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
- Complement
- Autoantibodies
- 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. 

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. 

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.  

Platelets are known to express several complement proteins and receptors and they promote and actively initiate complement activation on the platelet surface. 

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.  

Platelets also contain antigens and damage-associated molecular patterns, known to be involved in SLE pathogenesis. 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, but there is no clear consensus regarding the role of platelet size in SLE. Platelets from SLE patients also show signs of being degranulated, depleted in serotonin, and are a major source of microparticles. Decreased-sized platelets are also associated with antiphospholipid antibodies. The mechanism responsible for the decreased platelet size in SLE is not known, but increased rates of apoptosis can reduce platelet size presenting a possible explanation of the smaller size. This explanation would also be consistent with the abnormal apoptosis patterns observed in other circulating cell types in patients with SLE. 

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,
Whole blood from all patients with SLE and HC were collected in ACD tubes (BD) and centrifuged at 200 g for 15 minutes 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). Included patients received oral and written information, and all had Caucasian heritage. All individuals were selected as controls. Their mean age was 44 years, 80% were female.

**Table 1** Clinical characteristics of SLE patients

| Parameter                        | Value           |
|----------------------------------|-----------------|
| Number (n)                       | 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 (n)                   | 7/23            |
| Anti-C1q (n)                     | 0/23            |
| Anti-β2GP1 (n)                   | 3/23            |
| Anti-CL (n)                      | 1/23            |
| ANA (ever) (n)                   | 23/23           |
| SLEDAI score, median (range)     | 2 (0–10)        |
| Pharmacologic treatment          |                 |
| HCQ/CQ (n)                       | 17              |
| Aza (n)                          | 4               |
| Beli (n)                         | 4               |
| Mtx (n)                          | 4               |
| MMF (n)                          | 3               |
| Rtx (n)                          | 1               |
| Cic (n)                          | 1               |
| GC (n, mean dose)                | 14 (5.7 mg)     |

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 female, 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 μM. To minimize platelet activation and aggregation, PSG buffer with PGE1 was used in all washing procedures throughout the 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/IIa 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 SLElow) and the five with highest FSC (referred to as SLEnor). Prior to mass spectrometry analysis, platelets were purified from contaminating white and red blood cells using negative selection. 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 μL 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 μg) 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 μL 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 Trivid 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 μg of the peptides was injected into the LC-MS. Peptides were concentrated on an Acclaim PepMap 100 C18 precolumn (75 μm × 2 cm, Thermo Scientific, Waltham, Massachusetts, United States) and then separated on an Acclaim PepMap RSLC column (75 μm × 25 cm, nanoViper, C18, 2 μm, 100 Å) 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 × 10^5 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 PRIDE 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 (log2), 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 log2-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.5 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
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 (SLEnor) had equal levels compared with HC, while the five with the lowest FSC (SLElow) 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 SLEnor platelets had significantly lower levels of complement C3 and C4 (t-test p-value, 0.04 and 0.009) (►Table 2).

The proteomes from SLElow and SLEnor 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 SLElow (n = 5) and SLEnor (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 |
|----|-----|---------|--------|----------------|---------------|----------|------------|--------|----------|----------|--------------|--------------|-----------|
| SLElow 1 | F | 54 | 16 | 228 | 5.8 | 124 | 0.6 | 97 | 1.01 | 0.18 | 4 | Proteinuria | HCQ, MMF, GC 7.5 mg |
| SLElow 2 | F | 27 | 7 | 271 | 4.3 | 137 | 0.6 | 80 | 1.09 | 0.15 | 2 | DNA | HCQ, Aza |
| SLElow 3 | F | 42 | 14 | 189 | 7.0 | 125 | 0.7 | 120 | 0.88 | 0.15 | 8 | Pyuria, arthritis | Bel, Aza, GC 2.5 mg |
| SLElow 4 | F | 47 | 29 | 150 | 10.3 | 151 | 0.6 | 87 | 0.96 | 0.19 | 0 | N/A | HCQ, Cicl, Bel, GC 10 mg |
| SLElow 5 | M | 57 | 8 | 230 | 4.6 | 146 | 0.7 | 138 | 1.00 | 0.19 | 2 | Rash | HCQ |
| SLEnor 1 | F | 48 | 29 | 165 | 10.6 | 151 | 0.6 | 110 | 0.74 | 0.13 | 2 | LC | Bel, CQ, GC 7.5 mg |
| SLEnor 2 | F | 69 | 10 | 165 | 4.8 | 136 | 0.6 | 59 | 0.48 | 0.02 | 2 | LC | HCQ |
| SLEnor 3 | F | 46 | 4 | 284 | 7.5 | 138 | 0.9 | 107 | 0.68 | 0.10 | 4 | LC, DNA | Mtx, Bel, GC 10 mg |
| SLEnor 4 | F | 47 | 21 | 239 | 3.5 | 110 | 8.0 | 87 | 1.07 | 0.13 | 0 | N/A | Aza, HCQ, NSAID |
| SLEnor 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.
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<sub>low</sub> and SLE<sub>nor</sub>. 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<sub>low</sub> and SLE<sub>nor</sub>, 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<sub>low</sub> or SLE<sub>nor</sub>, 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<sub>low</sub> and SLE<sub>nor</sub> Proteomes: Indications of Degranulation and Skewed Hemostatic Function

An unbiased evaluation of annotated protein function in proteins with higher abundance in SLE<sub>low</sub> and SLE<sub>nor</sub> showed a significant enrichment of several biological processes, compared with the expected from chance alone (Fig. 4).
Additionally, we observed a relative accumulation of galectin-1 in SLE<sub>low</sub> relative to SLE<sub>nor</sub> 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 microparticles 25 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<sub>nor</sub> (► Table 3). Compared with SLE<sub>low</sub>, SLE<sub>nor</sub> had a 4.4-fold higher levels of Ig heavy constant mu (IGHM), 3.0-fold higher of Ig heavy constant α 1 (IGHA1), and 2.1-fold higher levels of Ig heavy constant gamma 3 (IGHC3). 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<sub>low</sub> and SLE<sub>nor</sub> displayed a consistent overrepresentation of plasma protease C1 inhibitor (SPR-ING1), 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<sub>low</sub> and SLE<sub>nor</sub> was detected although the SLE<sub>nor</sub> 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, β2GPI) had an equally increased abundance in SLE<sub>low</sub> and SLE<sub>nor</sub>. β2GPI 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<sub>low</sub> 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<sub>low</sub> and SLE<sub>nor</sub>.

Increased Presence of Common Plasma Proteins in the SLE<sub>low</sub> and SLE<sub>nor</sub> Proteomes

One set of common plasma proteins, including neutrophil defensin 1 (DEFA1), hemoglobin subunits α and β (HBA1 and HBB), galectin-3-binding protein (LGAL3B3), apolipoprotein E (Apo E), and apolipoprotein B-100 (APOB), was detected in significantly higher levels in SLE<sub>low</sub> compared with SLE<sub>nor</sub>, while other traditional plasma proteins, such as angiotensinogen, apolipoprotein A-I (Apo AI),

The network of proteins with increased abundance in SLE<sub>low</sub> and SLE<sub>nor</sub> 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 × 10<sup>−16</sup>. 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<sup>−16</sup>, suggesting that SLE<sub>low</sub> and SLE<sub>nor</sub> 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 antithrombin-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<sub>low</sub> and SLE<sub>nor</sub> have an altered hemostatic function, compared with platelets from HC.

Fig. 3 Overlapping and different protein levels in platelet proteome of SLE<sub>nor</sub>, SLE<sub>low</sub>, 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 SLE<sub>nor</sub> and SLE<sub>low</sub> had 20 proteins with higher levels than both SLE groups, 67 compared with SLE<sub>nor</sub> only, and 32 compared with SLE<sub>low</sub>.
ceruloplasmin (CP) and α-1-antitrypsin (α1AT), had increased abundance in SLEnor (►Table 3). A more detailed presentation of the relative difference between SLElow and SLEnor 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 SLElow and SLEnor

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^{-15} (►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^{-15} 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, 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 and LBP has also been detected in the platelet proteome. 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 SLEnor and 3.7-fold higher levels compared with SLElow. 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
| Accession | Description                                           | Gene symbol | SLE<sub>nor</sub> vs. HC (FD) | t-test | SLE<sub>low</sub> vs. HC (FD) | t-test | Top biological process |
|-----------|-------------------------------------------------------|-------------|-------------------------------|--------|-------------------------------|--------|------------------------|
| P01876    | Immunoglobulin heavy constant α 1                     | IGHA1       | 14.0                          | 0.0000 | 4.7                           | 0.0000 | n/a                    |
| P0DOY2    | Immunoglobulin lambda constant 2                       | IGLC2       | 6.2                           | 0.0000 | 3.2                           | 0.0000 | n/a                    |
| P0DOX5    | Immunoglobulin gamma-1 heavy chain                     | N/A         | 4.6                           | 0.0000 | 3.1                           | 0.0000 | n/a                    |
| P0DOX8    | Immunoglobulin lambda-1 light chain                    | N/A         | 3.6                           | 0.0000 | 2.8                           | 0.0000 | n/a                    |
| P01859    | Immunoglobulin heavy constant gamma 2                  | IGHG2       | 2.9                           | 0.0000 | 2.5                           | 0.0013 | n/a                    |
| P01871    | Immunoglobulin heavy constant mu                       | IGHM        | 10.5                          | 0.0000 | 2.4                           | 0.0014 | n/a                    |
| P01860    | Immunoglobulin heavy constant gamma 3                  | IGHG3       | 3.4                           | 0.0000 | 1.7                           | 0.0021 | n/a                    |
| P05155    | Plasma protease C1 inhibitor                           | SERPING1    | 4.8                           | 0.0000 | 5.8                           | 0.0000 | #1, #2, #3             |
| P0C0L5    | Complement C4-B                                        | C4B         | 3.1                           | 0.0000 | 3.6                           | 0.0000 | #2, #3                 |
| P01024    | Complement C3                                         | C3          | 2.4                           | 0.0000 | 2.6                           | 0.0000 | #2, #3                 |
| P08603    | Complement factor H                                    | CFH         | 1.9                           | 0.0004 | 2.4                           | 0.0000 | #2, #3                 |
| P00751    | Complement factor B                                    | CFB         | 2.5                           | 0.0000 | 2.1                           | 0.0014 | #2, #3                 |
| P19827    | Inter-α-trypsin inhibitor heavy chain H1               | ITIH1       | 11.1                          | 0.0000 | 8.3                           | 0.0000 | #2, #3                 |
| P19823    | Inter-α-trypsin inhibitor heavy chain H2               | ITIH2       | 9.0                           | 0.0000 | 6.8                           | 0.0000 | #2, #3                 |
| P01023    | Alpha-2-macroglobulin                                  | A2M         | 7.2                           | 0.0000 | 5.6                           | 0.0000 | #1, #2, #3             |
| P01008    | Antithrombin-III                                       | SERPINC1    | 5.1                           | 0.0000 | 4.9                           | 0.0000 | #2, #3                 |
| P01009    | Alpha-1-antitrypsin                                    | SERPINA1    | 4.2                           | 0.0000 | 2.7                           | 0.0000 | #1, #2, #3             |
| P08697    | Alpha-2-antiplasmin                                    | SERPINF2    | 2.5                           | 0.0000 | 2.5                           | 0.0008 | #1, #2, #3             |
| P05546    | Heparin cofactor 2                                     | SERPIND1    | 2.3                           | 0.0052 | 2.1                           | 0.0228 | #2, #3                 |
| Q14624    | Inter-α-trypsin inhibitor heavy chain H4               | ITIH4       | 1.5                           | 0.0000 | 1.6                           | 0.0006 | #1, #2, #3             |
| P06727    | Apolipoprotein A-IV                                    | APOA4       | 6.0                           | 0.0000 | 7.9                           | 0.0000 | #1, #2, #3             |
| P02647    | Apolipoprotein A-I                                     | APOA1       | 9.8                           | 0.0000 | 5.7                           | 0.0000 | #1, #2, #3             |
| P02649    | Apolipoprotein E                                       | APOE        | 1.9                           | 0.0139 | 4.1                           | 0.0000 | b                      |
| P02652    | Apolipoprotein A-II                                    | APOA2       | 4.7                           | 0.0000 | 3.6                           | 0.0000 | b                      |
| P04114    | Apolipoprotein B-100                                   | APOB        | 1.9                           | 0.0005 | 3.3                           | 0.0000 | b                      |
| P02768    | Serum albumin                                          | ALB         | 4.1                           | 0.0000 | 3.3                           | 0.0000 | #1, #2, #3             |
| P69905    | Hemoglobin subunit α                                   | HBA1        | 1.2                           | 0.4322 | 2.9                           | 0.0038 | b                      |
| P02787    | Serotransferrin                                        | TF          | 3.8                           | 0.0000 | 2.8                           | 0.0000 | #1, #2, #3             |
| P68871    | Hemoglobin subunit β                                   | HBB         | 1.0                           | 0.8209 | 2.6                           | 0.0036 | b                      |
| P02774    | Vitamin D-binding protein                              | GC          | 2.5                           | 0.0000 | 2.2                           | 0.0000 | b                      |
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 α1, 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 α1 ($r = -0.847$, p-value = 0.003) and heavy constant gamma 3 ($r = -0.798$, p-value = 0.002).

### 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 | *                        |
| P02790    | Hemopexin                     | HPX         | 11.6                  | 0.00000 | 7.8                   | 0.00000 | #1                      |
| P19652    | Alpha-1-acid glycoprotein 2   | ORM2        | 3.7                   | 0.00000 | 4.5                   | 0.00000 | #1                      |
| P42224    | Signal transducer and activa- | STAT1       | 2.2                   | 0.00000 | 2.8                   | 0.00015 | #1                      |
| P00738    | Haptoglobin                   | HP          | 3.0                   | 0.00000 | 2.3                   | 0.00000 | #1                      |
| P59665    | Neutrophil defensin 1         | DEFA1       | −1.7                  | 0.00087 | 2.2                   | 0.02729 | #1                      |
|           | Phosphatidylycerine (PS)      |             |                       |         |                       |         |                        |
| 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                     |
|           | 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 | #1                     |
| 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                     |
| Q8IWV2    | Contactin-4                   | CNTN4       | 1.9                   | 0.00000 | 1.7                   | 0.00113 | #1                     |
| 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 | #1                     |
| Q16822    | Phosphoenolpyruvate car-      | PCK2        | 1.9                   | 0.01447 | 2.4                   | 0.00172 | #1                     |
|           | boxykinase [GTP], mitochondrial|             |                       |         |                       |         |                        |
| P02765    | Alpha-2-HS-glycoprotein       | AHSG        | 2.5                   | 0.00000 | 2.2                   | 0.00002 | #1, #2, #3             |
| P51114    | Fragile X mental retardation  | FXR1        | 1.6                   | 0.00038 | 1.9                   | 0.00005 | #1, #2, #3             |
| P01019    | Angiotensinogen                | AGT         | 3.1                   | 0.00002 | 1.6                   | 0.00312 | #2, #3                 |
| Q9P035    | Very-long-chain (3R)-3-        | HACD3       | 1.3                   | 0.71545 | 1.6                   | 0.00368 | #1                     |
|           | hydroxyacyl-CoA dehydro-      |             |                       |         |                       |         |                        |
|           | tase 3                         |             |                       |         |                       |         |                        |
| Q16799    | Reticulon-1                   | RTN1        | 1.3                   | 0.08735 | 1.6                   | 0.00006 | #1                     |
| Q02218    | 2-oxoglutarate dehydro-       | OGDH        | 1.8                   | 0.00004 | 1.2                   | 0.76368 | #1                     |
|           | nase, mitochondrial           |             |                       |         |                       |         |                        |

Abbreviation: FD, fold difference; SLE, systemic lupus erythematosus.
Note: #1: platelet degranulation. #2: negative regulation of endopeptidase activity. #3: negative regulation of proteolysis.
*Significantly higher in SLE_{nor} vs. SLE_{low} and HC.
**Significantly higher in SLE_{low} vs. SLE_{nor} and HC.
No correlation between serum levels of complement protein C1q was found (data not shown). The most striking difference between SLENor and SLELow was the difference in relative levels of immunoglobulins. Immunoglobulin protein heavy constant mu, heavy constant α1, heavy constant gamma 3, and lambda constant 2 had significantly higher levels in SLENor compared with SLELow platelets, and platelet sizes in SLE groups were also positively correlated to these immunoglobulins (Fig. 6). No correlation between serum levels of complement protein C1q was found (data not shown). The most striking difference between SLENor and SLELow was the difference in relative levels of immunoglobulins. Immunoglobulin protein heavy constant mu, heavy constant α1, heavy constant gamma 3, and lambda constant 2 had significantly higher levels in SLENor compared with SLELow platelets, and platelet sizes in SLE groups were also positively correlated to these immunoglobulins (Fig. 6). 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. They are furthermore recognized as a major source of circulating microparticles that contain cytoskeletal proteins. 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. SLELow and SLENor platelets were enriched in proteins

![Fig. 5](https://source.unsplash.com/random/1200x800) Network of proteins with higher levels in HC compared with SLE. Twenty proteins had higher levels in HC compared with SLE (SLELow and SLENor) with a PPI enrichment p-value of 1.65e-09. 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.
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 SLElow and SLEnor, 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 immunoactive 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

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**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 alpha 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.
immunoglobulin gamma Fc region receptor IIA (CD32) that, when targeted by IgG-containing ICs, can stimulate platelet activation and internalization of ICs. 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 FcYRIIA.

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. C1 inhibitor is a major complement regulator, found to be secreted from platelet α-granules, but also present on the surface of activated platelets. 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. CFH is a complement regulator that can be stored and released from platelet α-granules, competing with CFB attachment to C3b, limiting formation of the alternative pathway C3 convertase C3bBb. The main activators of the classical complement pathway are ICs of IgG and IgM type and ICs of IgG, IgM, and IgA type are commonly found to be elevated in patients with SLE with increased disease activity. 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.

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 FcYRIIA. Since increased levels of ICs are considered a leading cause of acquired complement deficiency in SLE, 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 μ, 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. It inhibits platelet activation and binds PS on apoptotic cells, aiding their phagocytic removal by macrophages. A similar role has been attributed to annexin A5 that binds specifically to PS, promoting clearance by phagocytes. Exposure of PS serves as an “eat-me” signal, supporting noninflammatory clearance by phagocytes. Interestingly, in addition to its well-recognized role in hemostasis, prothrombin (F2) also has a strong affinity to PS on apoptotic cells. Unlike most other cells however, platelets may expose PS during both activation and apoptosis, 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. Annexin A5 is also a C1q ligand and a possible biomarker in SLE. Apoptosis is also evident in other circulating cell types 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. Interestingly, in addition to being associated with apoptosis, annexin A5, β2GPI, and prothrombin are also identified as autoantibody targets linked to APS.

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...
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.

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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. R.K. reports no conflict of interests. C.W. 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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**What is known about this topic?**
- Platelets’ non-haemostatic properties could play a role in the pathogenesis of SLE.
- Platelets from SLE patients display an activated phenotype.
- Morphological 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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