Received: 5 April 2017 | Accepted: 14 May 2017

DOI: 10.1002/rth2.12017

REVIEW ARTICLE

WILEY rpth research and practice in thrombosis and haemostasis | ISTH

Minor plasma lipids modulate clotting factor activities and may affect thrombosis risk

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Funding information
Support was provided, in part, by grants HL021544, HL052246, HL133728 (J.H.G.) and UL1RR025774 and 8UL1 TR000109 (CTSA, PI: E. Topol) from the National Institutes of Health and funding from the Scripps Health (D.J.E.). We gratefully acknowledge members of the Griffin laboratory for their helpful discussions. We apologize to our respected colleagues whose important publications we were not able to cite or discuss due to space limitations.

Abstract:

Different minor abundance plasma lipids significantly influence thrombin generation in vitro and significant differences in such lipids are linked to risk for venous thrombosis. Some plasma sphingolipids including glucosylceramide, lyso-sulfatide and sphingosine have anticoagulant properties whereas, conversely, some plasma phospholipid derivatives, including certain lyso-phospholipids and ethanolamides, have procoagulant properties. Plasma metabolite profiling of venous thrombosis patients showed association of venous thrombosis with decreased plasma long-chain acylcarntines, leading to discovery of their anticoagulant activity as inhibitors of factor Xa. Inhibition of factor Xa by acylcarnitines does not require the protein's Gla-domain, emphasizing an expanded framework for the paradigm for lipid-clotting factor interactions. Overall, whether by genetics or environment, alterations in the dynamics of lipid metabolism linked to an altered lipidome may contribute to regulation of blood coagulation because imbalances between physiologic procoagulant and anticoagulant lipids may contribute to excessive thrombin generation that augments risk for thrombosis.

KEYWORDS
blood coagulation, lipids, phospholipids, sphingolipids, thrombin, venous thromboembolism

Essentials

- Circulating blood contains hundreds of lipids, many of which might influence blood coagulation.
- Recent discoveries about circulating lipids that can affect blood coagulation are reviewed.
- Minor abundance plasma lipids can modulate thrombin generation via direct effects on factor Xa.
- Variations in minor abundance plasma lipids can influence thrombin generation and thrombosis risk.

1 | THROMBOSIS, BLOOD COAGULATION AND THROMBIN GENERATION

Thrombotic diseases contribute to morbidity and mortality in deep vein thrombosis and pulmonary embolism (VTE), acute myocardial infarction, and ischemic stroke. VTE in the USA annually results in >500 000 hospitalizations and circa 100 000 deaths.[1–4] Morbidity and mortality of cardiovascular diseases are often linked to hypercoagulability which is linked to excessive thrombin generation. Both retrospective and prospective studies report associations of initial or recurrent VTE risk with elevated peak thrombin in endogenous thrombin generation (ETP) in vitro assays.[5–10] Excess amounts of thrombin also contribute to arterial disease.[11–13] Enhanced thrombin generation in thrombotic diseases may be correlated with other risk factors, such as lipoproteins and lipids.[5,11–13]

Thrombin generation is central to regulation of hemostasis and thrombosis and to the pathogenesis of thrombosis. Thrombin, the major product of the blood coagulation pathways (Figure 1), involves sequential enzymatic activations of serine protease zymogens enhanced by nonenzymatic cofactors, tissue factor (TF), factors Va and VIIIa.[14,15] Thrombin is generated by the "prothrombinase" complex (factors Xa:Va:Ca^{++}) which activates prothrombin, and the thrombin can ultimately provide feedback downregulation of its generation through the activated protein C (APC) pathway via thrombomodulin and the endothelial protein C receptor.[16] Canonical prothrombinase components include factors Va and Xa, prothrombin, calcium and magnesium ions and phospholipids.[14,15] Phosphatidylserine (PS) is a potent prothrombinase lipid cofactor. In purified biochemical systems, model membrane phospholipids (PLs), comprising, in part, PS, bind the gamma-carboxyglutamic acid (Gla) domains of prothrombin and factor Xa and the C1 and C2 domains of factor Va such that multiple PL-clotting factor interactions promote and stabilize the prothrombinase complex formation and function.[17–19] The Gla domain is a common domain in the structures of vitamin K-dependent coagulation factors, i.e, of prothrombin, factor X, factor IX, factor VII, protein C, and protein S. The Gla domain is located at the N-terminus of the clotting factor and its synthesis involves the unusual post-translational modification of glutamic acid residues by adding an extra carboxyl group to the gamma-carbon of glutamic acid residues. Warfarin blocks the post-translational modifications of the N-terminal domain by inhibiting vitamin K epoxide reductase and decreases vitamin K hydroquinone[20] such that the liver produces less active coagulation factors, thereby acting as an anticoagulant. Much evidence supports the physiological relevance for the Gla domains as PL-binding sites for clotting factors on cell membranes where reactions central to thrombosis and hemostasis can occur–thus, the canonical binding sites on clotting factors for lipids are their Gla domains. In silico models for the protein-protein structures of the prothrombinase complex have appeared,[21,22] but few convincing models exist for prothrombinase-lipid interactions and their structures. The complete structural basis for regulation of prothrombinase activity by lipids is not apparent from the limited number of interesting available structures.

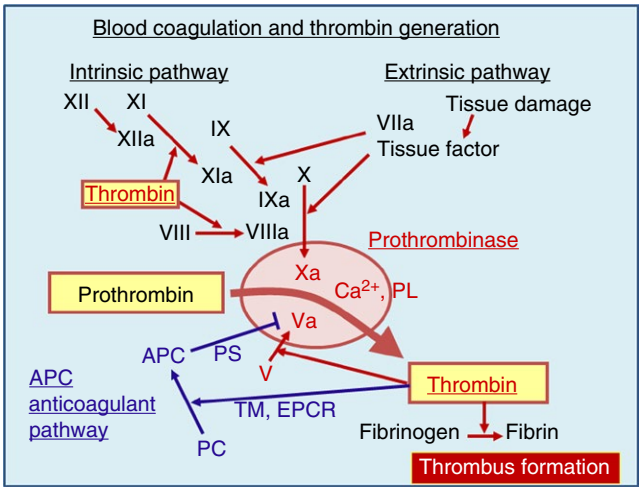

FIGURE 1 Blood coagulation and protein C pathways. Thrombin is the major product of the blood coagulation pathways involving sequential enzymatic activations of serine protease zymogens enhanced by nonenzymatic cofactors, factors Va and VIIIa.[14,15] Thrombin is generated by the "prothrombinase" complex which is formed by binding of factor Xa to factor Va on a phospholipid (PL) surface in the presence of Ca^{2+} ions. Small amount of thrombin can be generated by tissue factor with activation of factors VII and X via the extrinsic pathway or following activation of factors XI, VIII, IX, and X via the intrinsic pathway. Once generated, thrombin activates platelets and factor V, factor VIII, and factor XI, thereby stimulating multiple steps in the intrinsic pathway and amplifying thrombin generation. For negative feedback downregulation of thrombin generation, thrombin generates the potent anticoagulant, activated protein C (APC), when it is bound to thrombomodulin (TM) and protein C (PC) is bound to the endothelial protein C receptor (EPCR)[16]

Plasma lipids and lipoproteins can influence both procoagulant and anticoagulant reactions in plasma, implying that imbalances of lipids and lipoproteins may be linked to atherothrombosis and also to VTE,[4,23–27] although some conflicting data are reported.[28] Remarkably, plasma contains many lipids present in the nmol/L to mmol/L ranges (e.g, some PLs and cholesterol plasma levels can exceed 1 mmol/L) (Table S1). Each of the 3 major classes of lipoproteins (VLDL, LDL, and HDL) contains neutral lipid-rich cores comprising cholesteryl esters, triglycerides, etc. with various major and minor apolipoproteins residing on the hydrophilic surfaces that also contain many different polar or charged lipids, such as PLs, sphingolipids, lyso-lipids, and cholesterol. Heterogeneous lipoprotein particles and albumin carry numerous less abundant lipids which can exert a wide range of bioactivities. Based on the spectrum of lipids in plasma and their multiple activities, blood coagulation and thrombin generation can be physiologically or pathologically directly modulated by plasma lipids (Figure 2A). Imbalances of thrombin generation caused by imbalances in plasma lipids may affect hypercoagulability and be associated with thrombosis risk. This review summarizes literature concerning modulation of blood clotting reactions by various minor abundance lipids and their levels in clinical studies of thrombosis patients.

Studies that challenged the widely accepted, simple paradigm for PL-Gla-domain lipid effects on prothrombinase structure and activity involved synthetic, soluble dicaproyl-PS that was reported to promote prothrombinase activity by binding to factor Xa outside the Gla-domain (Figure 2B).[29,30] Similarly, recent publications both extend and challenge the simple classical paradigm for PL-Gla-domain-factor Xa interactions because it was shown that several plasma lipids carried by lipoproteins or proteins such as albumin and/or lipid binding proteins (e.g, sphingosine, sphinganine, acyl-carnitines [ACs], and lyso-sulfatide [LSF]), so called "soluble" lipids, bind factor Xa and alter its procoagulant activity (Figure 2B),[31–33] supporting and extending the challenge of the classical, simple paradigm for lipid-clotting factor interactions. In summary, innovative findings show that certain, minor abundance plasma lipids can bind directly to factor Xa outside the Gla domain and modulate thrombin generation by the prothrombinase complex (Figure 2B), leading to an expanded framework for the paradigm for lipid-clotting factor interactions.[32,33]

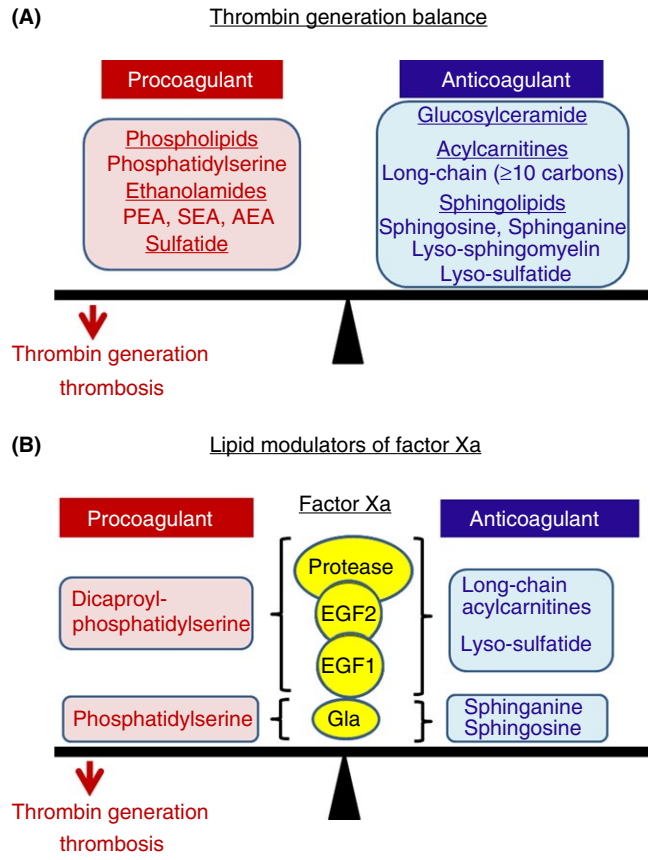

FIGURE 2 Plasma lipids can exert procoagulant or anticoagulant activity. (A) Thrombin generation balance. Anticoagulant and procoagulant physiologic plasma lipids of minor abundance can alter the balance for thrombin generation and altered levels of some of these lipids are found in VTE patients. (B) Lipid modulators of factor Xa. The physiologic plasma sphingolipids, long chain acylcarnitines and lyso-sulfatide, are anticoagulant and bind to factor Xa whereas the synthetic lipid, dicaproyl-PS (C6PS), is prothrombotic. In a potential paradigm shift for how lipids regulate factor Xa activity, the factor Xa region that binds anticoagulant long chain acylcarnitines and LSF is not the Gla domain. In contrast, sphingosine inhibition of factor Xa requires its Gla-domain. Color highlighting indicates whether a particular lipid inhibits thrombin generation (blue) or enhances thrombin generation (pink). PEA, palmitoyl-ethanolamide; SEA, stearoyl-ethanolamide; and AEA, arachidonoyl-ethanolamide

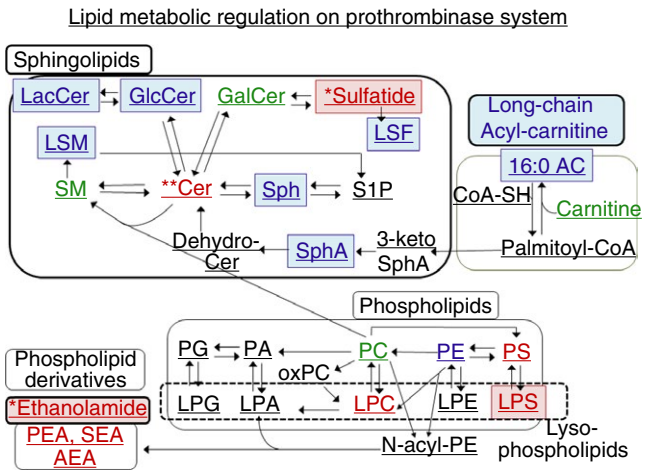

FIGURE 3 Lipid metabolic regulation on coagulation system. The dynamic metabolic balances among various sphingolipid and phospholipid metabolites may be shifted by a variety of factors and influences, and such changes in the relative concentrations of sphingolipids or phospholipids might regulate inflammatory events such as cell proliferation and also might down-regulate blood coagulation and thrombin generation. A variety of enzymes that regulate sphingolipid metabolism are capable of shifting the balance between sphingosine and ceramide, sphingosine and sphingosine-1-phosphate, GlcCer and ceramide, etc.[37,38] Similarly, a variety of enzymes that regulate PL metabolism are capable of shifting the balance between PLs, LPLs, and ethanolamides.[53,54,60,61] More detailed information including related metabolic enzymes is presented in Figure S1 and Table S2. *Indicates the effect is on the contact phase; **Indicates the effect is on vWF release. Color highlighting indicates whether a particular lipid inhibits thrombin generation (blue) or enhances thrombin generation (red) or has no effect (green). Square boxes with colored background indicates lipids whose effects on coagulation are discussed in this review. Sph, sphingosine; GlcCer, glucosylceramide; LacCer, lactosylceramide (CD17), GalCer, galactosylceramide; SF, sulfatide; LSF, lyso-sulfatide; SM, sphingomyelin; LSM, lyso-sphingomyelin; Cer, ceramide; sph, sphingosine; sphA, sphinganine; S1P, sphingosine-1-phosphate; 16:0 AC, 16:0 acylcarnitine (palmitoylcarnitine); PC, phosphatidylcholine; PG, phosphatidylglycerol; PA, phosphatidic acid; PS, phosphatidylserine; PE, phosphatidylethanolamine; LPC, lyso-phosphatidylcholine; LPG, lyso-phosphatidylglycerol; LPA, lyso-phosphatidic acid; LPS, lyso-phosphatidylserine; LPE, lyso-phosphatidylethanolamine; oxPC, oxidized phosphatidylcholine; PEA, palmitoyl-ethanolamide; SEA, stearoyl ethanolamide; AEA, arachidonoyl ethanolamide

Three different classes of minor abundance plasma lipids can affect coagulation reactions and thrombin generation (Figure 1A) and this review summarizes the association of blood coagulation with sphingolipids, phospholipid derivatives, and long-chain acylcarnitines in 3 subsections below.

1.1 | Plasma sphingolipids, blood coagulation and VTE

Plasma lipoproteins contain sphingolipids as well as phospholipids,[34–36] and the metabolism of these lipids is notably complex (Figure 3, Figure S1, Table S1). Glycolipids play critical roles as bioregulators of a variety of processes such as cell proliferation, cell mobility and apoptosis.[36]

Some physiologic sphingolipids were studied for their anticoagulant or procoagulant activities and some common sphingolipids were discovered as potent anticoagulant lipids (see below).

1.2 | Sphingolipid metabolism

Sphingolipids are defined by the presence of a long-chain sphingoid backbone (Figure 4), and the anticoagulant sphingolipids, glucosylceramide (glucocerebroside, GlcCer) and sphingosine, are dynamically metabolized directly from ceramide (Figure 3).[37,38] A variety of enzymes that regulate sphingolipid metabolism are capable of shifting

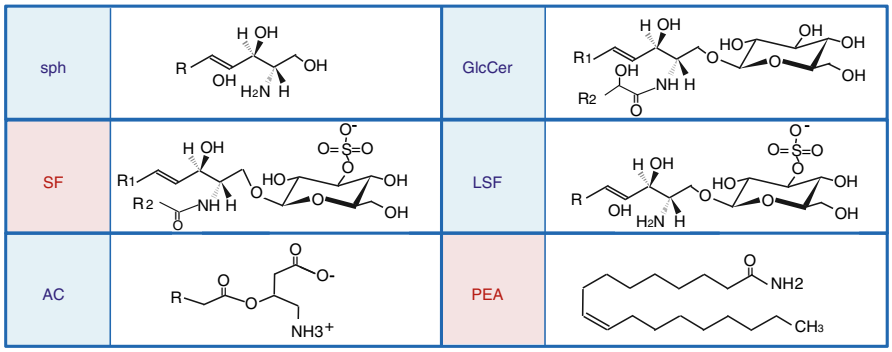

FIGURE 4 Structures of some plasma minor abundance lipids. Color highlighting indicates whether a particular lipid can contribute to inhibition of thrombin generation (blue) or to enhancement of thrombin generation (red). Sph, sphingosine; GlcCer, glucosylceramide; SF, sulfatide; LSF, lyso-sulfatide; AC, acylcarnitine; and PEA, palmitoyl-ethanolamide

the balance between sphingosine and ceramide, sphingosine and sphingosine-1-phosphate, GlcCer and ceramide, etc.[37,38] These metabolic changes in the relative concentrations of sphingolipids might regulate inflammatory events such as cell proliferation and also might down-regulate blood coagulation and thrombin generation. Certain sphingolipid imbalances might be linked to risk of thrombotic diseases and certain sphingolipid metabolic imbalances might actually be causally associated with thrombosis based on the findings that multiple sphingolipids can exert either procoagulant or anticoagulant activities, as described below.

1.3 | GlcCer as an anticoagulant

Glycosphingolipids found in plasma include inter alia, the neutral lipids, GlcCer, lactosylceramide (CD17, LacCer), globotriaosylceramide (CD77, Gb3Cer), and globotetraosylceramide (Gb4Cer),[39] as well as various gangliosides (GM3, GD1a, GM2, GT1b, GD1b, GQ1b) and sulfatides.[37,38] GlcCer contains a hydrophilic glucose moiety that links to the hydroxyl group of ceramide (Figure 4). GlcCer is a key metabolic precursor of several distinct subclasses of glycosphingolipids and is synthesized intracellularly by GlcCer synthase which utilizes UDP-Glc and ceramide as substrates (Figure 3).[37,38] Glycolipids are important components of cell membranes, and glycolipid molecules present their highly varied saccharide residues on cell surfaces as well as on the surface of lipoprotein particles, exposing saccharides in the outer lipid leaflet for interactions with cells, antibodies, bacterial toxins, and viral envelope proteins.[36]

GlcCer, LacCer, Gb3Cer, and Gb4Cer have levels of approximately 1-10 μmol/L in plasma,[39] and these glycolipids can contribute to prolongation of plasma clotting times in a similar concentration range as their physiological plasma concentrations.[40] GlcCer exerts substantial anticoagulant activity acting via the protein C pathway as an activated protein C (APC) cofactor in purified protein assays,[40,41] probably by increasing the affinity of APC for phospholipid vesicles.[42] Other neutral glycolipids (e.g, LacCer and Gb3Cer) also possess anticoagulant activity as APC-cofactors,[40–42] and the anticoagulant activity of these glycolipids demonstrates specificity for the conformation and composition of the saccharide moieties. Anticoagulant APC cofactor activity appears to be, at least in part, specific for *d*-Glc linked covalently to ceramide because neither *d*-Glc nor ceramide nor galactosylceramide (an isoform of GlcCer) alone show anticoagulant activity.

Thus, certain glycosphingolipids can enhance the anticoagulant protein C pathway and thereby decrease thrombin generation in plasma. The APC-cofactor anticoagulant activity of GlcCer is particularly striking in the context of lipid-clotting factor interactions because GlcCer is an uncharged (neutral) lipid, and neutral lipids are not otherwise known to stimulate the activity of procoagulant or anticoagulant clotting factors.

1.4 | Lyso-sulfatide as an anticoagulant

Lyso-sulfatide (LSF) (Figure 4, Table 1) is an anticoagulant plasma lipid.[32] The amount of LSF in HDL was estimated to be 5 μg per mg of HDL.[43] Assuming a HDL concentration of 1 mg/mL, LSF may be present in the lower micromolar range in plasma which is a concentration at which LSF can begin to prolong clotting times (Table 1). LSF inhibits thrombin generation by prothrombinase in purified reaction mixtures (factor Xa, factor Va, prothrombin and Ca^{2+}) in the presence of PL.[32] LSF also inhibits thrombin generation by prothrombinase when modified factor Xa or modified prothrombin lacking Gla domains (Gla-domainless [des-Gla]-factor Xa or factor Xa/des-Gla-prothrombin) are used for purified system assays.[32] Nonetheless, LSF does not inhibit thrombin generation by factor Xa and factor Va in the absence of PLs,[32] a pattern which differs from factor Xa inhibition by sphingosine or long-chain ACs (see below). PLs alter the properties of factor Xa, so a requirement for phospholipid to alter factor Xa activity is not surprising. Surface plasmon resonance binding studies show that LSF binds both to factor Xa and to desGla-factor Xa. Thus, the binding of LSF to factor Xa which does not require the Gla-domain of factor Xa does correlate with inhibition of Gla-domainless-factor Xa, implying that other domains of factor Xa bind lipids that inhibit its ability to generate thrombin, as also shown for acylcarnitines (see below). As described in the "Thrombosis, Blood Coagulation and Thrombin Generation" section, lipid-Gla domain interactions provide the canonical paradigm for how lipids modulate the coagulation system; hence, binding of physiological lipids to clotting factor domains outside the Gla-domain may provoke a potential paradigm shift. Since this anticoagulant activity can be observed only in the presence of PL, we assume that PL binding to factor Xa causes conformational changes, resulting in greater affinity for LSF. Similarly, PL-induced conformational changes in factor Xa are necessary for binding protein S.[44]

TABLE 1 Summary of soluble sphingolipid and phospholipid effects on thrombin generation

LIPID	Thrombin generation in presence of: plasma	FXa+FVa	FXa alone	Plasma concentration	µmol/L[a]	Reference
Sphinganine	↓	↓	–	0.5 µmol/L	2.5	[31]
Sphingosine	↓	↓	–	0-70 nmol/L	2.5	[31]
Lysosulfatide	↓	↓	↓	~5 µmol/L	4	[32]
Long-chain acylcarnitine	↓	↓	↓	1-2 µmol/L	5-10	[33]
PEA, SEA, AEA	↑	–	–	1-6 nmol/L	0.0002	[59]

The Table summarizes the effects of certain lipids on thrombin generation in plasma or in purified prothrombinase reaction mixtures comprising prothrombin and PL/Ca^{2+} with either factors Xa+Va or only factor Xa (without factor Va). Downward blue arrows indicate inhibition of thrombin generation. Black bars (▬) indicate no effect on thrombin generation in prothrombinase assays. Upward red arrow indicates enhancement of thrombin generation in plasma. Color highlighting in the background indicates whether the lipid inhibits thrombin generation (blue) or enhances thrombin generation (pink). PEA, palmitoyl-ethanolamide; SEA, stearoyl ethanolamide; and AEA, arachidonoyl ethanolamide. Plasma concentrations are indicated as well as the approximate lipid concentrations minimally required for showing their effect in plasma thrombin generation assays.

[a]Indicates minimum concentration required to exert procoagulant or anticoagulant activities in plasma.

It is also notable that sulfatide, the precursor of LSF, may act as a prothrombotic factor[45] and is procoagulant by enhancing the contact activation system.[46] However, sulfatides interact with a variety of proteins which can affect hemostasis and thrombosis, and so no simple single reaction involving sulfatides can be accepted for this lipid's likely physiologic or pathophysiologic effects.[45]

1.5 | Sphingosine and its derivatives as anticoagulants

In 1989, sphingosine was reported to decrease thrombin generation by inhibiting the extrinsic pathway of coagulation.[47] Subsequently sphingosine was shown to inhibit specifically factor Xa at notably lower concentrations.[31] Sphingosine and sphinganine, but not ceramide or sphingosine-1-phosphate, potently inhibit thrombin generation in plasma clotting assays when their concentrations are >1.5 µmol/L.[31] When thrombin generation is monitored either in purified reactions mixtures containing factor Xa, factor Va and prothrombin plus other reactants, sphingosine and sphinganine inhibit prothrombin activation.[31] Similarly, in studies using prothrombinase assays, thrombin generation is also inhibited by glucosylsphingosine (lyso-GlcCer), phytosphingosine (plant sphingosine), lyso-sphingomyelin and primary alkylamines (i.e, an amine attached to a fatty acid side chain) with >10 carbons[31] but not by the related sphingolipids, ceramide or sphingosine-1-phosphate. Acylation of the amino group of several of these anticoagulant lipids ablated anticoagulant activity, implicating the need for a positive charge for anticoagulant activity. Binding studies showed that sphingosine binds to fluorescein-labeled factor Xa but not to fluorescein-labeled, Gla-domainless-factor Xa; so sphingosine binding and inhibition require the Gla-domain. Thus, lipid-factor Xa interactions that cause inhibition of thrombin generation differ for the 3 classes of lipids, i.e, sphingosines, LSF, and acylcarnitines. Hence, distinct mechanisms are involved for each class of anticoagulant lipids.

1.6 | Clinical association

When the association of VTE with GlcCer and PE levels was studied, VTE was associated with plasma GlcCer deficiency but not with any changes in PE plasma levels.[40] Subsequently, we replicated this finding in 2 other VTE case-control studies involving the Scripps VTE Registry (105 cases and 105 controls) and the Valencia Spain VTE Registry (316 cases and 320 controls).[48] The Scripps and Valencia cohorts show that lower plasma GlcCer levels are strongly linked with VTE; e.g, the OR values for 10%-ile cut off were 5.5 (95% CI, 2.4-12) and 2.1 (95% CI, 1.3-3.3), respectively. Thus, low levels of plasma GlcCer are associated with VTE thrombosis, implying GlcCer could be a biomarker for VTE.[48] Mechanistically, this association was hypothesized by Deguchi.[40] to be based on the reduced ability of plasma GlcCer to contribute to APC's antithrombotic activity, leading to hypercoagulability and an increased risk for thrombosis. Decreased immunomodulatory and anti-inflammatory functions of GlcCer[49,50] might also contribute to an increased thrombosis risk.

Additionally or alternatively, the association of low plasma GlcCer level with VTE could be a secondary effect. The dynamic sphingolipid metabolism may be shifted by a variety of factors, including various enzymes that determine levels of sphingosine, ceramide, sphinganine and sphingosine-1-phosphate, GlcCer and ceramide, etc. Low GlcCer association with VTE might reflect altered levels of other sphingolipids which contribute to thrombosis risk. In either case, plasma GlcCer levels might be used for a future biomarker of VTE risk, although more systemic studies are warranted and needed before this could occur.

Discovery that certain sphingolipids exert notable anticoagulant activity implies the association of sphingolipid imbalances with VTE risk. To date, no clinical study has linked alterations in plasma levels of sphingosine or sphinganine with thrombosis. The clinically relevant influences of these lipids on coagulation might be an issue for local

fluctuations in these lipids which are not mirrored in their circulating plasma levels. Further studies of sphingolipids and thrombotic risk are warranted.

2 | PLASMA PHOSPHOLIPID DERIVATIVES, BLOOD COAGULATION, AND VTE

2.1 | Lyso-phospholipid metabolism

Thrombin generation is classically promoted by phospholipid bilayers or membranes containing PS, and prothrombinase activity may be enhanced by PE when membranes have low levels of PS or cardiolipin.[51,52] Plasma contains multiple soluble PL derivatives, e.g, lyso-phospholipids (LPLs) and ethanolamides. LPLs are plasma membrane-derived bioactive lipid composed of a single acyl carbon chain attached to a polar headgroup. The acyl moieties of glycerophospholipids are hydorolyzed into LPLs by hydrolytic enzymes such as phospholipase A2 and phospholipase A1.[53] In contrast, the lysophospholipid acyltransferase family is a putative enzyme superfamily which catalyzes the transfer of acyl-CoAs to LPLs to produce different classes of phospholipids.[54] The most abundant LPL is lysophosphatidylcholine (LPC) present at >100 μmol/L, and most plasma LPLs are reversibly associated with albumin or lipoprotein particles. LPC in liposomes was reported to enhance prothrombinase activity, although the potency for this activity is very modest, only two-fold or so.[55]

Lysophosphatidic acid (LPA) is a dynamic lipid and autotaxin (phospholipase D) generates LPA by hydrolyzing LPCs. Aberrant LPA generation, receptor expression and signaling can lead to initiation, progression and metastasis of cancers. The physiological functions of other LPLs has not been determined, but the average concentrations of other plasma LPLs are ~0.25, 7, 0.25, and 0.06 μmol/L for LPA, lyso-phosphatidylethanolamine, lyso-phosphatidylglycerol and lyso-phosphatidylserine.[56] Their plasma levels can reach 1.5, 35, 1.5, and 1.6 μmol/L in acute coronary syndrome patients.[56] The observation that overexpression of Type V soluble phosphodiesterase A2, which produces more lyso-phospholipids, enhances thrombus formation in mice, might suggest the association of LPLs with thrombus formation.[57] Future studies of the effects of various LPLs on thrombin generation and thormbosis are well warranted.

2.2 | LPLs as procoagulant plasma lipids

The direct association of soluble LPLs with coagulation reactions has not been extensively studied. As far as we know, only a single study of lyso-phosphatidylserine was reported which showed that this lipid enhances prothrombinase activity.[58]

2.3 | Ethanolamides as procoagulants

An untargeted metabolomic study of subjects taking warfarin led to the surprising discovery that ethanolamides are procoagulant.[59] Ethanolamides are naturally occurring saturated N-acylethanolamines (e.g, PEA in Figure 4). N-acyltransferase transfers an acyl group from the sn-1 position of a phospholipid to PE such as PC to form N-acylphosphatidylethanolamine, a precursor of ethanolamides. Several pathways act upon N-acylphosphatidylethanolamine to produce ethanolamides (Figure 3, Figure S1, Table S2).[60–62] Ethanolamide with a longer unsaturated side chain (20:4), arachidonoyl ethanolamide (anandamide, AEA), is one of the most studied ethanolamides as it binds to the cannabinoid receptor.[63] Palmitoyl-ethanolamide (PEA) and stearoyl ethanolamide (SEA), structurally related to AEA, are devoid of affinity for cannabinoid receptors. The role of PEA in inflammation and nociception via a variety of molecular mechanisms has been documented.[64]

In coagulation reaction studies, PEA enhanced TF-induced and recalcification-induced thrombin generation in plasma and the enhancement was observed at a concentration as low as 0.17 nmol/L.[59] When the extrinsic pathway was blocked by anti-factor VII antibodies, PEA still enhaced recalcification-induced thrombin generation, suggesting PEA can act on factors outside of the extrinsic pathway of coagulation system.[59] Other mechanistic studies implied that the enhancement of thrombin generation by PEA is dependent of the contact activation.[59]

The plasma normal concentrations of PEA, SEA, and AEA are approximately 6, 1.5, and 1 nmol/L, respectively,[65,66] and the procoagulant effects of ethanolamides are apparent below or within their plasma concentration ranges. These data suggest that the ethanolamide family of lipids possibly contributes to enhance thrombin generation in plasma. The precursor molecule of PEA (glycerophospho-N-palmitoyl ethanolamine) and analogs of PEA with longer side chains (stearoyl-ethanolamide [SEA]) and AEA also enhance thrombin generation. Other ethanolamide analogs, palmitoyl N-isopropylamide and N-palmitoyl taurine, which are lacking a hydroxyl group in the head group showed little enhancement of thrombin generation, suggesting that that the free hydroxyl group in the head group appears to be a key component for the observed procoagulant activity of certain ethanolamides. The levels of certain ethanolamides are decreased in plasma by warfarin,[59] and an anticoagulant consequent effect of warfarin by decreasing the ethanolamides in plasma might be a minor, but potentially additional anticoagulant property of warfarin.

2.4 | Soluble dicaproyl-PS as procoagulant

Synthetic, soluble dicaproyl-PS (C6PS) potently promotes prothrombinase activity by binding to factor Xa outside the Gla-domain.[29,30,58,67,68] Both the C1 and C2 domains of factor Va bind C6PS, and binding to C1 domain can influence prothrombinase complex assembly.[68] C6PS also appears to promote dimer formation of factor Xa and such dimerization may prevent factor Xa binding to factor Va due competition.[69,70] Furthermore, this short chain lipid binds to factor IXa[71] and protein Z,[72] possibly affecting functions of these molecules. Short chain phospholipids might be produced by oxidation, but this has not been reported to our knowledge; so the pathophysiologic relevance of short chain PLs like C6PS remains unknown.

2.5 | Clinical associations

Ovarian cancer patients who exhibit plasma hypercoagulability and elevated VTE risk[73] have elevated plasma levels of LPA[74,75] implying a potential association of LPA with coagulability and VTE. For instance, the LPA level increases to 43 μmol/L which might contribute to a large increase in thrombin generation. However, the procoagulant activities of LPA and the association of plasma LPA levels with thrombosis remains to be further evaluated as do the relationships between various ethanolamides and thrombosis.

2.6 | Discovery of anticoagulant acylcarnitine lipids related to VTE

Genomics, transcriptomics, proteomics, metabolomics, lipidomics and epidemiological data provide different angles to our understanding of gene-environment interactions and the determinants of disease and health. Metabolomics is a rapidly growing research area and a systems approach for comprehensive and quantitative analysis of the global metabolites in a biological matrix. Plasma contains thousands of metabolites including lipids, hormones, sugars, nucleotides, organic acids, and amino acids. Only minimal data are available for the association of plasma levels of lipids or metabolites with VTE. Studies using mass spectrometry-based metabolomics[76–78] were used to study VTE and discover novel anticoagulant lipids, as described below.

2.7 | Global metabolomics for VTE

Because we had found that certain plasma sphingolipids including glucosylceramide, sphingosine, and LSF are anticoagulant lipids whereas, conversely, some plasma lipids can exert procoagulant properties at physiological levels (see above), we hypothesized that certain soluble plasma lipids or other metabolites that have modest or low abundance might be associated with risk for or protection against VTE. To assess whether patients with VTE have imbalances in such lipids or other metabolites, a mass spectrometry-based, global metabolomics methodology which can detect >3500 plasma metabolomic features[76] was employed to study VTE patients and matched controls in the Scripps VTE Registry. Among key findings, it was discovered that 10:1 and 16:1 ACs are low in plasmas of the VTE patient group compared to matched controls.[79] Data from subsequent targeted metabolomics studies show that several long-chain ACs (10:1, 12:0, 12:2, 18:1, and 18:2) are lower in the VTE group. Conversely, higher level (>75%-ile) of decenoyl carnitine (10:1), dodecadienoylcarnitine (12:2), tetradecadienoylcarnitine (14:2), oleylcarnitine (18:1), and linoleylcarnitine (18:2) were associated with reduced occurence of VTE.[79]

2.8 | AC metabolism

Total long-chain ACs (acyl chains >10 carbons) circulate in plasma, with reported concentrations ranging between 1 and 4 μmol/L,[33,80–82] and their levels can reach to 10-30 μmol/L under certain metabolic conditions.[83–86] Long-chain ACs are lipid metabolites with a hydrophobic side chain and a free amine like sphingosine (Figure 4), but they belong to the long-chain AC family which plays an important role in energy metabolism through mitochondrial β-oxidation of fatty acids.[87] The acylcarnitines and Co-A are produced by the reaction in which acyl moieties are transferred to carnitine from acyl-CoA by carnitine palmitoyl transferase-I (Figure 3, Figure S1, Table S2).[87] Once the fatty acid-carnitine is inside the matrix, carnitine palmitoyl transferase -II then converts the long-chain acylcarnitine and CoA back to long-chain acyl-CoA and carnitine (Figure 3).[87] Although the physiological regulatory mechanisms of plasma ACs levels are poorly understood, studies using peroxisome proliferator activated receptors agonists, e.g, fibrate, suggest that the β-oxidation enhancement in mitochondria (e.g, increase of carnitine palmitoyl transferase-II activity) promoting catabolism of ACs by peroxisome proliferator activated receptors could be one mechanism to reduce plasma ACs level.[88,89]

2.9 | Long chain ACs as anticoagulants

Studies of ACs using thrombin generation assays show that multiple ACs exert remarkable anticoagulant activity. Long chain ACs prolonged clotting in factor Xa-1-stage clotting assays when their concentration was ≥2 μmol/L.[79] Longer acyl chains were more potent anticoagulants than ACs with shorter acyl chains (e.g, the potency ranking for chain length was 18, 16>14, 10>6 acyl chain carbons).[79] Since plasma contains a spectrum of ACs, their ensemble, i.e, the set of long-chain ACs, could potentially influence thrombin generation. Indeed, 16:0 AC inhibited prothrombinase activity in purified reaction mixtures both in the presence and absence of procoagulant PL vesicles.[79] Neither carnitine alone without any side chain nor AC with only a 4 carbon long chain had any prothrombinase inhibitory activity, suggesting that a minimum number of carbons in the aliphatic chain is required for anticoagulant activity.[79] Surprisingly, 16:0 AC inhibited thrombin generation by Gla-domainless (des-Gla)-factor Xa.[79] Surface plasmon resonance binding studies showed that the anticoagulant 16:0 AC was bound to Gla-domainless-factor Xa.[79] The binding affinity of 16:0 AC for factor Xa matched the range of lipid levels that doubled clotting times (circa 10-23 μmol/L). Thus, there are functionally important lipid binding sites on factor Xa located outside the Gla-domain's canonical lipid-binding sites which markedly affect this clotting factor's activity. Although 16:0 AC's anticoagulant activity does not require the Gla domain of either factor Xa or II, it is distinguishable from LSF because PL is required for LSF's anticoagulant effects. Thus, 16:0 AC seems to act, at least in part, via different binding sites in factor Xa than does LSF. As noted above, this mechanism for AC's anticoagulant effect differs from that for anticoagulant sphingosine which requires the factor Xa Gla-domain.

2.10 | Clinical association

Multiple ACs are lower in VTE cases than controls, as noted above.[79] Now there is an obvious need to replicate these findings in different cohorts to establish very firmly the association of lower ACs with VTE risk.

3 | CONCLUDING REMARKS

A variety of minor abundance soluble plasma lipids manifest either procoagulant or anticoagulant properties by affecting thrombin generation (Figure 2A). Biochemical studies of plasma lipids and thrombin generation show that functionally important lipid binding sites are located on factor Xa outside its N-terminal Gla-domain[32,33] (Factor 2B) which historically has been to only region on factor Xa widely accepted to bind lipids. Thus, the canonical paradigm for clotting factor-lipid interactions needs to evolve to include functionally important lipid-protein interactions independent of membrane binding sites. This insight may lead to explorations of whether or how lipid metabolites may affect blood coagulation reactions and risks for thrombosis. Some case-control studies[33,40,48] suggest that plasma GlcCer or long-chain ACs levels might possibly be useful as new biomarkers for VTE risk, although such findings still need replication by clinical studies with larger cohorts. Further, this potential new concept might lead to the future developments of new classes of anticoagulant drugs. Traditionally, the anticoagulant warfarin therapy targeted only the Gla domain of clotting factors.[20] There is increasing interest in direct inhibitors of factor Xa which are recently becoming more widely used for anticoagulant therapy.[90] Since lipid metabolism is so dynamic in vivo, the anticoagulant lipids themselves might not be suitable as drug candidates. However, modified anticoagulant lipid molecules with longer half-lifes which maintain anticoagulant activity might be developed as drug candidates and studied. The binding regions on coagulation factors for anticoagulant or procoagulant lipids might provide novel targets for further efforts for rationale drug development. For instance, antibodies or other small molecules targeting factor Xa binding sites for ACs might merit exploration.

The pathophysiologic relevance of minor abundance plasma lipids that alter clotting assays in vitro remains a very open issue for future preclinical and clinical studies and discussions. For some of these lipids, the systemic circulating level is in the concentration range for potentially having effects on thrombin generation. Moreover, the circulating level of a metabolite is not the only determinant of a metabolite's effects. It remains possible, if not likely, that local concentrations of certain lipids could markedly fluctuate. For example, lipid levels near a dying cell or near cell-derived enzymes could vary greatly. Thus, given current systemic levels and the possibilities for locally increased lipid levels, one can entertain the thought that these lipids could exert physiologic pathophysiologic actions.

The various clotting activities of certain plasma lipids reviewed here may suggest new mechanisms for cross-talk between inflammation and coagulation involving alterations of these lipids. For example, redistributions in levels of certain lipid metabolites via the highly dynamic sphingolipid-phospholipid metabolic networks[37,38] (Figure 3, Figure S1, Table S2) might act to regulate inflammatory events such as cell proliferation at the same time they act to alter thrombin generation. Notably, a variety of enzymes that regulates lipid metabolism are capable of shifting the balances between sphingosine and ceramide, sphingosine and sphingosine-1-phosphate, glucosylceramide and ceramide, etc.[37,38] The sphingolipid rheostat which influences the balance between cell survival and cell death might also provide a rheostat for lipids which influence thrombin generation and/or the activity of activated protein C — 2 proteins which themselves can influence inflammation, cell survival and apoptosis.[16,91] Lipid-dependent cross-talk might thus contribute to integrate inflammation and coagulation.

AUTHOR CONTRIBUTIONS

J.H. Griffin, D.J. Elias, and H. Deguchi wrote the manuscript and each gave final approval to the revised manuscript.

RELATIONSHIP DISCLOSURES

None of the authors have any disclosures relevant to this paper.

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How to cite this article: 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. https://doi.org/10.1002/rth2.12017