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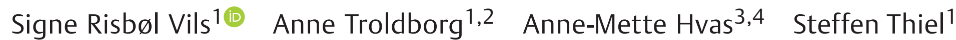
Platelets and the Lectin Pathway of Complement Activation in Patients with Systemic Lupus Erythematosus or Antiphospholipid Syndrome



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Abstract

Background Patients with systemic lupus erythematosus (SLE) have an increased risk of thrombosis even when they do not have antiphospholipid syndrome (APS). Interactions between complement activation and activated platelets have been suggested in SLE and APS and could play a role in the increased thrombosis risk.

Objectives To explore factors potentially related to the prothrombotic pathophysiology in patients with SLE, primary APS, and healthy controls, by investigating lectin pathway proteins (LPPs), complement activation, platelet aggregation, and platelet activation.

Methods This cross-sectional cohort study included 20 SLE patients, 17 primary APS, and 39 healthy controls. Flow cytometry and light transmission aggregometry were used to assess platelet activation and aggregation. Using time-resolved immunofluorometric assays, the plasma concentrations of 11 LPPs and C3dg, reflecting complement activation, were measured.

Results H-ficolin plasma concentrations were higher in SLE and APS patients than in controls ($p = 0.01$ and $p = 0.03$). M-ficolin was lower in SLE than in APS ($p = 0.01$) and controls ($p = 0.03$). MAp19 was higher in APS patients than in SLE patients ($p = 0.01$) and controls ($p < 0.001$). In APS patients, MASP-2 and C3dg correlated negatively with platelet activation. Platelet-bound fibrinogen after agonist stimulation and C3dg concentrations correlated negatively with platelet activation.

Conclusion We observed significant differences between SLE and APS patients regarding complement proteins and platelet activation. Particularly the negative correlations between MASP-2 and C3dg with platelet activation only observed in APS patients suggest that interactions between complement activation and platelets differ in SLE and APS.

Keywords

- platelets
- complement activation
- lectin
- systemic lupus erythematosus
- antiphospholipid syndrome

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Introduction

Systemic lupus erythematosus (SLE) and antiphospholipid syndrome (APS) are chronic autoimmune diseases. APS is characterized by venous and/or arterial thromboses, or placenta-mediated pregnancy complications, combined with the persistent presence of antiphospholipid antibodies (aPLs).[1] APS can be primary and secondary. Secondary APS is related to autoimmune diseases like SLE.[2] Among patients with SLE, 30 to 40% have aPLs, and 10 to 15% have clinical APS.[3] SLE primarily affects young women. It is a heterogeneous disease with manifestations in multiple organ systems, including the kidneys, skin, central nervous system, lungs, joints, and cardiovascular system.[4]

Thromboembolic disease is one of the leading causes of death among patients suffering from SLE or APS.[5,6] Patients suffering from SLE develop thromboembolic disease earlier and more frequently than the general population. They have a three to four times higher risk of developing venous thromboembolism than age- and gender-matched healthy individuals.[7] In a cohort of 1,000 APS patients from 13 different countries, 31.7% experienced deep vein thrombosis, 9.1% pulmonary emboli, and 13.1% experienced stroke.[3]

The increased thromboembolic risk in SLE and APS is strongly associated with the presence of aPL.[8] However, despite the strong association, the mechanism of aPL-mediated thrombosis has not been fully elucidated.[9] New research suggests a multifactorial mechanism of thrombosis, and experimental animal models and in vitro studies indicate the involvement of multiple cellular and inflammatory systems, including platelets and the complement system.[10,11]

Complement activation occurs through three distinct pathways, the classical, the lectin, and the alternative pathway, ultimately leading to the cleavage of C3 to bioactive C3a and C3b. This initiates the common terminal pathway, leading to the membrane attack complex.[12] Activation of the complement system leads to inflammation, opsonization, phagocytosis, and adaptive immune system activation.[13] Although normally well-regulated, the system can be both inefficient, contributing to susceptibility to infections, and overstimulated, furthering autoimmunity, inflammation, and possibly thrombosis.[13]

The lectin pathway of the complement system includes five pattern-recognition molecules (PRMs), including mannan-binding lectin (MBL; mannose-binding lectin), M-ficolin (ficolin-1), L-ficolin (ficolin-2), H-ficolin (ficolin-3), and CL-LK. CL-LK is found in plasma as a heteromer of the two CL-L1 and CL-K1 polypeptide chains. MBL-associated serine proteases 1, 2, and 3 (MASP-1, MASP-2, and MASP-3) are in complex with the PRMs and are activated upon binding of the PRMs to a fitting target, e.g., microorganisms or altered self-structures. Additionally, MBL-associated proteins 44 (MAp44) and 19 (MAp19) are associated with the PRMs.[14]

The role of the complement system in SLE is well-known, and its importance is underlined by its inclusion in the SLE classification criteria.[15] In APS, recent studies reported hypocomplementemia and increased levels of complement activation products,[16] and animal models of aPL-induced thrombosis showed diminished thrombus formation in complement-deficient rats.[16,17]

Increased activation of platelets has been found in SLE and APS. Thrombocytopenia correlates with disease activity in SLE, and platelet immune-modulating responses have been suggested to contribute to SLE pathogenesis.[18] In APS, aPLs bind to and activate platelets and potentiate agonist-stimulated aggregation.[19]

Several studies have demonstrated complement–platelet interactions.[20,21] In SLE, complement deposition on platelets has been associated with vascular events, both venous and arterial, and the association was strengthened by the presence of aPLs.[22] Moreover, Peerschke et al found complement fixation on platelets to associate with the presence of aPLs and increased platelet activation, suggesting an aPL-mediated link between platelet–complement interactions.[23]

This leads to the hypothesis that crosstalk between complement and platelet plays a role in the thrombotic pathophysiology in SLE and APS. However, studies focusing on the lectin pathway of complement and its interactions with platelets are sparse.

To further explore the prothrombotic pathophysiology in SLE and APS, the present study investigated the correlations between lectin pathway protein (LPP) concentrations, complement activation, platelet aggregation, and platelet activation in SLE, APS, and healthy controls.

Methods

Study Population and Design

This cross-sectional cohort study was conducted at Aarhus University Hospital, Denmark, from October 2019 to August 2020. Patients with SLE were included at the Rheumatology outpatient clinic, and patients with APS were included at the outpatient clinic at the Thrombosis and Haemostasis Clinic. Healthy controls were recruited at the Blood Bank, Aarhus University Hospital.

Inclusion criteria for the patients were age $\geq$18 years with either a diagnosis of SLE or APS. SLE diagnosis required at least 4 out of 11 criteria from the American College of Rheumatology's revised classification criteria of 1997 to be fulfilled.[24] APS diagnosis required the Sydney Classification Criteria for Definite Antiphospholipid Syndrome to be fulfilled.[25] Patients were excluded if they met one or more of the following criteria: active cancer, liver failure ($>$ Child–Pugh class A), pregnancy, use of anticoagulants or antiplatelet drugs, except for low-molecular-weight heparin (LMWH). LMWH was never administered on the day of blood sampling. SLE patients were excluded if they met both clinical and biochemical criteria for secondary APS, as they all received regular anticoagulant treatment.

Clinical information regarding diagnosis, disease activity, medical history, and clinical manifestations, including previous thromboembolic disease, was systematically collected through interviews supplemented by electronic medical and pharmacy records.

The inclusion criteria for healthy controls were age between 17 and 75 years. Exclusion criteria were autoimmune

disease of the muscle, joints, skin, or connective tissue, hemophilia or coagulation defects, active cancer, and use of anticoagulants or antiplatelet drugs.

The study was approved by the Central Denmark Region Committees on Health Research Ethics (case no. 1-10-72-91-19) and the Danish Data Protection Agency (case no. 1-16-02-346-19). Written informed consent was obtained from all patients before inclusion.

Blood Sampling and Laboratory Analyses

Blood was drawn from the antecubital vein through a 21-gauge needle. The first tube was discarded.

For the APS cohort, aPL antibodies were detected at two times with at least 12 weeks apart, before project blood samples were acquired, and blood samples were acquired at least 3 months after any clinical event.

Samples for hemoglobin, platelet count, immature platelet fraction, immature platelet count, and leucocytes were collected in 2 mL ethylenediaminetetraacetic acid (EDTA) tubes and measured by Sysmex XN9000 (Sysmex, Kobe, Japan). Likewise, C3 and C4 concentration samples were collected in EDTA tubes and determined by turbidimetry using ADVIA Chemistry XPT Systems (Siemens, Chicago, Illinois, United States). C-reactive protein (CRP) and creatinine were collected in 4 mL lithium-heparin tubes and analyzed employing a Cobas 6000 (Roche Diagnostics, Indianapolis, Indiana, United States). Samples for tests of fibrinogen (functional, Clauss method), fibrin D-dimer, activated partial thromboplastin time (aPTT), and international normalized ratio (INR) were collected in 1.8 mL citrate 3.2% tubes and analyzed using a Sysmex CS 5100i (Sysmex, Kobe, Japan).

Antinuclear antibodies (ANAs) were determined in serum by fluoroenzyme-immunoassay using a Phadia 250 (Thermo Scientific, Uppsala, Sweden) or indirect immunofluorescence with human epithelial cells (Hep-2 cells) as substrate. A positive ANA was defined as a fluoroenzyme-immunoassay ratio above 1 or a Hep-2 assay with a dilution ratio above or equal to 1:160.

Lupus anticoagulant (LA) was analyzed by the dilute Russell Viper Venom test and a lupus-sensitive aPTT using a CS 2100i Sysmex (Siemens/Dade Behring, Marburg, Germany). The concentration of immunoglobulin G (IgG) and IgM anticardiolipin antibodies (aCLs) was determined using a Bio-Flash (Inova Diagnostics, San Diego, United States), and IgG and IgM anti-β_2GPI antibodies were determined using a Phadia 250 (Thermo Scientific, Uppsala, Sweden). aPL positivity was defined as either an LA1/LA2 ratio of or above 1.4, a measurement of anti-β_2GPI antibodies above 10^*10^3 int. units/L, and measurement of aCL antibodies above 40^*10^3 int. units/L, as defined by the APS criteria.[25]

Platelet Aggregation

Blood was collected in sodium citrate 3.2% tubes and analyzed within 1 hour of sampling by light transmission aggregometry (Bio/Data Corporation, Pennsylvania, United States). Calf Skin Collagen agonist (type 1) lyophilized collagen (0.19 mg/mL, Bio/Data Corporation, Pennsylvania, United States) and adenosine diphosphate (ADP) (2 µM, Bio/Data Corporation, Pennsylvania, United States) were used as agonists. The final concentrations were 0.19 mg/mL (collagen) and 200 µM (ADP). Platelet-rich and platelet-poor plasma were prepared using PDQ Platelet Function Centrifuge (Bio/data Corporation, Pennsylvania, United States). Platelet aggregation was monitored for 6 minutes. Results were expressed as maximum aggregation (%).

Platelet Activation

Blood was collected in sodium citrate 3.2% tubes and rested for 1 hour. Flow cytometry measurement of platelet activation was performed on whole blood employing Navios EX Flow Cytometer (Beckman Coulter, Miami, United States). Fluorescent-labelled antibodies specific for P-selectin (eBioscience, San Diego, California, United States), CD63 (PE Cy7, BD Bioscience, San Jose, California, United States), and bound fibrinogen (FITC, Polyclonal chicken, Diapensia HB, Linköping, Sweden) were used.

P-selectin is an indicator of α-granule secretion, whereas CD63 is an indicator of δ-granule secretion, both being activation-dependent markers. The glycoprotein IIb–IIIa complex is only capable of binding to fibrinogen following a conformational change after activation. Consequently, the use of specific antibodies against fibrinogen allows us to explore platelet activation-dependent surface changes.

The four agonists were ADP (140 µM, Sigma-Aldrich, St. Louis, United States), arachidonic acid (AA; 7.5 mM, Sigma-Aldrich, St. Louis, United States), collagen-related peptide, platelet GpVI ligand (collagen) (1.5 µg/mL, University of Cambridge, United Kingdom), and thrombin receptor activating peptide (TRAP; 371 µM, JPT, Berlin, Germany).[26] The final concentrations of the four agonists were 10.8 µM (ADP), 0.58 mM (AA), 0.12 µg/mL (collagen), and 28.6 µM (TRAP). A pool of 35 µL HEPES buffer, 5µL agonist, and 5 µL off each antibody was made. 5 µL blood was added, and samples were incubated for 10 minutes at room temperature in darkness, giving a final dilution of 1:13. Fixation was performed using 2 mL 0.2% PFA-PBS. Preparation and fixation were completed within 2 hours of sampling. Platelet activation was expressed as the percentage of positive platelets within a gate (%-positive platelets) and median fluorescence intensity (MFI) of all platelets within the gate. For each sample, gates were set to include 1 to 2% positive events for CD63 and bound fibrinogen and 0.1 to 0.2% for P-selectin on the negative control using single-stained platelets and matching isotype controls, as previously optimized in our lab.[27] The gating strategy is shown in ►**Supplementary Fig. S1**. Daily quality control of particle size and fluorescence was performed according to the manufacturer's instructions using Flow-Check Pro and Flow-set Pro (Beckman Coulter, Florida, United States). In performance of the flow cytometric analysis, the MIFlowCyt guideline, as outlined by Lee et al,[28] was followed including compensation, type of flow cell, and signal characteristics, with the information also being available in our protocol publication.[27]

Lectin Pathway Protein Concentrations

Plasma was frozen after collection and stored at −80°C until analysis. The concentrations of LPPs (MBL, CL-L1, CL-K1, M-ficolin, H-ficolin, MASP-1, -2 and -3, MAp19 and MAp44) and the complement activation fragment C3dg were determined in EDTA plasma using time-resolved immunofluorometric assays at the Department of Biomedicine, Aarhus University, employing protocols previously described.[29,30] Briefly, microtiter wells were coated with mannan (for the MBL assay), acetylated bovine serum albumin (for the H-ficolin assay), or relevant capture antibodies for the remaining assays. Plasma was thawed and diluted in assay-specific buffers and added to the coated microtiter wells. Biotinylated assay-specific antibodies followed by europium-labelled streptavidin were used to detect proteins bound in the wells. Signals were compared to a standard curve with known protein concentration, and three quality controls were used for each microtiter plate. Intra- and interassay coefficients of variation were below 15% for all assays. The concentration of L-ficolin was measured by a commercial immunoassay, following the instructions of the manufacturer (HK336-02, HycultBiotech, Uden, The Netherlands). As mentioned in the introduction, CL-LK is a heteromer of the two CL-L1 and CL-K1 polypeptide chains. In the present report we measure the concentration of both of the CL-L1 and CL-K1 polypeptide chains.

Statistical Analyses

Data were visually assessed for Gaussian distribution by QQ-plots and histograms. Data were log-transformed if Gaussian distribution could not be assumed on the normal scale. For comparison across all three groups, one-way ANOVA (analysis of variance) or the Kruskal–Wallis test was used for data following and not following Gaussian distribution. When a significant difference, defined as a p-value <0.05, was found across groups, a post-hoc unpaired t-test was performed between groups for data following Gaussian distribution and Mann–Whitney U-test for data not following Gaussian distribution. Spearman's rank correlation coefficient was calculated for correlation analyses.

As this was considered an exploratory study, no corrections for multiple testing were made.

CRP values below 4 mg/L were noted as 4 mg/L, INR values below 1 were noted as 1, and fibrin D-dimer values below 0.25 mg/L were noted as 0.25 mg/L.

Data were described with median and interquartile range (IQR) and presented graphically as dot plots with bars marking median and IQR unless otherwise stated.

Statistical analyses were performed using Stata Statistical Software version 16.1, and graphical illustrations were created using GraphPad Prism version 8.4.3.

Results

Study Population

In total, 20 SLE patients, 17 patients with APS, and 39 age-matched healthy controls were included. Age was similar across the three groups (►**Table 1**).

Regarding hereditary thrombophilia, only three SLE patients had been systematically examined for hereditary thrombophilia, and one of these was heterozygous for factor V Leiden. All APS patients were investigated at the time of APS diagnosis, and three patients were heterozygous for Factor V Leiden.

One SLE and one APS patient in the cohort were triple aPL positive.

Besides the eight SLE patients receiving prednisolone treatment on the day of inclusion, one APS patient also received prednisolone treatment due to rheumatic polymyalgia. Seven patients with APS received LMWH outside the day of blood sampling. APS patients did not take any other medication.

The SLE patients had low disease activity, indicated by a median SLEDAI score of 4, and were managed on hydroxychloroquine (HCQ; 85%) and low-dose prednisolone (40%) (►**Supplementary Table S1**).

Three arterial and seven venous thromboembolic events had previously occurred in the APS cohort (►**Table 1**). All arterial cases were ischemic strokes. Of the seven patients with APS-related venous thromboembolic events, two were in vena porta and one in vena jugularis. The remaining four patients had deep venous thrombosis, and three had a simultaneous pulmonary embolism.

In the SLE cohort, four patients had experienced unexplained fetal death after the 10th week of gestation, and one experienced three or more consecutive spontaneous abortions before the 10th week of gestation. For the APS cohort, two patients had experienced unexplained fetal death after the 10th week of gestation, two patients experienced three or more spontaneous abortions before the 10th week of gestation, and three patients experienced premature birth before the 34th week of gestation due to severe pre-eclampsia.

Lectin Pathway Protein Levels in Patients with SLE or APS and Healthy Controls

Levels of LPPs are illustrated in ►**Fig. 1**, and all values are presented in ►**Supplementary Table S2**. Four LPPs (L-, M-, H-ficolin, and MAp19) differed in concentrations between patients with SLE, APS, and healthy controls. Higher concentrations of L-ficolin were found in patients with APS than in patients with SLE ($p = 0.004$), and higher H-ficolin concentrations were found in both SLE ($p = 0.025$) and APS ($p = 0.004$) patients compared to healthy controls. The concentration of M-ficolin was lower in the SLE cohort compared to the APS cohort ($p = 0.004$) and healthy controls ($p = 0.032$), and the MAp19 concentration was higher in APS patients than in SLE patients ($p = 0.007$) and healthy controls ($p < 0.001$).

A positive correlation between C3dg and M-ficolin was found in the APS population ($r = 0.50$, $p = 0.043$). No other significant correlations were observed between C3dg and the proteins with a concentration difference.

Platelet Aggregation and Activation in SLE and APS Patients and Healthy Controls

We found no differences in platelet aggregation between the three cohorts, except for a significantly lower ADP-induced

Table 1 Baseline characteristics for patients with SLE or APS and healthy controls

Demographics	SLE, $n = 20$	APS, $n = 17$	Healthy controls, $n = 39$
Age at inclusion, y	44 [35; 55]	43 [32; 51]	44 [33; 55]
Age at diagnosis, y	40 [28; 49]	43 [31; 51]	
Gender, female	20 (100)	8 (47)	28 (72)
Race, white	18 (90)	17 (100)	
Body mass index, kg/m^2	23 [21; 24]	27 [26; 31]	
Smoker status			
Current smoker	1 (5)	3 (18)	
Previous smoker	10 (50)	8 (47)	
Biochemical data at day of inclusion			*Reference interval*
Hemoglobin, mM	8.0 [7.6; 8.5]	8.8 [8.1; 9.3]	7.3–10.5[a]
Platelet count, 10^9/L	244 [202; 293]	238 [199; 285]	145–400[a]
Immature platelet fraction	0.032 [0.023; 0.048]	0.024 [0.015; 0.043]	0.016–0.126
Immature platelet count, 10^9/L	9.4 [6.8; 12.5]	5.7 [4.6; 8.8]	4.4–26.7
Fibrinogen, μmol/L	8.1 [7.2; 9.5]	9.9 [8.2; 12.0]	5.5–12.0
Fibrin D-dimer, mg/L	0.3 [0.3; 0.6]	0.3 [0.3; 0.4]	< 0.5[b]
aPTT, s	25 [22; 27]	24 [22; 25]	20–29
INR	1.1 [1.0; 1,2]	1.0 [1.0; 1.0]	< 1.2
White blood cell count, 10^9/L	4.4 [3.7; 5.6]	6.6 [5.0; 7.9]	3.3–10.0
Creatinine, μmol/L	57 [49; 64]	68 [62; 73]	45–105[a]
CRP, mg/L	4 [4; 5]	4 [4; 6]	< 8
Positive ANA	20 (100)	2 (12)	
aPL status			
aPL present at any time	4 (20)	17 (100)	
aPL present 2 times 12 weeks apart	2 (10)	17 (100)	
aPL subtype (present 2 times 12 weeks apart)			
Lupus anticoagulant	2 (10)	15 (88)	
Anticardiolipin IgM or IgG	1 (5)	3 (18)	
Anti-beta2-glycoprotein-I IgM or IgG	1 (5)	1 (6)	
aPL-related complications			
Arterial thrombosis	0 (0)	3 (18)	
Venous thrombosis	1 (5)	7 (41)	
Obstetric complications	4 (20)	7 (88)	
Concomitant disease			
Concomitant chronic disease[c]	4 (20)	3 (18)	
Hereditary thrombophilia[d]	1 (5)	2 (12)	

Abbreviations: ANA, antinuclear antibodies; APS, antiphospholipid syndrome; aPTT, activated partial thromboplastin time; CRP, C-reactive protein; INR, international normalized ratio; SLE, systemic lupus erythematosus.
Note: Results are provided as median [IQR] or *n* (%).
[a]Combined RI for females and males.
[b]Reference interval increases with 0.1 mg/L per 10 years of age, from age 55 and above.
[c]Including the autoimmune or inflammatory diseases: Sjögren's syndrome, rheumatoid or juvenile chronic arthritis, autoimmune thrombocytopenia, autoimmune hypo- or hyperthyroidism, poly- or dermatomyositis, myasthenia gravis, coeliac disease, fibrosing alveolitis, chronic active hepatitis, chronic urticarial, psoriasis, colitis ulcerosa, asthma requiring systemic treatment.
[d]Hereditary thrombophilia includes factor V Leiden, prothrombin G20210A mutation, protein S, C, or antithrombin deficiency.

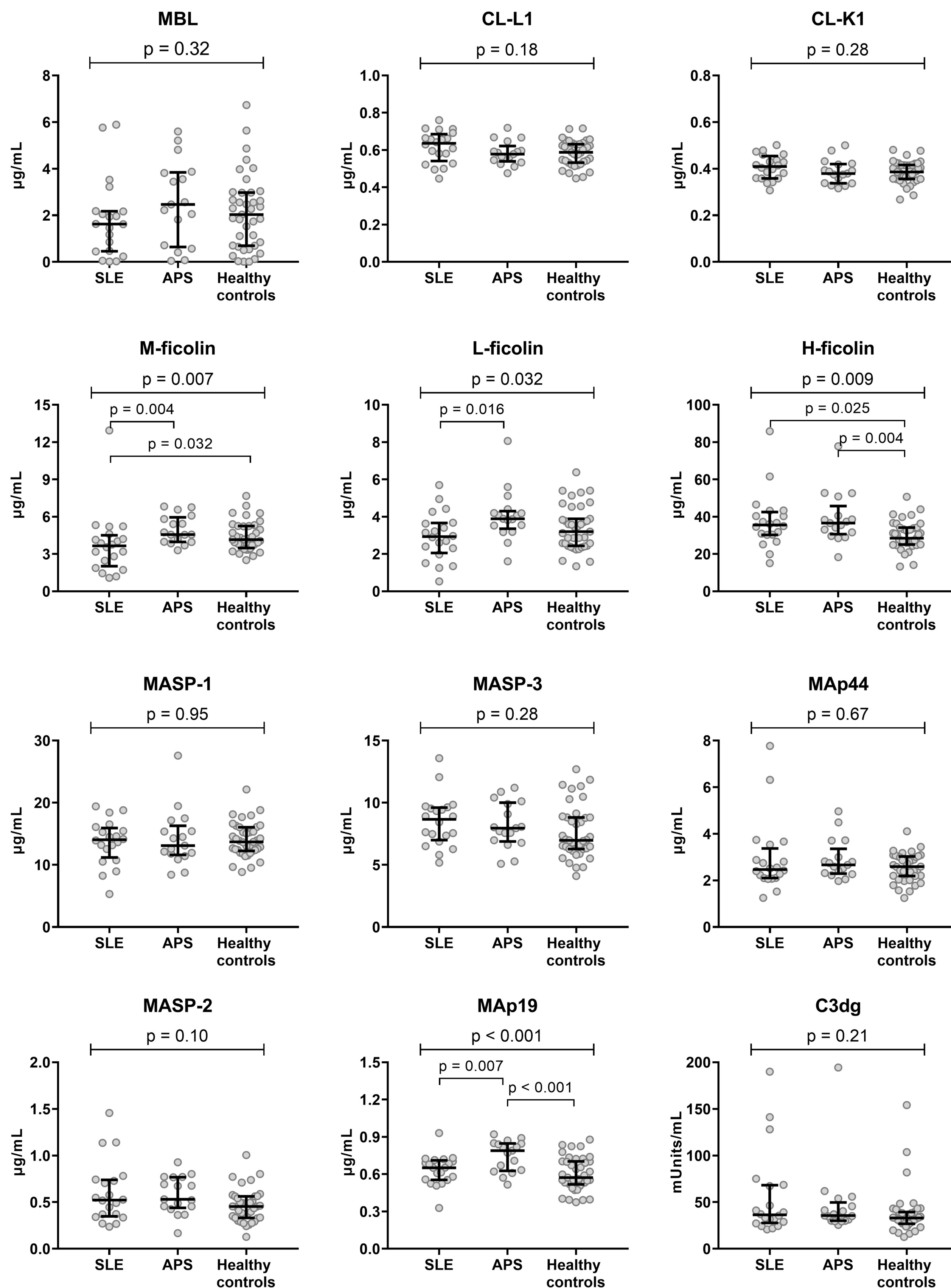

Fig. 1 Concentrations of lectin pathway proteins and C3dg in patients with SLE ($n = 20$), patients with APS ($n = 17$), and healthy controls ($n = 39$). Bars indicate median and interquartile range. Capped lines indicate *p*-values across all three groups. Tick-down lines indicate *p*-values between two groups when significant. APS, antiphospholipid syndrome; CL-K1; collectin kidney 1; CL-L1, collectin liver 1; MAp19/44, MBL-associated protein of 19/44 kDa; MASP, MBL-associated protease; MBL, mannose-binding lectin; SLE, systemic lupus erythematosus.

platelet aggregation in the SLE cohort than in healthy controls ($p = 0.009$) (►**Supplementary Fig. S2** and ►**Supplementary Table S2**).

Platelet activation is also illustrated in ►**Fig. 2** and presented in ►**Supplementary Table S2**. The %-positive platelets were significantly higher in the SLE cohort than in healthy controls when using AA as an agonist for bound fibrinogen ($p = 0.001$), CD63 ($p = 0.007$), and P-selectin ($p < 0.001$). A similar increase in AA-induced %-positive platelets was found in the APS cohort; however, only significant for the surface marker-bound fibrinogen ($p = 0.014$). The %-positive platelets for bound fibrinogen, CD63, and P-selectin did not differ significantly (p-values > 0.05). The median (IQR) preactivation, as assessed by %-positive platelets for P-selectin

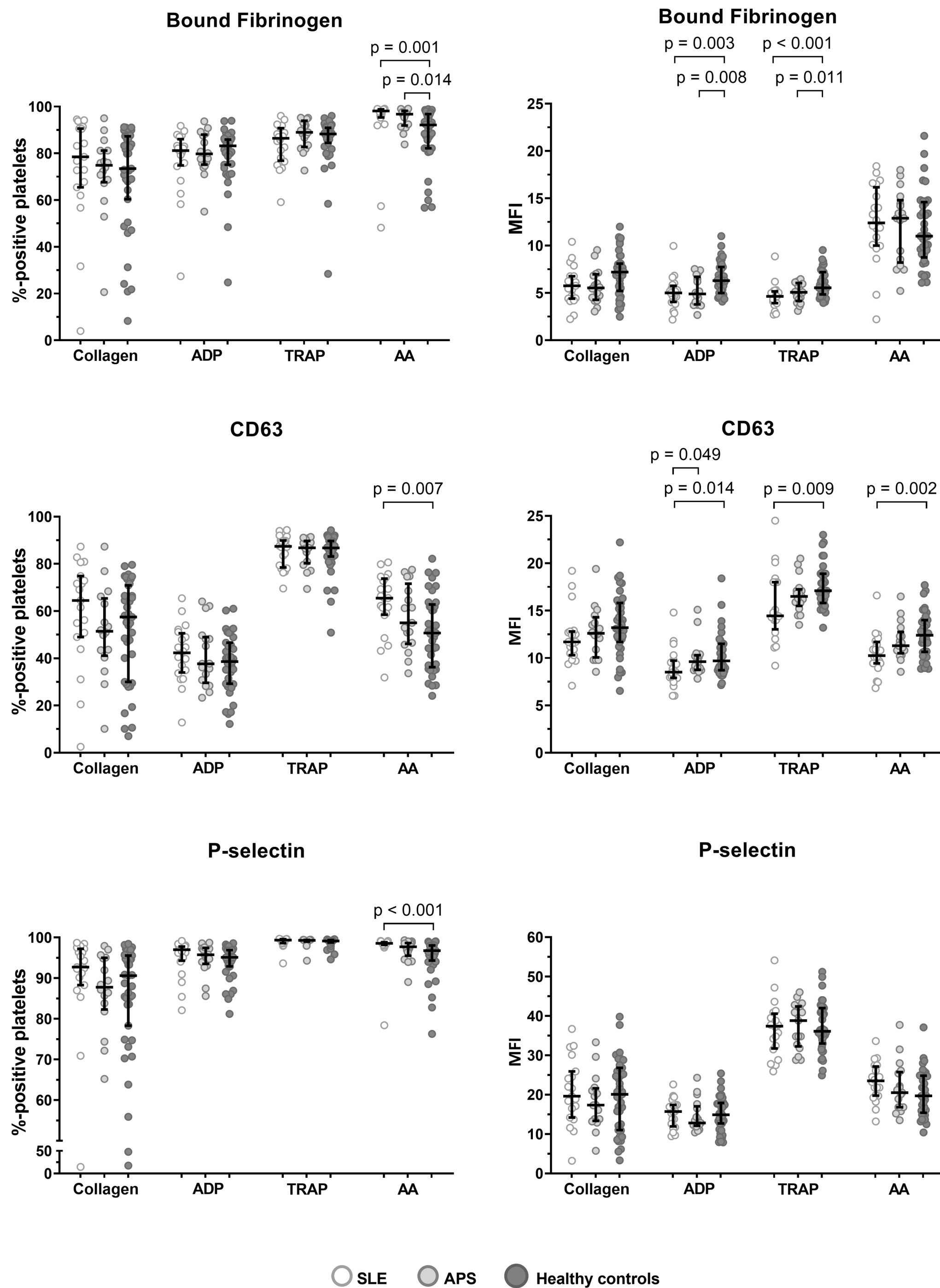

Fig. 2 Flow cytometry analyses of platelet activation in patients with SLE ($n = 20$), patients with APS ($n = 17$), and healthy controls ($n = 39$). Expressions of activation-dependent platelet surface markers bound fibrinogen, CD63, and P-selectin are shown. Graphs to the left illustrate the %-positive platelets. Graphs to the right illustrate the MFI of the platelet surface markers. Bars indicate median with IQR. The tick-down lines indicate p-values between two groups when significant. AA, arachidonic acid; ADP, adenosine diphosphate; APS, antiphospholipid syndrome; Collagen, collagen-related peptide; MFI, median fluorescence intensity; SLE, systemic lupus erythematosus; TRAP, thrombin receptor activating peptide-6.

after the addition of HEPES buffer, was 6% (5–11%), 7% (5–9%), and 10% (9–14%) for the SLE, APS, and healthy controls, respectively.

Lower MFIs were found in SLE patients compared to healthy controls. The MFI of bound fibrinogen was significantly lower using ADP ($p = 0.003$) and TRAP ($p < 0.001$) as agonists. For CD63, the MFI was significantly lower using ADP ($p = 0.014$), TRAP ($p = 0.009$), and AA ($p = 0.002$) as agonists.

Similarly, lower MFIs of bound fibrinogen were found in APS patients than in healthy controls when using ADP ($p = 0.008$) and TRAP ($p = 0.011$) as agonists, but this was not the case for MFIs of CD63. When comparing APS and SLE patients, the MFIs of bound fibrinogen, CD63, and P-selectin did not differ significantly, except for CD63 with ADP as an agonist, where SLE patients showed lower MFI than patients with APS ($p = 0.049$).

SLE patients not treated with prednisolone ($n = 12$) had a higher percentage of platelets positive for bound fibrinogen ($p = 0.002$), CD63 ($p < 0.001$), and P-selectin ($p < 0.001$) when using AA as an agonist compared to healthy controls. Similar results were found when comparing SLE patients with and without prednisolone treatment. However, this was only significant for the platelet marker CD63 ($p = 0.01$) (►**Fig. 3**). No significant differences were found for MFI.

In the platelet aggregation and activation analyses, a few low outliers can be seen in ►**Fig. 2** and ►**Supplementary Fig. S2**. A systematic review of outlying data points in the analyses was performed to clarify whether these outliers could be explained by the use of drugs inhibiting platelet function (suppression of platelet function under AA stimulation could indicate the use of aspirin or nonsteroidal anti-inflammatory drug (NSAID), and suppression of platelet function during ADP stimulation could indicate the use of P2Y12s inhibitors).

One SLE patient showed low platelet activation following stimulation but not in platelet aggregation. The patient did not report any use of aspirin or NSAID, and no drugs were administered, according to the medical database.

One APS patient showed low platelet activation and aggregation following stimulation by ADP and TRAP. According to medical records, the patient did not use any ADP inhibitors or ASA-containing drugs at the time of inclusion.

To inspect the possible effect of LMWH, all patients' aPTT was reviewed. One of the seven patients receiving LMWH had a prolonged aPTT of 38 as well as prolonged thrombin time. As so, we cannot rule out an effect of LMWH on the remaining analyses in this individual.

Correlation between LPPs and Platelet Activation in APS Patients

In the APS cohort, MASP-2 concentrations correlated negatively with both %-positive platelets and MFI for bound fibrinogen with all agonists (all $r < -0.3$), except for %-positive platelets when using collagen as an agonist. No significant correlation between MASP-2 and plasma fibrinogen was found in the APS cohort ($r = 0.34$, $p = 0.18$). No correlation was found in the SLE or control cohorts (►**Fig. 4** and ►**Supplementary Table S3**).

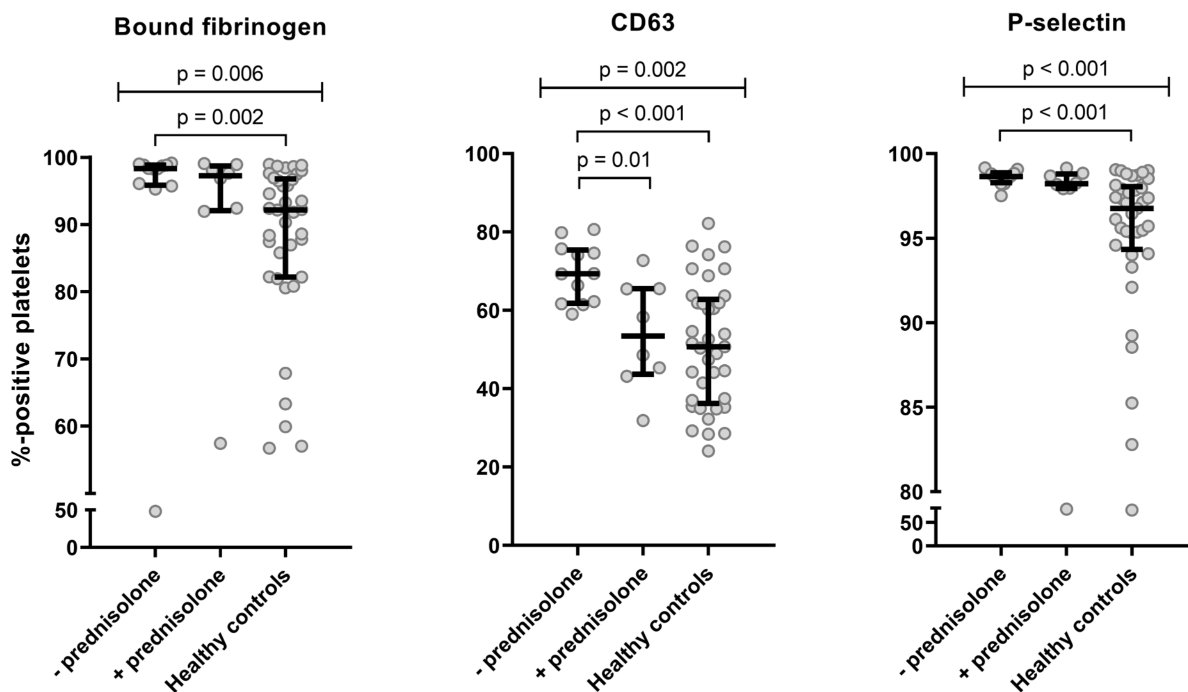

Fig. 3 Flow cytometry analyses of platelet activation, measured as %-positive platelets for bound fibrinogen, CD63, and P-selectin, using arachidonic acid as an agonist, in patients suffering from SLE not receiving prednisolone treatment ($n = 12$), patients suffering from SLE receiving prednisolone treatment ($n = 8$), and healthy controls ($n = 39$). Bars indicate median with IQR. Capped lines indicate p-values across all three groups. Tick-down lines indicate p-values between two groups when significant. APS, antiphospholipid syndrome; SLE, systemic lupus erythematosus.

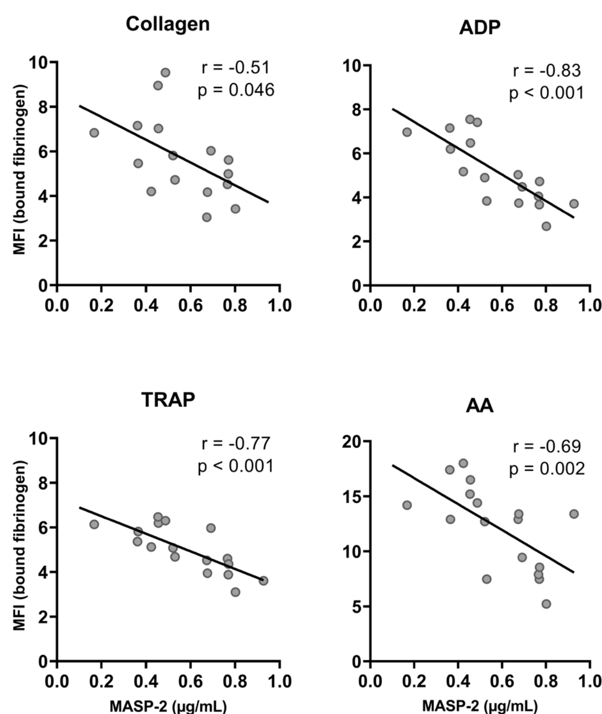

Fig. 4 Correlations between MASP-2 concentrations and MFI of platelet-bound fibrinogen after agonist stimulation in patients with APS ($n = 17$). The agonist is defined in the titles. AA, arachidonic acid; ADP, adenosine diphosphate; APS, antiphospholipid syndrome; Collagen, collagen-related peptide; MASP, MBL-associated protease; MFI, median fluorescence intensity; TRAP, thrombin receptor activating peptide-6.

Correlation between Complement Activation and Platelet Activation in APS Patients

C3dg concentrations correlated negatively with both %-positive platelets and MFI across all surface markers and agonists in the APS cohort, except for MFI of CD63 after collagen stimulation (►**Fig. 5**). The strongest correlations were found between C3dg and P-selectin expression, measured as both MFI and %-positive platelets (r between −0.18 and −0.82). However, only three out of eight correlation coefficients were significant ($p < 0.05$).

No strong correlations between platelet activity and C3dg were found in healthy controls or the SLE cohort (►**Supplementary Table S4**).

Discussion and Conclusion

This study is the first to investigate and compare LPP levels, complement activation, and platelet function in three distinct cohorts of patients with SLE, APS, and healthy controls.

The main finding was that LPP concentrations and platelet activation differed between patients with SLE or APS. We demonstrated that increased platelet activation in the SLE cohort was only observed in patients without prednisolone treatment. We showed that the concentration of MASP-2 and C3dg correlated negatively with platelet activation in the APS cohort. These findings suggest that complement and platelet activation interactions occur in both SLE and APS, but the interactions are different.

The two patient cohorts in the present study varied significantly in gender distribution, with the SLE cohort consisting solely of women. Previous studies from our lab demonstrated no significant differences in platelet function across gender.[31] However, several LPPs vary between gender, with higher L- and H-ficolin, MASP-2 and -3 concentrations, and lower M- ficolin and MASP-1 concentrations in women.[32] Regarding other demographic parameters, the two cohorts were comparable.

It was intended to include a third patient cohort consisting of SLE patients with secondary APS. However, these patients received lifelong anticoagulant therapy and were excluded according to the project exclusion criteria.

Our group has previously quantified the concentrations of LPPs in a large cohort of SLE patients. Overall, our findings align with those previously reported.[29] However, our previous studies showed increased levels of C3dg in patients with SLE, which was not found in the current study, suggesting that the SLE patients had low disease activity.

Interestingly, comparisons of LPP concentrations between the SLE and APS cohorts showed significant differences in M- and L-ficolin as well as MAp19, whereas both cohorts had increased levels of H-ficolin compared with healthy controls. This could indicate genetic differences in LP regulating genes in the two cohorts or that LP proteins serve other immune functions in patients unrelated to complement activation.

Only a few previous studies have quantified the concentrations of LPPs in a cohort with APS or persistent presence of aPLs. In 2014, Breen et al investigated the concentration of MBL, L- and H-ficolin in a cohort of 100 patients, of which 69 patients had primary APS and 31 had isolated aPL antibodies present but no clinical APS outcomes. They found no difference in LPP concentrations between the patient cohort APS and healthy controls.[33] An important difference in Breen et al's approach is their use of serum for sampling collection. In contrast, we used EDTA plasma to avoid further complement activation by Ca^{2+} and Mg^{2+} chelation. Further, complement enzymes are less prone to auto-activation in EDTA compared to serum.[34] A previous study from our laboratory determined LPP concentrations in serum and plasma and showed serum concentrations of H-ficolin significantly higher than plasma concentrations.[32] Likewise, previous studies have described L-ficolins' ability to bind to the silica particles in serum tubes, leading to lower concentrations in serum than plasma.[35] Thus, the comparison of L- and H-ficolin concentrations between our study and the study by Breen et al is hampered by sampling differences.

APS patients had significantly higher levels of MAp19 than healthy controls and SLE patients. MAp19 is suggested to be a modulator of the lectin pathway; however, the biological functions of MAp19 are yet to be elucidated.[14] Increased levels of MAp19 in plasma could be due to increased production, decreased usage, or reduced ability to bind to cell surfaces. MAp19 and MASP-2 are splice variants from the same gene.[36] As MASP-2 was not found to be increased in APS patients, high levels of MAp19 are presumably not due to

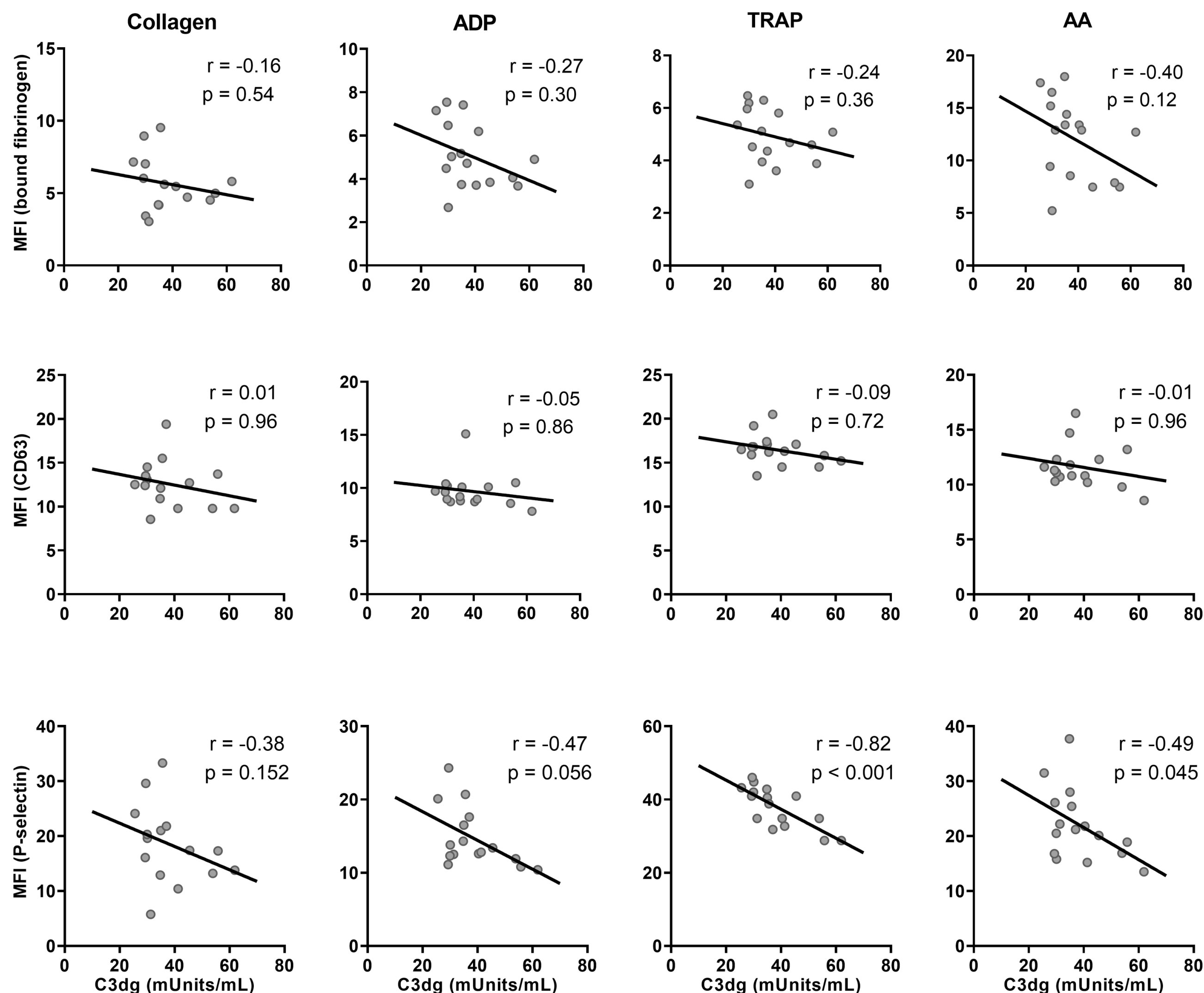

Fig. 5 Correlations between C3dg concentrations and MFI of platelet surface markers after agonist stimulation in patients with APS ($n = 17$). One outlier with C3dg concentrations at 194 mUnits/mL was excluded in the graphical illustrations, as it did not affect the overall conclusions. See ►**Supplementary Table S4** for further correlation analyses. AA, arachidonic acid; ADP, adenosine diphosphate; APS, antiphospholipid syndrome; Collagen, collagen-related peptide; MFI, median fluorescence intensity; TRAP, thrombin receptor activating peptide-6.

increased transcription but could be due to increased alternative splicing of the gene. Concentrations of MAp19 have been measured in a few other cohorts. In a cohort of septic shock patients, no significant differences in MAp19 concentrations were found between septic shock patients and healthy controls.[37] In patients suffering out-of-hospital cardiac arrest, Bro-Jeppesen et al demonstrated a significantly lower concentration of MAp19 compared with healthy controls, and low MAp19 levels were associated with increased mortality.[38]

Previous studies have shown increased platelet activation in patients with SLE.[39] Contrarily, our study shows an equal or decreased platelet activation and aggregation in SLE compared to healthy controls on almost all parameters, except %-positive platelets when stimulated with AA. A recent study by Cornwell et al found a significant reduction in platelet aggregation and decreased P-selectin expression in SLE patients receiving HCQ compared to SLE patients not receiving HCQ.[40] As 17 out of 20 patients in our cohort were treated with HCQ, this could be a factor worth considering when interpreting our results.

SLE patients not receiving prednisolone had increased platelet activation to agonist stimulation compared to patients treated with prednisolone and healthy controls, indicating platelet activation is inhibited by prednisolone treatment. Two previous studies on the in vitro effect of prednisolone on platelets found that prednisolone interacts with glucocorticoid platelet receptors leading to reduced platelet adhesion and aggregation.[41]

Both cohorts showed decreased platelet activation measured as MFI. This could be due to reduced platelet size, as previously found in an SLE cohort,[42] leading to fewer receptors per platelet. It has been theorized that constant in vivo platelet activation can lead to partial degranulation and exhausted platelets with decreased responsiveness to agonist stimulation ex vivo. Previous studies have found platelets with a high degree of in vivo activation to have a reduced in vitro function, supporting the theory of exhausted

platelets.[43] Such constant activation in the circulation has been suggested in both SLE and APS,[44,45] and several reasons, including the presence of platelet-activating autoantibodies like double-stranded DNA antibodies and aPLs, have been proprosed.[11,46]

In our APS cohort, we found a reverse correlation between platelet activation and complement activation. This does not comply with previous studies. Peerschke et al proposed that circulating aPL antibodies form immune complexes on the surface of platelets, leading to activation of the classical pathway and deposition of complement proteins on the surface of platelets, followed by complement-mediated platelet activation.[47] Likewise, complement fragments generated during activation of the complement system can bind directly to platelet receptors. C3dg, measured in the present report, binds to complement receptor 2 on platelets, leading to platelet activation.[48] Recently, Lonati et al measured increased platelet-bound C4d, a measure of complement activation, in APS patients and found positive correlations between platelet-bound C4d and aPL concentrations. Additionally, they showed in vitro binding of recombinant anti-β2GPI antibodies to activated platelets, inducing C4d binding to the surface.[49] Svenungsson et al showed C4d deposition on platelets in SLE and an association with vascular thrombotic events.[22] A plausible explanation for our results could be that constant aPL-mediated activation of platelets and platelet-related complement activation is followed by C3dg generation and exhaustion, leading to a decreased in vitro platelet activation potential.

Our study found a negative correlation between platelet activation, measured as bound fibrinogen, and concentrations of MASP-2 in the APS cohort. Increased deposition of MASP-2 has previously been demonstrated on activated platelets.[50] The lower concentration of MASP-2 with high platelet activation could represent deposition on platelets and activation of the lectin pathway in APS. In our study, this observation was APS-specific and could not be replicated in the SLE or control cohort. A study of MASP-2s proteolytic effect on the coagulation proteins demonstrated MASP-2's ability to mediate fibrinogen turnover through activation of prothrombin.[51] Kozarcanin et al showed that activated platelets bind ficolins, which is associated with MASP-1 and -2 activation. Further, the cleavage product from fibrinogen and fibrin could bind and activate both MASPs.[50] Our results support the theory of a link between the LPPs, coagulation proteins such as fibrinogen, and platelet activation in APS.

Some advantages to our study are the well-characterized patient groups and the exclusion of patients in antithrombotic treatment, making it possible to perform detailed investigations of platelet aggregation and stimulus activation.

We recognize that our study has limitations. Our control cohort is age-matched to both patient cohorts. Still, due to significant gender variations between the two cohorts, the control cohort is possibly not an optimal match for either. It cannot be excluded that gender is a potential confounding factor for our complement and platelet results. As it is an exploratory study, multiple parameters were tested without correcting for multiple testing. Also, our correlations are primarily hypothesis-generating and require more studies to elucidate the interactions between complement and platelets and the pathological role in thrombosis. Measurement of platelet activation during nonstimulated conditions would have been relevant to elucidate platelet activation in circulation further. We lack a measure of mean corpuscular volume of the platelets, as this measurement has been associated with disease activity and platelet activation in SLE cohorts.[52] There is a possible selection bias in the SLE cohort, as SLE patients with a history of thromboembolic disease would be on antithrombotic treatment and excluded from our study. Likewise, our SLE cohort had low disease activity, measured as SLEDAI, limiting our results to SLE patients with low disease activity and excluding potential correlations and associations with high disease activity. Although information regarding medical use was collected through medical records and interviews, the use of over-the-counter drugs cannot be ruled out, which could affect the platelet function analyses.

In summary, the LP represents a potential link between complement activation and platelet activation.[16,50] However, complement and platelet interactions are different in the two diseases we studied. We found reduced platelet aggregation and platelet activation in SLE patients compared to healthy controls, which could partly explain the use of prednisolone. In the APS cohort, we found negative correlations between MASP-2 and C3dg concentrations and platelet activation. The findings suggest that complement and platelet interactions occur in both SLE and APS although the mechanisms behind the interactions differ.

What Is Known about This Topic?

- Patients with SLE or APS have an increased thromboembolic risk.
- Interactions between platelets and the complement system have been suggested as a possible risk factor.

What Does This Paper Add?

- Differences between APS and SLE patients, observed in both platelet activation and complement activation, indicate that the prothrombotic state in these conditions has different driving mechanisms.
- In patients with APS, platelet activation showed a negative correlation with both C3dg and MASP-2, implicating a role of the lectin pathway in platelet activation in APS.

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Conflict of Interest
None declared.

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