-97.2)	.38
1-3	48.1 (34.7-78.3)	
>3	43.8 (26.8-103.8)	
Pulmonary embolism		
Yes	44.1 (28.6-89.1)	.28
No	49.5 (35.2-103)	
Factor V Leiden		
Yes	44.7 (28.3-72.9)	.87
No	43.5 (30.3-61.3)	
Prothrombin G20210A		
Yes	39.6 (27.7-65.5)	.62
No	43.8 (28.9-66.9)	
Warfarin use		
Yes	48.1 (32.3-97.2)	.87
No	45.7 (30.8-88.4)	
Hormone use (female only)		
Yes	45.3 (32.5-69.4)	.28
No	52.3 (38.6-76.1)	
	Correlation with SAA4 levels	
	Spearman *r*	***P* value**
Age at blood drawing	−.008	.90
Age at event	.03	.79
BMI	.007	.92
VLDL particles	.03	.69
LDL particles	−.03	.65
HDL particles	.15	.023
SAA1 + SAA2	.28	<.0001
CRP	.15	.025

Note: Subgroup analysis for plasma SAA4 levels by gender, years at blood sampling from the VTE event, pulmonary embolism occurrence, carrier of factor V Leiden or prothrombin G20210A, warfarin use, or hormone use were performed to test if these are influences on plasma SAA4 levels. The median values for each subgroup and their difference (*P* values) are shown. The SAA4 levels were also analyzed for correlation with age, BMI, lipoprotein particles (VLDL, LDL, and HDL particles), CRP and acute-phase SAA1 + SAA2; and the Spearman *r* and *P* values are shown. N = 113 subjects, except data for CRP value for 1 VTE subject was missing.

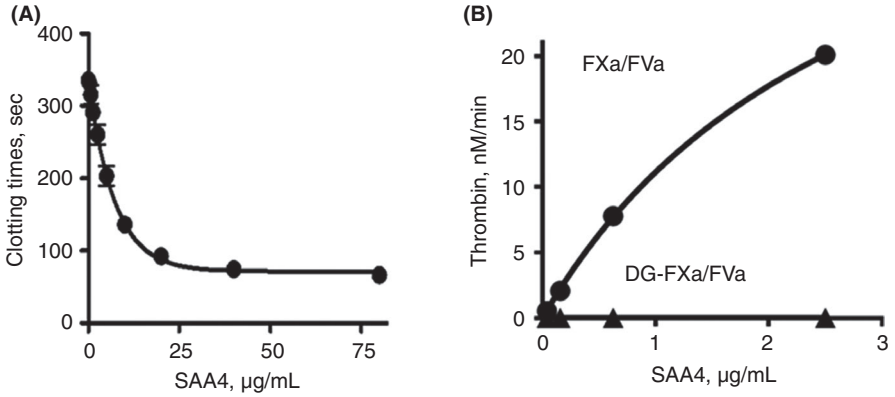

FIGURE 2 Coagulation assays show the procoagulant activity of recombinant SAA4. (A) FXa-1-stage plasma assays were performed as followed. For FXa 1-stage clotting assays, citrated plasma (25 μL) was incubated for 180 s at 37°C with 25 μL of FXa (34 nmol/L), and 25 μL of rat SAA4 at varying concentrations. Clotting was initiated with 25 μL of 30 mmol/L $CaCl_2$. Clotting times were measured using an Amelung KC4 micro coagulometer (Sigma Diagnostic, St Louis, MO, USA). Recombinant SAA4 shortened the clotting time from 320 s to 55 s in a dose-dependent fashion. (B) SAA4-dependent enhancement of prothrombin activation by FXa/FVa (solid circle) or by Gla-domainless (DG)-FXa/FVa (solid triangle) were determined as followed. Prothrombin (0.76 μM final) activation by purified FXa (0.125 nmol/L final) and Va (6.25 nmol/L final) plus or minus varying concentrations of SAA4 was assayed in the absence of exogenously added phospholipids. Reactants were mixed and incubated at room temperature for 5 min to allow pro-thrombin activation before the reaction was quenched by EDTA, and then the amidolytic activity of thrombin was quantified using Pefachrome TH thrombin chromogenic substrate. SAA4 enhanced thrombin generation by FXa/FVa but not by Gla-domainless-FXa/FVa. FVa, factor Va; FXa, factor Xa; SAA4, serum amyloid A4

with VTE occurrence (Table 3). VTE patients were <55 years old at first event. The retrospective-based analysis of antigen levels from these studies does not distinguish causality from coincidence, but the procoagulant properties of SAA4 does provide some reasonable degree of likelihood for potential prothrombotic actions of SAA4. Given that the patients were mainly Caucasians, the findings here remain to be evaluated in other racial groups.

In summary, these results show that elevated monomeric plasma levels of SAA4 are associated with VTE in adults <55 years old and that SAA4 itself is a potential enhancer of thrombin generation in plasma. These results support the hypothesis that SAA4 may act as a prothrombotic agent in vivo. In vivo proof-of-concept studies of SAA4's prothrombotic property are warranted, and if they were very successful, then reagents targeting SAA4 might become potential drug candidates.

RELATIONSHIP DISCLOSURE

The authors report nothing to disclose. No external funding was received to perform the study.

ACKNOWLEDGMENT

We are grateful to Lacthu Tonnu for skillful technical assistance.

AUTHOR CONTRIBUTIONS

JAF, HD, and JHG participated in the conception of the study. JAF and HD were responsible for SAA4 measurements and in vitro experiments. Statistical analyses were performed by JAF and HD. DJE was responsible for organizing the Scripps VTE Registry, consenting the patients, and obtaining blood specimens. JAF, HD, and JHG were responsible for writing the manuscript. All authors were involved in the interpretation of data and gave final approval.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Fernández JA, Deguchi H, Elias DJ, Griffin JH. Serum amyloid A4 is a procoagulant apolipoprotein that it is elevated in venous thrombosis patients. *Res Pract Thromb Haemost*. 2020;4:217–223. https://doi.org/10.1002/rth2.12291