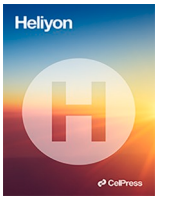
Heliyon 10 (2024) e36131

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Autophagy-enabled protein degradation: Key to platelet activation and ANGII production in patients with type 2 diabetes mellitus

Qiang Wu [a,c,d], Siwen Yu [d], Shufei Zang [e], Kangkang Peng [c], Zhicheng Wang [b,*]

[a] Department of Clinical Laboratory Medicine, Huashan Hospital, Shanghai Medical College, Fudan University, Shanghai, 200040, China
[b] Department of Transfusion Medicine, Huashan Hospital, Fudan University, Shanghai, 200040, China
[c] Department of Clinical Laboratory Medicine, Sijing Hospital of the Songjiang District of Shanghai, Shanghai, 201601, China
[d] Department of Clinical Laboratory Medicine, Shanghai Fifth People's Hospital, Fudan University, Shanghai, 200240, China
[e] Department of Endocrinology, Shanghai Fifth People's Hospital, Fudan University, Shanghai, 200240, China

ARTICLE INFO

Keywords:
Angiotensinogen
Autophagy
Lysosome
Platelet
Thrombosis
Type 2 diabetes mellitus (T2DM)

ABSTRACT

Background: Type 2 diabetes mellitus (T2DM) presents a thrombotic environment, contributing to diabetic macroangiopathy and microangiopathy. In this study, the regulation of microthrombosis in T2DM was assessed.

Methods: Platelets from T2DM patients and healthy controls were analyzed using 4D label-free proteomics and bioinformatics. The role of autophagy in T2DM platelet activation and conversion of platelet-derived angiotensinogen (AGT) was investigated.

Results: The results showed that complement and coagulation cascades, platelet activation, metabolic pathways, endocytosis, autophagy, and other protein digestion-related pathways were enriched. The levels of the key protein AGT were increased in T2DM platelets. Chloroquine (CQ) inhibited ADP- or arachidonic acid (AA)-stimulated platelet aggregation and granule release in a dose-dependent manner, while the effects were less pronounced or even reversed for the proteasome inhibitor PYR-41 and the endocytosis inhibitor Pitstop 2. This indicated the dependence of platelet activation and the accompanying protein digestion on the autophagy-lysosome pathway. Mitophagy occurred in fresh T2DM platelets and ADP- or storage-stimulated platelets; mitophagy was inhibited by CQ. However, the mitophagy inhibitor Mdivi-1 failed to show effects similar to those of CQ. AGT, which could be transformed into ANGII in vitro by ADP-stimulated platelets, was upregulated in T2DM platelets and in MEG-01 cell-derived platelets cultured in a high-glucose medium. Finally, microthrombosis was alleviated as indicated by a reduction in the levels of red blood cells in the liver, spleen, heart, and kidney tissues of db/db mice treated with CQ or valsartan.

Conclusion: In platelets, macroautophagy promotes protein digestion, subsequently facilitating platelet activation, ANGII-mediated vasoconstriction, and microthrombosis. Our results suggested that lysosome is a promising therapeutic target for antithrombotic treatment in T2DM.

* Corresponding author. Department of Transfusion Medicine, Huashan Hospital, Fudan University, No. 12 Middle Urumqi Road, Shanghai, 200040, China.
E-mail address: wangzhicheng@huashan.org.cn (Z. Wang).

https://doi.org/10.1016/j.heliyon.2024.e36131
Received 6 May 2024; Received in revised form 1 August 2024; Accepted 9 August 2024
Available online 10 August 2024

1. Introduction

Type 2 diabetes mellitus (T2DM) affects about 5282 per 100,000 people worldwide [1], and its incidence has increased among youths [2]. Cardiovascular disease affects about 32.2 % of T2DM patients and accounts for nearly half of all deaths of T2DM patients [3]. Other macro- and micro-vascular complications, including cerebrovascular and peripheral arterial lesions, as well as, diabetic nephropathy, retinopathy, and neuropathy are also highly prevalent and detrimental among diabetic patients [4].

The pathology of diabetic complications is quite similar and is mainly attributed to macro- and microcirculatory disorders. Microcirculatory disorders are mainly characterized by endothelial damage, thickening of the vascular basement membrane, and the presence of microthrombosis [5]. Platelets strongly influence microcirculatory disorders. Diabetic platelets have functional and morphological abnormalities, such as impaired carbohydrate and lipid metabolism, stronger adhesion and aggregation, and hypersensitivity to subthreshold stimuli [6]. Promoted by the enhanced thrombotic environment, inflammatory cytokines, hyperglycemia, damaged endothelium, and impaired fibrinolysis, platelets in T2DM patients are highly procoagulant [7–10]. The functional alteration of platelets in T2DM patients facilitates the formation of microthrombus, which is the key event in microcirculatory disorders. Antithrombotic therapy is mainly considered secondary prevention in T2DM patients to improve vascular outcomes. However, the duration of T2DM, the presence of complications, and the risk of bleeding need to be considered to balance the benefits and risks during antithrombotic therapy [7]. Therefore, new antithrombotic targets need to be urgently identified to develop more efficacious therapy to manage T2DM patients.

Proteomics is an effective tool in medical research as it is an inexpensive technique with high throughput and sensitivity [11]. As platelets are cell nucleus-free particles with limited transcription capacity, proteomics techniques are even more important to characterize the functional alterations and signal transduction changes [12]. In this study, platelets from five T2DM patients and five healthy volunteers were analyzed by proteomics; their differentially expressed proteins (DEPs) were characterized, enriched with Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG), and the protein-protein interactions were analyzed. The bioinformatics-characterized mechanism and key proteins responsible for platelet activation and microthrombosis formation in T2DM patients were validated in vitro and in db/db mice.

2. Materials and methods

2.1. Patients and collection of blood samples

This study was approved by the ethics committee of Shanghai Fifth People's Hospital, Fudan University [approval number: (2021) LSD (103), approval date: Apr 26, 2021], and performed following the guidelines of the Declaration of Helsinki. Written informed consent was obtained from all participants. Whole blood samples were collected from five T2DM patients and five healthy volunteers for proteomics analysis, as well as, six T2DM and five healthy control samples were collected in medical anticoagulant tubes to validate protein expression by the Inpatient Department of Endocrinology and the Physical Examination Centre of Shanghai Fifth People's Hospital. The inclusion criteria were as follows: Type 2 diabetes was diagnosed according to the World Health Organization criteria [13], with normal electrocardiogram, sitting blood pressure (BP) less than 150/90 mmHg, no history or symptoms of cardiovascular disease, alanine aminotransferase (ALT) levels $<$60 U/L, and serum creatinine levels $<$1.25 mg/dL, no pathogenic infection, no acute diabetic complications, and the age range was 30–70 years. The exclusion criteria were as follows: onset of cerebrovascular disease or diabetes complications within 14 days, liver and kidney insufficiency, taking anticoagulants or other drugs that affect platelet function within two weeks, and incomplete clinical data. All healthy volunteers passed the oral glucose (75 g) tolerance test.

2.2. Sample preparation

For proteomics analysis, protein digestion, liquid chromatography-tandem mass spectrometry (LC-MS/MS), and primary bioinformatics analysis were performed by Hoogen Biotech (Shanghai, China). After the samples were centrifuged at 200 *g* for 15 min, 2 mL of the platelet-rich supernatant was collected. The supernatant was further centrifuged at 120 *g* for 6 min to remove white blood cells, followed by centrifugation at 1500*g* for 10 min to obtain a relatively pure platelet precipitate. The platelet precipitate was washed with Tyrode's solution (Solarbio, T1420, Beijing, China) and centrifuged at 120 *g* for 4 min to obtain purified platelet samples. Filter-aided sample preparation was applied to prepare platelet samples with SDT reagents (4 % (w/v) SDS, 100 mM Tris/HCl (pH 7.6), and 0.1 M DTT) [14]. The platelet samples were incubated with SDS buffer at 95 °C for 10 min, followed by ultrasound treatment for 60 s, and centrifugation at 16,500 *g* for 10 min to harvest the supernatant. The supernatant obtained was incubated, filtered, and centrifuged after adding the lysis buffer (8 M urea, pH 8.0, one cocktail, and 1 mM PMSF). The peptides were incubated at 37 °C with urea buffer containing recombinant lysyl endopeptidase overnight, followed by trypsin (1:100) for 16 h. The digests were desalted in C_{18} cartridges, and the freeze-dried digests were quantified (BCA method, OD280) and dissolved in 40 μL dissolution buffer before analysis.

For other tests, platelets were centrifuged at 200 *g* for 15 min to collect the platelet-rich supernatant. After washing twice with Tyrode's solution, the resuspended platelet precipitate was considered platelet-rich plasma (PRP).

2.3. Data collection with label-free peptides using LC-MS/MS

For each sample, 20 μg of peptide mixture was loaded on a NanoElute HPLC system (A: 1 % formic acid, B: acetonitrile (84 %) aqueous solution; loading column: Thermo Scientific Acclaim PepMap100, 100 μm × 2 cm, nanoViper C18; separating column:

Thermo scientific EASY column, 10 cm, ID75 μm, 3 μm, C18-A2; Flow: 300 nL/min). The peptides were transferred to the chromatography-tandem mass spectrometer Tims TOF Pro (4D), followed by ionization and detection (1.5 kV, 100–1700 *m/z*, TOF detector). A parallel accumulation serial fragmentation (PASEF) model was used for data collection.

2.4. Bioinformatics analysis

The raw LC-MS/MS file was analyzed using MaxQuant (v1.5.5.1) to obtain qualitative and quantitative results, by referring to the UniProt_HUMAN_20221203 dataset (FDR $\leq$0.01, Match between runs = 2 min, Peptide Mass Tolerance = $\pm$ 20 ppm).

The characterized proteins were annotated by referring to the UniProt database. DEPs were defined as 1.5-fold or higher expression differences between the two groups and $p < 0.05$ by *t*-test. For proteins with 1–2 missing values, fold change (FC), and p-values were calculated, while proteins with three or more missing values were ignored. The data were analyzed using R (v3.4.2), and volcano plots were constructed using the ggplot2 package. The GO enrichment of the DEPs, including biological process (BP), cellular component (CC), and molecular function (MF), as well as, KEGG enrichment, were verified by Fisher's exact test performed using the R package clusterProfiler and visualized using the ggplot2 package. The protein-protein interaction (PPI) was analyzed by STRING (http://string-db.org/), and the interaction of the hub proteins was filtered using the STRING online database (V12.0, https://cn.string-db.org/).

2.5. In vitro tests with isolated platelets

The isolated PRP were transferred to Petri dishes for treatment. Whole blood samples were pretreated upon sample collection if needed, and other treatments were performed within 30 min with PRP. For the in vitro tests that involved co-treatment of chloroquine (CQ, 0.1 and 1 mM for protein tests, 0.1 and 0.5 mM for aggregation and granule release tests, HY-17589A, MCE) and adezmapimod (8 μM, HY-10256, MCE)/Mdivi-1 (10 μM, HY-15886, MCE), CQ was supplemented 10 min after the other compounds were added.

To conduct aggregation tests, PYR41 (10 and 30 μM, HY-13296, MCE) and Pitstop 2 (40 and 80 μM, HY-115604, MCE) were simultaneously added with adenosine diphosphate (ADP, 20 μM, HY-W010918, MCE) or arachidonic acid (AA, 100 mM, HY-109590, MCE) in 500 μL of PRP and immediately tested on a CHRONO-LOG700 platelet aggregator. Endocytosis and phagosome were inhibited using Pitstop 2, as phagosome is also clathrin-dependent [15].

For enzyme-linked immunosorbent assay (ELISA), the supernatant of the reaction mix was tested using related kits (PF4 ELISA Kit, ml059961; β-TG ELISA Kit, ml057852, Shanghai Enzyme-linked Biotechnology Co., Ltd.), following the manufacturer's instructions. Then, proteins were isolated from the remaining platelets, and P-selectin was tested to confirm the granule release.

For Western blotting analysis, the platelets (about 1×10^6 platelets for each sample) were centrifuged at 1500 *g* for 10 min, lysed with RIPA lysis buffer, and loaded in 12 % SDS-PAGE gel for protein separation in an ice-cold fresh electrophoresis buffer. Primary and secondary antibodies were incubated according to the instructions of the manufacturers. The antibodies used included AGT (11992-1-AP, 1:2000, Proteintech), ANGII (1:500, 18302-1-AP, Proteintech), P-selectin (1:5000, 15052-1-AP, Proteintech), PI3K (60225-1-Ig, Proteintech, Rosemont, USA), COXIV (66110-1-Ig, Proteintech), β-tubulin (AB0039, Abways, Shanghai, China), β-actin (AB0035, Abways), Beclin-1 (11306-1-AP, Proteintech), ATG7 (67341-1-Ig, Proteintech), ATG3 (11262-2-AP, Proteintech), LC3B (14600-1-AP, Proteintech), Parkin (AF0235, Affinity, Victoria, Australia), GAPDH (AB2000, Abways), Goat Anti-Rabbit IgG (H + L) HRP (AB0101, Abways), and Goat Anti-Mouse IgG (H + L) HRP (AB0102, Abways). The blots were analyzed using ImageJ software (NIH).

2.6. Transmission electron microscopy (TEM)

The platelets collected were fixed in a fixative solution (G1102, Servicebio, Wuhan, China) at 4 °C for 2 h. After washing with 0.1 M PBS, the platelets were further fixed with a 1 % OsO_4 solution in 0.1 M PBS for 2 h at room temperature. The samples were dehydrated with graded ethanol solutions (50 %, 70 %, 80 %, 90 %, 95 %, 100 %, and 100 %; 15 min for each gradient), permeated with acetone Head-EPON™ 812 mix (Hedebio, Beijing, China), and embedded in pure 812 mix for 48 h at 60 °C. Then, sections (60–80 μm thick) were obtained using a Leica UC7 ultramicrotome (Leica, Wetzlar, Germany), followed by incubation with 2 % uranium acetate and lead citrate solutions for 15 min, respectively. The sections were analyzed using a transmission electron microscope (Tecnai G2 20 TWIN, FEI, The Netherlands, USA) after drying overnight at room temperature.

2.7. Immunofluorescence

For immunofluorescence and confocal microscopy, platelets fixed in 4 % paraformaldehyde (PFA, P0099, Beyotime, Shanghai, China) were centrifuged and attached on poly-l-lysine coverslips (VWR; 600×*g*, 5 min). The attached platelets were then treated with PBS containing 0.2 % Triton, 1 % bovine serum albumin (BSA), and 2 % donkey serum, followed by incubation with primary antibodies (COXIV, 66110-1-Ig; LC3B, 14600-1-AP, Proteintech) and secondary antibodies (Alexa Fluor® 488, ab150077, ab150113; Alexa Fluor® 647, ab150115, ab150075, Abcam, Cambridge, UK). The coverslips with samples were observed under an ECHO microscope (RVL-100-G, CA, USA). Analysis of fluorescence positive cells or fluorescence positive area was conducted using Fiji Software.

2.8. Cell culture and platelet induction in vitro

To assess the influence of hyperglycemia on the expression of AGT, platelets were collected from MEG-01 cells (CL-0498, Pricella, China) and cultured in high- and low-glucose (25 mM and 5 mM) RPMI 1640 media (c3010-0500, Viva Cell) containing 10 % FBS (BI,

04-001-1ACS). Insulin (1 mL/L, P3376–100IU, Beyotime) was added if required. Platelets were induced by treatment with thrombopoietin (Human, CHO, His, 100 ng/mL) and PMA (HY-18739, MCE, 10 nM) for 72 h. After the supernatant underwent centrifugation at 800*g* for 15 min, the platelets were resuspended to a density of about 1×10^7 cells/mL and treated as PRP.

2.9. Animal experiments

The animal experiments were approved by the Animal Welfare and Ethics Committee of Fudan University Experimental Animal Science Department [Approval Number: 2021JSWY-012, Approval Date: Feb 25, 2021]. All experiments were performed following the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. Nine db/db mice (male, 28 ± 5 g, nine weeks old, GemPharmatech Co., Ltd, Shanghai) were used in the experiments. Three hungry mice were orally administered saline, valsartan (40 mg/kg), or CQ (80 mg/kg) in 5 mL of saline. The mice were sacrificed by cervical dislocation 12 h after administering treatment, and transcardiac perfusion with 4 % PFA was performed before harvesting the liver, spleen, kidney, and heart tissues for fixation in 4 % paraformaldehyde for 24 h. Finally, H&E staining was performed using these tissues.

2.10. Statistical analysis

The data were expressed as the mean ± standard deviation. Bar charts were plotted using GraphPad Prism 6 (GraphPad Software Inc., California, USA). All differences were determined by one-way ANOVA followed by Tukey's multiple comparisons. All results were considered to be statistically significant at $P < 0.05$. Tests were conducted in triplicate unless otherwise mentioned.

3. Results

3.1. T2DM platelets are partly activated, with reduced cellular components and altered metabolic profiles

Platelets from five healthy volunteers and five T2DM patients were used in the LC-MS/MS analysis. The clinical characteristics of the participants, including those for validation, are listed in Table 1. The blood glucose and triglyceride levels were higher, while high-density lipoprotein levels were lower in T2DM patients. In total, 3239 proteins were identified from both healthy control and T2DM platelet samples via LC-MS/MS (Table S1). The variance between the samples was determined by PCA (Fig. 1A), which confirmed the consistency of the samples. Among these, 211 proteins were only found in diabetic platelets, and 497 proteins were only found in healthy controls (Fig. 1B). With criteria of $P < 0.05$ and $|\log2FC| > 0.58$, 260 co-expressed proteins were found to be significantly highly expressed in diabetic platelets, while 906 proteins were highly expressed in healthy platelets; these proteins were defined as DEPs (Table S1, Fig. 1C). The heat map of all DEPs is shown in Fig. 1D. Overall, the within-group variance for DEPs in the T2DM group was larger than that in the HC group. Typically, ITIH1, A2M, AGT, SERPIND1, and APOB are the most significantly up-regulated DEPs, which are related with complement and coagulation cascades and blood coagulation. In contrast, ITGB6, DDX1, HNRNPA2B1, HMGB1, and QDPR are among the most significantly down-regulated DEPs, which are related with metabolic pathways and others.

Enrichment was performed with the DEPs (Table S2). The most significantly enriched GO terms were cellular components (CCs) including cytosol, cytoplasm, and extracellular exosome (Fig. 2A). Additionally, complement and coagulation cascades, metabolic

Table 1
Clinical characteristics of the enrolled T2DM patients and healthy volunteers.

Item	Healthy Control (N = 10)		T2DM (N = 11)		*P*
	Mean	SD	Mean	SD	
Gender (Male/Female)	5/5	–	7/4	–	0.670
Age (year)	39.900	7.824	44.800	8.334	0.195
Height (cm)	166.950	8.293	164.300	10.161	0.573
Weight (kg)	66.390	11.695	69.150	10.958	0.637
BMI (kg/m^2)	23.669	2.402	25.587	3.506	0.212
Insulin (pM)	50.643	16.981	60.804	40.294	0.507
C-peptide (nM)	0.579	0.111	0.703	0.310	0.247
FBG (mM)	5.165	0.456	9.123	5.236	***0.011**
2hPBG (mM)	6.113	1.037	13.788	8.425	***0.006**
Creatinine (μM)	64.453	16.001	88.100	56.334	0.257
Blood cholesterol (mM)	4.454	0.776	5.460	1.618	0.069
HDL (mM)	1.242	0.159	0.920	0.177	***<0.001**
LDL (mM)	2.825	0.675	3.362	1.263	0.307
TG (mM)	1.251	0.621	3.338	2.019	***0.004**
Uric acid (μM)	328.355	75.418	236.385	151.698	0.162
AST (U/L)	27.748	23.777	28.700	11.649	0.985
ALT (U/L)	24.076	11.311	21.400	5.076	0.471
MPV (fl)	10.614	0.894	10.270	0.823	0.484

Note: 2hPBG, 2-h postprandial blood glucose; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; BMI, Body Mass Index; FBG, Fasting blood glucose; HDL, high-density lipoprotein; LDL, Low-density lipoprotein; MPV, Mean platelet volume; TG, Triglyceride.

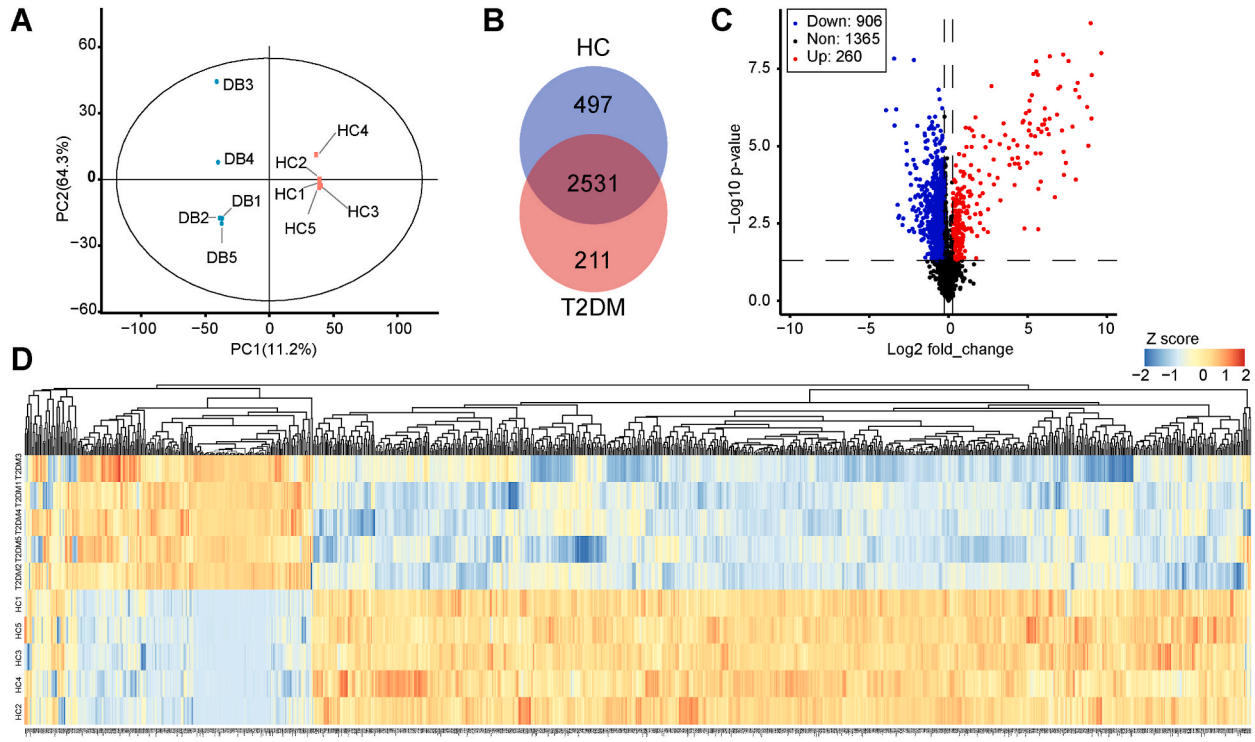


Fig. 1. Identification of DEPs in healthy control and diabetic patients. **(A)** A PCA plot for the LC-MS/MS characterized platelet samples is shown. **(B)** The distribution of the characterized proteins in both groups is illustrated. **(C)** The volcano plot shows the co-expressed DEGs, which were characterized based on the threshold $|\log2FC| > 0.58$ and $P < 0.05$. **(D)** The heat map shows the characterized co-expressed proteins.

pathways, endocytosis, platelet activation, oxidative phosphorylation, phagosome, autophagy, the insulin signaling pathway, and some platelet-activation-related diseases were significantly enriched with KEGG (Fig. 2B).

Next, GSEA was performed with all co-expressed proteins (Table S2). Complement and coagulation cascades, and platelet activation were among the most significantly enriched pathways. Moreover, phagosome was upregulated, while autophagy, endocytosis, the insulin signaling pathway, metabolic pathways, and oxidative phosphorylation were downregulated (Fig. 2C). The enrichment was consistent with the observation that fewer proteins were characterized in T2DM platelets, and cellular components were different for the two kinds of platelets. As T2DM is a metabolic disease, and because metabolic pathways were enriched with KEGG in this study, the interaction of the significantly upregulated DEPs ($|\log2FC| > 1$), as well as, the proteins unique to T2DM (characterized in all T2DM platelets but not identified in any HC platelets) that were enriched in the metabolic pathways were analyzed by STRING (Fig. 2D). Angiotensinogen (AGT) was one of the most significantly upregulated proteins.

The bioinformatics analysis showed that platelets from T2DM patients were partly activated, with reduced cellular components and altered metabolic profiles. AGT levels were significantly higher in the T2DM platelets, probably due to an altered metabolic profile.

3.2. *Platelet activation primarily depends on the autophagy-lysosome pathway in T2DM platelets*

Autophagy, which plays a key role in platelet activation [16,17], was enriched and might be responsible for the altered protein expression profile in T2DM platelets. Other protein degradation processes, including proteasome, endocytosis, and phagosome [18], were also enriched. Thus, their role in platelet activation was tested. In platelets activated by AA (Fig. 3A and C), Pitstop 2-mediated inhibition of endocytosis and phagosome promoted coagulation to some degree, and the influence of proteasome was not significant. In ADP-activated platelets (Fig. 3B and C), Pitstop 2 inhibited coagulation, however, the effect was less significant in the high-dose group. The influence of proteasome was also not significant in ADP-stimulated platelets. For both AA- and ADP-treated platelets, CQ inhibited coagulation in a dose-dependent manner.

Granule release was further tested both in AA- and ADP-activated T2DM platelets (Fig. 3D). Similarly, the influence of Pitstop 2 and PYR-41 on the release of PF4 and β-TG were either not significant or irregular. CQ significantly (in a dose-dependent manner) reduced the release of PF4 and β-TG. The release of granules was also confirmed with the platelet-reserved P-selectin, as CQ most significantly inhibited the increase in P-selectin levels in platelets (Fig. 3E).

These results supported that although proteasome, endocytosis, and phagosome are involved, although to different degrees, in protein degradation and platelet activation, the degradation and activation of platelets primarily depend on the autophagy-lysosome pathway.

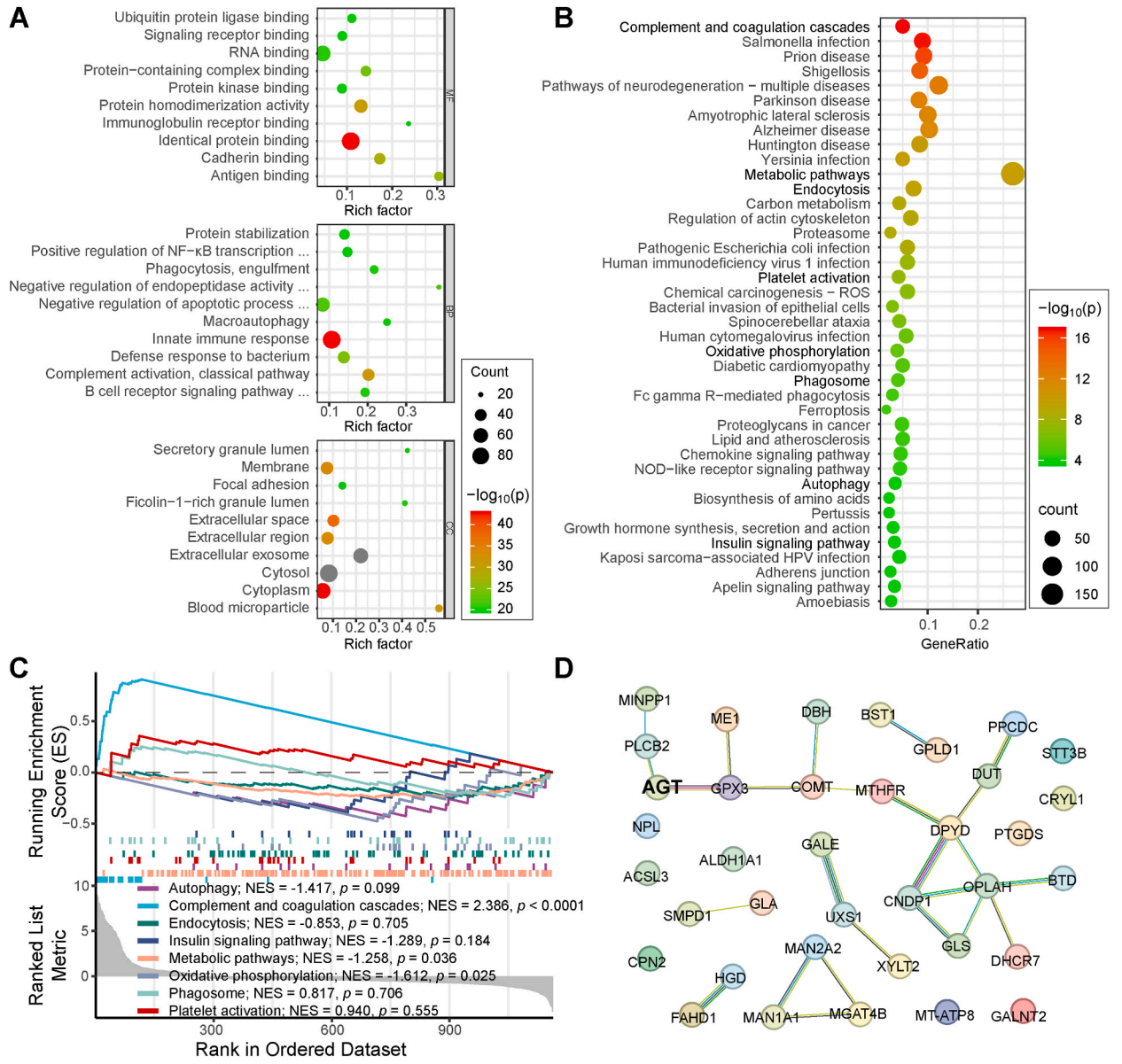


Fig. 2. Bioinformatics analysis of the DEPs. **(A)** The top 10 enriched molecular functions (MFs), biological processes (BPs), and cellular components (CCs) of the DEPs were enriched with GO. **(B)** The top 40 enriched KEGG pathways are shown. **(C)** The GSEA plot of typical enrichments. **(D)** Protein-protein interaction of the significantly upregulated proteins (|log2FC| > 1) and T2DM unique proteins that belong to the metabolic pathways is presented.

3.3. Macroautophagy is necessary for platelet activation

We evaluated the role of autophagy and mitophagy in the activation of platelets (Fig. 4A and B). The levels of Beclin 1, ATG7, ATG3, total LC3B, and Parkin increased in T2DM platelets. Adding CQ increased the total protein content in a dose-dependent manner in PRP stored for 24 h (Fig. 4C). LC3B/COX4 immunofluorescence was also observed in T2DM platelets (Fig. 4D). The pseudopodia, alpha granules and dense granules were reduced in T2DM platelets as observed with TEM. Autophagy was confirmed by the presence of fewer platelet contents and the occurrence of more autophagosomes in T2DM platelets (Fig. 4E).

Autophagy and mitophagy were confirmed in stored T2DM platelets and in ADP-activated HC platelets (Fig. 5A). CQ increased Pink 1, Parkin, COX4, total LC3B, Beclin 1, and β-tubulin levels in stored T2DM platelets in a dose-dependent manner. Also, the levels of these proteins were reduced with ADP in HC platelets but reverted with CQ. However, Mdivi-1 could not restore these ADP-reduced proteins (Fig. 5B); additionally, the increase in LC3B/COX4 immunofluorescence intensity by CQ was not achieved with Mdivi-1 (Fig. 5C).

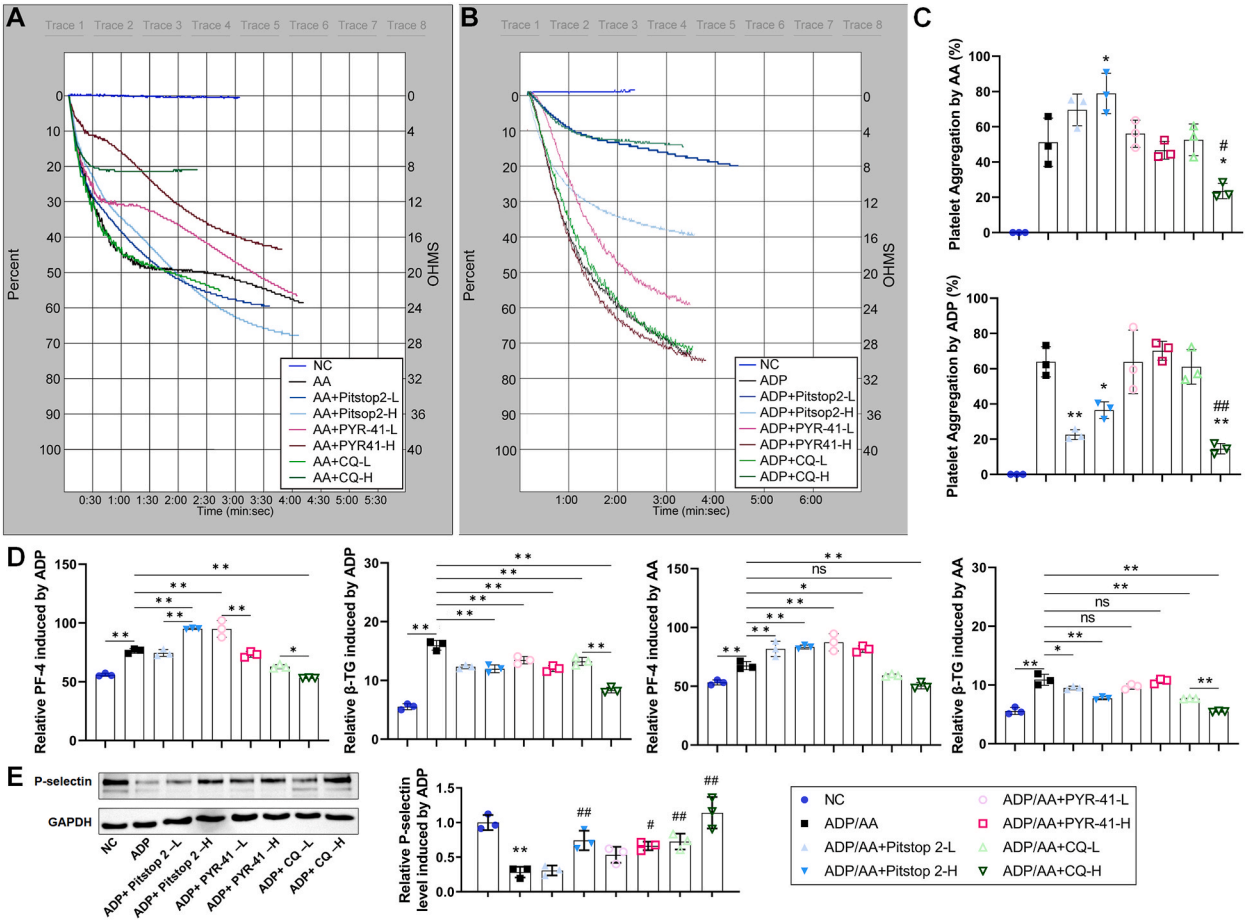


Fig. 3. The role of proteasome, endocytosis, phagosome, and autophagy-lysosome pathway in the activation and granule release of T2DM platelets. Proteasome, endocytosis/phagosome, and the autophagy-lysosome pathway were inhibited by PYR-41, Pitstop 2, and CQ, respectively, in platelet-rich plasma (PRP). Platelet aggregation after activation by **(A)** AA and **(B)** ADP. **(C)** Platelet aggregation was compared; *P < 0.05 and **P < 0.01 vs. the AA or ADP group; #P < 0.05 and ##P < 0.01 vs. the related low-dose group by one-way ANOVA. **(D)** PF4 and β-TG released into the supernatant were tested by ELISA in AA- and ADP-activated PRP. **(E)** The platelet retained P-selectin after ADP activation, as determined by Western blotting analysis; N = 3. For D and E, *P < 0.05 and **P < 0.01 vs. the NC or line-marked group; #P < 0.05 and ##P < 0.01 vs. the ADP or AA groups, as determined by one-way ANOVA and Tukey's multiple comparisons test.

Overall, these results suggested that autophagy and mitophagy occurred during platelet activation for HC and T2DM platelets, although mitophagy only played a minor role.

3.4. Lysosomal degradation-mediated overexpression of AGT in platelets promotes coagulation in T2DM

Initially, the level of AGT was evaluated in platelets. Then, the effect of autophagy on AGT activation, and the in vivo effects of AGT and autophagy were evaluated. T2DM platelets expressed more AGT, which was converted to ANGII following ADP activation in vitro (Fig. 6A). High levels of AGT were also induced in platelets originating from MEG-01 cells that were treated with high-glucose (Fig. 6B). AGT and ANGII were influenced by CQ rather than captopril in vitro (Fig. 6C). Finally, the possible effects of autophagy and AGT activation on microvascular coagulation were investigated in db/db (T2DM) mice, which was indicated by the tissue-retained red blood cells (RBCs) after transcardiac perfusion. Both CQ and valsartan decreased RBC counts in the liver, spleen, heart, and kidney tissues of db/db mice, and the effects of CQ were prominent (Fig. 6D). Additionally, microvasoconstriction was inhibited in CQ-treated and valsartan-treated groups, as indicated by less extrusion of RBCs. These results suggested that AGT may be induced with high glucose in T2DM platelets, and its activation in platelets was lysosome-dependent.

4. Discussion

Changes in platelet function in T2DM influence microthrombus formation, a key event in diabetic vasculopathy. Microcirculation disorders are primarily characterized by endothelial injury, thickening of the vascular basement membrane, and microthrombosis

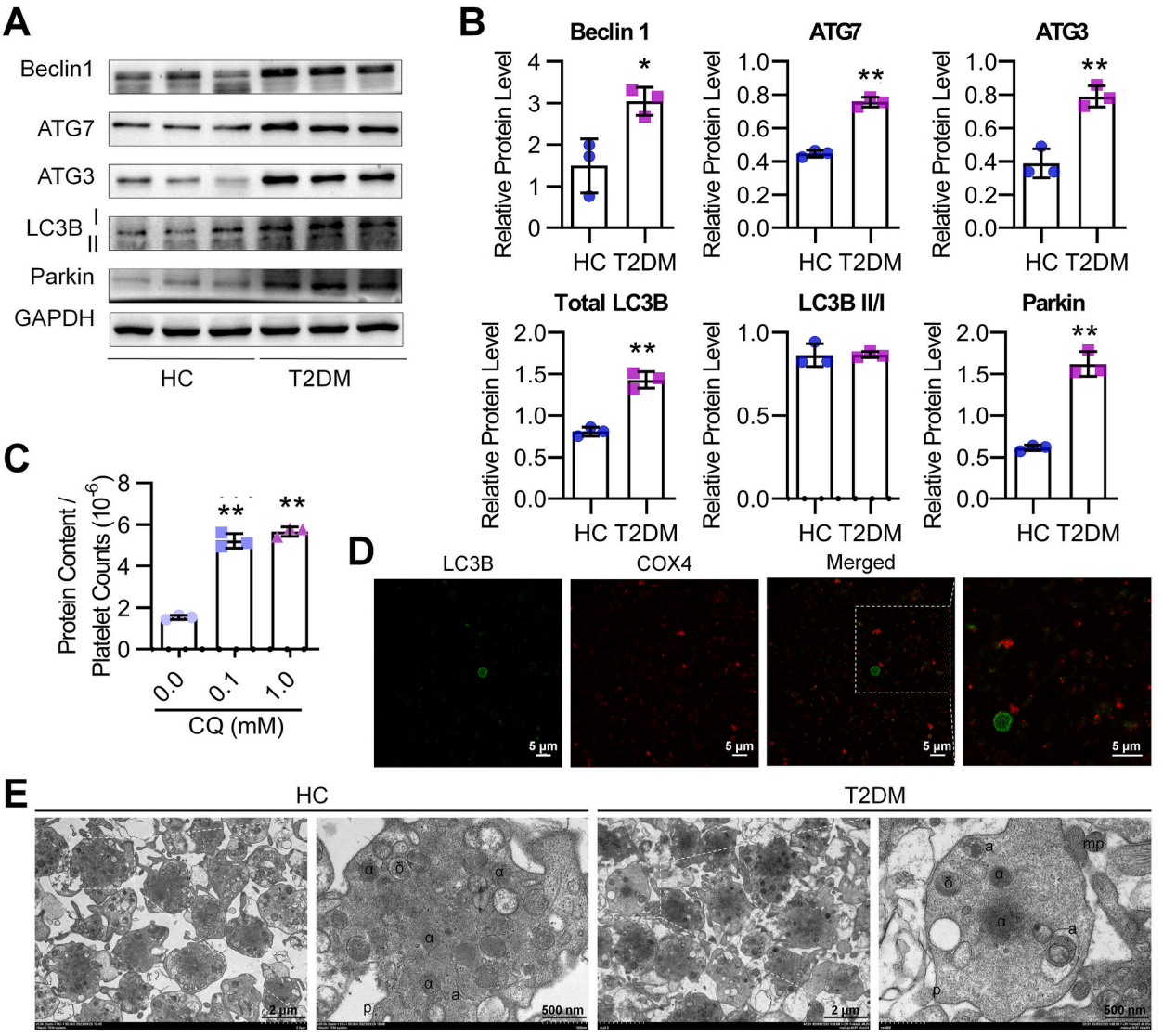


Fig. 4. Characterization of autophagy after platelet activation. **(A)** Autophagy- and mitophagy-related proteins were tested in platelets from healthy volunteers and T2DM platelets. **(B)** The protein levels were evaluated using ImageJ and compared. **(C)** The protein content of storage-stimulated T2DM platelets was determined after CQ treatment. **(D)** LC3B and COX4 immunofluorescence of T2DM platelets was recorded. **(E)** TEM analysis of the HC and T2DM platelets; α, alpha granules; δ, dense granules; a, autophagosome; p, pseudopodia; mp, extracellular microparticles. N = 3; *P < 0.05 and **P < 0.01, as determined by the *t*-test or one-way ANOVA and Tukey's multiple comparisons test.

[19]. According to Virchow's theory of thrombosis, the mechanism of thrombosis in diabetic vasculopathy is primarily associated with vascular endothelial damage, abnormalities in the coagulation-fibrinolytic system, alterations in blood rheology, and platelet activation [20]. Approximately 98 % of endothelial cells in humans are found in microvessels [21], making them highly conducive to coagulation under the influence of increased platelet activation and endothelial dysfunction in T2DM. Coagulation time is positively correlated with hematocrit, blood flow velocity, and blood vessel size. Microvessels and capillaries exhibit a low specific volume [22], the slowest blood flow rate [23], and the smallest diameter, all of which greatly favor thrombosis [24]. Moreover, elevated shear forces in capillaries further promote platelet aggregation [25]. Additionally, smaller vessels may be more susceptible to injuries [26]. These factors collectively indicate that patients with T2DM may experience widespread and uncharacterized microvascular coagulation [27], which might play a key role in the pathology of T2DM. We also found that platelet activation in T2DM was promoted by macroautophagy, which in turn activated AGT and promoted microthrombosis.

The mechanism underlying the overactivation of T2DM platelets was analyzed by 4D label-free LC-MS/MS. The results showed that the DEPs were mainly distributed on the HC side. Most downregulated proteins displayed minor fold changes. In contrast, although fewer proteins were upregulated, many of them exhibited substantial differences in expression. Similar findings were reported in another study on proteomic investigations [28]. Moreover, the characteristic proteins identified in T2DM platelets in our study closely

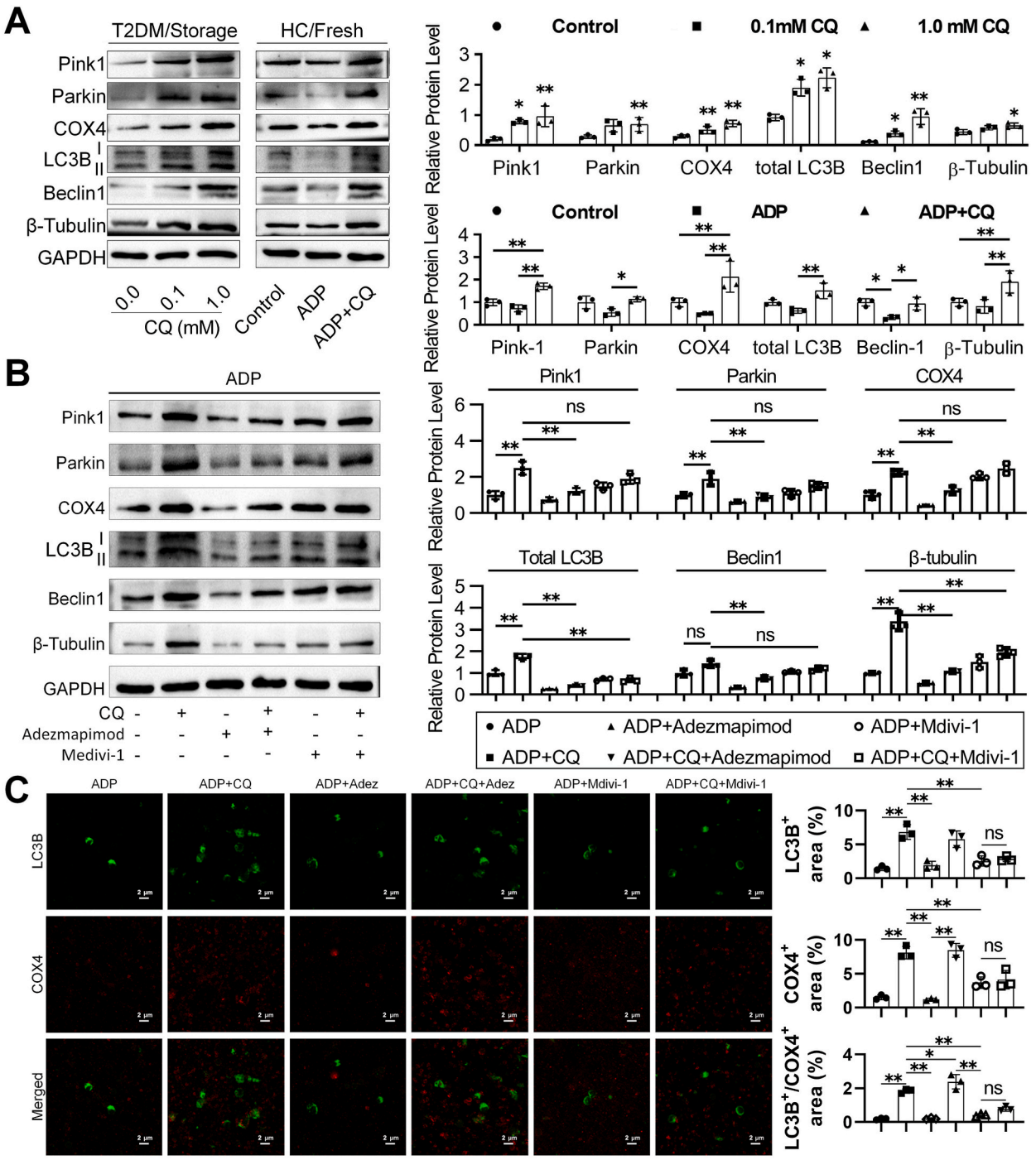


Fig. 5. Activation of autophagy and mitophagy in T2DM platelets. **(A)** The levels of proteins related to autophagy and mitophagy in 24-h-stored platelet-rich plasma (PRP) of T2DM patients and ADP-activated (30 min) HC PRP were tested by Western blotting assays and semi-quantified by ImageJ; $*P < 0.05$ and $**P < 0.01$ vs. Control; $\#P < 0.05$ vs. ADP, as determined by one-way ANOVA. **(B)** The autophagy- and mitophagy-related proteins in T2DM platelets were treated with the autophagy/mitophagy activator adezmapimod, mitophagy inhibitor Mdivi-1, and lysosomal inhibitor CQ. CQ was added 10 min after the other compounds were added to the PRP reaction mix. **(C)** LC3B and COX4 immunofluorescence of T2DM platelets; N = 3 for each group. For B and C, $*P < 0.05$ and $**P < 0.01$, as determined by one-way ANOVA and Tukey's multiple comparisons test.

resembled those found in another study involving T2DM platelets [29]. Our findings discarded the possibility of experimenter and sample selection bias. The uneven distribution observed suggested an overall depletion of cytosolic proteins in T2DM platelets, probably due to the uneven activation of these T2DM platelets [30]. The depletion of cytosolic proteins in T2DM platelets was also

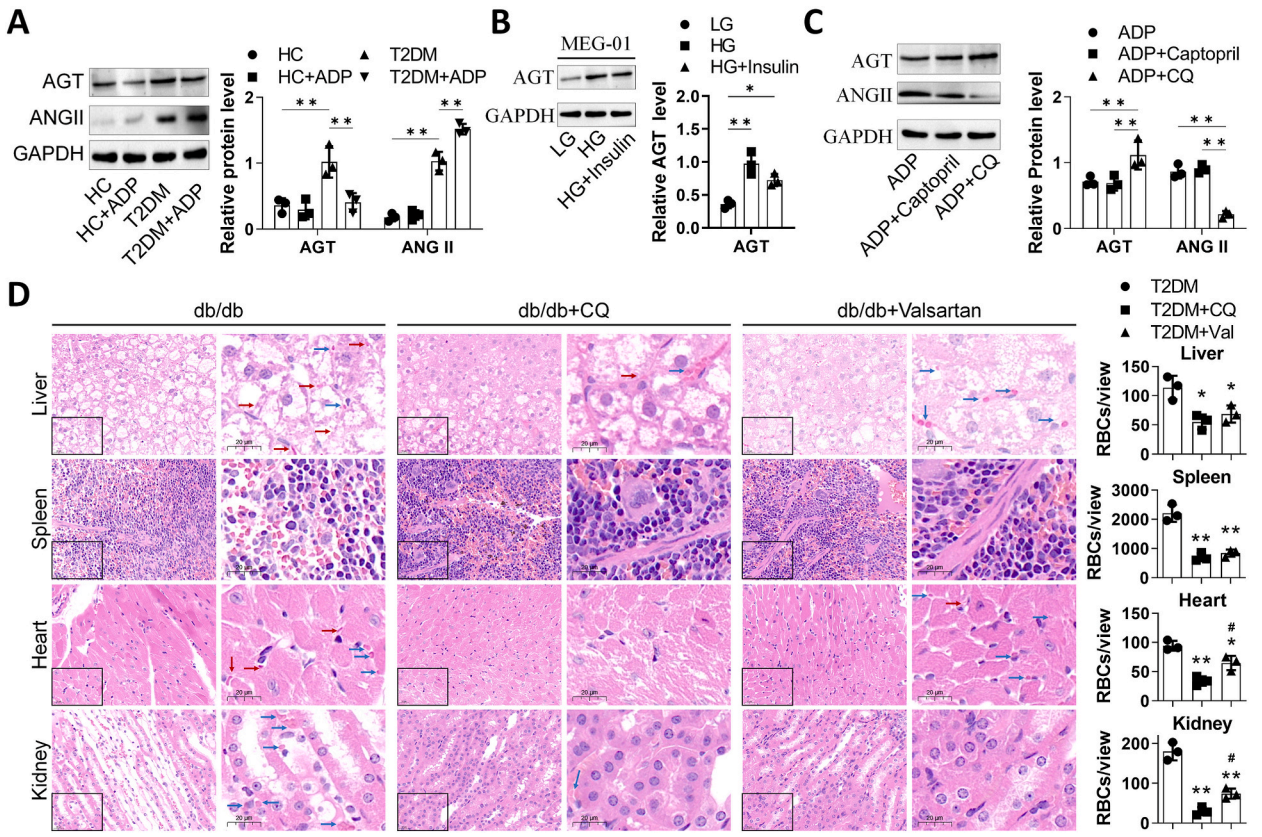


Fig. 6. Role of AGT and autophagy in microthrombosis. **(A)** The levels of AGT and ANGII in HC and T2DM platelets were evaluated after ADP activation. **(B)** The AGT high-expression platelets were induced by MEG-01 cells cultured with a high-glucose medium. **(C)** The influence of angiotensin-converting enzyme inhibitor captopril and lysosomal inhibitor CQ on AGT activation was assessed. **(D)** The effect of CQ and the ANGII receptor blocker valsartan on tissue-retained red blood cells (RBCs) in the liver, spleen, heart, and kidney after treatment for 12 h was evaluated by H&E staining; the normal RBCs were indicated by blue arrows, the squeezed and deformed RBCs were indicated with red arrows, $N = 3$; $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. the line-marked group or db/db group, as determined by one-way ANOVA followed by Tukey's multiple comparisons test. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

supported with the loss of platelets granules by TEM and reduced total protein content by BCA assay. Among the KEGG-enriched pathways, complement and coagulation cascades were significantly upregulated. Most other pathways, including platelet activation, were less significant, as determined by GSEA. Most of the enriched pathways were downregulated, which occurred probably due to platelet activation in T2DM, rather than due to the downregulation of these pathways in newly formed platelets [12]. Other pathways, especially endocytosis, proteasome, phagosome, and autophagy were also enriched with KEGG. The enrichment of these protein digestion processes highlighted their importance in platelet activation, most of which can be inhibited by CQ.

Autophagy involves lysosome-dependent non-selective or selective degradation of intracellular components, molecules, and organelles [31]. It strongly influences platelet activation and thrombosis, as determined by impaired hemostasis and thrombosis in vitro and in vivo with platelet-specific deletion of Atg7, a key regulator of autophagy [32]. Upon activation, the volume and light transmittance of platelets increase considerably [33]. Lysosomes, serving as major intracellular reservoirs of hydrolytic enzymes, play a key role in these changes. Intracellular proteins and organelles are protected by the cell membrane and remain inaccessible. However, once exposed, they may trigger autoimmune reactions and inflammation. In platelets, extensive degradation of intracellular proteins by lysosomes may mitigate this effect [34]. However, the role of autophagy, particularly the autophagy lysosomal pathway, in platelet granule release, aggregation, and protein degradation remains unclear. In a study, the release of granules increased the extracellular procoagulant components, while $HAuCl_4$ (100 μM) failed to inhibit platelet aggregation in vitro despite reducing the secretion of dense granules and lysosomes [35]. This suggested that the release of granules may not be necessary for platelet activation. Our results showed that CQ significantly inhibited platelet aggregation and the release of granules, whereas the effects of PYR-41 and Pitstop 2 were either not significant or unstable. Thus, platelet aggregation and release of granules primarily rely on the autophagy lysosomal pathway, while other protein degradation pathways may be secondary. As platelet granules are lysosome-like organelles, CQ may directly act on them.

Mitophagy is a novel therapeutic target for platelet activation [36]. Hypoxia can promote mitophagy activation in platelets, leading to platelet activation [37]. Under physiological conditions, at the onset of platelet activation, oxygen consumption increases for a short

duration, which may provide energy for coagulation [38] and promote ROS production [39]. However, we found that compared to the CQ group, the mitochondrial inhibitor Mdivi-1 did not significantly increase protein expression in platelets or alter the co-localization of LC3B and COX4. Our results suggested that macroautophagy plays a key role in platelet activation and thrombosis.

The only source of ANGs in the body is AGT, which plays a key role in regulating vasoconstriction [40]. Some studies have found that the ANGII type 1 receptor (AT1R) signaling pathway plays a pivotal role in the interaction among platelets, leukocytes, and endothelial cells in cerebral microvessels. In a procoagulant process stimulated by ANGII, aggregation of platelets and leukocytes happens in pial venules to adhere to the endothelium [41]. By inducing blood vessel constriction post-activation, elevating blood pressure, and increasing shear force, high AGT expression may be associated with the hypercoagulable state in T2DM patients. We found elevated AGT expression in platelets from T2DM patients and in platelets derived from MEG-01 cells cultured in high-glucose, further highlighting the potential for excessive production of AGT in response to metabolic abnormalities in T2DM. Activated platelets carry AGT and release ANGII in blood vessels, exerting a vasoconstrictive effect near the site of coagulation. Although this effect promotes coagulation, it does not induce abnormal vessel constriction in unrelated areas. Hence, platelet AGT may be a crucial component of its coagulation function. This effect might explain the inhibition of platelet aggregation by ANGII-AT1 receptor blockers [42]. Additionally, we found that platelet AGT activation does not depend on ACE but relies on lysosomal degradation. Thus, inhibiting lysosomal activity can suppress platelet protein degradation and platelet activation without interfering with ANGII signals in the circulatory system of the patient. It can also prevent the conversion of AGT in platelets to ANGII, thereby inhibiting vasoconstriction. CQ analogs can reduce the MA value [43] while preserving normal coagulation function at a controlled dose [44]. This preserves the normal coagulation function while preventing excessive coagulation. Therefore, CQ analogs and their preparations that target platelet lysosomes are promising agents for developing anticoagulants for T2DM patients. In a procoagulant microenvironment, slower movement or even immobility of RBCs rather than clotting can be easily observed [45]. We then observed the tissue-retained RBCs post-perfusion in the db/db mouse model of T2DM. Consequently, CQ and Valsartan reduced tissue-retained RBCs in the liver, spleen, heart, and kidney. Interestingly, we also observed that microvasoconstriction was inhibited in the liver and heart by CQ and Valsartan, as the squeezed and deformed RBCs observed in the control group were almost absent in the CQ and Valsartan groups. Our findings support that the overexpressed AGT can be activated by lysosomal degradation in platelets to promote coagulation in T2DM, which may contribute to the platelet activation, hypercoagulable state, and microthrombosis in T2DM patients.

Collectively, we showed that autophagy-enabled protein degradation is key to platelet activation and ANGII production, which is possibly responsible for the hypercoagulable state and enhanced microthrombosis in T2DM patients. Our results provide new knowledge in the field and shed light on a promising therapeutic strategy for T2DM. However, the expression of AGT in T2DM platelets requires support from large-scale clinical investigations. Implications for other protein degradative processes were not extensively tested. The mechanism by which AGT is upregulated in T2DM platelets and the activation of platelet-derived ANGII in vivo also require further exploration.

To summarize, we found that activation of the platelets from T2DM patients primarily depends on macroautophagy and lysosomal degradation of its proteins. Additionally, AGT expression in T2DM platelets was high, and it was converted into ANGII via lysosomal degradation. The targeting of lysosomes by CQ can inhibit microthrombosis by obstructing autophagy efflux and preventing the release of ANGII.

Funding

This study was supported by Health Profession Clinical Research Funds of Shanghai Municipal Health Commission (No.201940257) and the Natural Science Foundation of Shanghai (No.22ZR1409400).

Data availability statement

The mass spectrometry proteomics data have been deposited to the Proteome Xchange Consortium (http://proteomecentral.proteomexchange.org/cgi/GetDataset?ID=PXD054086) via the iProX partner repository with the dataset identifier (https://www.iprox.cn/page/project.html?id= IPX0009154000).

Ethics and consent statement

This study was reviewed and approved by the Ethics Committee of Shanghai Fifth People's Hospital, Fudan University with the approval number: (2021)LSD(103), dated: Apr 26, 2021, and was applied in accordance with the Helsinki Declaration II. All participants provided written informed consent to participate in the study and for their data to be published. The animal experiments were approved by the Animal Welfare and Ethics Group of Fudan University Experimental Animal Science Department with the approval number: 2021JSWY-012, dated: Feb 25, 2021, and was carried out in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

CRediT authorship contribution statement

Qiang Wu: Writing – original draft, Software, Resources, Methodology, Formal analysis, Data curation. **Siwen Yu:** Writing – review & editing, Visualization, Software, Resources, Formal analysis, Data curation. **Shufei Zang:** Writing – review & editing, Visualization, Software, Resources, Methodology, Formal analysis. **Kangkang Peng:** Writing – review & editing, Visualization, Software,

Resources, Methodology. **Zhicheng Wang:** Writing – review & editing, Validation, Supervision, Project administration, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

The language of this study was professionally edited by ExEditing.com.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e36131.

References

[1] N.W.S. Chew, C.H. Ng, D.J.H. Tan, G. Kong, C. Lin, Y.H. Chin, et al., The global burden of metabolic disease: data from 2000 to 2019, Cell Metabol. 35 (3) (2023) 414–428.e3, https://doi.org/10.1016/j.cmet.2023.02.003.
[2] E.J. Mayer-Davis, J.M. Lawrence, D. Dabelea, J. Divers, S. Isom, L. Dolan, et al., Incidence trends of type 1 and type 2 diabetes among youths, 2002-2012, N. Engl. J. Med. 376 (15) (2017) 1419–1429, https://doi.org/10.1056/NEJMoa1610187.
[3] T.R. Einarson, A. Acs, C. Ludwig, U.H. Panton, Prevalence of cardiovascular disease in type 2 diabetes: a systematic literature review of scientific evidence from across the world in 2007–2017, Cardiovasc. Diabetol. 17 (1) (2018) 83, https://doi.org/10.1186/s12933-018-0728-6.
[4] R. Liu, L. Li, C. Shao, H. Cai, Z. Wang, The impact of diabetes on vascular disease: progress from the perspective of epidemics and treatments, J. Diabetes Res. 2022 (2022) 1531289, https://doi.org/10.1155/2022/1531289.
[5] Y. Li, Y. Liu, S. Liu, M. Gao, W. Wang, K. Chen, et al., Diabetic vascular diseases: molecular mechanisms and therapeutic strategies, Signal Transduct. Targeted Ther. 8 (1) (2023) 152, https://doi.org/10.1038/s41392-023-01400-z.
[6] C. Watala, M. Boncler, P. Gresner, Blood platelet abnormalities and pharmacological modulation of platelet reactivity in patients with diabetes mellitus, Pharmacol. Rep. 57 (Suppl) (2005) 42–58.
[7] R.A. Ajjan, N. Kietsiriroje, L. Badimon, G. Vilahur, D.A. Gorog, D.J. Angiolillo, et al., Antithrombotic therapy in diabetes: which, when, and for how long? Eur. Heart J. 42 (23) (2021) 2235–2259, https://doi.org/10.1093/eurheartj/ehab128.
[8] M.A. Christou, P.A. Christou, C. Kyriakopoulos, G.A. Christou, S. Tigas, Effects of hypoglycemia on cardiovascular function in patients with diabetes, Int. J. Mol. Sci. 24 (11) (2023) 9357, https://doi.org/10.3390/ijms24119357.
[9] E. Chow, A. Iqbal, E. Walkinshaw, F. Phoenix, I.A. Macdonald, R.F. Storey, et al., Prolonged prothrombotic effects of antecedent hypoglycemia in individuals with type 2 diabetes, Diabetes Care 41 (12) (2018) 2625–2633, https://doi.org/10.2337/dc18-0050.
[10] M. Edvardsson, M. Oweling, P. Järemo, Small procoagulant platelets in diabetes type 2, Thromb. Res. 195 (2020) 1–7, https://doi.org/10.1016/j.thromres.2020.06.033.
[11] A.T. Rajczewski, P.D. Jagtap, T.J. Griffin, An overview of technologies for MS-based proteomics-centric multi-omics, Expet Rev. Proteonomics 19 (3) (2022) 165–181, https://doi.org/10.1080/14789450.2022.2070476.
[12] H.E. Allan, M.A. Hayman, S. Marcone, M.V. Chan, M.L. Edin, T. Maffucci, et al., Proteome and functional decline as platelets age in the circulation, J. Thromb. Haemostasis 19 (12) (2021) 3095–3112, https://doi.org/10.1111/jth.15496.
[13] WHO, HEARTS D: Diagnosis and Management of Type 2 Diabetes, World Health Organization, 2020.
[14] J.R. Wiśniewski, A. Zougman, N. Nagaraj, M. Mann, Universal sample preparation method for proteome analysis, Nat. Methods 6 (5) (2009) 359–362, https://doi.org/10.1038/nmeth.1322.
[15] C.E. Lancaster, A. Fountain, R.M. Dayam, E. Somerville, J. Sheth, V. Jacobelli, et al., Phagosome resolution regenerates lysosomes and maintains the degradative capacity in phagocytes, J. Cell Biol. 220 (9) (2021) e202005072, https://doi.org/10.1083/jcb.202005072.
[16] T.Y. Lee, W.J. Lu, C.A. Changou, Y.C. Hsiung, N.T.T. Trang, C.Y. Lee, et al., Platelet autophagic machinery involved in thrombosis through a novel linkage of AMPK-MTOR to sphingolipid metabolism, Autophagy 17 (12) (2021) 4141–4158, https://doi.org/10.1080/15548627.2021.1904495.
[17] M. Banerjee, Y. Huang, M.M. Ouseph, S. Joshi, I. Pokrovskaya, B. Storrie, et al., Autophagy in platelets, Methods Mol. Biol. 1880 (2019) 511–528, https://doi.org/10.1007/978-1-4939-8873-0_32.
[18] L. Zhao, J. Zhao, K. Zhong, A. Tong, D. Jia, Targeted protein degradation: mechanisms, strategies and application, Signal Transduct. Targeted Ther. 7 (1) (2022) 113, https://doi.org/10.1038/s41392-022-00966-4.
[19] M. Fuentes, R. Araya-Maturana, I. Palomo, E. Fuentes, Platelet mitochondrial dysfunction and mitochondria-targeted quinone-and hydroquinone-derivatives: review on new strategy of antiplatelet activity, Biochem. Pharmacol. 156 (2018) 215–222, https://doi.org/10.1016/j.bcp.2018.08.035.
[20] J.A. Davies, The pre-thrombotic state, Clin. Sci. 69 (6) (1985) 641–646, https://doi.org/10.1042/cs0690641.
[21] S.P. Jackson, R. Darbousset, S.M. Schoenwaelder, Thromboinflammation: challenges of therapeutically targeting coagulation and other host defense mechanisms, Blood 133 (9) (2019) 906–918, https://doi.org/10.1182/blood-2018-11-882993.
[22] B. Klitzman, B.R. Duling, Microvascular hematocrit and red cell flow in resting and contracting striated muscle, Am. J. Physiol. 237 (4) (1979) H481–H490, https://doi.org/10.1152/ajpheart.1979.237.4.H481.
[23] C. Gopalan, E. Kirk, Chapter 2 - the blood vessels, in: C. Gopalan, E. Kirk (Eds.), Biology of Cardiovascular and Metabolic Diseases, Academic Press, 2022, pp. 35–51, https://doi.org/10.1016/B978-0-12-823421-1.00004-4.
[24] X. Xu, J. Geng, X. Teng, Monitoring the blood coagulation process under various flow conditions with optical coherence tomography, J. Biomed. Opt. 19 (4) (2014) 046021, https://doi.org/10.1117/1.Jbo.19.4.046021.
[25] J.J. Hathcock, Flow effects on coagulation and thrombosis, Arterioscler. Thromb. Vasc. Biol. 26 (8) (2006) 1729–1737, https://doi.org/10.1161/01.ATV.0000229658.76797.30.
[26] T.B. Wu, S. Wu, M. Buoni, T. Orfeo, K. Brummel-Ziedins, M. Cohen, et al., Computational model for hyperfibrinolytic onset of acute traumatic coagulopathy, Ann. Biomed. Eng. 46 (8) (2018) 1173–1182, https://doi.org/10.1007/s10439-018-2031-9.
[27] E. Karampini, R. Bierings, J. Voorberg, Orchestration of primary hemostasis by platelet and endothelial lysosome-related organelles, Arterioscler. Thromb. Vasc. Biol. 40 (6) (2020) 1441–1453, https://doi.org/10.1161/atvbaha.120.314245.

[28] D.L. Springer, J.H. Miller, S.L. Spinelli, L. Pasa-Tolic, S.O. Purvine, D.S. Daly, et al., Platelet proteome changes associated with diabetes and during platelet storage for transfusion, J. Proteome Res. 8 (5) (2009) 2261–2272, https://doi.org/10.1021/pr800885j.
[29] H. Yu, Y. Liu, T. He, Y. Zhang, J. He, M. Li, et al., Platelet biomarkers identifying mild cognitive impairment in type 2 diabetes patients, Aging Cell 20 (10) (2021) e13469, https://doi.org/10.1111/acel.13469.
[30] P. Soma, A.C. Swanepoel, J.N. du Plooy, T. Mqoco, E. Pretorius, Flow cytometric analysis of platelets type 2 diabetes mellitus reveals 'angry' platelets, Cardiovasc. Diabetol. 15 (1) (2016) 52, https://doi.org/10.1186/s12933-016-0373-x.
[31] H. Schwertz, E.A. Middleton, Autophagy and its consequences for platelet biology, Thromb. Res. 231 (2023) 170–181, https://doi.org/10.1016/j.thromres.2022.08.019.
[32] M.M. Ouseph, Y. Huang, M. Banerjee, S. Joshi, L. MacDonald, Y. Zhong, et al., Autophagy is induced upon platelet activation and is essential for hemostasis and thrombosis, Blood 126 (10) (2015) 1224–1233, https://doi.org/10.1182/blood-2014-09-598722.
[33] J. Chung, D. Jeong, G.-h. Kim, S. Go, J. Song, E. Moon, et al., Super-resolution imaging of platelet-activation process and its quantitative analysis, Sci. Rep. 11 (1) (2021) 10511, https://doi.org/10.1038/s41598-021-89799-9.
[34] E. Boilard, M. Bellio, Platelet extracellular vesicles and the secretory interactome join forces in health and disease, Immunol. Rev. 312 (1) (2022) 38–51, https://doi.org/10.1111/imr.13119.
[35] T. Misztal, T. Rusak, J. Brańska-Januszewska, M. Gąsowska, B. Szynaka, A. Gołaszewska, et al., Aquaporins in human platelets: intracellular localization and possible role in granule and lysosome secretion, Acta Biochim. Pol. 65 (4) (2018) 555–566, https://doi.org/10.18388/abp.2018_2621.
[36] E. Fuentes, D. Arauna, R. Araya-Maturana, Regulation of mitochondrial function by hydroquinone derivatives as prevention of platelet activation, Thromb. Res. 230 (2023) 55–63, https://doi.org/10.1016/j.thromres.2023.08.013.
[37] W. Zhang, The mitophagy receptor FUN14 domain-containing 1 (FUNDC1): a promising biomarker and potential therapeutic target of human diseases, Genes Dis 8 (5) (2021) 640–654, https://doi.org/10.1016/j.gendis.2020.08.011.
[38] P.P. Kulkarni, M. Ekhlak, D. Dash, Energy metabolism in platelets fuels thrombus formation: halting the thrombosis engine with small-molecule modulators of platelet metabolism, Metabolism 145 (2023) 155596, https://doi.org/10.1016/j.metabol.2023.155596.
[39] P. Yadav, S.K. Beura, A.R. Panigrahi, T. Bhardwaj, R. Giri, S.K. Singh, Platelet-derived microvesicles activate human platelets via intracellular calcium mediated reactive oxygen species release, Blood Cell Mol. Dis. 98 (2023) 102701, https://doi.org/10.1016/j.bcmd.2022.102701.
[40] H. Lu, L.A. Cassis, C.W.V. Kooi, A. Daugherty, Structure and functions of angiotensinogen, Hypertens. Res. 39 (7) (2016) 492–500, https://doi.org/10.1038/hr.2016.17.
[41] M. Ishikawa, E. Sekizuka, N. Yamaguchi, H. Nakadate, S. Terao, D.N. Granger, et al., Angiotensin II type 1 receptor signaling contributes to platelet-leukocyte-endothelial cell interactions in the cerebral microvasculature, Am. J. Physiol. Heart Circ. Physiol. 292 (5) (2007) H2306–H2315, https://doi.org/10.1152/ajpheart.00601.2006.
[42] L. Kalinowski, T. Matys, E. Chabielska, W. Buczko, T. Malinski, Angiotensin II AT1 receptor antagonists inhibit platelet adhesion and aggregation by nitric oxide release, Hypertension 40 (4) (2002) 521–527.
[43] B.A. Boone, P. Murthy, J. Miller-Ocuin, W.R. Doerfler, J.T. Ellis, X. Liang, et al., Chloroquine reduces hypercoagulability in pancreatic cancer through inhibition of neutrophil extracellular traps, BMC Cancer 18 (1) (2018) 678, https://doi.org/10.1186/s12885-018-4584-2.
[44] V. Jancinova, R. Nosal, M. Petriková, On the inhibitory effect of chloroquine on blood platelet aggregation, Thromb. Res. 74 (5) (1994) 495–504, https://doi.org/10.1016/0049-3848(94)90270-4.
[45] J. Li, Z. Li, Y. Zhu, H. Peng, Z. Du, S. Ru, et al., Bisphenol S remodels red blood cell membrane lipids by altering plasma lipid levels, causing the risk of venous thrombosis in SD rats and zebrafish embryos, Environ. Int. 182 (2023) 108331, https://doi.org/10.1016/j.envint.2023.108331.