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

José A. Fernández | Hiroshi Deguchi | Darlene J. Elias | John H. Griffin

Department of Molecular Medicine, The Scripps Research Institute, La Jolla, CA, USA

Correspondence
John H. Griffin, Scripps Research Institute, IMM-316, 10550 N. Torrey Pines Rd, La Jolla, CA 92037.
Email: jgriffin@scripps.edu

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
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. An association between VTE and risk factors for atherosclerotic vascular diseases is emerging and may help identifying new risk factors for VTE.

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. These factors include changes in levels of C-reactive protein and acute-phase serum amyloid A (SAA). Recent studies show that acute-phase SAA can directly bind to fibrin and affect coagulation by promoting amyloid formation in fibrin. The SAA gene family has 4 members. SAA 1-3 are acute-phase apolipoproteins that may rise >100-fold in acute inflammation. Constitutive SAA4, where “constitutive” denotes its level is not subject to remarkable alterations, is linked to a certain group of HDL particles (HDL3). More recent studies have identified a minor portion of SAA4 that is in the LDL and VLDL subfractions. SAA4 comprises >90% of the total serum SAA proteins 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). SAA4 expression is implicated during inflammation including atherosclerosis; notably, SAA4 lacks expression in normal arteries but is expressed in carotid lesions. Thus, SAA4 is a histologic and serologic biomarker for atheromatous lesions. However, the specific physiological role of SAA4 has not been well defined.

Previously, we reported that increased levels of acute-phase SAA is associated with the risk of VTE. 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. 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).
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 CaCl₂.

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
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, SAA1 and SAA2, was elevated in VTE patients. 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 recombinant SAA4 was added to purified prothrombinase assays, consisting of purified FXa, FV,a, prothrombin, and Ca\(^{2+}\), SAA4 promoted clotting from 320 seconds to 55 seconds (Figure 2A). When recombinant SAA4 was added to purified prothrombinase assays, consisting of purified FXa, FV,a, prothrombin, and Ca\(^{2+}\), SAA4 promoted clotting from 320 seconds to 55 seconds (Figure 2A). When recombinant SAA4 was added to purified prothrombinase assays, consisting of purified FXa, FV,a, prothrombin, and Ca\(^{2+}\), SAA4 promoted clotting from 320 seconds to 55 seconds (Figure 2A). When recombinant SAA4 was added to purified prothrombinase assays, consisting of purified FXa, FV,a, prothrombin, and Ca\(^{2+}\), SAA4 promoted clotting from 320 seconds to 55 seconds (Figure 2A). When recombinant SAA4 was added to purified prothrombinase assays, consisting of purified FXa, FV,a, prothrombin, and Ca\(^{2+}\), SAA4 promoted clotting from 320 seconds to 55 seconds (Figure 2A). When recombinant SAA4 was added to purified prothrombinase assays, consisting of purified FXa, FV,a, prothrombin, and Ca\(^{2+}\), SAA4 promoted clotting from 320 seconds to 55 seconds (Figure 2A). When recombinant SAA4 was added to purified prothrombinase assays, consisting of purified FXa, FV,a, prothrombin, and Ca\(^{2+}\), SAA4 promoted clotting from 320 seconds to 55 seconds (Figure 2A). When recombinant SAA4 was added to purified prothrombinase assays, consisting of purified FXa, FV,a, prothrombin, and Ca\(^{2+}\), SAA4 promoted clotting from 320 seconds to 55 seconds (Figure 2A). When recombinant SAA4 was added to purified prothrombinase assays, consisting of purified FXa, FV,a, prothrombin, and Ca\(^{2+}\), SAA4 promoted clotting from 320 seconds to 55 seconds (Figure 2A).
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.

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 2
| Percentile of SAA4 | Odds ratio (95% CI) | 90th | 75th | 67th |
|-------------------|--------------------|------|------|------|
| Cutoff values     |                    |      |      |      |
| 80.0 μg/mL        | 3.8 (1.8-8.0)      | 2.5 (1.4-4.4) | 1.9 (1.1-3.3) |
| 52.7 μg/mL        | 4.6 (2.1-10)       | 3.2 (1.7-5.8) | 2.4 (1.3-4.2) |
| 48.0 μg/mL        | 3.7 (1.7-7.8)      | 2.4 (1.4-4.3) | 1.8 (1.1-3.2) |

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), which detects acute-phase SAA. CRP values were obtained using CRP high-sensitivity ELISA (Calbiotech, Inc, El Cajon, CA, USA).

### Table 3
| 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.
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.

RELEVANCE 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.

ORCID
José A. Fernández https://orcid.org/0000-0002-0804-1865

REFERENCES
1. Rinde LB, Lind C, Smabrekke B, Njolstad I, Mathiesen EB, Wilsgaard T, et al. Impact of incident myocardial infarction on the risk of venous thromboembolism: the Tromso Study. J Thromb Haemost. 2016;14:1183–91.
2. Prandoni P. Venous and arterial thrombosis: is there a link? Adv Exp Med Biol. 2017;906:273–83.
3. Poredos P. Interrelationship between venous and arterial thrombosis. Int Angiol. 2017;36:295–8.
4. Lowe GD. Arterial disease and venous thrombosis: are they related, and if so, what should we do about it? J Thromb Haemost. 2006;4:1882–5.
5. Lippi G, Favaloro EJ. Venous and arterial thromboses: two sides of the same coin? Semin Thromb Hemost. 2018;44:239–48.
6. Keller K, Hobohm L, Munzel T, Ostad MA. Impact of symptomatic atherosclerosis in patients with pulmonary embolism. Int J Cardiol. 2019;278:225–31.
7. Mineo C, Deguchi H, Griffin JH, Shaul PW. Endothelial and anti-thrombotic actions of HDL. Circ Res. 2006;98:1352–64.
8. Deguchi H, Pecheniuk NM, Elias DJ, Averell PM, Griffin JH. High-density lipoprotein deficiency and dyslipoproteinemia associated with venous thrombosis in men. Circulation. 2005;112:893–9.
9. Deguchi H, Elias DJ, Navarro S, Espana F, Griffin JH. Elevated serum amyloid A is associated with venous thromboembolism. Thromb Haemost. 2013;109:358–9.
10. Page MJ, Thomson GJA, Nunes JM, Engelbrecht AM, Nell TA, de Villiers WJS, et al. Serum amyloid A binds to fibrinogen, promoting fibrin amyloid formation. Sci Rep. 2019;9:3102.
11. Sun L, Ye RD. Serum amyloid A1: structure, function and gene polymorphism. Gene. 2016;583:48–57.
12. Ducret A, Bruun CF, Bures EJ, Marhaug G, Husby G, Aebersold R. Characterization of human serum amyloid A protein isoforms separated by two-dimensional electrophoresis by liquid chromatography/electrospray ionization tandem mass spectrometry. Electrophoresis. 1996;17:866–76.
13. De Buck M, Gouwy M, Wang JM, Van Snick J, Opdenakker G, Struyf S, et al. Structure and expression of different serum amyloid A (SAA) variants and their concentration-dependent functions during host insults. Curr Med Chem. 2016;23:1725–55.

14. Uhlar CM, Whitehead AS. Serum amyloid A, the major vertebrate acute-phase reactant. Eur J Biochem. 1999;265:501–23.

15. Whitehead AS, de Beer MC, Steel DM, Rits M, Lelias JM, Lane WS, et al. Identification of novel members of the serum amyloid A protein superfamily as constitutive apolipoproteins of high density lipoprotein. J Biol Chem. 1992;267:3862–7.

16. Steel DM, Donoghue FC, O’Neill RM, Uhlar CM, Whitehead AS. Expression and regulation of constitutive and acute phase serum amyloid A mRNAs in hepatic and non-hepatic cell lines. Scand J Immunol. 1996;44:493–500.

17. de Beer MC, Yuan T, Kindy MS, Asztalos BF, Roheim PS, de Beer FC. Characterization of constitutive human serum amyloid A protein (SAA4) as an apolipoprotein. J Lipid Res. 1995;36:526–34.

18. Savinova OV, Fillaus K, Jing L, Harris WS, Shearer GC. Reduced apolipoprotein glycosylation in patients with the metabolic syndrome. PLoS ONE. 2014;9:e104833.

19. Krishnan S, Huang J, Lee H, Guerrero A, Berglund L, Anuurad E, et al. Combined high-density lipoprotein proteomic and glycomic profiles in patients at risk for coronary artery disease. J Proteome Res. 2015;14:5109–18.

20. Yamada T, Kakihara T, Kamishima T, Fukuda T, Kawai T. Both acute phase and constitutive serum amyloid A are present in atherosclerotic lesions. Pathol Int. 1996;46:797–800.

21. Hrzenjak A, Arlt A, Knipping G, Kostner G, Sattler W, Malle E. Silent mutations in secondary Shine-Dalgarno sequences in the cDNA of human serum amyloid A4 promotes expression of recombinant protein in Escherichia coli. Protein Eng. 2001;14:949–52.

22. Langley SR, Willeit K, Didangelos A, Matic LP, Skroblin P, Barallobre-Barreiro J, et al. Extracellular matrix proteomics identifies molecular signature of symptomatic carotid plaques. J Clin Investig. 2017;127:1546–60.

23. Deguchi H, Navarro S, Payne AB, Elias DJ, Dowling NF, Austin HD, et al. Low level of the plasma sphingolipid, glucosyceramide, is associated with thrombotic diseases. Research and practice in thrombosis and haemostasis. 2017:1–33–40.

24. Deguchi H, Elias DJ, Griffin JH. Minor plasma lipids modulate clotting factor activities and may affect thrombosis risk. Res Pract Thromb Haemost. 2017;1:93–102.

25. Pechlaner R, Tsimikas S, Yin X, Willeit P, Baig F, Santer P, et al. Very-low-density lipoprotein-associated apolipoproteins predict cardiovascular events and are lowered by inhibition of APOC-III. J Am Coll Cardiol. 2017;69:789–800.

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 is elevated in venous thrombosis patients. Res Pract Thromb Haemost. 2020;4:217–223. https://doi.org/10.1002/rth2.12291