




Enrichment of Complement, Immunoglobulins, and Autoantibody Targets in the Proteome of Platelets from Patients with Systemic Lupus Erythematosus

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Abstract

Background Systemic lupus erythematosus (SLE) is a complex disease characterized by autoimmunity toward apoptotic cells, excessive amounts of circulating immune complexes, and complement activation. A decreased platelet size has been observed in SLE and their nonhemostatic functions may play an active role in the disease. The main objective of this study was to find clues that could explain their decreased size and functional role, analyzing the entire platelet proteome.

Methods Platelets were isolated from 23 patients with SLE. The five individuals with the highest and lowest average platelet forward scatter were selected for further analysis. Platelet protein content was analyzed using liquid chromatography with tandem mass spectrometry (LC-MS/MS) and compared with platelets from five healthy controls. Data are available via ProteomeXchange with identifier PXD031202.

Results Out of 2,572 proteins identified, 396 had significantly different levels (ANOVA q -value ≤ 0.01). Forty proteins, including immunoglobulin-, complement- and phosphatidylserine-binding proteins had higher abundance in platelets from SLE patients, largely independent of size (fold difference of ≥ 1.5 and a t -test p -value of ≤ 0.05 as cut-off). Functional characterization revealed increased degranulation and skewed hemostatic balance in platelets from SLE patients. In the SLE proteome, immunoglobulin proteins were negatively correlated to serum complement C3 and C4 and the highest relative levels were detected in platelets of normal size.

Conclusion Platelets from SLE patients shared a specific protein profile, including immunoglobulins, complement proteins, and autoantigens, largely independent of the platelet size and in agreement with an integrated role for platelets in SLE.

Keywords

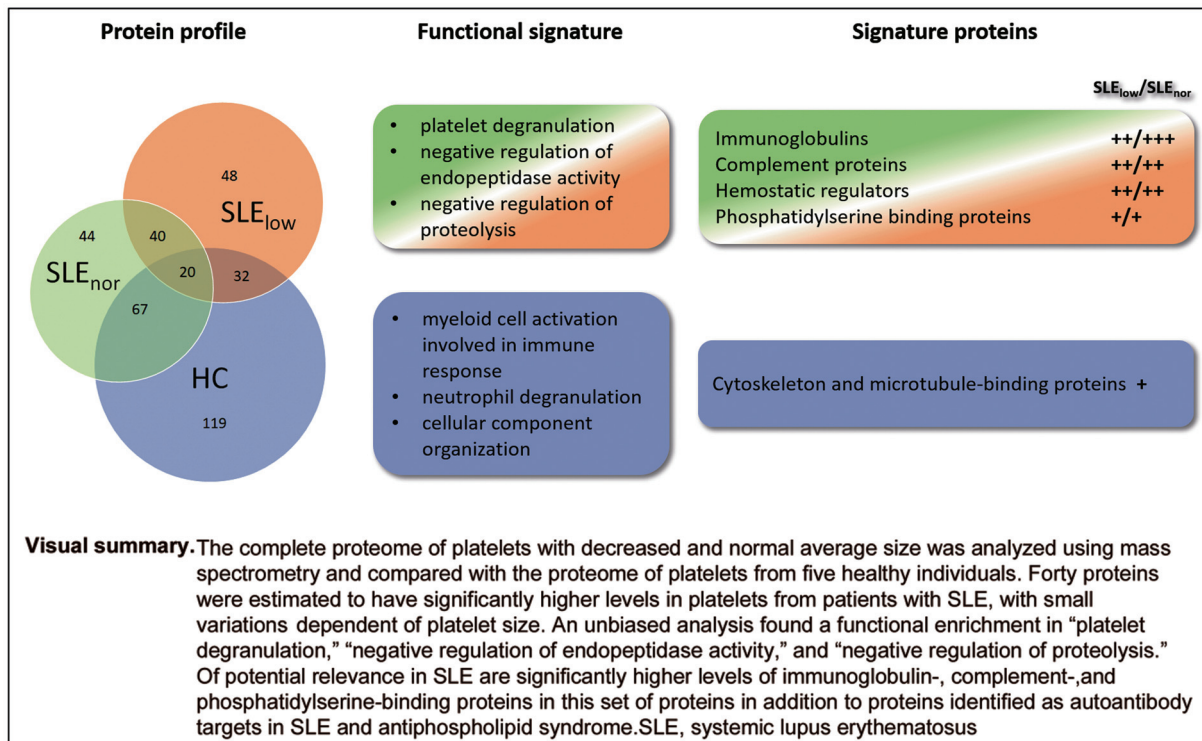
- ▶ SLE
- ▶ immunoglobulins
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- ▶ platelet

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Introduction

Platelets are small circulating anucleate fragments derived from megakaryocyte progenitors in the bone marrow. In addition to their role in hemostasis and thrombosis, they are active immune cells and may also be involved in processes leading to chronic inflammation and autoimmunity.¹ This view of platelets as cells participating in inflammation and immunity is based on observations, showing for example that platelets interact directly with classical immune cells and that mediators contained in platelet granules as well as de novo produced platelet derived cytokines can modulate both innate and adaptive immunity.^{2,3} The role of these mediators and interactions in systemic lupus erythematosus (SLE), including interactions with immune complex (IC), complement, and shedding of CD40 ligand (CD40L), are being investigated.¹ Platelets are known to express several complement proteins and receptors and they promote and actively initiate complement activation on the platelet surface.⁴

SLE is characterized by antibodies directed toward intracellular antigens, resulting in circulating ICs consisting of autoreactive immunoglobulins and antigens. Studies on platelets from SLE patients clearly demonstrate both morphologic changes and signs of increased platelet activation.^{5,6} Platelets also contain antigens and damage-associated molecular patterns, known to be involved in SLE pathogenesis.^{1,7} Others and we have observed that platelet sizes determined by flow cytometry forward scatter (FSC) and mean platelet volume (MPV) are decreased in patients with SLE,⁸⁻¹⁰ but there is no clear consensus regarding the role of platelet size in SLE.^{11,12} Platelets from SLE patients also show signs of being degranulated, depleted in serotonin,

and are a major source of microparticles.^{13,14} Decreased-sized platelets are also associated with antiphospholipid antibodies.⁸ The mechanism responsible for the decreased platelet size in SLE is not known, but increased rates of apoptosis can reduce platelet size¹⁵⁻¹⁷ presenting a possible explanation of the smaller size.¹ This explanation would also be consistent with the abnormal apoptosis patterns observed in other circulating cell types in patients with SLE.^{18,19} In this project, we investigated the proteome of platelets of differing average sizes from patients with SLE and from healthy control (HCs). Our aim was to characterize potential differences in protein levels that could explain these size differences and help determine the potential role of platelets in SLE.

Methods

Patients

A total of 23 consecutive patients with SLE were included during routine visits at the outpatient clinic, Department of Rheumatology, Skåne University Hospital in Lund. All were antinuclear antibody (ANA) positive and fulfilled at least four of the 1982 American College of Rheumatology criteria. Their mean age was 54 years, 87% were females and all had Caucasian ancestry. Clinical evaluation and Systemic Lupus Erythematosus Disease Activity Index 2000 (SLEDAI-2K) scoring was performed by specialist physicians. Routine blood tests, including total platelet count (PLT), MPV, white blood cell count (WBC), and hemoglobin levels (Hb), in addition to serological testing and serum levels of complement C1q, C3, and C4 were analyzed by the local accredited clinical laboratories at the Skåne University Hospital. Blood samples from each patient, including routine tests,

Table 1 Clinical characteristics of SLE patients

Number (<i>n</i>)	23
Female (%)	87%
Age (y), median (range)	54 (27–74)
Clinical laboratory parameters	
PLT ($10^9/L$), median (range)	253 (150–385)
WBC ($10^9/L$), median (range)	6.9 (3.1–11.1)
Hb (g/L), median (range)	139 (110–151)
CRP (mg/L), median (range)	0.88 (0.6–18)
Complement	
C1q (%), median (range)	111 (59–161)
C3 (g/L), median (range)	1.01 (0.48–1.58)
C4 (g/L), median (range)	0.16 (0.02–0.31)
Autoantibody profile	
Anti-dsDNA (<i>n</i>)	7/23
anti-C1q (<i>n</i>)	0/23
anti- β 2GPI (<i>n</i>)	3/23
anti-CL (<i>n</i>)	1/23
ANA (ever) (<i>n</i>)	23/23
SLEDAI score, median (range)	2 (0–10)
Pharmacologic treatment	
HCQ/CQ (<i>n</i>)	17
Aza (<i>n</i>)	4
Beli (<i>n</i>)	4
Mtx (<i>n</i>)	4
MMF (<i>n</i>)	3
Rtx (<i>n</i>)	1
Cic (<i>n</i>)	1
GC (<i>n</i> , mean dose)	14 (5.7 mg)

Abbreviations: Aza, azathioprine; Bel, belimumab; Cic, ciclosporin A; CQ, chloroquine; GC, glucocorticoids (prednisolone); HCQ, hydroxychloroquine; MMF, mycophenolate mofetil; Mtx, methotrexate; PLT, platelet count; SLE, systemic lupus erythematosus; WBC, white blood cell count.

complement, serological testing, and whole blood for platelet isolation were collected at one single occasion. Ten healthy individuals were selected as controls. Their mean age was 44 years, 80% were females, and all had Caucasian heritage. All patients and controls were included upon informed consent (→Table 1).

Ethical Consideration

Included patients received oral and written information, prior to signing their consent to participate in this study. Ethical approval to perform the study was given by the local ethics board, (Dnr 2018/131).

Platelet Isolation and Purification

Whole blood from all patients with SLE and HC were collected in ACD tubes (BD) and centrifuged at 200 g for 15 minutes

to obtain platelet-rich plasma (PRP). Platelets were resuspended in PIPES-saline-glucose (PSG) buffer, with prostaglandin E1 (Sigma) to a final concentration 0.6 μ M. To minimize platelet activation and aggregation, PSG buffer with PGE1 was used in all washing procedures throughout the entire protocol. To define the platelet size, PRP was centrifuged at 1,130 g for 10 minutes before being washed and average FSC and side scatter (SSC) determined by a flow cytometer (Accuri C6, BD). Gating was based on FSC, using FITC-labeled CD41 and APC-labeled CD61 as a reference (see →Supplementary Fig. S1 for representative gating, available in the online version). The percentage expression and mean fluorescence intensity (MFI) of CD40 Ligand (CD154), activated GPIIb/IIIa complex (PAC1), low-affinity immunoglobulin gamma Fc region receptor II b (CD32), proteinase-activated receptor 1 (PAR1), P-selectin (CD62P), and surface phosphatidylserine (PS) exposure (Annexin V [AV]) were determined to evaluate platelet functional characteristics. From the cohort of 23 patients, 10 PRP samples were selected for further analysis by mass spectrometry (→Supplementary Table S1, available in the online version). To obtain potential clues that could explain the difference in average size, we used PRP from the five patients with the smallest (referred to as SLE_{low}) and the five with highest FSC (referred to as SLE_{nor}). Prior to mass spectrometry analysis, platelets were purified from contaminating white and red blood cells using negative separation. PRP was incubated with biotinylated antibodies against CD45 (BD) and CD235 (BioLegend) for 15 minutes, then centrifuged at 1,130 g for 10 minutes. The pellet was resuspended and incubated with streptavidin-coupled Dynabeads (Invitrogen) and contaminated cells were separated magnetically from the platelets.

Mass Spectrometry

In-Solution Digestion with Trypsin

Cell pellets were lysed with 100 μ L Ripa buffer (R0278, Sigma Aldrich). They were placed in a Bioruptor Plus (Diagenode), using the settings: 20 cycles of 30 seconds on and 30 seconds off. Samples were then centrifuged 14,000 g for 10 minutes at 4°C. Supernatants were collected and proteins determined by MicroBCA Protein Assay Kit (Thermo Scientific) according to the manufacturer instructions. Samples (100 μ g) were reduced with 10 mM dithiothreitol at 56°C for 30 minutes followed by alkylation with 20 mM iodoacetamide for 30 minutes at room temperature in the dark. Proteins were precipitated with ice cold ethanol to a final concentration of 90% overnight at –20°C. The samples were centrifuged 16,000 g for 10 minutes at 4°C. The ethanol was removed and the samples were resuspended in 50 mM ammonium bicarbonate and then digested with trypsin (Promega, Madison, Wisconsin, United States) in a 1:50 w/w ratio (enzyme: proteins) overnight at 37°C. The digestion was stopped by adding 2 μ L 10% trifluoroacetic acid (TFA). The samples were dried using a Speed Vac and resolved in 2% ACN/0.1% TFA. To normalize for platelet size differences, the same amount of total peptides from each platelet fraction was injected into the liquid chromatography with tandem mass spectrometry

(LC-MS/MS). In all groups, three biological replicates from each sample were analyzed.

Mass Spectrometry Acquisition

The LC-MS detection was performed on Tribrid mass spectrometer Fusion equipped with a NanoEASY source and coupled with an EASY-nLC 1000 ultrahigh pressure liquid chromatography pump (Thermo Fischer Scientific). For the analysis, 1 μ g of the peptides was injected into the LC-MS. Peptides were concentrated on an Acclaim PepMap 100 C18 precolumn (75 μ m \times 2 cm, Thermo Scientific, Waltham, Massachusetts, United States) and then separated on an Acclaim PepMap RSLC column (75 μ m \times 25 cm, nanoViper, C18, 2 μ m, 100 \AA) at the temperature of 45°C and with a flow rate of 300 nL/min. Solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile) were used to create a nonlinear gradient to elute the peptides. For the gradient, the percentage of solvent B was maintained at 3% for 3 minutes, increased from 3 to 30% for 90 minutes, and then increased to 60% for 15 minutes and then increased to 90% for 5 minutes, and then kept at 90% for another 7 minutes to wash the column.

The Orbitrap Fusion was operated in the positive data-dependent acquisition (DDA) mode. Full MS survey scans from m/z 350–1,350 with a resolution of 120,000 were performed in the Orbitrap detector. The automatic gain control (AGC) target was set to 4×10^5 with an injection time of 50 milliseconds. The most intense ions (up to 20) with charge states 2 to 5 from the full scan MS were selected for fragmentation in the Orbitrap. The MS2 precursors were isolated with a quadrupole mass filter set to a width of 1.2 m/z . Precursors were fragmented by high-energy collision dissociation at a normalized collision energy of 30%. The resolution was fixed at 30,000 and for the MS/MS scans, the values for the AGC target and injection time were 5×10^4 and 54 milliseconds, respectively. The duration of dynamic exclusion was set to 45 seconds and the mass tolerance window was 10 ppm. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE²⁰ partner repository with the dataset identifier PXD031202.

Data Analysis

The raw DDA data were analyzed with Proteome Discoverer 2.2 (PD 2.2) software (Thermo Fisher Scientific). Peptides were identified using SEQUEST HT against UniProtKB human database (release 20190124). The search was performed with the following parameters applied: static modification: cysteine carbamidomethylation. Precursor tolerance was set to 15 ppm and fragment tolerance was set to 0.05 ppm. Up to two missed cleavages were allowed and Percolator was used for peptide validation at a q -value of maximum 0.01. Filter settings at the protein level: Master is equal to Master, Protein Unique Peptides is greater than or equal to 2, Found in samples has confidence High in every sample holding a value. Extracted peptides were used to identify and quantify them by label-free relative quantification. The extracted chromatographic intensities were used to compare peptide

abundance across samples. The STRING software (<https://string-db.org>, version 11.0b) analyzed potential protein interactions. Functional enrichment of proteins is calculated based on the level of protein annotations with a particular term within a network, related to the number that would be expected by chance. The false discovery rate (FDR) p -values assigned to the top Biological Process (GO) describes how significant the enrichment is using the Benjamini–Hochberg procedure. Biological processes were also rated by Strength, an enrichment effect describing the ratio between the number of proteins in the network annotated with a term and the number of proteins expected to be annotated with this term in an equal-sized random set of proteins. Data transformation (\log_2), filtering (filter out the proteins for missing value in one of the samples), normalization (subtract median), and statistical analysis were performed in Perseus 1.6.6.0. Significant differently expressed proteins were extracted after the ANOVA (analysis of variance) test and the cluster analysis heat map was plotted in RStudio by using the z -score. The distance calculation method used was maximum and clustering method was ward.D.

Statistical Analysis

Differences in abundance levels of each protein identifier were analyzed using ANOVA. To correct for multiple-hypothesis testing, significant hits of the ANOVA test were truncated at a permutation-based FDR threshold of 0.01 (250 randomizations). To allow direct comparisons of relative protein levels between groups, we performed t -test analysis of \log_2 -transformed values using means of triplicates for each sample. A fold difference (FD) of ≥ 1.5 and a p -value of ≤ 0.05 were used as cut-off values when comparing estimated protein levels between groups. Correlations between estimated protein levels of immunoglobulins, serum levels of complement proteins, FSC, and other flow cytometry values were analyzed by Spearman correlation and the Mann–Whitney test, using the GraphPad Prism software (version 7.04).

Results

Patient Characteristics and Platelet Size

Patients in this study had a median SLEDAI-2K score of 2 (0–10) and all had a normal platelet count. Complement C1q, C3, and C4 median levels were in the normal range, but two patients had decreased levels of C1q, five had decreased levels of C3, and three patients had decreased levels of C4 (**Table 1**). FSC is one of several methods to determine platelet size,²¹ previously shown to be decreased and strongly associated with MPV in patients with SLE.⁸ Using FSC to determine platelet size, we observed large variations in average platelet FSC, with significantly lower levels in SLE compared with HC (**Fig. 1A, B**). SSC values also differed significantly between SLE and HC, being highest in platelets from HC and lowest in small-sized platelets from SLE patients. Compared with HC, platelets from SLE patients had significantly higher levels of CD41-positive cells, higher MFI values of CD154, and lower levels of PAR1, but

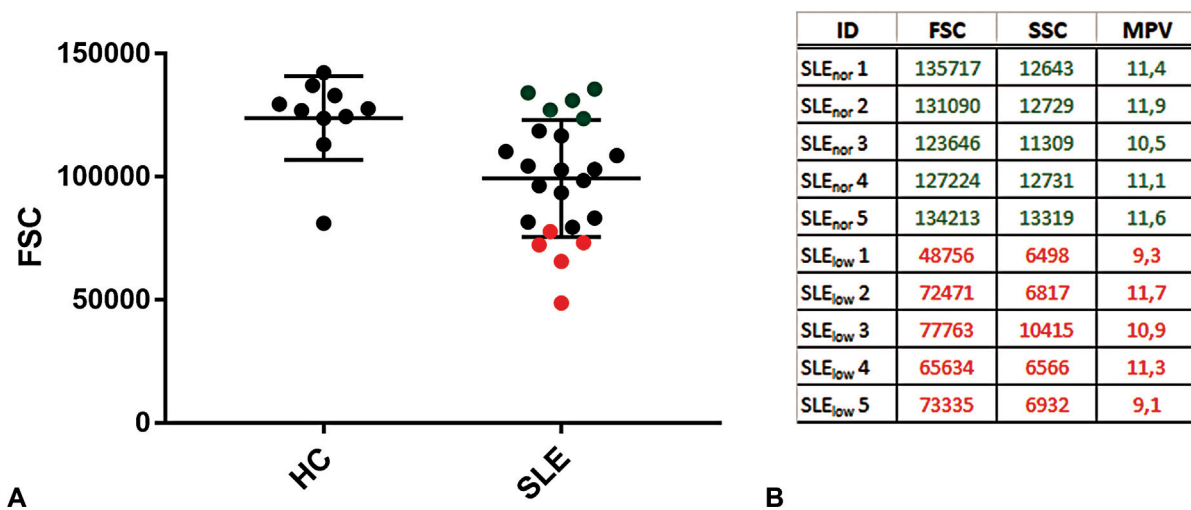


Fig. 1 Average platelet size in healthy controls and patients with SLE. Distribution of flow cytometry FSC in platelets from HC ($n = 10$) and SLE ($n = 23$) (A). FSC, SSC, and MPV values from the five SLE patients with highest platelet FSC (SLE_{nor}) and the five with lowest (SLE_{low}) are shown (B). FSC, forward scatter; HC, healthy control; MPV, mean platelet volume; SLE, systemic lupus erythematosus; SSC, side scatter.

these differences were independent of platelet size (► **Supplementary Fig. S2**, available in the online version). We selected samples from the five patients with highest average FSC and from the five patients with the lowest average FSC for further analysis of the platelet proteome. These were compared with the platelet proteome of five healthy individuals. The five patients with the highest FSC (SLE_{nor}) had equal levels compared with HC, while the five with the lowest FSC (SLE_{low}) were decreased by more than

two standard deviations compared with average HC values (► **Supplementary Table S1**, available in the online version). Patients did not differ significantly in age, gender, disease duration, treatment or SLEDAI-2K score, but patients with SLE_{nor} platelets had significantly lower levels of complement C3 and C4 (t -test p -value, 0.04 and 0.009) (► **Table 2**).

The proteomes from SLE_{low} and SLE_{nor} and five HCs were analyzed in parallel. A total of 396 proteins with significantly different abundance levels were identified from the three

Table 2 Clinical characteristics of SLE_{low} ($n = 5$) and SLE_{nor} ($n = 5$)

ID	Sex	Age (y)	DD (y)	PLT ($10^9/L$)	WBC ($10^9/L$)	Hb (g/L)	CRP (mg/L)	C1q (%)	C3 (g/L)	C4 (g/L)	SLEDAI score	SLEDAI units	Treatment
SLE _{low} 1	F	54	16	228	5.8	124	0.6	97	1.01	0.18	4	Proteinuria	HCQ, MMF, GC 7.5 mg
SLE _{low} 2	F	27	7	271	4.3	137	0.6	80	1.09	0.15	2	DNA	HCQ, Aza
SLE _{low} 3	F	42	14	189	7.0	125	0.7	120	0.88	0.15	8	Pyuria, arthritis	Bel, Aza, GC 2.5 mg
SLE _{low} 4	F	47	29	150	10.3	151	0.6	87	0.96	0.19	0	N/A	HCQ, Cikli, Bel, GC 10 mg
SLE _{low} 5	M	57	8	230	4.6	146	0.7	138	1.00	0.19	2	Rash	HCQ
SLE _{nor} 1	F	48	29	165	10.6	151	0.6	110	0.74	0.13	2	LC	Bel, CQ, GC 7.5 mg
SLE _{nor} 2	F	69	10	165	4.8	136	0.6	59	0.48	0.02	2	LC	HCQ
SLE _{nor} 3	F	46	4	284	7.5	138	0.9	107	0.68	0.10	4	LC, DNA	Mtx, Bel, GC 10 mg
SLE _{nor} 4	F	47	21	239	3.5	110	8.0	87	1.07	0.13	0	N/A	Aza, HCQ, NSAID
SLE _{nor} 5	F	33	1	266	7.2	139	0.6	118	0.73	0.10	4	LC, DNA	HCQ, MMF, GC 5 mg

Abbreviations: Aza, azathioprine; Bel, belimumab; Cic, ciclosporin; CQ, chloroquine; DD, disease duration; DNA, increased DNA binding; F, female; GC, glucocorticoids (prednisolone); HCQ, hydroxychloroquine; LC, low complement; M, male; MMF, mycophenolate mofetil; Mtx, methotrexate; NSAID, nonsteroidal anti-inflammatory drug; PLT, platelet count; SLE, systemic lupus erythematosus; WBC, white blood cell count.

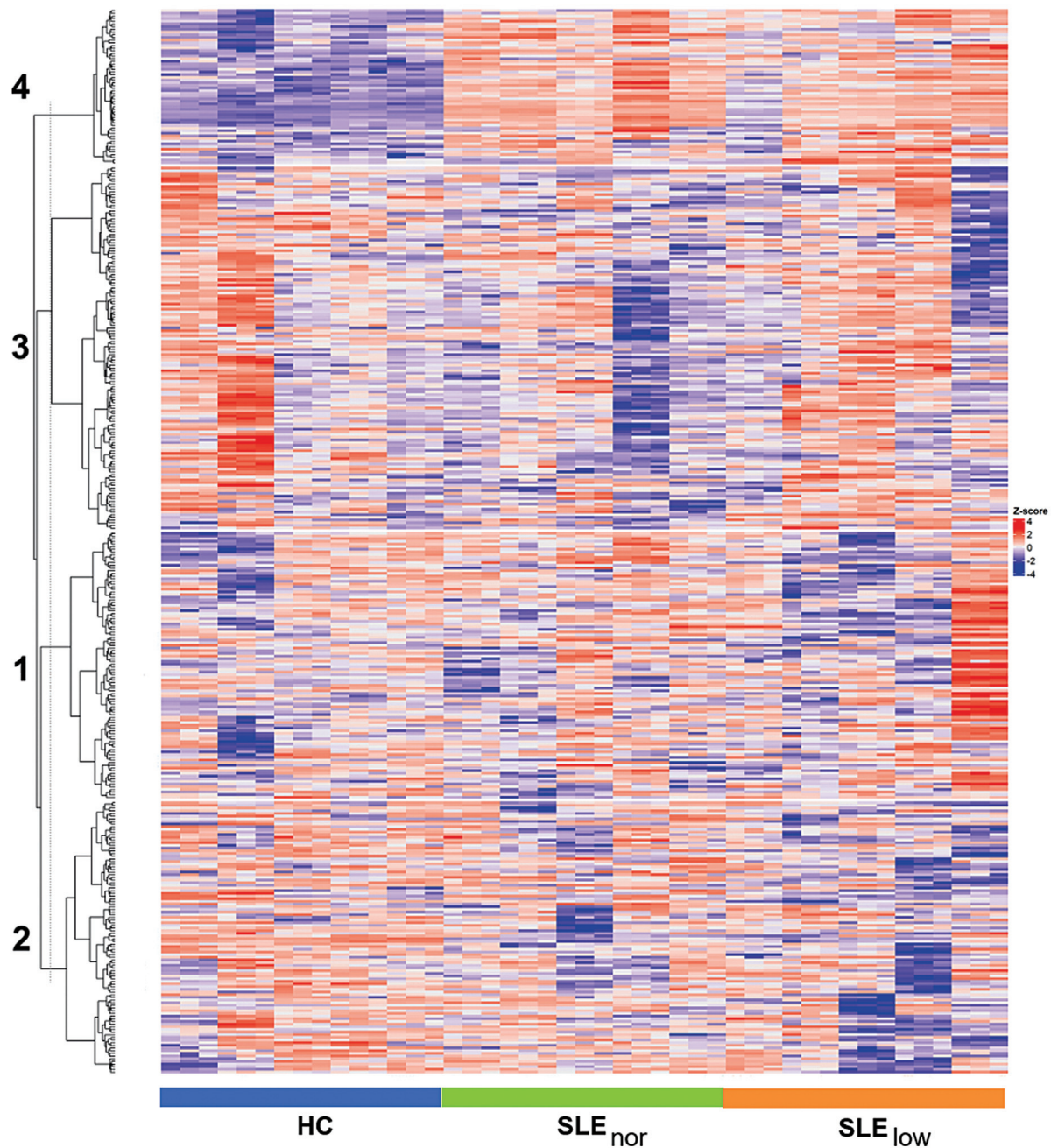


Fig. 2 Figure depicting a hierarchical clustering heat map. All 396 proteins identified as differentially expressed by ANOVA were analyzed using hierarchical clustering. The heat map represents log₂ fold differences (FDs) of the quantitative data in biological triplicates (blue: lowest abundance and red: highest abundance).

groups, ANOVA (FDR, 0.01). An unbiased hierarchical clustering grouped these proteins into four clusters, with a clear separation between SLE groups and HC in cluster 4 (► **Fig. 2**).

The abundance level of proteins was compared between all groups and significant differences evaluated using a *t*-test. There was a substantial overlap between the SLE_{low} and SLE_{nor}. Out of 52 proteins with relatively higher abundance levels in either of the SLE groups, 40 proteins (77%) shared a higher level in both SLE_{low} and SLE_{nor}, compared with HC. Additionally, 50 out of 52 proteins were present in cluster 4 (► **Supplementary Table S2**, available in the online version), confirming the separation observed in this cluster. Platelets

from HC on the other hand had 119 proteins with significantly higher levels compared with either SLE_{low} or SLE_{nor}, but only 20 had higher levels compared with both SLE groups. An overview of overlaps and differences between groups is shown in ► **Fig. 3**.

The SLE_{low} and SLE_{nor} Proteomes: Indications of Degranulation and Skewed Hemostatic Function

An unbiased evaluation of annotated protein function in proteins with higher abundance in SLE_{low} and SLE_{nor} showed a significant enrichment of several biological processes, compared with the expected from chance alone (► **Fig. 4**).

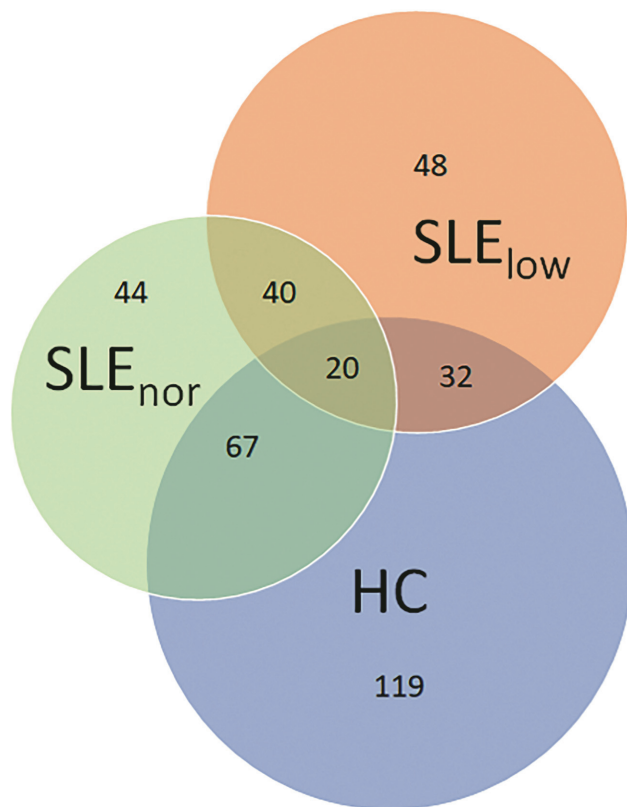


Fig. 3 Overlapping and different protein levels in platelet proteome of SLE_{nor}, SLE_{low}, and HC. 44 proteins from platelets of normal size and 48 proteins from platelets of decreased size had significantly higher abundance than HC. Of these, 40 proteins were shared between SLE_{nor} and SLE_{low}. HC had 20 proteins with higher levels than both SLE groups, 67 compared with SLE_{nor} only, and 32 compared with SLE_{low}. HC, healthy control; SLE, systemic lupus erythematosus.

The network of proteins with increased abundance in SLE_{low} and SLE_{nor} also had a higher degree of interactions than expected from an equal number of randomly selected proteins, with a protein–protein interaction (PPI) enrichment p -value of $<1.0 \times 10^{-16}$.²² Among these 33 proteins (the seven immunoglobulins were excluded from this analysis), the top three most significantly overrepresented gene ontology (GO) terms of Biological Process were: “platelet degranulation,” “negative regulation of endopeptidase activity,” and “negative regulation of proteolysis” (–Table 3). The network of proteins associated with platelet degranulation had the highest significance, with a FDR value of 1.66×10^{-16} , suggesting that SLE_{low} and SLE_{nor} platelets have an increased level of degranulation compared with platelets from HC. The proteins associated with negative regulation of endopeptidase activity and negative regulation of proteolysis, on the other hand, were essentially overlapping. These groups included several serine protease inhibitors from the superfamily of Serpins, named after their ability, to inhibit serine proteases. Serpins, including anti-thrombin-III (SERPINC1), alpha-1-antitrypsin (SERPINA1), heparin cofactor 2 (SERPIND1), and alpha-2-antiplasmin (SERPINF2) have important hemostatic functions,²³ suggesting that platelets from SLE_{low} and SLE_{nor} have an altered hemostatic function, compared with platelets from HC.

Additionally, we observed a relative accumulation of galectin-1 in SLE_{low} relative to SLE_{nor} and HC. Galectin-1 is a β -galactoside-binding protein necessary for primary hemostasis.²⁴ Galectin-1 may also be involved in platelet activation and shedding of platelet microparticles²⁵ and proapoptotic features are reported in other cell types.²⁶ A manually curated categorization furthermore revealed that platelets from SLE patients shared an increased level of immunoglobulin proteins, complement-associated proteins, PS-binding proteins, and plasma proteins. Except for the immunoglobulin proteins, the protein profile of these categories was largely independent of platelet size (–Table 3).

Accumulation of Immunoglobulins

While both SLE groups had significantly higher levels of seven immunoglobulin proteins compared with HC with FDs ranging from 1.7 to 14.0, the relative abundance of four immunoglobulin proteins was even more pronounced in SLE_{nor} (–Table 3). Compared with SLE_{low}, SLE_{nor} had a 4.4-fold higher levels of Ig heavy constant μ (IGHM), 3.0-fold higher of Ig heavy constant α 1 (IGHA1), and 2.1-fold higher levels of Ig heavy constant gamma 3 (IGHG3). While immunoglobulins are common plasma proteins, their presence in human platelets has been known for decades.²⁷

Complement Proteins and Complement Regulators

The proteome of SLE_{low} and SLE_{nor} displayed a consistent overrepresentation of plasma protease C1 inhibitor (SERPING1), complement C4-B (C4B), complement C3 (C3), complement factor H (CFH), and complement factor B (CFB) relative to HC (FD ranging from 5.8 to 1.9) (–Table 3). No statistical difference in abundance level between SLE_{low} and SLE_{nor} was detected although the SLE_{nor} group included individual patients displaying decreased serum levels of complement proteins C3 (4/5), C4 (3/5), and C1q (1/5).

Increased Presence of PS-Binding Proteins

The plasma protein beta-2-glycoprotein 1 (APOH, β 2GPI) had an equally increased abundance in SLE_{low} and SLE_{nor}. β 2GPI has been attributed to a wide diversity of functions, including binding of PS on the surface of apoptotic cells.²⁸ Additionally, it contains the main antigen in the antiphospholipid syndrome (APS).²⁹ Annexin A5 with increased presence in SLE_{low} compared with HC is also associated with PS binding on the surface of activated or apoptotic platelets.³⁰ Although mainly recognized for its role in coagulation and hemostasis, prothrombin is also associated with PS binding and apoptosis.³¹ Prothrombin had increased presence in platelets from SLE_{low} and SLE_{nor}.

Increased Presence of Common Plasma Proteins in the SLE_{low} and SLE_{nor} Proteomes

One set of common plasma proteins, including neutrophil defensin 1 (DEFA1), hemoglobin subunits α and β (HBA1 and HBB), galectin-3-binding protein (LGALS3BP), apolipoprotein E (Apo E), and apolipoprotein B-100 (APOB), was detected in significantly higher levels in SLE_{low} compared with SLE_{nor},³² while other traditional plasma proteins, such as angiotensinogen, apolipoprotein A-I (Apo AI),

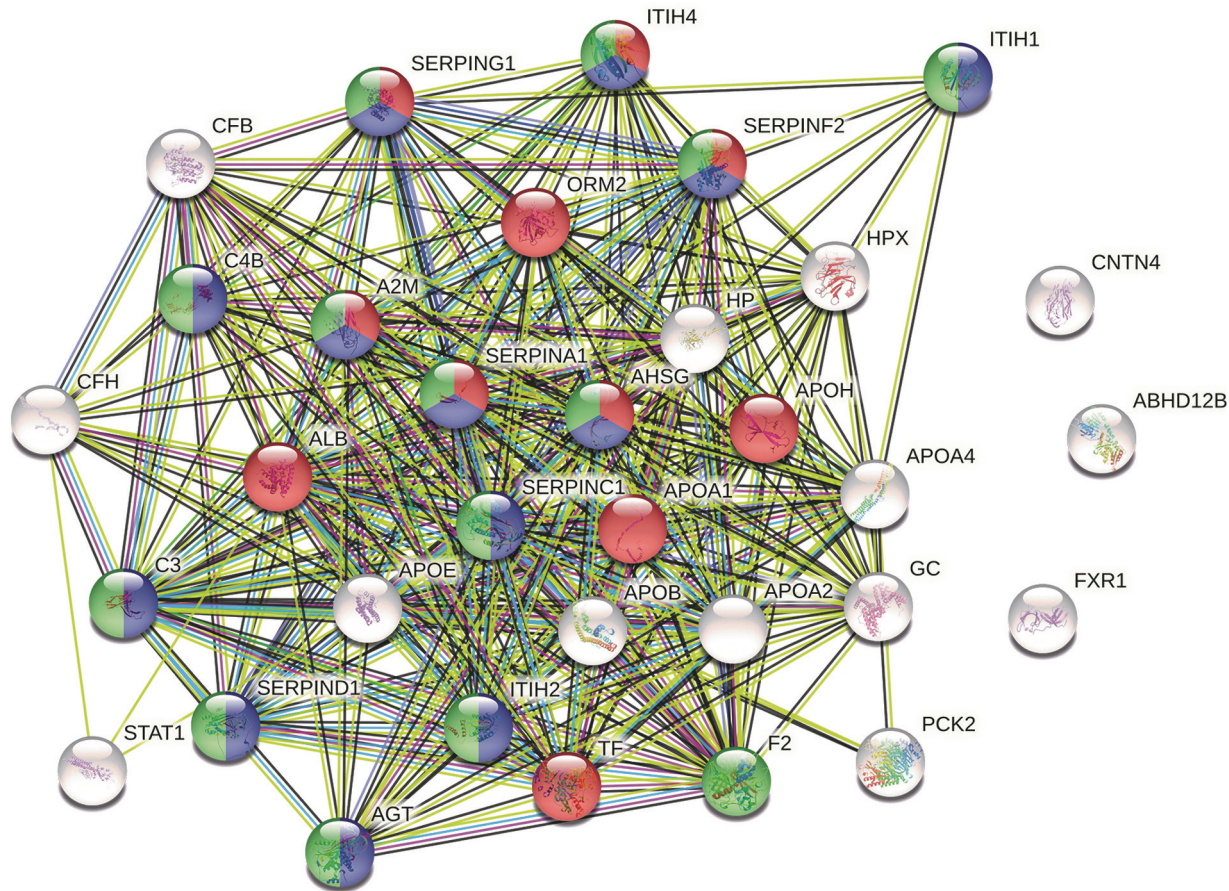


Fig. 4 Proteins with higher levels in SLE compared with HC 33* of 40 proteins that had higher levels in SLE (SLE_{low} and SLE_{nor}) compared with HC, with a PPI enrichment p -value of $<1.0e^{-16}$. The top three most overrepresented biologic processes (GO) were: (1) platelet degranulation (red), FDR 1.73×10^{-13} , strength 1.7; (2) negative regulation of endopeptidase activity (blue), FDR 1.73×10^{-13} , strength 1.5; and (3) negative regulation of proteolysis (green), FDR 1.73×10^{-13} , strength 1.4. (*seven immunoglobulin proteins were among the 40 proteins that had higher levels in SLE_{low} and SLE_{nor} , but they were not identified in the STRING database and therefore not included in this network. FDR, false discovery rate; HC, healthy control; PPI, protein-protein interaction; SLE, systemic lupus erythematosus.

ceruloplasmin (CP) and α -1-antitrypsin (α 1AT), had increased abundance in SLE_{nor} ³² (► **Table 3**). A more detailed presentation of the relative difference between SLE_{low} and SLE_{nor} proteomes can be found in ► **Supplementary Tables S3** and **S4** (available in the online version).

The HC Proteome: Higher Levels of Structural Proteins Compared with SLE_{low} and SLE_{nor}

Twenty proteins shared an increased level in HC compared with SLE groups and these proteins also had a higher degree of expected interactions than an equal sized random set of proteins, with a PPI enrichment p -value of 1.65×10^{-09} (► **Fig. 5**). The three most enriched biological processes were: “myeloid cell activation involved in immune response”; “neutrophil degranulation”; and “cellular component organization.” The highest enriched biological process had a FDR of 2.41×10^{-5} and the others had similar FDR values. There was a considerable overlap between the included proteins however, especially tubulin and dynein proteins were included in all enriched processes. Isoforms of α - and β -tubulin are found in abundance in human platelets,³³ forming the microtubules that along with actin make up the major structural polymer systems. The cytoskeleton

and microtubule-binding dynein proteins are involved in maintaining platelet shape and shape change, but also in the intracellular transportation of vesicles and organelles along microtubules. Three proteins involved in inflammation and immunity; C-X-C chemokine receptor type 4 (CXCR4), the cytokine like protein macrophage migration inhibitory factor (MIF), and lipopolysaccharide-binding protein (LBP) had higher levels in HC compared with SLE_{low} and SLE_{nor} (► **Supplementary Table S5**, available in the online version). CXCR4 and MIF are known to be expressed by platelets^{34,35} and LBP has also been detected in the platelet proteome.³⁶ While CXCR4 and MIF had detection levels close to the cut-off limit of FD 1.5, LBP had over fivefold higher levels in HC compared with SLE_{nor} and 3.7-fold higher levels compared with SLE_{low} . LBP is involved in the recognition of lipopolysaccharide and toll-like receptor 4 (TLR4) signaling.

Inverse Correlation between Serum Complement Proteins and Immunoglobulin Proteins in Patients with SLE

Complement proteins C1q, C3, and C4 are routinely used as biomarkers in SLE and decreased levels are associated with active disease. If levels of platelet-associated

Table 3 Signature proteins in platelets from SLE patients

Accession	Description	Gene symbol	SLE _{nor} vs. HC (FD)	t-test	SLE _{low} vs. HC (FD)	t-test	Top biological process	
	Immunoglobulins							
P01876	Immunoglobulin heavy constant α 1	IGHA1	14.0	0.00000	4.7	0.00000	n/a	^a
P0DOY2	Immunoglobulin lambda constant 2	IGLC2	6.2	0.00000	3.2	0.00000	n/a	^a
P0DOX5	Immunoglobulin gamma-1 heavy chain	N/A	4.6	0.00000	3.1	0.00000	n/a	
P0DOX8	Immunoglobulin lambda-1 light chain	N/A	3.6	0.00000	2.8	0.00006	n/a	
P01859	Immunoglobulin heavy constant gamma 2	IGHG2	2.9	0.00015	2.5	0.00131	n/a	
P01871	Immunoglobulin heavy constant mu	IGHM	10.5	0.00000	2.4	0.00014	n/a	^a
P01860	Immunoglobulin heavy constant gamma 3	IGHG3	3.4	0.00000	1.7	0.00021	n/a	^a
	Complement proteins and complement regulators							
P05155	Plasma protease C1 inhibitor	SERPING1	4.8	0.00000	5.8	0.00000	#1, #2, #3	
P0C0L5	Complement C4-B	C4B	3.1	0.00003	3.6	0.00000	#2, #3	
P01024	Complement C3	C3	2.4	0.00000	2.6	0.00000	#2, #3	
P08603	Complement factor H	CFH	1.9	0.00049	2.4	0.00000		
P00751	Complement factor B	CFB	2.5	0.00000	2.1	0.00014		
	Protease inhibitors							
P19827	Inter- α -trypsin inhibitor heavy chain H1	ITIH1	11.1	0.00000	8.3	0.00000	#2, #3	
P19823	Inter- α -trypsin inhibitor heavy chain H2	ITIH2	9.0	0.00000	6.8	0.00000	#2, #3	
P01023	Alpha-2-macroglobulin	A2M	7.2	0.00000	5.6	0.00000	#1, #2, #3	
P01008	Antithrombin-III	SERPINC1	5.1	0.00000	4.9	0.00000	#2, #3	
P01009	Alpha-1-antitrypsin	SERPINA1	4.2	0.00000	2.7	0.00000	#1, #2, #3	^a
P08697	Alpha-2-antiplasmin	SERPINF2	2.5	0.00007	2.5	0.00008	#1, #2, #3	
P05546	Heparin cofactor 2	SERPIND1	2.3	0.00528	2.1	0.02285	#2, #3	
Q14624	Inter- α -trypsin inhibitor heavy chain H4	ITIH4	1.5	0.00001	1.6	0.00006	#1, #2, #3	
	Lipid transport							
P06727	Apolipoprotein A-IV	APOA4	6.0	0.00000	7.9	0.00000		
P02647	Apolipoprotein A-I	APOA1	9.8	0.00000	5.7	0.00000	#1	^a
P02649	Apolipoprotein E	APOE	1.9	0.01392	4.1	0.00000		^b
P02652	Apolipoprotein A-II	APOA2	4.7	0.00000	3.6	0.00000		
P04114	Apolipoprotein B-100	APOB	1.9	0.00055	3.3	0.00000		^b
	Transport proteins							
P02768	Serum albumin	ALB	4.1	0.00000	3.3	0.00000	#1	
P69905	Hemoglobin subunit α	HBA1	1.2	0.43227	2.9	0.00038		^b
P02787	Serotransferrin	TF	3.8	0.00000	2.8	0.00000	#1	
P68871	Hemoglobin subunit β	HBB	1.0	0.82095	2.6	0.00036		^b
P02774	Vitamin D-binding protein	GC	2.5	0.00000	2.2	0.00000		

Table 3 (Continued)

Accession	Description	Gene symbol	SLE _{nor} vs. HC (FD)	t-test	SLE _{low} vs. HC (FD)	t-test	Top biological process	
P00450	Ceruloplasmin	CP	2.3	0.00000	1.4	0.00001		^a
	Antiviral, antibacterial, acute phase							
P02790	Hemopexin	HPX	11.6	0.00000	7.8	0.00000		
P19652	Alpha-1-acid glycoprotein 2	ORM2	3.7	0.00000	4.5	0.00000	#1	
P42224	Signal transducer and activator of transcription 1- α /beta	STAT1	2.2	0.00000	2.8	0.00015		
P00738	Haptoglobin	HP	3.0	0.00000	2.3	0.00000		
P59665	Neutrophil defensin 1	DEFA1	-1.7	0.00087	2.2	0.02729		^b
	Phosphatidylserine (PS) binding							
P02749	Beta-2-glycoprotein 1	APOH	2.2	0.00000	2.2	0.00004	#1, #2	
P08758	Annexin A5	ANXA5	-1.0	0.54821	1.6	0.00013	#1	^b
	Coagulation and hemostasis							
P00734	Prothrombin	F2	1.8	0.00000	2.1	0.00000	#3	
P09382	Galectin-1	LGALS1	-1.1	0.74120	1.7	0.03659		^b
P01042	Kininogen-1	KNG1	1.5	0.01146	1.3	0.09360	#1, #2, #3	
	Cell-contact and adhesion							
Q08380	Galectin-3-binding protein	LGALS3BP	1.5	0.30258	3.4	0.00560	#1	^b
Q8I WV2	Contactin-4	CNTN4	1.9	0.00000	1.7	0.00113		
P04004	Vitronectin	VTN	1.6	0.00034	1.4	0.00418	#2, #3	
	Other							
Q7Z5M8	Protein ABHD12B	ABHD12B	5.6	0.00000	3.9	0.00000		
Q16822	Phosphoenolpyruvate carboxykinase [GTP], mitochondrial	PCK2	1.9	0.01447	2.4	0.00172		
P02765	Alpha-2-HS-glycoprotein	AHSG	2.5	0.00000	2.2	0.00002	#1, #2, #3	
P51114	Fragile X mental retardation syndrome-related protein 1	FXR1	1.6	0.00038	1.9	0.00005		
P01019	Angiotensinogen	AGT	3.1	0.00002	1.6	0.00312	#2, #3	^a
Q9P035	Very-long-chain (3R)-3-hydroxyacyl-CoA dehydratase 3	HACD3	1.3	0.11545	1.6	0.00368		
Q16799	Reticulon-1	RTN1	1.3	0.08735	1.6	0.00006		
Q02218	2-oxoglutarate dehydrogenase, mitochondrial	OGDH	1.8	0.00004	1.2	0.16368		^a

Abbreviation: FD, fold difference; SLE, systemic lupus erythematosus.

Note: #1: platelet degranulation. #2: negative regulation of endopeptidase activity. #3: negative regulation of proteolysis.

^aSignificantly higher in SLE_{nor} vs. SLE_{low} and HC.

^bSignificantly higher in SLE_{low} vs. SLE_{nor} and HC.

immunoglobulins reflect levels of circulating ICs, we would expect levels of platelet-associated immunoglobulins to be inversely correlated with serum complement. To investigate this, we performed a correlation analysis of complement proteins C1q, C3, and C4 in serum compared with immunoglobulin heavy constant α 1, lambda constant 2, gamma-1 heavy chain, lambda-1 light chain, heavy constant gamma 2,

heavy constant mu, and heavy constant gamma 3. Indeed, serum levels of complement protein C3 were negatively associated with platelet heavy constant gamma 2 ($r = -0.65$, p -value = 0.049), while serum levels of C4 were inversely correlated with platelet heavy constant mu ($r = -0.644$, p -value = 0.049), heavy constant α 1 ($r = -0.847$, p -value = 0.003) and heavy constant gamma 3 ($r = -0.798$, p -value

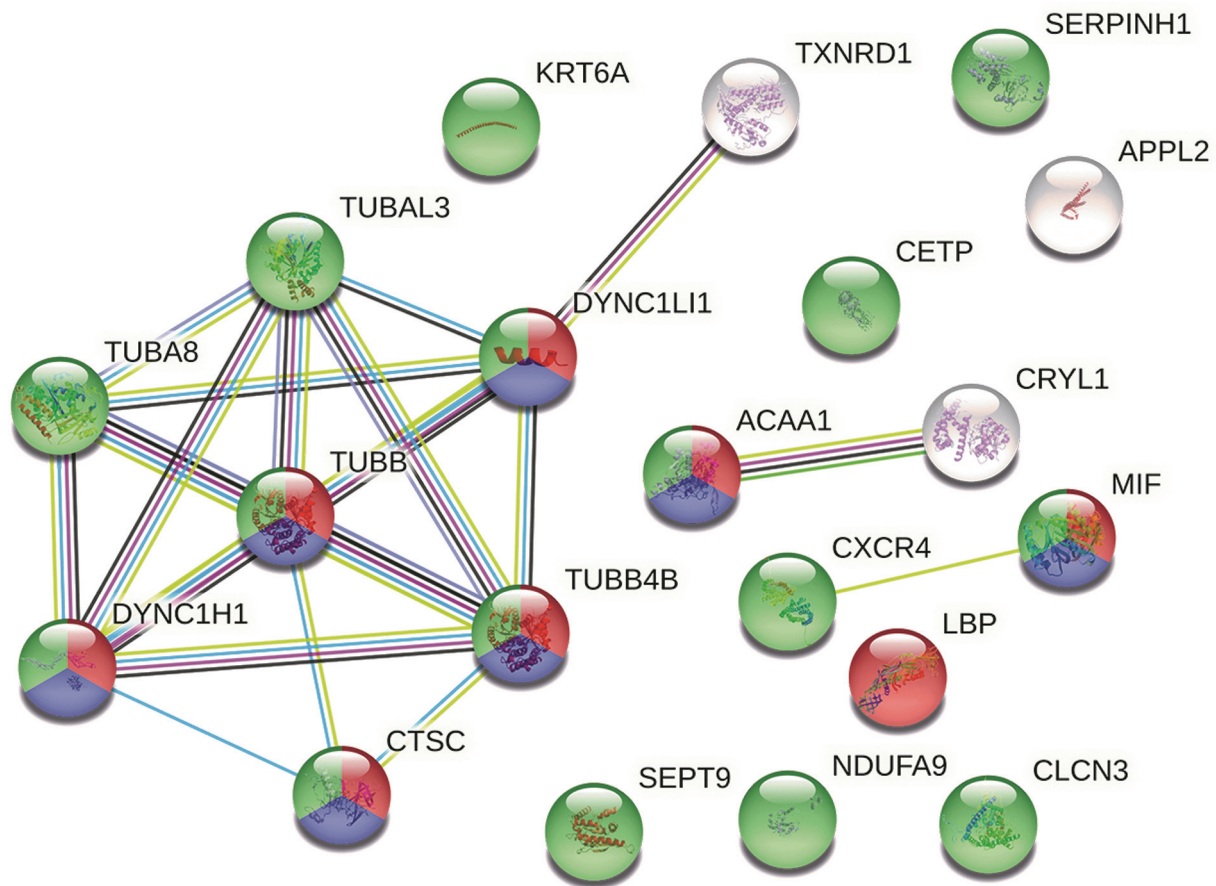


Fig. 5 Network of proteins with higher levels in HC compared with SLE. Twenty proteins had higher levels in HC compared with SLE (SLE_{low} and SLE_{nor}) with a PPI enrichment p -value of 1.65×10^{-9} . The top three biologic process (GO) were: (1) myeloid cell activation involved in immune response (red), FDR 2.41×10^{-5} , strength 1.2; (2) neutrophil degranulation (blue), FDR 6.90×10^{-5} , strength 1.2; and (3) cellular component organization (green), FDR 7.09×10^{-5} , strength 0.5. FDR, false discovery rate; HC, healthy control; PPI, protein–protein interaction; SLE, systemic lupus erythematosus.

0.008) (► **Fig. 6**). No correlation between serum levels of complement protein C1q was found (data not shown). The most striking difference between SLE_{nor} and SLE_{low} was the difference in relative levels of immunoglobulins. Immunoglobulin protein heavy constant mu, heavy constant α 1, heavy constant gamma 3, and lambda constant 2 had significantly higher levels in SLE_{nor} compared with SLE_{low} platelets, and platelet sizes in SLE groups were also positively correlated to these immunoglobulins (► **Supplementary Fig. S3**, available in the online version).

Discussion

To our knowledge, this is the first study to use high-throughput proteomics to address the protein characteristics of platelets in patients with SLE, providing new insights into the protein composition and their possible role. We aimed not only to characterize possible differences between platelets from SLE patients and healthy individuals, but also sought to find clues that could explain the reduced platelet size observed in SLE, using mass spectrometry. To achieve this, we selected five platelet isolates with the smallest (SLE_{low}) and five with the largest (SLE_{nor}) average platelet size, out of a cohort of 23 patients with SLE. The analysis

showed that, despite being selected based on their size difference, platelets from SLE patients had a similar protein profile, sharing significant differences compared with the platelets from HCs. The core of this profile consisted of 40 proteins with functions pointing to increased degranulation and a skewed hemostatic balance. Distinct differences in individual protein groups with potential implications in SLE were also observed, including increased levels of immunoglobulins, complement proteins, and proteins associated with PS binding and apoptosis. The overrepresentation of proteins involved in degranulation in platelets from SLE patients is consistent with observations of platelets in SLE being chronically activated and degranulated.¹ They are furthermore recognized as a major source of circulating microparticles that contain cytoskeletal proteins.³⁷ It is tempting to speculate that this could explain the relative difference in proteins related to the cell integrity and dynamics the cytoskeleton (► **Fig. 5**), i.e., if platelets from SLE patients lose structural proteins during microparticle release, it could potentially explain this difference in protein profile. Thrombosis is a major cause of morbidity and mortality in SLE and the increased risk for thrombotic events is at least partially attributed to increased platelet activation.³⁸ SLE_{low} and SLE_{nor} platelets were enriched in proteins

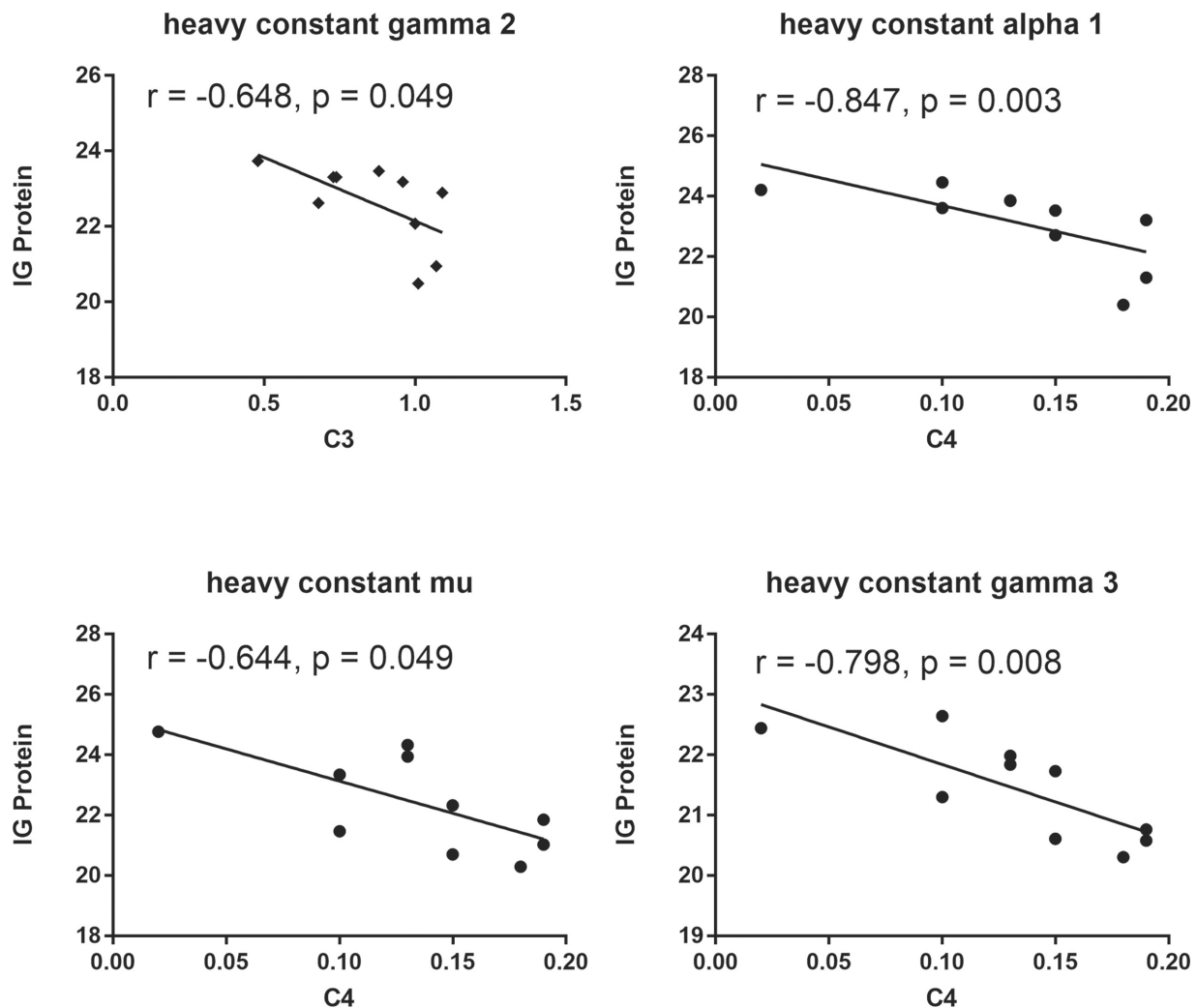


Fig. 6 Negative correlations between relative IG (MS) and complement levels (serum). Negative correlations between C3 and heavy constant gamma 2 ($r = -0.648, p = 0.049$), C4 and heavy constant α 1 ($r = -0.847, p = 0.003$), heavy constant mu ($r = -0.644, p = 0.049$) and heavy constant gamma 3 ($r = -0.798, p = 0.008$) were found (Spearman correlation, $p < 0.05, n = 10$). IG, immunoglobulin; MS, mass spectrometry.

associated with hemostatic functions (→ Fig. 4). Among these were proteins of the large family of serpins, including antithrombin (SERPINC1), alpha-1-antitrypsin (SERPINA1), alpha-2-antiplasmin (SERPINF2), and heparin cofactor 2 (SERPIND1). Serpins have both pro- and anticoagulatory effects, balancing hemostasis and fibrinolysis through key regulatory functions in coagulation, the protein C pathway, and fibrinolysis²³ (→ Table 3). Additionally, platelets from SLE patients were also enriched in complement proteins, implicated in thrombotic events in SLE patients.³⁹ While we are unable to determine the exact functional implications of this enrichment, it can be concluded that the maintenance of hemostatic balance is skewed in platelets from SLE patients, exemplified by the 4.9- and 5.1-fold higher level of antithrombin versus HC, in SLE_{low} and SLE_{nor}, respectively.

Levels of seven immunoglobulin proteins were increased in both SLE groups, but the relative difference was greatest in normal-sized platelets, ranging from 2.9- to 14-fold higher levels compared with HC. There are several possible explanations to this interesting observation. First, it should be noted that platelets have a “sponge-like” surface, adsorbing plasma

components through perfusion of the open canalicular system⁴⁰ and vesicles,⁴¹ making a total removal of plasma components complicated and therefore not possible to rule out completely. However, the disproportionate distribution of immunoglobulins relative to other proteins commonly found in plasma argues against this as the only explanation. Second, subsets of platelets from healthy individuals have been identified as more prone to act as vehicles for circulating proteins including immunoglobulins and other immunoreactive proteins,⁴² consistent with the proposed role for platelets in SLE¹ and it has been known for decades that subcellular immunoglobulin G (IgG) is present in platelet α -granules.²⁷ In patients diagnosed with immune thrombocytopenic purpura, on the other hand, platelets are targeted by antiplatelet antibodies and both increased levels of complement factor C3 and C4, and immunoglobulins are detected on the platelet surface.⁴³ Thus, platelet from SLE patients may harbor subcellular immunoglobulins, but considering the observed accumulation of known autoantigens in platelets one might also have to consider contribution from autoantibodies or direct interaction with ICs. Platelets express the low-affinity

immunoglobulin gamma Fc region receptor IIA (CD32) that, when targeted by IgG-containing ICs,⁴⁴ can stimulate platelet activation and internalization of ICs.⁴⁵ Thus, if increased levels of platelet-associated immunoglobulins are indeed associated with higher levels of circulating ICs, we would expect to find an increased level of platelet activation and degranulation, mediated by FcγRIIA.⁴⁶

Both SLE groups showed increased levels of plasma protease C1 inhibitor, complement C4B, complement C3, CFH, and CFB. Complement proteins are believed to interact extensively with platelets and evidence suggests that these interactions may promote key pathogenic mechanisms in SLE, including vascular inflammation and thrombosis.¹ C1 inhibitor is a major complement regulator, found to be secreted from platelet α-granules, but also present on the surface of activated platelets.⁴⁷ Complement protein C4 is a member of the classical pathway of complement activation and C3 is common for the classical, alternative, and the lectin pathways. Upon activation of the classical complement pathway, C4 is cleaved, resulting in C4a and C4b. C4a diffuses away, while the highly reactive C4b rapidly forms a covalent bond to a nearby protein or carbohydrate.⁴⁸ CFH is a complement regulator that can be stored and released from platelet α-granules,⁴⁷ competing with CFB attachment to C3b, limiting formation of the alternative pathway C3 convertase C3bBb.⁴⁹ The main activators of the classical complement pathway are ICs of IgG and IgM type⁵⁰ and ICs of IgG, IgM, and IgA type are commonly found to be elevated in patients with SLE with increased disease activity.⁵¹ Despite that patients in this study had a low median disease activity, their platelet proteome showed increased levels of C4 and future studies will have to determine if this can be related to exposure to ICs or an ongoing activation of the classical pathway. A split product of C4, C4d, has been found in increased levels on SLE patient platelet surfaces and is associated with increased disease activity, thrombosis and antiphospholipid antibodies.⁵²

SLE_{nor} differed from SLE_{low} in significantly higher immunoglobulin levels, and it is tempting to speculate that this at least partially reflects levels of circulating ICs, potentially bound by the FcγRIIA. Since increased levels of ICs are considered a leading cause of acquired complement deficiency in SLE,⁵³ we would expect to find an association with circulating complement. Indeed, serum levels of C3 were negatively associated with heavy constant gamma 2 and levels of C4 inversely correlated with heavy constant α 1, heavy constant mu, and heavy constant gamma 3 (→ **Fig. 6**). This supports the concept that immunoglobulin levels on platelets at least partially may reflect levels of circulating ICs.

β2GPI is prevalent in plasma and has been associated with a wide range of functions.⁵⁴ It inhibits platelet activation and binds PS on apoptotic cells, aiding their phagocytic removal by macrophages.⁵⁵ A similar role has been attributed to annexin A5 that binds specifically to PS, promoting clearance by phagocytes.⁵⁶ Exposure of PS serves as an “eat-me” signal, supporting noninflammatory clearance by phagocytes.⁵⁷ Interestingly, in addition to its well-recognized role in hemostasis, prothrombin (F2) also has a strong affinity to PS on apoptotic cells.³¹ Unlike most other cells however,

platelets may expose PS during both activation and apoptosis,³⁰ making interpretation of the accumulation of annexin A5, β2GPI, and prothrombin less clear. Potentially in favor of apoptosis, nonetheless, is the overrepresentation of complement (C3, C4, and factor H), known to be involved in opsonization of apoptotic cells.⁴⁹ Annexin A5 is also a C1q ligand⁵⁸ and a possible biomarker in SLE.^{59–61} Apoptosis is also evident in other circulating cell types⁶² in SLE and it is known that apoptotic cells, including platelets, undergo distinct morphological changes, such as cell shrinkage and plasma membrane blebbing and increased release of microparticles.^{16,63,64} Interestingly, in addition to being associated with apoptosis, annexin A5, β2GPI, and prothrombin are also identified as autoantibody targets linked to APS (→ **Table 3**).^{60,65–67} Clearance of apoptotic cells is tightly regulated and chiefly dependent on signals on the cell surface.⁶⁸ It has been shown that a disturbed apoptotic process and membrane changes can promote a breach in tolerance¹⁸ and an increase in reactivity of antiphospholipid antibodies,⁶⁹ but the accumulation of APS-related autoantigens in platelets is to our knowledge a novel finding, suggesting that platelets may be an overlooked source of autoantigens in SLE.¹ It should be noted, however, that no patient in this study had APS (one from the SLE_{low} and one from the SLE_{nor} group were positive for anti-β2GPI antibodies). SLE_{low} had a significant overrepresentation of annexin A5 compared with SLE_{nor} and slightly higher level of complement proteins, providing some support to the hypothesis that the decreased average platelet size in SLE could be at least partially dependent on the increased level of apoptosis. Considering this enrichment of PS-binding proteins in platelets from SLE patients, a corresponding increase in AV-positive cells might be expected. The functional characterization using flow cytometry showed a tendency in support of this, with a higher level of AV-positive cells from patients with SLE, but the difference was not statistically significant and there was no difference in MFI values, nor did we detect any difference between small-sized and normal-sized platelets from SLE patients (→ **Supplementary Fig. S2**, available in the online version), including no size related difference in AV-positive cells. Thus, further studies are needed, to determine the location of these antigens and their potential relation to platelet size and apoptosis. A potential limitation to this study is the relatively small patient cohort, which increases the risk of it not being representative and generalizable. Additionally, our method of selecting samples is relative and observation-based. Although sufficient to meet our objectives in this study, the method has not been validated or described previously. Therefore, we emphasize the need to replicate our findings in other cohorts, before attempting to generalize these results to a wider selection of SLE patients. Future studies may also be able determine if observations presented here are exclusive to platelets from patients with SLE or if these changes are universal to platelets from patients with other autoimmune conditions.

Moreover, the overrepresentation of hemoglobin subunits α and β in SLE_{low} may raise questions and concerns about

possible hemolysis during sample preparation (→ **Table 3**). We find this unlikely, however. Hemoglobins are common in plasma and other studies have shown a high level of these proteins in the platelet proteome, determined free from contamination.³⁶ It is also noteworthy that other highly abundant erythrocyte proteins, such as hemoglobin subunit gamma (estimated at 40 million copies per erythrocyte),⁷⁰ were absent from the SLE_{low} proteome.

Platelets from the two subgroups of SLE patients included in this study shared a large majority of their differences, compared with platelets from HC. This included accumulation of complement proteins and immunoglobulins, in keeping with previous observations made by us and other groups. We furthermore show that SLE patients' platelet size is largely independent of protein content, also supported by functional characterization, showing no differences in platelet activation markers between normal and decreased sizes. This study supports the idea that platelets have an integrated role in SLE pathogenesis and potential interactions between platelets and ICs. However, the hemostatic function of platelets from SLE patients may also be significantly altered, as suggested by the enrichment in hemostatic regulators in platelets from SLE patients. The accumulation of autoantigens associated with apoptosis in platelets from SLE patients also strengthens the need to clarify the role of platelets in autoimmunity and SLE.

What is known about this topic?

- Platelets' nonhemostatic properties could play a role in the pathogenesis of SLE.
- Platelets from SLE patients display an activated phenotype.
- Morphologic changes including decreased average platelet size have been observed in SLE, but the functional role of this has not been determined.

What does this paper add?

- Characterization of the functional properties of the proteome of platelets from SLE patients of decreased and normal sizes revealed significant differences compared with healthy controls, suggesting increased degranulation, skewed homeostatic balance, and loss of structural proteins.
- Specifically, platelets from SLE patients were enriched in immunoglobulin proteins, complement proteins, and known autoantigens in SLE.
- Except an even greater enrichment in immunoglobulin proteins in platelets from SLE patients of normal compared with decreased size, the platelet proteome from SLE patients was largely independent of platelet size.

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Conflict of Interest

C.P.L. reports no conflict of interests. A.J. reports no conflict of interests. H.T. reports no conflict of interests. B.G. reports no conflict of interests. H.Y. reports no conflict of interests. C.W. reports no conflict of interests. R.K. reports no conflict of interests. A.J. reports, outside the submitted work, consulting fees from Astra Zeneca and GlaxoSmithKline. J.W.S. reports no conflict of interests. A.A.B. reports no conflict of interests.

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