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BRIEF REPORT

Platelet lipidomics indicates enhanced thrombocyte activation in patients with antiphospholipid syndrome *in vivo*

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Abstract

Background: Antiphospholipid syndrome (APS) is an autoimmune disorder characterized by the presence of antiphospholipid antibodies in patients with thromboembolic/thromboinflammatory events and/or obstetric complications.

Objectives: The aim of this study was to examine whether there are alterations in the platelet lipidome of APS patients in comparison with patients affected by thromboembolism without APS (control) and healthy volunteers.

Methods: We applied quantitative mass spectrometry-based lipidomics to investigate the platelet lipidome of isolated resting and thrombin-stimulated platelets as well as platelet release in patients with APS, controls, and healthy volunteers.

Results: Lipidomic data revealed an increase in lysophospholipids (LPLs) in platelets from APS patients, specifically in lysophosphatidylcholine and lysophosphatidylethanolamine species. As LPLs are cleavage products generated by phospholipase A (PLA) from the corresponding phospholipid precursor, LPL/phospholipid ratios may be employed as surrogates for PLA1 and PLA2 activities. The surrogate ratios for PLA2, which participates in the release of arachidonic acid during platelet activation, were significantly increased in APS in both resting platelets and upon thrombin-induced activation for phosphatidylcholine and phosphatidylethanolamine. The phosphatidylcholine-PLA2 surrogate ratio was found to correlate with serum levels of anti–β2-glycoprotein I and anticardiolipin immunoglobulin G. Finally, receiver operator characteristic analysis demonstrated excellent discrimination of patients with APS from controls and healthy volunteers.

Conclusion: These findings provide substantial evidence that platelet activation is enhanced in APS *in vivo*, involving the activation of PLA2.

KEYWORDS
antiphospholipid syndrome, blood platelets, lipidomics, platelet activation, platelet function tests

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Gerhard Liebisch and Christina Hart contributed equally to this study.

1 | INTRODUCTION

Antiphospholipid syndrome (APS) is an immune-mediated, thromboinflammatory disorder characterized by the presence of antiphospholipid (aPL) antibodies (Ab) in patients with thromboembolic events and/or obstetric complications (reviewed by Giannakopoulos and Krilis [1] and Knight et al. [2]). aPL-Ab comprising anticardiolipin (aCL)-Ab, anti–β2-glycoprotein I (aβ2GPI)-Ab, and the functionally measured lupus anticoagulant (LA), are the key drivers of APS pathophysiology and responsible for the elevated risk of thromboembolism that is highest in patients who are triple positive for all 3 standard assays (reviewed by Arreola-Diaz et al. [3]). In the context of hypercoagulability, a variety of mechanisms affecting the activation of endothelial cells, monocytes, neutrophils, and platelets mainly by aβ2GPI have been described [2]. In particular, platelets are thought to be the major target of aPL-Ab (reviewed by Salet et al. [4]), and platelet activation may play a crucial role in the development of arterial and venous thromboembolism in APS. Despite strong *in vitro* evidence for aβ2GPI-mediated platelet activation [5–7], limited *in vivo* data are available. Here, we used quantitative mass spectrometry-based lipidomics to investigate alterations in the platelet lipidome, in particular lipid markers of platelet activation, in patients with APS.

2 | METHODS

2.1 | Patients' characteristics and ethical approval

Platelets were obtained from patients and healthy volunteers in whom the use of antiplatelet drugs had to be paused for at least 10 days. Patients with APS had to have at least 1 venous or arterial thromboembolic event diagnosed at least 12 weeks before study inclusion and had to have a stable presence of moderate to high titer aCL and aβ2GPI of immunoglobulin G (IgG) or immunoglobulin M (IgM) isoform and the detection of a functionally measured LA according to the classification criteria and the laboratory criteria for APS [8,9]. According to the modified Sapporo criteria, all patients were classified as triple-positive [9], and 85% of all patients fulfilled the criteria for triple positivity when using the American College of Rheumatology/

TABLE 1 Main characteristics of the patient population and the healthy volunteers.

	Patients with APS	Thrombosis patients without APS (controls)	Healthy volunteers
No. of participants	20	20	16
Sex, *n* (%)			
Female	15 (75)	10 (50)	10 (62.5)
Male	5 (25)	10 (50)	6 (37.5)
Age (y)			
Mean	46.3	45.8	41.1
SD	14.0	13.1	9.8
Range	23-64	27-61	27-56

APS, antiphospholipid syndrome.

TABLE 2 Patients' characteristics based on thromboembolic events, anticoagulation, and laboratory values.

	Patients with APS	Thrombosis patients without APS (controls)
No. of participants	20	20
Clinical manifestation of hypercoagulability, *n* (%)		
Venous thromboembolism	14	19
1 event	7 (50)	19 (95)
2 events	5 (36)	
3 events	2 (14)	
Arterial thromboembolism	9	1 (5)
In addition to a venous thromboembolic event	3 (33)	
Solely arterial thromboembolism	6 (67)	1 (100)
Anticoagulation at the time of blood sampling, *n* (%)		
Yes	19 (95)	19 (95)
VKA	16 (84)	2 (11)
DOXa-I	2 (11)	16 (84)
LMWH	1 (5)	1 (5)
Laboratory values		
Thrombocytes per nL	262 (102-353)	262 (154-417)
Anticardiolipin IgG (CU), median, range	232 (5-4926)	[b]
Anticardiolipin IgM (CU), median, range	10 (2-2085)	[c]
aβ2GP IgG (CU), median, range	682 (23-11 449)	[c]
aβ2GP IgM (CU), median, range	7 (1-631)	[c]
Presence of lupus anticoagulant,[a] *n* (%)	20 (100)	0 (0)

aβ2GP, anti–β2-glycoprotein; APS, antiphospholipid syndrome; CU, chemiluminescent unit; DOXa-I, direct oral Xa inhibitor; IgG, immunoglobulin G; LMWH, low-molecular-weight heparin; VKA, vitamin K antagonist.

[a] Laboratory testing was performed according to the laboratory criteria for APS [1,2].

[b] Two patients had values slightly above the cutoff, whereas the values in all other patients were under the cutoff (<20 CU) of the manufacturer's instructions.

[c] All laboratory values were under the cutoff (<20 CU) of the manufacturer's instructions.

European Alliance of Associations for Rheumatology criteria, published in 2023 [10]. In 90% of the patients with APS, there was no underlying disease, and the APS occurred as a primary condition. Patients affected by venous thromboembolism with no clinical or laboratory evidence for APS were additionally included in the study. Nineteen out of 20 patients (95%) with APS and without APS, respectively, were under anticoagulation at the time of blood sampling. Patients with APS were mostly treated with the vitamin K antagonist phenprocoumon (16/19; 84%). The intake of direct oral anticoagulants (DOACs) had to be paused for at least 48 hours before blood sampling; otherwise, the plasma sample was pretreated with DOAC Stop (Haematex) to remove the interference of DOACs on

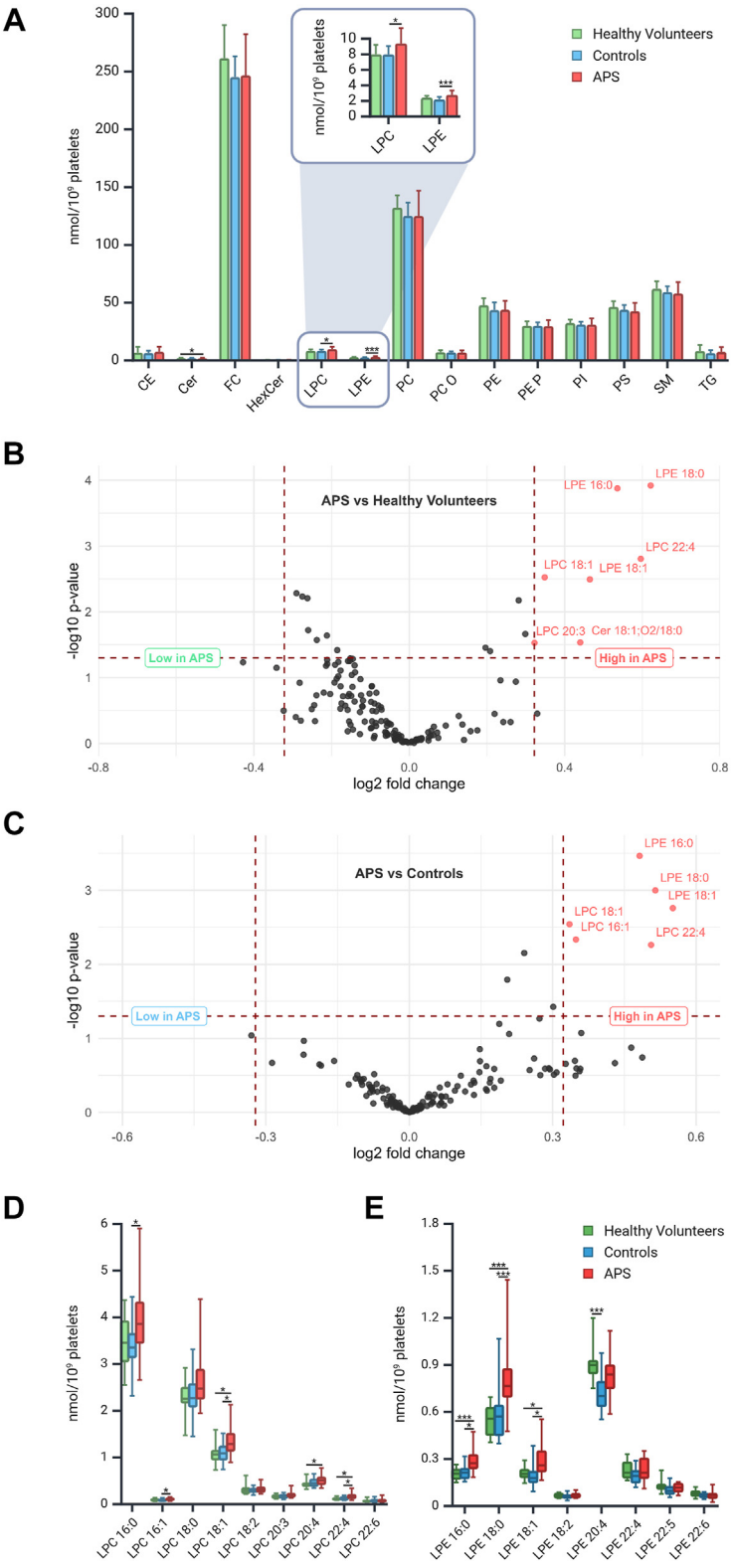

FIGURE 1 Lysophospholipid concentrations are increased in platelets of antiphospholipid syndrome (APS) patients. Platelets of healthy volunteers (n = 16), thrombosis patients without APS (controls; n = 20), and APS patients (n = 20) were isolated by washing and subjecting to mass spectrometry-based quantitative lipidomics. (A) Lipid class concentrations in resting platelets. Volcano plots comparing concentrations of all 166 measured lipid species in (B) APS patients and healthy volunteers and (C) APS patients and controls. The dashed lines indicate $P \leq .05$ (y-axis; Student's t-test) and fold changes ± 25% (x-axis). (D) Lysophosphatidylcholine (LPC) and (E) lysophosphatidylethanolamine (LPE) lipid species concentrations. Data are expressed as mean ± SD in bar graphs or as median ± min/max in box plots. Groups were compared using the Kruskal–Wallis test followed by the *Dunn–Bonferroni post hoc test*. The figure was created with BioRender.com. CE, cholesteryl ester; Cer, ceramides; FC, free cholesterol; HexCer, hexosylceramides; PC, phosphatidylcholine; PC O, PC ether; PE P, phosphatidylethanolamine-based plasmalogens; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; TG, triglycerides. *P < .05; ***P < .001.

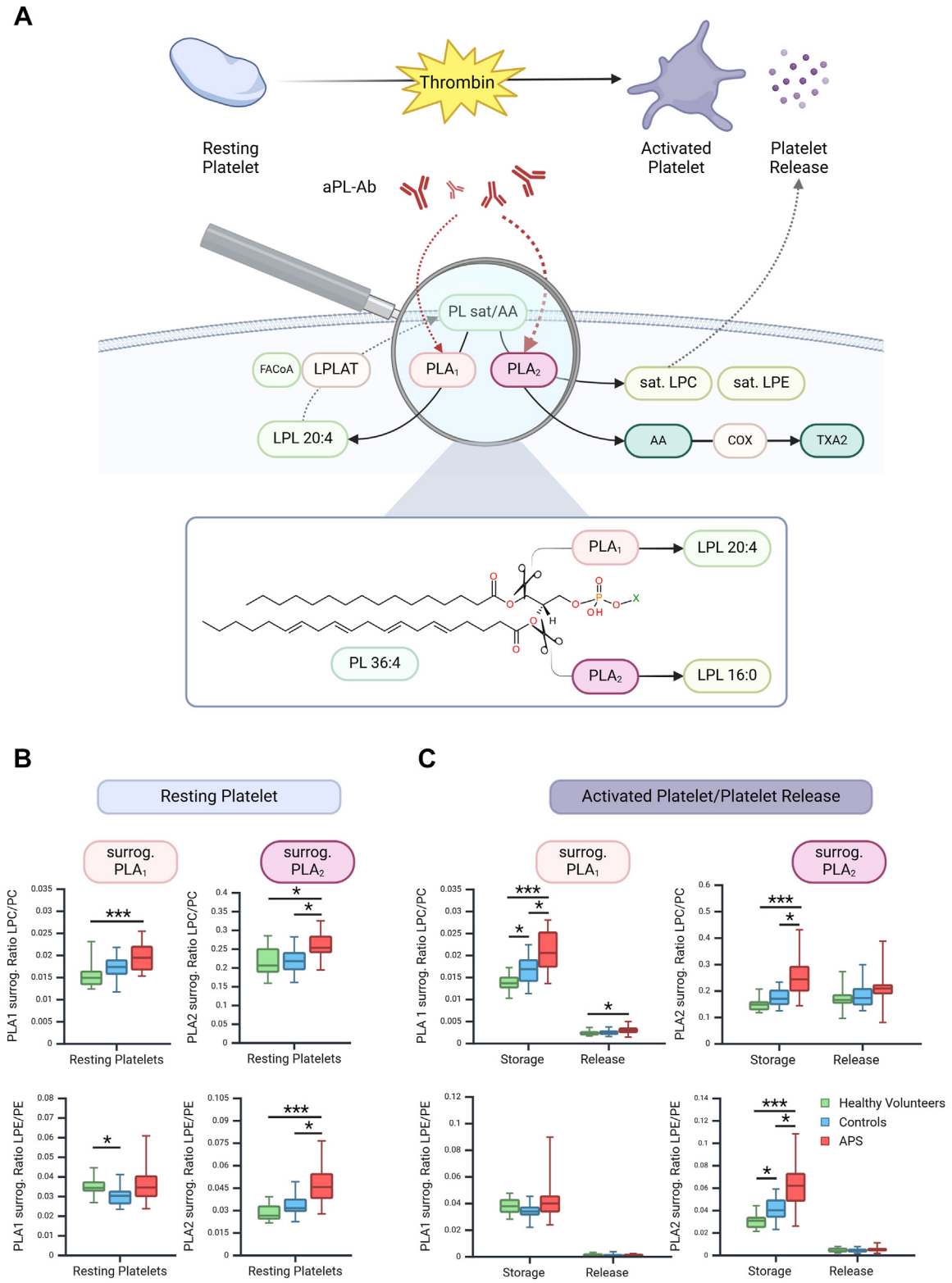

FIGURE 2 Phospholipase A (PLA) surrogate (surrog.) ratios are increased in resting and activated antiphospholipid syndrome (APS) platelets. (A) Analysis strategy associated with the APS model: quantitative lipidomes were analyzed from resting platelets, activated platelets, and platelet release after thrombin stimulation. It is hypothesized that platelets are stimulated by antiphospholipid antibodies (aPL-Ab), resulting in activation of PLA. PLA2 releases arachidonic acid (AA) as a precursor for cyclooxygenase (COX)-mediated thromboxane A2 (TXA2) generation from the *sn2* position of AA-containing phospholipids (PLs, saturated [sat]/AA) as well as sat lysophospholipids (LPL), including lysophosphatidylcholine (LPC) and lysophosphatidylethanolamine (LPE). Generated sat. LPC is released while sat LPE remains intracellular. PLA1 action leads to the formation of AA-containing LPL 20:4, which are not released but re-esterified with CoA-activated fatty acids (FACoA) by LPL acyltransferase (LPLAT) to serve as a source of AA. (B and C) LPL product and PL precursor ratios were calculated as surrog. ratios for

clotting assays [11]. Patients' characteristics are depicted in Tables 1 and 2.

The study was approved by the local Ethics Committee (Universitätsklinikum Regensburg, Ethikkommission der medizinischen Fakultät, proposal 20-1836-101). All subjects had given their informed consent to participate in the study. All research was performed in accordance with the relevant guidelines and regulations.

2.2 | Quantification of aPL and LA

aCL-Ab and aβ2GPI-Ab of IgG or IgM were quantified in serum on BIO-FLASH Chemiluminescent Analyzer (Inova Diagnostics, Werfen) using aCL IgM, aCL IgG, β2-glycoprotein I (β2GPI) IgM, and β2GPI IgG QUANTA Flash Reagents (Inova Diagnostics, Werfen) according to the manufacturer's instructions.

The presence of LA was detected by 2 different assay principles: diluted Russell viper venom time (LA1 Screening Reagent/LA2 Confirmation Reagent, Siemens Healthineers) and activated partial thromboplastin time (HemosIL APTT-SP/HemosIL SynthAFax, Instrumentation Laboratory, Werfen). All clotting tests were analyzed on BCS XP System (Siemens Healthineers).

2.3 | Blood count

Blood count including platelet count was analyzed on a hematology analyzer (XN-10/XN-20, Sysmex Corporation) according to the manufacturer's instructions.

2.4 | Donor platelet isolation

For platelet isolation, venous whole blood was collected into 3.2% sodium citrate tubes (S-Monovette, Sarstedt) by aspiration technique using 21-gauge butterfly needles. Platelet-rich plasma was obtained by centrifugation (139*g* for 10 minutes). Platelets were obtained by centrifugation at 900*g* for 10 minutes and at 800*g* for 10 minutes and resuspension in calcium-free Tyrode-Hepes buffer (138 mmol/L NaCl, 3 mmol/L KCl, 12 mmol/L $NaHCO_3$, 0.4 mmol/L NaH_2PO_4, 1 mmol/L $MgCl_2$, 5 mmol/L glucose, 10 mmol/L Hepes, 10 mmol/L ethylenediaminetetraacetic acid, and 0.5% [weight/volume] bovine serum lipid-free albumin, pH 7.4, sterile-filtered). The cell number of separated platelets was quantified by a hematology analyzer (XN-350, Sysmex Corporation) and was adjusted to 100/nL. One milliliter of the adjusted platelets was frozen immediately at −80 °C, and 1 mL was stimulated by thrombin described below.

2.5 | Platelet activation

One milliliter (corresponding to 100×10^6 platelets) of the adjusted platelets was recalcified with $CaCl_2$ and activated by incubation with 1 U/mL bovine thrombin for 15 minutes at 37 °C. After stimulation, platelets were pelleted by centrifugation at 5000*g* for 5 minutes and 10 000*g* for 1 minute and resuspended in Tyrode-Hepes buffer. The supernatant and resuspended pellet were stored at −80 °C until lipid analysis.

2.6 | Lipid mass spectrometry

Lipid extraction was performed according to the method of Bligh and Dyer [12] in the presence of not naturally occurring lipid species as internal standards. Platelet homogenates and lipids corresponding to 40×10^6 platelets were extracted.

The analysis of lipids was performed by direct flow injection analysis (FIA) using a triple quadrupole mass spectrometer (FIA-QQQ) and a hybrid quadrupole-Orbitrap (Thermo Fisher Scientific) mass spectrometer (FIA-FTMS).

FIA-QQQ was performed using the analytical setup and strategy described previously [13]. The following lipid classes were quantified by FIA-QQQ: phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), PE-based plasmalogens (PE P), lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), sphingosine-based ceramides (Cer), and hexosylceramides (HexCer).

The Fourier transform mass spectrometry (FIA-FTMS) setup is described in detail by Höring et al. [14]. The following lipid classes were quantified by FIA-FTMS: triglycerides (TG), cholesteryl ester (CE), phosphatidylcholine (PC), sphingomyelin (SM), and free cholesterol (FC) [15].

Lipid species were annotated according to the latest proposal for shorthand notation of lipid structures derived from mass spectrometry [16]. For FIA-QQQ, glycerophospholipid species annotation was based on the assumption of even-numbered carbon chains only.

Details of the lipidomics analysis are described in the Lipidomics Reporting Checklist [17], available online for FIA-FTMS (https://doi.org/10.5281/zenodo.14942994) and FIA-QQQ (https://doi.org/10.5281/zenodo.14942972).

2.7 | Statistical analysis

Data are expressed as mean ± SD in bar graphs or as median ± min/max in box plots. Statistical analysis was performed using IBM SPSS Statistics 22 software. Groups were compared using the Kruskal–Wallis test, followed by *Dunn's nonparametric comparison for post hoc testing*. The *P* values were corrected for multiple testing using

PLA activities. Surrog. ratios were calculated as the ratio of LPL 20:4 and the sum of LPL 16:0 and 18:0 to the sum of their precursors PL 36:4 and 38:4 for PLA1 and PLA2, respectively. The upper graphs show the ratio of LPC/phosphatidylcholine (PC), and the lower graphs show the ratio of LPE/phosphatidylethanolamine (PE) in (B) resting platelets, (C) activated platelets, and platelet release. Data are expressed as median ± minimum/maximum in box plots. Groups (healthy volunteers [n = 16], thrombosis patients without APS [controls; n = 20], and APS patients [n = 20]) were compared using the Kruskal–Wallis test followed by the *Dunn–Bonferroni post hoc test*. The figure was created with BioRender.com. $^*P < .05$; $^{***}P < .001$.

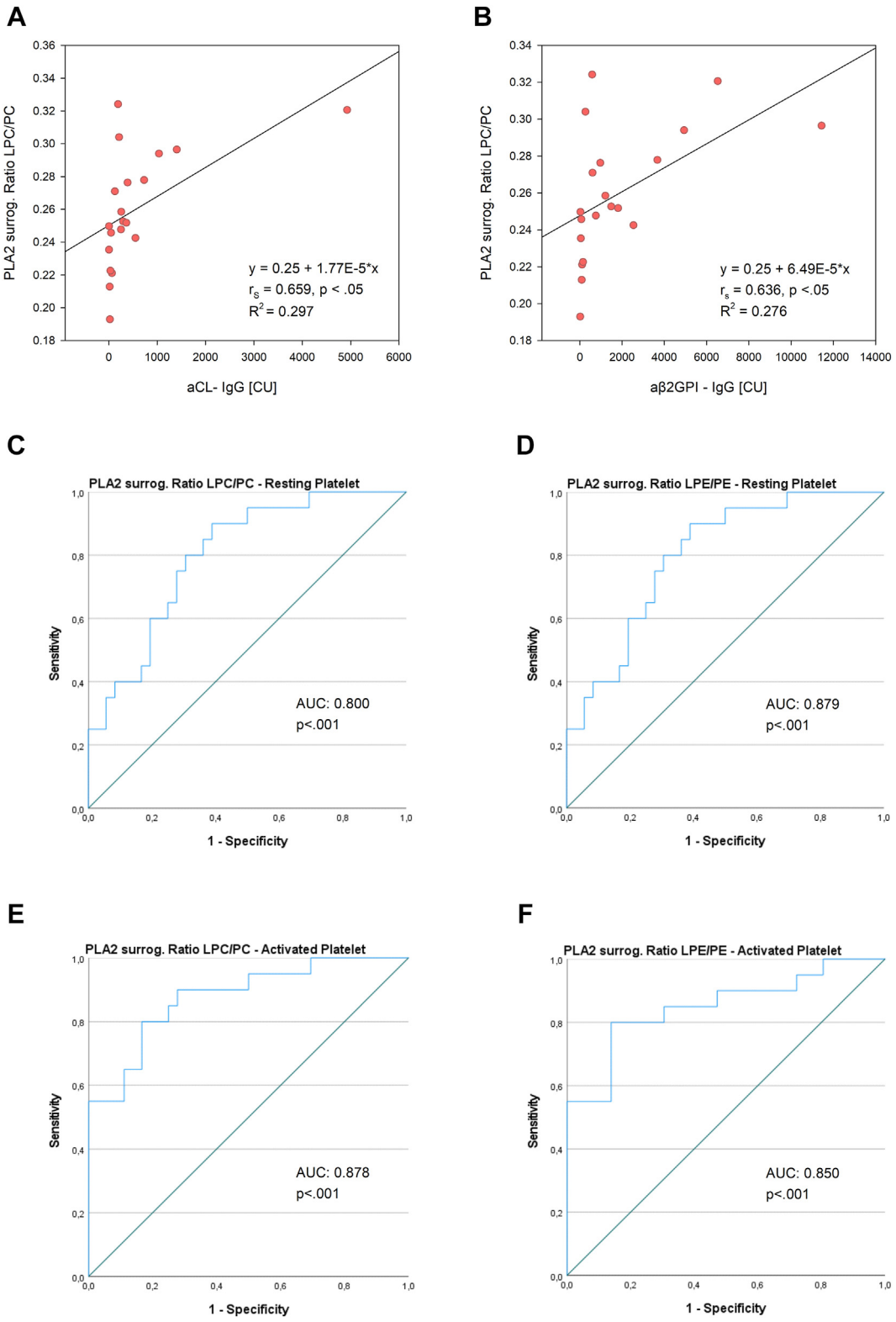

FIGURE 3 Increased phospholipase A2 (PLA2) surrogate (surrog.) ratio correlates with antiphospholipid antibodies and discriminates antiphospholipid syndrome. Correlation of lysophosphatidylcholine (LPC)/phosphatidylcholine (PC) PLA2 surrog. ratios in resting platelets with (A) aCL-Ab and (B) aβ2GPI-Ab. Receiver operator characteristic curve discrimination of antiphospholipid syndrome patients from healthy volunteers and controls using PLA2 surrog. ratios for (C) LPC/PC in resting platelets, (D) lysophosphatidylethanolamine (LPE)/phosphatidylethanolamine (PE) in resting platelets, (E) LPC/PC in activated platelets, and (F) LPE/PE in activated platelets. Correlations were analyzed by Spearman's method. Receiver operator characteristic curves were generated using IBM SPSS Statistics 22 software. AUC, area under the curve; CU, chemiluminescent unit; IgG, immunoglobulin G.

the Bonferroni procedure. Correlations were analyzed by Spearman's method. Receiver operator characteristic curves were generated with IBM SPSS Statistics 22 software. A P value <.05 was considered statistically significant.

Volcano plots were generated with RStudio (version 2023.6.2; Integrated Development Environment for R. RStudio) using R (version 4.1.2; A Language and Environment for Statistical Computing. R Foundation for Statistical Computing). A Student's t-test was performed for the calculation of P values.

3 | RESULTS AND DISCUSSION

3.1 | Lysophospholipids are increased in APS platelets

Platelets were isolated from healthy volunteers (n = 16), thrombosis patients without APS (controls; n = 20), and triple-positive APS patients (n = 20) and subjected to quantitative lipidomics (Supplementary Figures S1-S14). Lipid class concentrations were largely similar in all 3 groups except for ceramides and the lysophospholipids (LPLs) lysophosphatidylcholine (LPC) and lysophosphatidylethanolamine (LPE), which were significantly increased in APS platelets (Figure 1A). In the next step, we evaluated the differences in all 166 quantified lipid species and classes (Figure 1B, C) and found a consistent significant increase in LPE 16:0, LPE 18:0, LPE 18:1, LPC18:1, and LPC22:4 concentrations in APS platelets compared with both healthy volunteers and control thrombosis patients (Figure 1D, E).

LPC and LPE are products of phospholipase A (PLA) action. In particular, PLA2 liberates arachidonic acid (AA) that is required for cyclooxygenase 1–mediated synthesis of thromboxane A2, which in turn leads to further platelet activation and vasoconstriction (reviewed by Badimon et al. [18]). As a surrogate for the PLA activity, we calculated the respective ratios of LPC and LPE to their most likely precursor phospholipids (PLs) PC and PE, ie, 36:4 and 38:4 for LPL 16:0 and 18:0, respectively. Typically, polyunsaturated acyl chains are in the *sn2* position, which is cleaved by PLA2 to give LPL 16:0/18:0 and AA, whereas PLA1 action leads to LPL 20:4 (Figure 2A). In principle, LPL/PL ratio could also be influenced by the reacylation rate of LPL. Although such an enzymatic activity has been found in platelet microsomes to preferentially synthesize AA-containing PLs [19], it is unclear whether LPL reacylation plays a role in activated platelets. The surrogate ratios for PLA2 activity in resting platelets were significantly increased in APS compared with thrombosis patients without APS and healthy individuals for both LPC/PC and LPE/PE. For PLA1, an elevated ratio in APS was found only for LPC/PC (Figure 2B).

3.2 | LPL/PL ratio is increased in APS platelets upon activation

Although the elevated PLA surrogate ratios indicated platelet stimulation *in vivo*, they did not prove that LPL are derived solely from platelet PLA activity because LPL such as LPC 16:0 and 18:0 are major plasma lipid species [20] that could be stored in platelets, eg, in the open canalicular system [21]. Therefore, isolated platelets were subjected to thrombin activation, and lipid changes/release in the activated platelets and supernatant were monitored using a previously validated assay [21] (Figure 2A).

Of note, we detected significantly increased PLA2 surrogate ratios for both LPC/PC and LPE/PE in activated platelets from APS patients (Figure 2C). In addition, the PLA1 surrogate ratio for PC and its product LPC 20:4 was elevated in APS platelets, which, in contrast to saturated LPC, was only scarcely released. This may be explained by the fact that LPC 20:4 serves as a valuable source of AA, which is channeled to re-esterification via the Lands cycle (reviewed by O'Donnell [22]).

Although the interpretation of lipid class-specific release and PLA activity will require additional studies, these data provide strong evidence for enhanced PLA2-mediated AA generation in APS platelets *in vivo* (Figure 2B) and upon activation (Figure 2C). These findings are consistent with *in vitro* data demonstrating an aPL-Ab mediated increase in thrombin-induced platelet activation and thromboxane B2 (the inactive metabolite of thromboxane A2) generation [23–25], as well as increased levels of the major thromboxane metabolite, 11-dehydrothromboxane B2, in the urine of APS patients [24,26]. Moreover, our data are in good accordance with data of Lauder et al. [27], demonstrating increased hydroxyeicosatetraenoic acid PLs as a potential marker of PLA activity in basal and thrombin-activated platelets of APS patients.

3.3 | Increased PLA2 surrogate ratio correlates with APS antibodies and has diagnostic potential in discriminating APS

The next step was to analyze whether PLA2 surrogate ratio in APS correlates with aPL-Ab levels. *In vitro* studies have shown that aβ2GPIs play a central role in platelet activation in APS. These antibodies induce dimerization of β2GPI, resulting in an increased affinity for PLs and adherence to platelets. This process is mediated by at least 2 receptors, the apolipoprotein E receptor 2' and glycoprotein Ibα [5,28,29]. Both receptors are required for platelet activation by dimeric β2GPI [7].

Therefore, we correlated aβ2GPI- and aCL-Ab levels with PLA2 surrogate ratios. In resting platelets, PLA2 ratios for LPC/PC correlated significantly with both aCL-IgG (r = 0.659; P = .002) and aβ2GPI-IgG (r = 0.636; P = .003; Figure 3A, B; Supplementary Table S1). Nevertheless, aβ2GPI-IgG has been demonstrated to be the only aPL antibody capable of activating platelets *in vitro* [30]. Given the strong correlation observed between aβ2GPI-IgG and aCL-IgG in our dataset (r = 0.965; P < .001), it is plausible that the correlation between LPC/PC ratios and aCL-IgG may be largely attributed to the influence of aβ2GPI-Ab rather than aCL-Ab. In contrast to LPC/PC species, no correlations were found with LPE/PE species, suggesting a more prominent role for PLA2-mediated PC degradation in APS pathophysiology.

Finally, we tested whether PLA2 surrogate ratios could be used to differentiate APS patients from healthy controls and patients with other causes of venous thromboembolism. Receiver operator characteristic curves for PLA2 surrogate ratios for LPC/PC and LPE/PE had an area under the curve (AUC) of 0.800 and 0.879 in resting platelets (Figure 3C, D) and 0.878 and 0.850 in activated platelets (Figure 3E, F), respectively. The superior AUC for LPC/PC in activated platelets may be related to residual plasma lipids trapped in the open canalicular system of resting platelets, which are likely to be released during platelet activation [21]. In contrast to LPE, LPC represents a major plasma lipidome fraction that may influence LPC/PC ratio and, consequently, the discrimination power. The high AUC observed for all PLA2 surrogate ratios highlights the enhanced thrombocyte activation as a feature of APS patients.

4 | CONCLUSION

In conclusion, quantitative platelet lipidomics provides strong evidence for aPL-Ab mediated platelet activation, specifically of PLA2, both *in vivo* and *ex vivo*, upon thrombin stimulation in APS patients. Surrogate PLA2 ratios discriminate APS patients not only from healthy controls but also from patients with thrombotic events but without APS. Larger study cohorts are needed to evaluate whether PLA2 surrogate ratios could be used for treatment stratification. *In vitro* studies are needed to understand PLA actions and their lipid class and species specificity in aPL-mediated pathophysiology. These findings support the necessity for future therapeutic strategies targeting platelet activation and thromboinflammation in patients with APS, with the objective of reducing the risk of venous and arterial thromboembolism.

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AUTHOR CONTRIBUTIONS

Conceptualization: C.H., S.H., G.L.; Methodology: R.B., M. Höring, G.L., A.S.; Formal analysis and investigation: M. Höring, S.H., G.L.; Writing—original draft preparation: S.H., G.L.; Writing—review and editing: M. Höring, R.B., M. Höpting, C.H.; Funding acquisition: G.L.; Resources: C.H., M. Höpting, S.H., R.B.; Supervision: C.H., S.H., G.L. All authors read and approved the final manuscript.

DECLARATION OF COMPETING INTERESTS

There are no competing interests to disclose.

DATA AVAILABILITY

For lipidomics raw data, please contact gerhard.liebisch@ukr.de. Lipidomics reporting checklist and further clinical and lipidomic data may be found in a data supplement available with the online version of this article.

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SUPPLEMENTARY MATERIAL

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