



Pôle de Recherche Cardiovasculaire (CARD)

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Linking platelet lipid metabolism and thrombosis:

Role of acetyl-CoA carboxylase

Marie Octave

Supervisor: Pr. Sandrine Horman

Co-supervisor: Pr. Christophe Beauloye

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JURY MEMBERS

<u>Supervisor:</u>	Sandrine Horman, professor Pole of Cardiovascular Research, Institute of Experimental and Clinical Research, Université Catholique de Louvain, Brussels, Belgium
<u>Co-supervisor:</u>	Christophe Beauloye, professor Pole of Cardiovascular Research, Institute of Experimental and Clinical Research, Université Catholique de Louvain, Brussels, Belgium
<u>President:</u>	Olivier Feron, professor Pole of Pharmacology and Therapeutics, Institute of Experimental and Clinical Research, Université Catholique de Louvain, Brussels, Belgium
<u>Internal members:</u>	Pierre Sonveaux, professor Pole of Pharmacology and Therapeutics, Institute of Experimental and Clinical Research, Université Catholique de Louvain, Brussels, Belgium Stefan Constantinescu, professor Pole of Cell Signaling, de Duve Institute, Université Catholique de Louvain, Brussels, Belgium
<u>External members:</u>	Jonathan Douxfils, professor Département de pharmacie, Université de Namur, Namur, Belgium Fabrice Cognasse, research supervisor INSERM 1059 – SAINBIOSE, Faculté de médecine, Université Saint-Etienne, Saint-Priest-en-Jarez, France

! Message de la plus haute importance !

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£ Laurent PERIGNON, *depuis que quelqu'un du labo l'a pris pour un obèse*, 2020

* Laurence PIROTON, *par e-mail et sous influence Bubulienne*, 2021 – 2022

Thibaut CRUL, *dès qu'il en a l'occasion*, 1992 –

\$ Youri CUMPS, *avant-pendant-après un match de frisbee*, 2018 –

Summary

Acetyl-CoA carboxylase (ACC) catalyzes the first step of de novo lipogenesis (DNL). Pharmacologic inhibition of ACC has been of interest for therapeutic intervention in a wide range of diseases, including type II diabetes and cancer. However, the impact of its inhibition on hemostasis remains under-investigated. We previously demonstrated that ACC1 activation promotes thrombus formation by increasing platelet AA-containing phosphatidylethanolamine plasmalogen (PEP) content and thromboxane A₂ generation. Inhibition of ACC activity decreases lipogenesis and increases acetyl-CoA content which can serve as a substrate for protein acetylation. This posttranslational modification plays a key role in the regulation of platelet aggregation, via tubulin acetylation. Therefore, we hypothesized that ACC inhibition or deletion may affect platelet functions via an alteration of lipid content and/or tubulin acetylation and that it could be a protective mechanism against thrombus formation, especially in prothrombotic diseases such as atherosclerosis. Treatment with CP640.186, a pharmacological ACC inhibitor, decreased platelet lipogenesis. However, the quantitative lipidomics analyses showed that 2 hours preincubation with the compound didn't affect global platelet lipid content. Interestingly, this short-term ACC inhibition was sufficient to increase tubulin acetylation level, at basal state and after thrombin stimulation. It was associated with an impaired platelet aggregation, in response to low thrombin concentration, while granules secretion wasn't affected. Mechanistically, we highlighted a decrease in Rac1 activity, associated with a reduced phosphorylation of its downstream effector PAK2. Surprisingly, the actin cytoskeleton was not impacted but we evidenced a significant decrease in ROS production which could result from a decreased NOX2 activity. The use of megakaryocyte/platelet-specific ACC1 deletion (ACC1 pKO) mouse model allowed us to evaluate the impact of a long-term ACC inhibition on platelet functions and thrombus formation. We revealed that this

enzyme is a tight regulator of saturated and polyunsaturated fatty acyl chain levels contained in platelet phospholipids. In particular, ACC1 pKO platelets have a decrease in AA-containing PEP content leading to a subsequent decrease in TxA₂ secretion and dense granules secretion. This results in a reduced thrombus formation on collagen-coated surfaces under flow conditions. Collectively, our data demonstrate that ACC1 is essential to maintain specific phospholipid pools required for platelet reactivity and might therefore constitute a novel potential target for anti-thrombotic applications.

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LIST OF ABBREVIATIONS

5-HT	Serotonin
12-HETE	12-hydroxyeicosatetraenoic acid
12-HPETE	12-hydroperoxyeicosatetraenoic acid
12-LOX	Platelet-type 12-lipoxygenase
α TAT1	alpha tubulin acetyltransferase 1
AA	Arachidonic acid
Abs	Antibodies
AC	Adenylate cyclase
ACC	Acetyl-CoA carboxylase
ACD	Acid citrate dextrose
ACL	ATP citrate lyase
ACS	Acute coronary syndrome
ADP	Adenosine diphosphate
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
ApoE	Apolipoprotein E
APT	Acyl protein thioesterase
Ata	Antimycin A
ATP	Adenosine triphosphate
ARC	Arthrogryposis, Renal dysfunction, and Cholestasis
BC	Biotin carboxylase
BCCP	Biotin carboxyl carrier protein
BM	Bone marrow
BMT	Bone marrow transfer
BRCA1	Breast cancer type 1 susceptibility protein
BSA	Bovine serum albumin
CAD	Coronary artery disease
CaIDAG-GEF1	Ca ²⁺ diacylglycerol guanine nucleotide exchange factor1
cAMP	Cyclic adenosine monophosphate
CCL5	C-C motif chemokine ligand 5
CDH1	Epithelial (E)-cadherin
CDP-Cho	Cytidine diphosphate-choline
CDP-Eth	Cytidine diphosphate-ethanolamine
CE	Cholesteryl ester
CER	Ceramide
CFSE	Carboxyfluorescein succinimidyl ester
Chol	Cholesterol
ChREBP	Carbohydrate response element binding protein
COX	Cyclooxygenase
CP640.186	(3R)-1'-(9-anthracenylcarbonyl)[1,4'-bipiperidin]-3-yl]-4-morpholinyl-methanone
CPDA	citrate, phosphate, dextrose, adenine
cPLA ₂	Cytosolic phospholipase A ₂
CPT	Carnitine palmitoyltransferase
CRP	Collagen-related peptide
Csk	C terminal Src kinase
CT	Carboxyl transferase

CTLs	Cytotoxic T lymphocytes
CXCL7	β -thromboglobulin
DAG	Diacylglycerol
DCER	Dihydroceramide
DGAT	Diacylglycerol acyltransferase
DHA	Docosahexaenoic acid
DHAP	Dihydroxyacetone phosphate
DKI	Double knock-in
DMEM	Dulbecco's Modified Eagle's Medium
DMS	Demarcation membrane system
DMSO	Dimethyl sulfoxide
DNL	<i>De novo</i> lipogenesis
DPA	docosapentaenoic acid
DTS	Dense tubular system
DTT	Dithiothreitol
ECAR	Extracellular acidification rate
ECP	Eosinophil cationic protein
EDTA	Ethylenediaminetetraacetic acid
eEF2	Eukaryotic translation elongation factor 2
EETs	Eosinophil extracellular traps
EGF	Epidermal growth factor
EGTA	Egtazic acid
ELISA	Enzyme-linked immunosorbent assay
EPA	Eicosapentaenoic acid
ERK1/2	extracellular signal-regulated kinase 1/2
ESAM	Endothelial cell-selective adhesion molecule
F1.2	Fragments 1.2
F1,6P	Fructose-1,6-bisphosphate
F6P	Fructose-6-phosphate
F-actin	Actin filament
FAK	Focal adhesion kinase
FAS	Fatty acid synthase
FCCP	Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
FDR	False discovery rate
FFA	Free fatty acid
FITC	Fluorescein isothiocyanate
G3P	Glyceraldehyde-3-phosphate
G6P	Glucose-6-phosphate
G-actin	Actin monomer
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GEF	Guanine nucleotide exchange factor
GFOGER	Glycine-phenylalanine-hydroxyproline-glycine-glutamic acid-arginine
GLUT3	Facilitated glucose transporter member 3
GP	Glycoprotein
GPCR	G protein-coupled receptor
GPO	Glycine-proline-hydroxyproline
GPS	Gray platelet syndrome
Gra	Granulocyte
H ₂ DCFDA	2',7'-dichlorodihydrofluorescein diacetate
HBSS	Hank's Balanced Salt Solution's
HCC	Hepatocellular carcinoma

HCER	Hydroxyceramide
HCT	Hematocrit
HDAC	Histone deacetylase
HDL	High-density lipoprotein
HGB	Hemoglobin
HRP	Horse radish peroxidase
ICAM	Intercellular adhesion molecule
IL-8	Interleukin 8
ILK	integrin-linked kinase
IP ₃	Inositol trisphosphate
IP ₃ R	Inositol trisphosphate receptor
ITAM	immunoreceptor tyrosine-based activation motif
JAM-A	Junctional adhesion molecule A
KAT	Acetyllysine transferase
KDACs	Lysine deacetylases
KI	knock-in
KO	knockout
LAT	Linker of activated T-cells
LDL	Low-density lipoprotein
LFA-1	Lymphocyte function-associated antigen-1
LIMK1	LIM domain kinase 1
LOX	Lipoxygenase
LPC	Lysophosphatidylcholine
LPE	Lysophosphatidylethanolamine
LPS	Lipopolysaccharide
LRP	LDL receptor-related protein
LTA4	Leukotriene A4
LXA4	Lipoxin A4
LXB4	Lipoxin B4
Lym	Lymphocyte
Mac-1	Macrophage-1 antigen
MAPK	Mitogen-activated protein kinase
MBP	Major basic protein
MCV	Mean cell volume
MEK	Mitogen-activated protein kinase kinase
MHC	Mean cell hemoglobin
MHCH	Mean cell hemoglobin concentration
Mid	Mid-range cell
MK	Megakaryocyte
MLC	Myosin light-chain
MLCK	Myosin light-chain kinase
MMPs	Matrix metalloproteinases
MPV	Mean platelet volume
MSTUS	MS total useful signal
MT	Microtubule
MYPT1	Myosin-phosphatase targeting subunit 1
n-3	Omega 3
n-6	Omega 6
NAD	Nicotinamide adenine dinucleotide
NAFLD	Nonalcoholic fatty liver disease
NAM	Nicotinamide

NASH	Nonalcoholic steatohepatitis
NETs	Neutrophils extracellular traps
NO	Nitric oxide
NOX	NADPH oxidase
OA	Oleic acid
OCR	Oxygen consumption rate
OCS	Open canalicular system
ORAI1	Calcium release-activated calcium channel protein 1
ORO	Oil red O
ox-LDL	Oxidized LDL
PA	Phosphatidic acid
P-ACC	ACC phosphorylation
PAF	Platelet-activating factor
PAI	Plasminogen activator inhibitor
PAK	P21-activated kinase
PAL	Palmitic acid
PAR	Protease-activated receptor
PATs	Palmitoyl acyltransferases
PC	Phosphatidylcholine
PCAF	p300/CBP-associated factor
PCP	Phosphatidylcholine plasmalogen
PD-ECGF	Platelet-derived endothelial growth factor
PDGF	Platelet-derived growth factor
PDK1	Phosphoinositide-dependent kinase-1
PE	Phosphatidylethanolamine
PEAR1	Platelet endothelial aggregation receptor-1
PECAM-1	Platelet endothelial cell adhesion molecule-1
PEO	Plasmanyphosphatidylethanolamine
PEP	Phosphatidylethanolamine plasmalogen
PF4	Platelet factor 4
PFA	Paraformaldehyde
PG	Phosphatidylglycerol
PGD ₂	Prostaglandin D ₂
PGE ₂	Prostaglandin E ₂
PGG ₂	Prostaglandin G ₂
PGH ₂	Prostaglandin H ₂
PI	Phosphatidylinositol
PI3K	Phosphoinositide 3-kinase
PIP ₂	Phosphoinositol bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G
PL	Phospholipid
PLA ₂	Phospholipase A ₂
PLT	Platelet
PMCA	Plasma membrane Ca ²⁺ -ATPase
PPARs	Peroxisome-proliferator-activated receptors
PRP	Platelet rich plasma
PS	Phosphatidylserine
PSGL-1	P-selectin glycoprotein ligand-1
PUFA	Polyunsaturated fatty acid

RBC	Red blood cell
RDW	Red cell distribution width
RIAM	Rap1-GTP-interacting adaptor molecule
RER	Rough endoplasmic reticulum
ROCK	Rho-associated protein kinase
ROS	Reactive oxygen species
RT	Room temperature
S1P	Sphingosine-1-phosphate
SA	Stearic acid
SAC	Surface area coverage
SCD	Stearoyl-CoA desaturase
SDF-1	Stromal-derived factor 1
SEM	Standard error of the mean
Ser	Serine
SER	Smooth endoplasmic reticulum
SERCA	Sarco(endo)plasmic reticulum Ca^{2+} -ATPase
SIRT	sirtuin
SLAM	Signaling lymphocytic activation molecule
SM	Sphingomyelin
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SNPs	Single nucleotide polymorphisms
SRBI	Scavenger receptor class B member 1
SREBP1c	Sterol regulatory element binding protein 1c
Syk	Spleen tyrosine kinase
TAG	Triacylglycerol
TATc	Thrombin anti-thrombin complex
TCA	Tricarboxylic acid
TF	Tissue factor
TGF- β	Transforming growth factor- β
TGRL	Triglyceride-rich lipoproteins
Thr	Thrombin
TOFA	5-(tetradecyloxy)-2-furoic acid
TP	Thromboxane A_2 receptor
tPA	tissue plasminogen activator
Treg	T regulator
TxA ₂	Thromboxane A_2
TxAS	Thromboxane synthase
uPA	urokinase plasminogen activator
VASP	Vasodilator-stimulated phosphoprotein
VCAM	Vascular cell adhesion molecule
VEGF	Vascular epidermal growth factor
VLA-4	Very late antigen-4
VLDL	Very low-density lipoprotein
vWF	Von Willebrand factor
WBC	White blood cell
WHO	World Health Organization
WT	Wild-type

INTRODUCTION

I. Platelets in hemostasis and diseases

1. Platelet origin

Platelets are small anucleate cell fragments derived from megakaryocytes (MKs). For platelet formation, MKs undergo a maturation process and restructure their cytoplasm. During maturation, MKs undertake endomitosis, increase their size to reach up to 100 μm , fill their cytoplasm with platelet granules and cytoskeletal proteins and develop an invaginated membrane system called demarcation membrane system (DMS). Once mature, MKs extend, into bone marrow (BM) sinusoids, long cytoplasmic protrusions (100-500 μm) called proplatelets. These proplatelets are released into peripheral blood and are fragmented into platelets. Their diameter is around 2-5 μm with a thickness of 0.5 μm and a mean platelet volume of 6-10 fL. Approximately 100×10^9 platelets are produced every day to maintain platelets count between 150 and 400×10^9 per liter in a human adult. Their lifespan is 7-10 days for humans and 4-5 days for rodents [2-4].

2. Platelet structure

Platelets can be structured in four theoretical zones: a peripheral zone, a sol-gel zone, an organelle zone, and membrane systems. A schematic overview of platelet structure is represented in **Figure 1**.

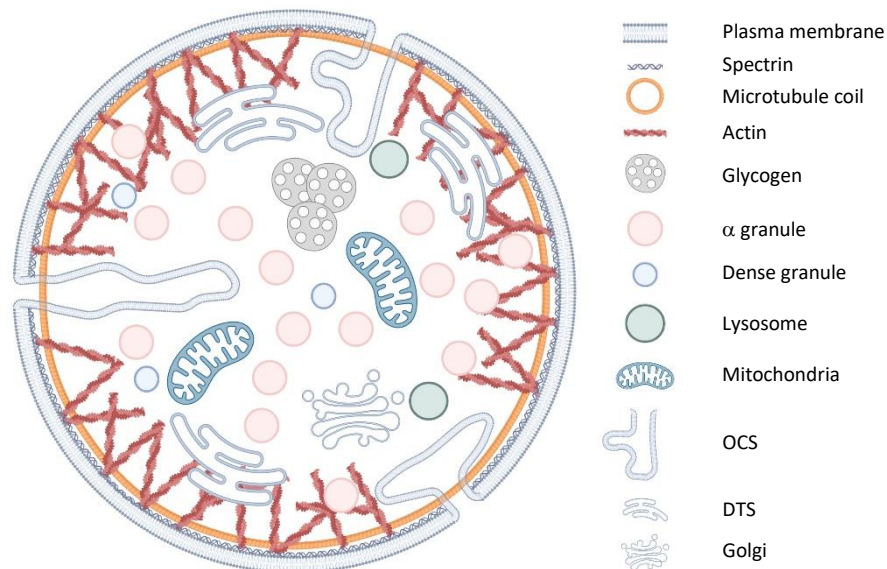


Figure 1. Platelet structure. Schematic overview of the organization of a resting platelet. Compared to other cells, platelets contain an organized spectrin-actin-microtubule cytoskeleton, different types of granules (α and dense), an open canalicular system (OCS) and a dense tubular system (DTS).

2.1. The peripheral zone

The peripheral zone contains the plasma membrane surrounded, above, by a layer of glycocalyx and, under, by a submembrane region containing the membrane contractile cytoskeleton. The glycocalyx is composed of glycoproteins (GPs) and glycolipids. The high number of GPs receptors (GPIb/IX/V, GPIV, GPVI, $\alpha 2\beta 1$, $\alpha IIb\beta 3$, $\alpha 5\beta 1$, $\alpha v\beta 3$, $\alpha 6\beta 1$) ensures platelet adhesion, activation and aggregation. Besides its role in hemostasis, glycocalyx, via its negative net charge, favors plasma component endocytosis, prevents spontaneous platelet aggregation, and regulates Ca^{2+} signaling via the capture of exported Ca^{2+} ions from platelets [4, 5]. The plasma membrane is composed of glycerophospholipid (phosphatidylcholine [PC] 37%, phosphatidylethanolamine [PE] 17%, phosphatidylserine [PS] 6%, phosphatidylinositol [PI] 3%, phosphatidylglycerol [PG] and phosphatidic acid [PA] 2%), sphingomyelin [SM] 7% and cholesterol (28%). In resting platelets, phospholipids (PLs) are asymmetrically distributed with PC and SM

mainly present at the outer leaflet of the plasma membrane whereas PE and PS are located at the inner leaflet (see section II.1.1). Upon activation, phospholipid scramblases, like TMEM16F, are activated and expose PS at the platelet surface, generating a procoagulant surface (see section II.1.4.) [6-8]. The submembrane area is characterized by the junction of the open canalicular system (OCS) with the plasma membrane and the association of cytoplasmic transmembrane receptors with cytoskeletal filament/proteins [4, 5].

2.2. The sol-gel zone

The sol-gel zone includes platelet cytoskeleton (a spectrin-based membrane skeleton, a microtubule (MT) coil and actin filaments), glycogen and secretory organelles. The spectrin membrane skeleton ensures the link between the plasma membrane and the actin network. The latter form a matrix in which organelles and structural compounds can tie. The MT coil, located below the spectrin skeleton, is formed from 3-24 MTs and is important for maintaining the discoid shape of resting platelets. Upon stimulation, platelet cytoskeleton helps granule relocation toward platelet center where they merge with OCS and secrete their content [4, 5].

2.3. The organelle zone

Platelet organelles include three different types of granules (α and dense granules and lysosomes), mitochondria, glycosomes, Golgi complexes, rough endoplasmic reticulum, smooth and coated vesicles, electron-dense chains and clusters and tubular inclusions. With 50 to 80 granules (200-500 nm each), α granule is the most frequent organelle and constitutes ~10% of platelet volume. It also contains numerous and diversified proteins (more than 200 were detected by mass spectrometry [9]) originating from MKs or uptake by endocytosis from the plasma. These proteins are implicated in

- platelet adhesion (endothelial cell-selective adhesion molecule [ESAM], platelet endothelial cell adhesion molecule-1 [PECAM-1], junctional adhesion molecule A [JAM-

A], fibronectin, von Willebrand factor [vWF], thrombospondin-1, fibrinogen, P-selectin, GPIIb α , GPIIb β , GPIX, GPV, integrin α IIb and β 3),

- coagulation (antithrombin III, factor V, IX and XIII)
- fibrinolysis (plasminogen, plasminogen activator inhibitor [PAI])
- angiogenesis (angiopoietin-1, platelet-derived growth factor [PDGF], vascular epidermal growth factor [VEGF], angiostatin, endostatin)
- cellular growth (epidermal growth factor [EGF], transforming growth factor- β [TGF- β])
- metabolism (facilitated glucose transporter member 3 [GLUT3])
- chemoattraction (β -thromboglobulin [CXCL7], platelet factor 4 [PF4], C-C motif chemokine ligand 5 [CCL5], interleukin 8 [IL-8]).
- immunity (complement C3 precursor, complement C4 precursor)

In humans, two inherited platelet disorders involve abnormal or absent α granules: gray platelet syndrome (GPS) and Arthrogryposis, Renal dysfunction, and Cholestasis (ARC) syndrome [4, 9, 10]. Dense granules differ from α granules in terms of number (3-8), size (150 nm) and content (Ca^{2+} , Mg^{2+} , K^{+} , ADP, ATP, serotonin, histamine, polyphosphate and pyrophosphate). A deficit in these granules is found in Hermansky-Pudlak Syndrome.

2.4. The membrane systems

Golgi complexes and rough endoplasmic reticulum (RER) are also included in membrane systems just like the plasma membrane, OCS and dense tubular system (DTS). Golgi complexes, mostly residues from MKs, and RER are rare in human platelets and mainly found in patients with hypogranular syndromes or immune thrombocytopenia, respectively [4]. OCS can be defined as an invagination of the plasma membrane, which takes up plasma proteins and distributes them into granules. It serves also as a reservoir for membrane extension during platelet spreading and allows granule content release upon stimulation [11]. DTS is the remains of MKs' smooth endoplasmic reticulum (SER) and is the main Ca^{2+} store in platelets. Unlike OCS,

DTS is not in direct contact with the plasma membrane but rather in platelet cytosol with few channels near the MT coil. It appears to participate in thromboxane A₂ (TxA₂) generation as arachidonic acid (AA) can be incorporated into DTS phospholipid membrane [12] and DTS has cyclooxygenase (COX) and thromboxane synthase (TxAS) activities [13].

3. The physiological role of platelets

At the end of the 19th century, Bizzozero discovered the physiological role of platelets (**Figure 2**): “Blood platelets, [...] are held up at the damaged spot as soon as they arrive at it. At first one sees only 2 to 4 to 6 (platelets); very soon the number climbs to hundreds. [...] Little by little the volume increases and soon the thrombus fills the lumen of the blood vessel, and impedes the blood stream more and more. [...] Fibrous material is deposited on the layer of platelets and as the flow continues it is laid down predominantly in long bundles of threads. [...] Blood platelet [...] has already provided a fundamental contribution to the explanation of the phenomena of thrombosis and blood clotting” [1]. The steps leading to thrombus formation are represented in **Figure 3** and detailed below.

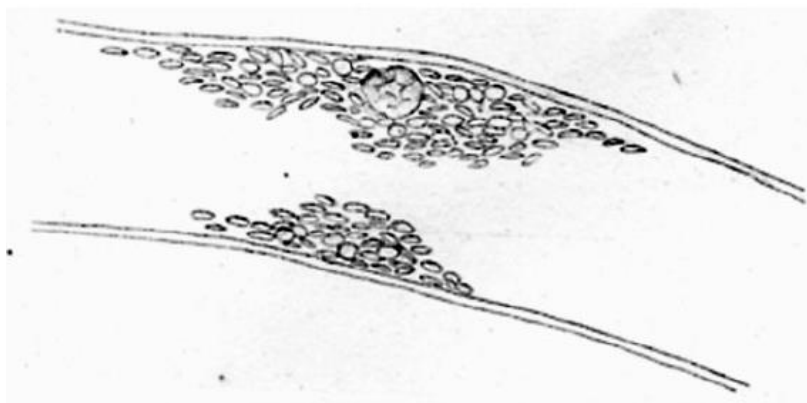


Figure 2. Platelet function discovery. Draw of thrombus formation in a small artery of a guinea pig by Bizzozero in 1882 [1].

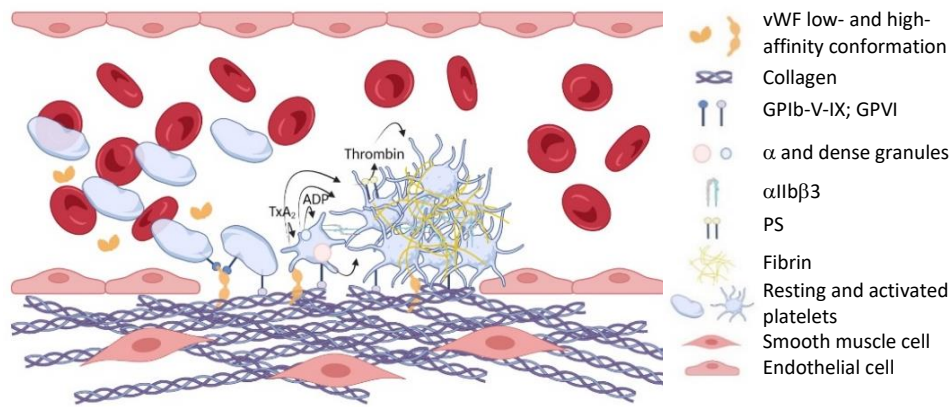


Figure 3. Thrombus formation. After a vessel injury, circulating vWF attach to collagen fibers and adopt a high-affinity conformation (activated state). Resting platelets adhere to the subendothelium via GPIIb/IIIa-activated vWF interaction followed by GPIIb/IIIa-collagen binding, both initiating platelet activation. Consequently, α and dense granules are released, TxA₂ is secreted, αIIbβ3 is activated and PS are exposed to the outer leaflet of the plasma membrane. ADP, from dense granule, and TxA₂, activate and recruit other platelets, αIIbβ3 contributes to platelet aggregation and PS exposure allows thrombin generation which result in fibrin formation to stabilize the clot. PS: phosphatidylserine; TxA₂: thromboxane A₂; vWF: von Willebrand factor.

3.1. Platelet adhesion

Following vascular injury, platelet adhesion to the subendothelium is the first step leading to clot formation. To do so, several platelet GP receptors, adhesive proteins and extracellular matrix components from the vessel wall act together to seal the vessel breach. Initial tethering is mediated by the binding of platelet GPIIb/IIIa, a subunit of GPIIb-V-IX receptor, to the activated immobilized vWF. The high association and dissociation rate of this bound makes platelet adhesion reversible and induces platelet rolling. This process allows a direct association between collagen (GPIIb/IIIa and α2β1) receptors and the exposed collagen, leading to a stable adhesion. Other receptors and adhesive components are also involved: α5β1 and fibronectin, α6β1 and laminin, CD36 and thrombospondin, αIIbβ3 and fibrinogen, αvβ3 and vitronectin. At a high shear rate ($> 500\text{s}^{-1}$ for human blood and $> 2000\text{s}^{-1}$ for mice blood), vWF is the only component capable of inducing platelet adhesion via its association with GPIIb/IIIa [14, 15] (**Figure 3**).

3.1.1. vWF – GPIb α interaction

vWF is found in Weibel-Palade bodies of endothelial cells and α granules. Endothelial cells secrete and tie ultra-large vWF that binds directly to platelet GPIb α receptor. In opposition, soluble vWF, found in plasma and originating from platelets, is smaller and has to be immobilized to extracellular matrix components (vWF or collagen) and activated (high-affinity conformation) to interact with platelets. First, soluble vWF binds to vWF or type I and III collagen, via its A3 domain, and type VI collagen via the A1 domain. Of note, type I and III collagen are mainly found in deep layer of the vessel and type VI in the subendothelium. Secondly, high shear stress influences and favors vWF binding to platelets as it induces its conformational change into a – so-called – activated form where the A1 domain of vWF is made accessible for the GPIb α subunit [14]. Besides high shear stress, GP receptor presence in lipid rafts [16] and association of the GPIb α cytoplasmic tail with 14-3-3 ζ [17] and filamin A are required for a firm vWF - GPIb α interaction and platelet adhesion [18]. Mutations in the vWF gene (known as vW disorder) cause a decrease in platelet adhesion and factor XIII binding or induce spontaneous association with GPIb α [19].

3.1.2. GPIb-V-IX receptor and signaling

In the middle of the 20th century, several cases of an autosomal recessive disorder characterized by macrothrombocytopenia were reported in patients suffering from severe bleeding. This disease, called Bernard-Soulier syndrome, is linked to mutations in GPIb α , β and GPIX subunits of the GPIb-V-IX complex [20]. With more than 25,000 copies/platelet, GPIb-V-IX is the second most abundant platelet receptor and it is composed of four subunits: 2 GPIb α , 4 GPIb β , 2 GPIX, and 1 GPV (**Figure 4**). To induce platelet adhesion, it associates with vWF through the GPIb α subunit (see section I.3.1.1). Of note, GPIb-V-IX can also bind to several other platelet agonists (thrombin, thrombospondin and collagen), coagulation factors (kininogen, factor XI and XII), integrin α M β 2, β 2-glycoprotein I or P-selectin [21].

The signaling cascade following vWF binding to GPIb α induces platelet activation, i.e. α IIb β 3 activation, TxA₂ and granule secretion. Upon platelet adhesion, Src and Lyn associate to GPIb-IX, and phosphoinositide 3-kinase (PI3K) binds 14-3-3 ζ , newly located at the cytoplasmic domain of GPIb α . Latter one, Lyn induces Akt phosphorylation via Rac1-induced PI3K activation [22]. Then, through the NO-cGMP-PKG pathway, Akt induces p38 and ERK activation [23, 24] leading to TxA₂ secretion, either via direct phosphorylation of cytosolic phospholipase A₂ (cPLA₂) or indirectly via LIMK1 activation [25, 26]. PKG-ERK signaling also leads to granule secretion and integrin α IIb β 3 activation [23]. Moreover, phospholipase C γ 2 (PLC γ 2), activated downstream of PI3K, cleaves phosphoinositol biphosphate (PIP₂) into inositol trisphosphate (IP₃) and diacylglycerol (DAG). IP₃ binding to its receptor (IP₃R) on DTS increases intracellular calcium (Ca²⁺) [27]. Both DAG and Ca²⁺ activate CalDAG-GEFI and protein kinase C (PKC), which are involved in integrin activation and granule secretion [28] (**Figure 4**).

3.1.3. Collagen - GPVI interaction and signaling

Collagen is considered the main ligand for GPVI even though it can also bind to fibrin, laminin, fibronectin or vitronectin. Among all types of collagen present in the vascular wall, type I and III account for 60% and 30% of total collagen, respectively. These two are the only ones that form large banded collagen fibers and bind to GPVI. The peptide sequence recognized by the receptor is glycine-proline-hydroxyproline (GPO) [14, 29, 30]. Of note, collagen-related peptide (CRP) is synthesized based on this motif, which makes it specific to GPVI receptor [31].

GPVI is a transmembrane GP that exists as mono-, di- and oligomers. Each monomer is linked with two FcR γ chains containing an immunoreceptor tyrosine-based activation motif (ITAM). GP dimerization is required to induce the downstream signaling cascade leading to platelet activation. The first event is tyrosine phosphorylation in the ITAM domain by Src family kinases, Fyn and Lyn. Spleen tyrosine kinase (Syk) associates with the phosphorylated residues, becomes activated and initiates signalosome formation. The intracellular framework of the signalosome includes linker of activated T-cells (LAT)

and the cytosolic adaptors SLP-76, Gads and Grb2. LAT signalosome is responsible for PI3K and PLC γ 2 activation and the downstream signaling cascade resulting from it (see section 3.1.2) [32, 33]. Of note, GPVI and GPIb α are physically associated at the plasma membrane [34] (**Figure 4**).

3.1.4. Collagen - α 2 β 1 interaction and signaling

The integrin α 2 β 1 binds to collagen type I and IV through a different peptide sequence than GPVI: glycine-phenylalanine-hydroxyproline-glycine-glutamic acid-arginine (GFOGER). It associates with spiral microfibrils rather than banded fibers [14]. Like other integrins, the activated state, i.e. high-affinity conformation, of α 2 β 1 is the more potent to interact with its ligand. The conformational change is due to the activation of the inside-out signaling [35]. Like GPVI, the signaling cascade activated downstream of α 2 β 1 involves Src kinases and PLC γ 2, and leads to platelet activation (see section I.3.1.3) [36, 37] (**Figure 4**).

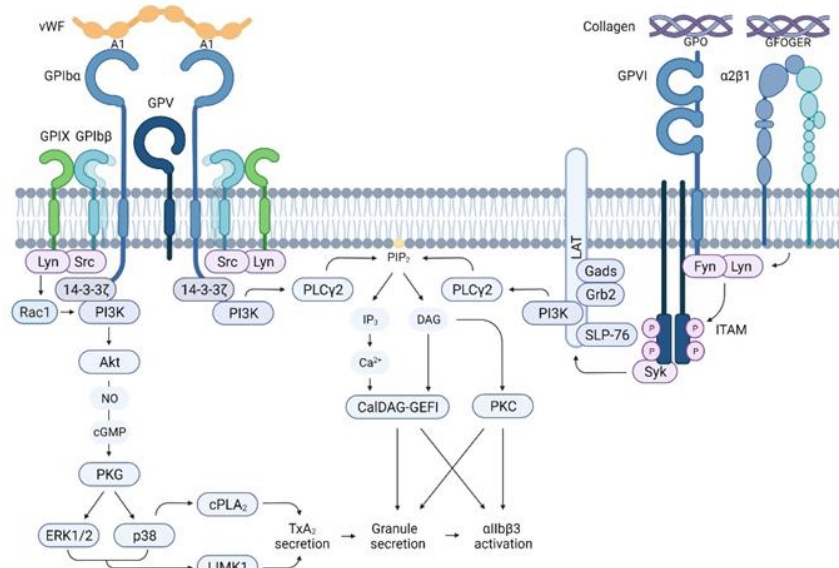


Figure 4. Signaling pathways of platelet adhesion receptors. Von Willebrand factor (vWF) binding to GPIIb α , from GPIb-V-IX receptor, triggers Src, Lyn and phosphoinositide-3 kinase (PI3K) activation. The latter leads to, on one hand, Akt-PKG-MAPKs activation and, on the other hand, PLC γ 2-induced PIP $_2$ cleavage to IP $_3$ and DAG. Both of them contribute to CalDAG-GEF1 and PKC activation, which participate in granule secretion and α IIb β 3 activation. Activation of MAPKs (ERK1/2 and p38) induces TxA $_2$ secretion either via cPLA $_2$ or LIMK1 activation; which, later on, triggers granule secretion and integrin activation. Collagen binding to GPVI leads to Fyn/Lyn-induced ITAM phosphorylation. Syk binds to the phosphorylated residues and initiates LAT signalosome formation. *In fine*, platelets are activated via the same pathway as vWF receptor, i.e. via PI3K-PLC γ 2 pathway. The signalling cascade activated downstream of α 2 β 1 includes Fyn/Lyn-Syk-PI3K-PLC γ 2 pathway. CalDAG-GEF1: Ca $^{2+}$ diacylglycerol guanine nucleotide exchange factor1; cPLA $_2$: cytosolic phospholipase A $_2$; DAG: diacylglycerol; ERK1/2: extracellular signal-regulated kinases; IP $_3$: inositol 1,4,5-trisphosphate; ITAM: immunoreceptor tyrosine-based activation motif; MAPKs: mitogen-activated protein kinases; PIP $_2$: phosphatidylinositol 4,5-bisphosphate; PKC: protein kinase C; PKG: protein kinase G; PLC γ 2: phospholipase C γ 2; TxA $_2$: thromboxane A $_2$.

3.2. Platelet spreading

The signaling cascade, activated downstream platelet adhesion, triggers a coordinated reorganization of MTs and actin cytoskeleton and activation of the actomyosin complex, features of platelet shape change and spreading. To go from a discoid to a spherical shape, spectrin-based membrane cytoskeleton reorganizes, and actin polymerizes. Elongation of F-actin gives rise to a finger-like structure called filopodia and lateral F-actin expansion called lamellipodia, localized between filopodia. It occurs

concurrently with OCS extrusion and granule centralization. Activation of the actomyosin complex participates, on one hand, in organelle centralization, which then fuse with OCS to secrete their content, and on the other hand, in circular or triangular stress fiber formation, characteristic of a fully spread platelet. In parallel, MTs condensate towards platelet center and depolymerize. A tiny MT ring persists at the center of the platelet and from there MT polymerization occurs into the cytosol (**Figure 5**) [5, 38, 39].

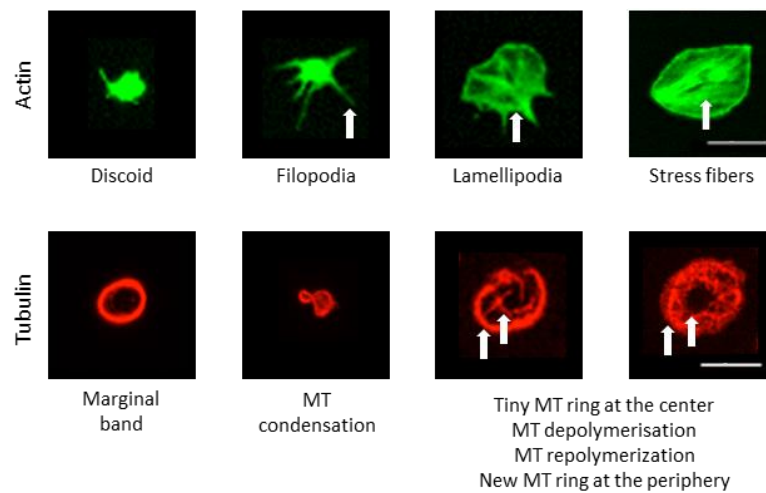


Figure 5. Platelet spreading. At resting state, platelets are discoid and MTs form the marginal band. During platelet spreading, F-actin elongates to give rise to finger-like structure called filopodia (white arrow) and MTs condensate towards the center. Then, lateral F-actin expansion results in lamellipodia formation (white arrow) and MTs depolymerize. Finally, platelets form stress fibers (white arrow) and a tiny MT ring persists at the center of the platelet (white arrow) and from there MT polymerization occurs into the cytosol and a new MT ring is formed at the periphery (white arrow). Scale bar: 5 μ M. MT: microtubule.

3.2.1. Actin polymerization

Actin polymerization occurs either from existing actin filament (F-actin) along with severing and uncapping proteins (gelsolin, CapZ, adducin and VASP) or *de novo* with the help of nucleating proteins (Arp2/3 complex and formins). Gelsolin activation initiates actin reorganization by severing F-actin and capping newly barbed ends to prevent the addition or removal of actin monomer (G-actin). Afterward, gelsolin and CapZ bind to

PIP₂ at the plasma membrane and α -adducin is phosphorylated. Both processes induce their dissociation from F-actin, creating free barbed ends available for actin polymerization. *De novo* actin polymerization requires G-actin formation. These monomers are formed due to gelsolin severing activity and act as substrates for Arp2/3 complex and formins to generate new F-actin [5, 38, 39].

3.2.2. Rho GTPases in platelet remodeling and their downstream signaling

Small Rho GTPases cdc42, Rac1 and RhoA play a key role in actin cytoskeleton remodeling. They are activated by collagen, via GPVI and α 2 β 1, by fibrinogen binding to its receptor, α IIb β 3, or by thrombin and TxA₂ via G protein-coupled receptor (GPCR) subunits G α q and G α 12/13.

GPVI signaling leads to Rac1 guanine nucleotide exchange factor (GEF) activation either via PI3K-PDK1 pathway or via Src-induced Syk phosphorylation [40]. Once in its activated form (i.e. GTP bound), Rac1 induces, on one hand, ORAI1 (calcium release-activated calcium channel protein 1) translocation to the plasma membrane which allows the entry of extracellular Ca²⁺ in platelets and, on the other hand, activation of WAVE-Arp2/3 complex, p21-activated kinase (PAK) and its effectors [41, 42]. The increased intracellular Ca²⁺, due to ORAI1 translocation and Rac1-induced PLC γ 2 activation, leads to 1) MLC phosphorylation, via Ca²⁺-calmodulin-induced MLCK activation, and 2) inside-out signaling, i.e. α IIb β 3 conformational change, and thereafter Rac1-activatory feedback loop [41-44]. Furthermore, Rac1-GTP induces cofilin phosphorylation via PAK2-induced LIM domain kinase 1 (LIMK1) activation and dephosphorylation via PAK4. Upon stimulation, cofilin is, first, dephosphorylated which triggers F-actin binding and severing and therefore promotes actin polymerization by forming newly barbed ends. Re-phosphorylation inhibits cofilin activity. Other PAK2 effectors, i.e. mitogen-activated protein kinase kinase (MEK), which phosphorylates extracellular signal-regulated kinase 1/2 (ERK1/2) and Akt, are also activated upon GPVI binding and participate in lamellipodia formation, F-actin polymerization, α IIb β 3 activation, granule secretion and aggregation [45, 46] (**Figure 6**).

Soluble platelet agonists, thrombin and TxA₂, bind GPCR protease-activated receptors (PARs) and TxA₂ receptor (TP), respectively, to activate cdc42, Rac1 and RhoA and reshape actin cytoskeleton. Gαq subunit induces PLCβ activation and subsequently a rise in Ca²⁺ level, resulting in the same downstream signaling cascade as GPVI [47, 48]. Moreover, Rac1-GTP is involved in reactive oxygen species (ROS) generation via its binding to a subunit of NADPH oxidase 2 (NOX2), p67phox, which induces its translocation to NOX2/p22phox complex, located at the plasma membrane [49, 50]. In addition to p67phox, translocation of p47phox and p40phox, mediated by phosphorylation, are also required for NOX2 activity. Several kinases trigger p47phox phosphorylation, namely PAK, ERK1/2, p38, Akt and PKC [51]. In platelets, rho-associated protein kinase (ROCK) also promotes p47phox phosphorylation. The subsequent ROS generation induces a direct activation of RhoA which participates in stress fiber formation via ROCK-induced myosin-phosphatase targeting subunit 1 (MYPT1) inhibition [49, 52]. The typical pathway leading to RhoA-ROCK activation downstream GPCR happens via Gα12/13. Recently, pyk2, a non-receptor tyrosine kinase of focal adhesion kinase (FAK) family, has been shown to regulate RhoA activation downstream of Gα13 [53]. Besides its role in MLC phosphorylation via MYPT1 inhibition, ROCK regulates F-actin content via the LIMK1-cofilin pathway [54] (**Figure 6**). Integrin α2β1 contributes to platelet cytoskeletal remodeling either, via Src activation and Rac-PAK downstream signaling cascade or, via PLCγ2 activation, independently of its phosphorylation by Src. PLCγ2-induced Ca²⁺-CalDAG-GEF1 activation is mediated through pyk2-induced PI3Kβ activation. Once activated, CalDAG-GEF1 triggers αIIbβ3 inside-out signaling via GDP/GTP exchange of Rap1b (see section I.3.5.1). As a result, the binding of fibrinogen to the activated αIIbβ3 triggers outside-in signaling (see section I.3.7) [37, 43, 55, 56] (**Figure 6**).

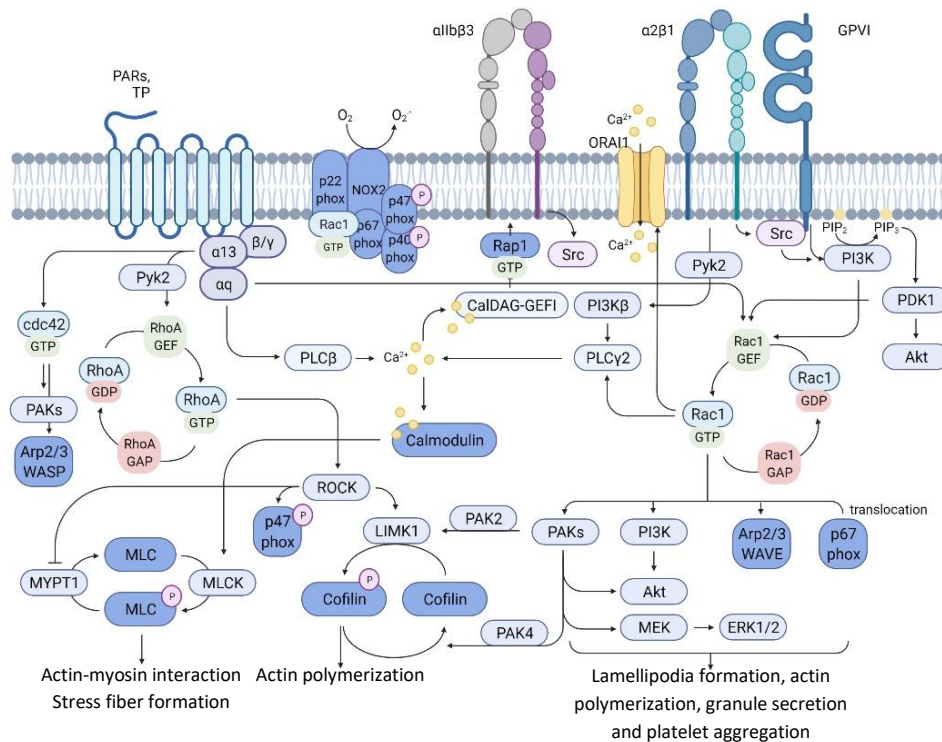


Figure 6. RhoGTPases signalling cascade upon platelet activation. GPVI downstream signalling leads to Rac1-GEF activation either via PI3K-PDK1 or Src-Syk-PI3K pathway. Rac1-GTP induces Ca²⁺ increased via 1) ORAI1 translocation to the plasma membrane or 2) PLCγ2 activation. Consequently, Ca²⁺ activates CalDAG-GEF1, which switches αIIbβ3 conformational state and binds to calmodulin that modulates MLC phosphorylation via MLCK activation. Once activated, αIIbβ3 triggers Rac1 activation through Src and via MLC participates in stress fiber formation. Rac1-GTP contributes to PAKs and its effectors (Akt, MEK, LIMK1), PI3K, Arp2/3-WAVE complex and NOX2 activation. All of them support platelet activation. α2β1 contributes to Rac1-GTP formation via Src-PI3K pathway and activates PLCγ2 via pyk2-PI3Kβ pathway. GPCRs (PARs or TP) downstream signaling activates cdc42, Rac1 and RhoA. Cdc42 participates in PAKs-Akt and Arp2/3-WASP activation. Gαq subunit triggers PLCβ activation and the downstream signaling due to an increase of Ca²⁺. Gα13 induces Rho-GTP formation that participates 1) in actin polymerization via ROCK-LIMK1-cofilin signaling and 2) NOX2 activation via p47phox phosphorylation. CalDAG-GEF1: Ca²⁺diacylglycerol guanine nucleotide exchange factor1; GPCR: G protein-coupled receptor; LIMK1: LIM domain kinase 1; MEK: mitogen-activated protein kinase kinase; MLC: myosin light-chain; MLCK: MLC kinase; NOX2: NADPH oxidase 2; PAK: p21-activated kinase; PAR: protease-activated receptor; PDK1: phosphoinositide-dependent kinase-1; PI3K: phosphoinositide-3 kinase; PLCγ2: phospholipase Cy2; ROCK: Rho-associated protein kinase; TP: thromboxane receptor.

3.3. Platelet secretion

In parallel with cytoskeleton remodeling, platelet activation causes granule secretion. This phenomenon is crucial for autocrine and paracrine platelet activation, platelet

recruitment at the lesion site and thrombus build-up. Indeed, granules contain secondary platelet agonists (ADP, ATP, 5-HT), vWF and the main component for interplatelet bridge formation, i.e. fibrinogen (see section 1.2.3) (**Figure 3**).

3.3.1. Granule fusion

α and dense granule secretion are differentially regulated as seen with mutated mouse models that only impair one of two types of granule secretion [57, 58]. Dense granules merge with OCS before α -granules and lysosomes. Of note, α -granule secretion kinetics varies, and its content is heterogeneous [59, 60]. Granule fusion happens with the help of soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins. The main vesicle or granule (v) SNAREs participating in granule release are VAMP 7 and 8. They interact with target membrane (t) SNARE, i.e. syntaxins and SNAP23/25, located on the plasma membrane. Association of VAMP 8, SNAP23 and syntaxin 11 is required for proper fusion of all types of granules whereas syntaxin 8 is only involved in dense granule secretion [61]. Some docking factors favor granule fusion such as Munc18b, STXBP5, Rab27a/b and SLP4. The small GTPase Rab27b is specifically involved in dense granule release [62]. In addition to regulate cytoskeleton remodeling, Rac1 and RhoA participate in granule secretion via the Rac1-PAK pathway and RhoA-ROCK-PKC δ signaling cascade [46, 53].

3.3.2. ADP signaling

ADP, released from dense granules, binds to its two purinergic receptors: P2Y1 and P2Y12. P2Y1 is a GPCR with G α_q subunit that triggers platelet shape change and initiates aggregation via PLC β activation, Ca²⁺ increase and PKC activation. The binding of ADP to P2Y12 allows full platelet aggregation and amplifies platelet activation initiated by other agonists. P2Y12 is coupled to G α_i2 , resulting in adenylate cyclase inhibition, and G $\beta\gamma$, leading to PI3K/Akt activation. cAMP-PKA-induced VASP phosphorylation is decreased downstream G α_i2 . VASP regulates actin cytoskeleton and

its dephosphorylation favors granule secretion. Integrin activation and TxA₂ secretion occur downstream Gβγ via PI3K-Rab1b or PI3K-ERK signaling events [63-65] (**Figure 7**).

3.3.3. ATP signaling

Another released nucleotide acting as a platelet agonist is ATP. It increases intraplatelet Ca²⁺ level by binding to its receptor P2X1, a ligand-gated Ca²⁺ channel, and mediates reversible shape change. Although it does not trigger platelet aggregation *in vitro*, it plays a role in thrombus formation *ex vivo*, on a collagen surface at a shear rate of 1000s⁻¹. The signaling cascade activated downstream Ca²⁺ entrance involved MEK-ERK and calmodulin-MLCK pathways [64, 66, 67].

3.3.4. Serotonin signaling

Serotonin (5-HT), also contained in dense granules, amplifies platelet activation via its binding to GPCR - Gq, 5-HT2A. Integrin activation and platelet aggregation occur via PLCβ-IP₃-Ca²⁺-PKC pathway [68].

3.4. TxA₂ generation

TxA₂ is a significant secondary mediator of platelet activation. It is generated from the cleavage of AA linked to PLs. AA is, first, oxidized into prostaglandin G₂ (PGG₂) by COX1 and then reduced into PGH₂. The latter is converted into TxA₂ by TxAS [69]. PLA₂ are the main enzymes responsible for AA release into the cytosol (see section II.2.2.2.1). TxA₂ binds to its GPCR receptors, i.e. TPα and TPβ, and amplifies platelet reactivity via Gαq-PLCβ and Gα12/13 – RhoA downstream signaling cascade [70] (**Figure 7**).

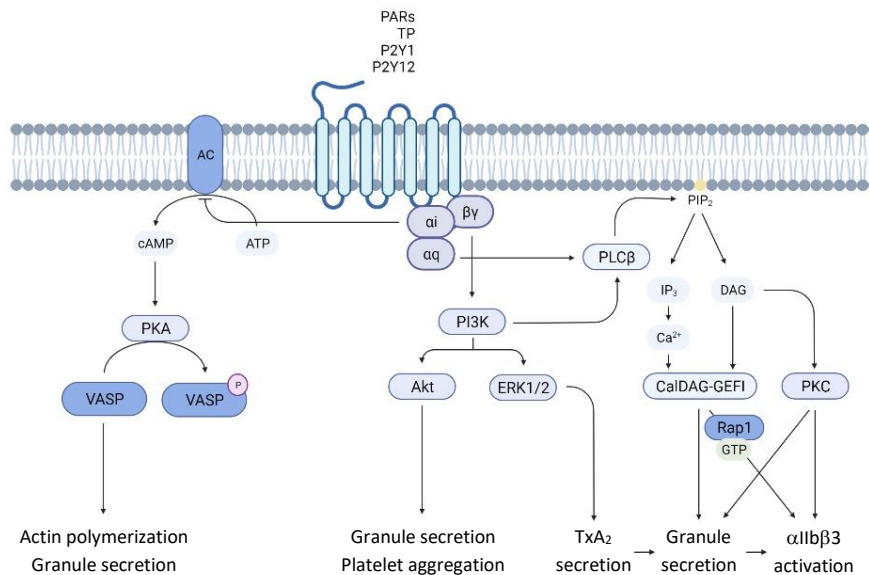


Figure 7. Signaling pathways of GPCR receptors. The subunit $G\alpha_i$ participates in actin cytoskeleton reorganisation and granule secretion via inhibition of AC and subsequently, cAMP-PKA-VASP pathway. $G\alpha_q$ contributes to granule secretion and platelet aggregation via $PLC\beta$ activation. The latter induces CalDAG-GEF1 and PKC activation via an increase in Ca^{2+} and DAG formation. $G\beta\gamma$ activates PI3K, which induces TxA_2 secretion, granule secretion and platelet aggregation via Akt, ERK1/2 or CalDAG-GEF1 activation. AC: adenylate cyclase; ATP: adenosine triphosphate; CalDAG-GEF1: Ca^{2+} diacylglycerol guanine nucleotide exchange factor1; cAMP: cyclic adenosine monophosphate; DAG: diacylglycerol; ERK1/2: extracellular signal-regulated kinases; GPCR: G protein-coupled receptor; IP_3 : inositol 1,4,5-trisphosphate; PKA: protein kinase A; PKC: protein kinase C; PIP_2 : phosphatidylinositol 4,5-bisphosphate; $PLC\beta$: phospholipase C β ; TxA_2 : thromboxane A_2 ; VASP: vasodilator-stimulated phosphoprotein.

3.5. Platelet aggregation

Once activated, platelets' fate is to form aggregates (**Figure 3**). The main receptor for platelet aggregation is $\alpha IIb\beta 3$. Indeed, inherited modifications in this receptor, leading to Glanzmann thrombasthenia disorder, prevent platelet aggregation [71, 72]. In order to form platelet connections, this integrin has to switch from an inactive (low binding affinity) to an active state (high binding affinity). This process is called inside-out signaling and is triggered by soluble (thrombin, ADP, 5-HT, TxA_2) and immobilized (vWF, collagen) platelet agonists. Interestingly, filopodia either enhance platelet aggregation, if their length is higher than usual, or prevent large aggregate formation if they are shorter [73, 74].

3.5.1. The inside-out signaling

The first step is DAG and IP₃ generation via PLC activation (β for GPCR and $\gamma 2$ for GP receptors). IP₃-induced Ca²⁺ increase activates CalDAG-GEF1, leading to GTP association to Rap1 and its translocation to the plasma membrane. It forms a complex with Rap1-GTP-interacting adaptor molecule (RIAM) that binds to talin-1 at integrin $\beta 3$ tail. Kindlins, along with talin, enhance integrin activation, i.e. high-affinity conformation (**Figure 8**). How they exactly cooperate is unknown but some studies suggest the participation of bridging proteins such as integrin-linked kinase (ILK), ADAP, paxillin, and Hic-5 [75].

3.5.2. Adhesive molecules in platelet aggregation

Platelet-platelet interaction can be further reinforced by adhesive molecules.

JAM-A and PECAM-1 are negative regulators of platelet aggregation and thrombus formation [76-78]. At resting state, JAM-A negatively regulates $\alpha IIb\beta 3$ outside-in signaling by recruiting C terminal Src kinase (Csk), a suppressor of Src activity, to the integrin. Upon stimulation, this association is lost and favors platelet aggregation and thrombus formation *in vivo* without affecting granule secretion and inside-out signaling [79, 80].

Close contact mediated secondary to integrin binding, notably by signaling lymphocytic activation molecule (SLAM), epithelial (E)-cadherin (CDH1) or by an interaction between Eph kinase A4 and B1 and their ligands, ephrin B1, positively regulate platelet aggregation and thrombus formation [81-84]. Like EphA4, some transmembrane protein expression, such as platelet endothelial aggregation receptor-1 (PEAR1), are increased at platelet surface upon stimulation. PEAR1 activation has been reported to amplify inside-out signaling and therefore plays a role in aggregation [85]. Interactions implicating CD40L and Gas6 receptors (Axl, Tyro3 and Mer) are not important for initiating platelet aggregation but rather participate in thrombus stabilization [86, 87].

3.6. Thrombus formation

Platelet aggregation leads to hemostatic plug formation with tightly packed and fully activated platelets in the core region and loosely packed and less activated platelets in the shell region. Secondary agonists, ADP and TxA₂, are recognized as drivers of platelet accumulation at thrombus shell. Indeed, mice lacking either TP receptors or Giα2 or mice fed with aspirin or injected with cangrelor, a P2Y₁₂ antagonist, only have an altered platelet accumulation in the shell area of the thrombus [88, 89]. Interestingly, the not fully activated platelets present at thrombus periphery extend filopodia. In a thrombus core, platelet activation is driven by thrombin. It is a protease that activates platelets by the cleavage of the N terminal domain of PARs receptors (3 and 4 in murine platelets; 1 and 4 in human platelets). The newly N terminal part binds to PARs receptors and induces a downstream signaling cascade leading, ultimately, to platelet activation (see section I.3.2.2) (**Figures 6-7**). Thrombin is generated via the coagulation cascade (see section I.3.8), and contributes also to the formation of a fibrin network that thickens the thrombus core [90, 91].

3.7. Clot retraction – the outside-in signaling

The thrombus core is consolidated and the gap between platelets is reduced via their contraction. The outside-in signaling, triggered after fibrinogen binding to αIIbβ₃, initiates this clot retraction. The first step consists in Gα₁₃ binding to β₃ cytoplasmic tail and c-Src activation. The latter phosphorylates β₃ tail, which then provides an anchor site for myosin II, promoting integrin-cytoskeleton connection. Moreover, c-Src and Syk phosphorylate a plethora of cytoskeletal-associated proteins including some RhoGEF that activate Rac1. Interestingly, c-Src induces RhoA inhibition by RhoGAP phosphorylation. This inhibition is relieved during thrombus formation by the cleavage of c-Src β₃ binding site and triggers the RhoA-ROCK-MLC signaling cascade. RhoA can also be activated, independently of c-Src, after the binding of VPS33B to β₃ tail [92, 93] (**Figure 8**).

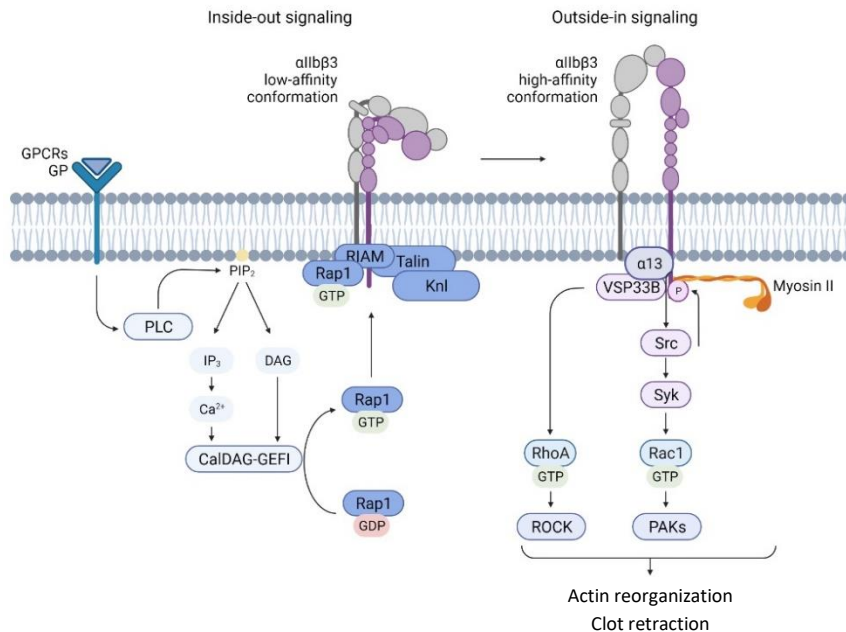


Figure 8. The inside-out and outside-in signaling pathways. Platelet stimulation leads to PLC activation which induces CalDAG-GEF1 activation via a Ca^{2+} increase and DAG formation. CalDAG-GEF1 exchanges GDP from Rap1 by GTP and allow its translocation to the plasma membrane where it forms a complex with RIAM at $\beta 3$ integrin. Consequently, talin-Knl binds to $\beta 3$ integrin and enhances $\alpha \text{IIb} \beta 3$ switch to a high-affinity conformation. This pathway is called inside-out signaling. Fibrinogen binding to $\alpha \text{IIb} \beta 3$, in turn, activates a signaling cascade leading to actin cytoskeleton reorganisation and platelet clot retraction, i.e. outside-in signaling. $\alpha 13$ and VSP33B associates with $\beta 3$ cytoplasmic tail and regulates RhoA-ROCK activation. Phosphorylation of $\beta 3$ by c-Src, activated upon fibrinogen binding, provides an anchor site for myosin II, promoting integrin-cytoskeleton interaction. Furthermore, Src activates Rac1-PAK pathway that contributes to actin reorganization. CalDAG-GEF1: Ca^{2+} diacylglycerol guanine nucleotide exchange factor1; DAG: diacylglycerol; GP: glycoprotein; GPCR: G protein-coupled receptor; IP_3 : inositol 1,4,5-trisphosphate; Knl: kindlin; PAK: p21-activated kinase; PIP_2 : phosphatidylinositol 4,5-bisphosphate; PLC: phospholipase C; RIAM: Rap1-GTP-interacting adaptor molecule; ROCK: Rho-associated protein kinase.

3.8. The coagulation cascade

Two ways initiate the coagulation cascade, the intrinsic pathway, via contact system, or the extrinsic pathway, via association between activated factor VII (FVIIa) and the exposed tissue factor (TF) of injured endothelial cells. Collagen, polyphosphates and neutrophil extracellular traps (NETs) activate FXII, initiating the intrinsic pathway. FXIIa leads to FXI and subsequently FIX activation. Tenase complex, i.e. FVIIIa.FIXa, cleaves

FX allowing the formation of FXa.FVa prothrombinase complex. The latter generates thrombin (FIIa) from prothrombin. The extrinsic pathway activates FX via TF.FVIIa complex, which also leads to prothrombinase complex and FIIa formation. Thrombin amplifies the coagulation cascade by activating FVIII and FV. Finally, thrombin converts fibrinogen to fibrin and activates FXIII, which participates in clot formation and catalyzes covalent fibrin interactions to stabilize the clot, respectively (**Figure 8**). Procoagulant platelets, i.e. with exposed PS, bind coagulation factors and serve as a platform for their activation [90, 91].

3.9. Fibrinolysis

To prevent any uncontrolled thrombus growth, the fibrin clot is degraded by a process called fibrinolysis. The serine protease plasmin is responsible for the cleavage of the cross-linked fibrin polymers. It is generated, from plasminogen, by tissue and urokinase plasminogen activator (tPA; uPA). Fibrinolysis can be downregulated by serpins. Some targets t and uPA, such as PAI-1, and other directly plasmin, such as α 2-antiplasmin (**Figure 9**). The level of fibrin degradation products, called D-dimers, are analyzed in clinics as an indirect marker of thrombin generation [94, 95].

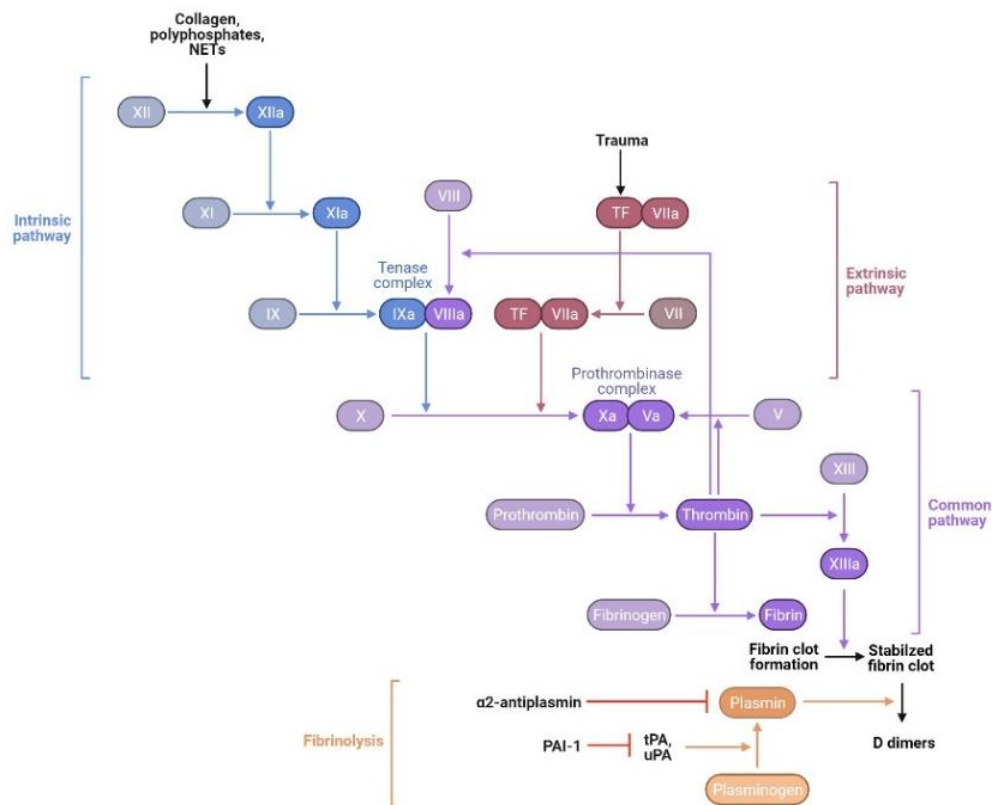


Figure 9. The coagulation cascade and fibrinolysis. The intrinsic pathway, triggered by activating surface (collagen, polyphosphates or NETs) activates FXII. FXIIa leads to XIa and, subsequently, IXa formation. Once IXa and VIIIa associate, they form the tenase complex which cleaves FX into Xa allowing prothrombinase complex formation. The latter generates thrombin (IIa) from prothrombin. The extrinsic pathway generates Xa via TF.FVIIa complex. Thrombin amplifies the coagulation cascade by activating FVIII and FV, induces fibrin formation from fibrinogen and stabilizes fibrin clot by generating XIIIa. Fibrinolysis is initiated by tPA or uPA cleaving plasminogen into plasmin. It ends with D dimers formation from cross-linked fibrin polymer degradation by plasmin. It is regulated by serpins, PAI-1, inhibiting t/uPA and α 2-antiplasmin, preventing plasmin action. NETs: neutrophil extracellular traps; PAI-1: plasminogen activator inhibitor-1; TF: tissue factor; t or u PA: tissue or urokinase plasminogen activator.

4. Platelet in atherosclerosis

4.1. Atherosclerosis

In 2019, the World Health Organization (WHO) estimated that 32% of deaths were due to cardiovascular diseases. Heart attack and stroke, mainly resulting from

atherothrombosis, account for ~80% of these deaths [96]. Dyslipidemia, due to elevated low-density lipoprotein (LDL) and triglyceride-rich lipoproteins (TGRL) and low high-density lipoprotein (HDL), and inflammation, due to high blood pressure or smoking, are the major triggers of atherosclerosis. Both participate in endothelial cell activation/dysfunction that promotes chemokine secretion and adhesion molecule expression at cell surface. Then, circulating monocytes are attracted and adhere to the activated endothelium via P-selectin glycoprotein ligand-1 (PSGL-1) - P-selectin interaction. Firm adhesion is mediated by macrophage-1 antigen (Mac-1) - Mac-1 or intercellular adhesion molecule/vascular cell adhesion molecule (ICAM/VCAM) - very late antigen-4/lymphocyte function-associated antigen-1 (VLA-4/LFA-1) interactions and results in endothelium disruption. Consequently, LDLs and monocytes enter into the intima where they turn into oxidized LDLs (ox-LDLs) and macrophages, respectively. Macrophages uptake ox-LDLs and are converted into foam cells, which secrete pro-inflammatory cytokines and growth factors. The inflammatory environment participates in leucocyte recruitment and smooth muscle cell migration and proliferation into the intima. Some smooth muscle cells form the fibrous cap and others uptake LDL aggregates through receptors of the LDL receptor-related protein (LRP) superfamily. Lipid-rich and necrotic core of atherosclerotic plaques is formed after macrophage and smooth muscle cell death and a defective efferocytosis, i.e. cell death clearance. Atherosclerotic plaque rupture exposes fibrillary collagen to circulating platelets leading, ultimately, to thrombus formation (**Figure 10**). An obstructive thrombus induces myocardial infarction and platelet aggregates embolization a stroke [97-99].

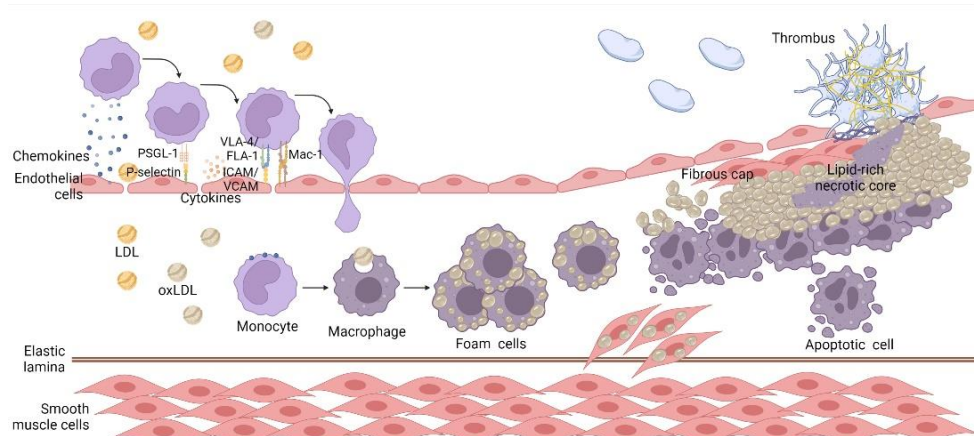


Figure 10. Atherosclerosis. Dyslipidemia and inflammation induce endothelial cell activation, leading to chemokine secretion and adhesion molecule expression (P-selectin, ICAM/VCAM, Mac-1). Circulating monocytes are attracted and adhere to endothelial cells via PSGL-1 - P-selectin interaction followed by firm adhesion mediated by VLA-4/FLA-1 – ICAM/VCAM and Mac-1 - Mac-1 interactions. Endothelial disruption allows monocyte transmigration. Once into the intima, monocytes become macrophages and uptake oxLDL, leading to foam cell formation. The pro-inflammatory environment induces cell death and smooth muscle cell migration leading to lipid-rich necrotic core (= atherosclerotic plaque) and fibrous cap formation, respectively. Plaque ruptured exposes fibrillary collagen to circulating platelets, which triggers thrombus formation. ICAM: intercellular adhesion molecule; LDL: low-density lipoprotein; LFA-1: lymphocyte function-associated antigen-1; Mac-1: macrophage-1 antigen; oxLDL: oxidized LDL; PSGL-1: P-selectin glycoprotein ligand-1; VCAM: vascular cell adhesion molecule; VLA-4: very late antigen-4.

4.2. Role of platelets in atherosclerosis progression

4.2.1. Platelets in atherosclerosis

Platelets do not only play a role in atherothrombosis but also take part in atherosclerosis progression. The relevance of platelets in this pathology is emphasized by studies showing that reducing the platelet count slows down atherosclerosis progression in diabetic and atherosclerotic (LDLR^{-/-}) mice. The underlying mechanism involves an altered monocyte recruitment [100, 101]. Moreover, increasing platelet activation enhances atherosclerotic lesions and blocking platelet adhesion to the endothelium decreases plaque formation [102-104].

4.2.2. Platelets – monocytes in atherosclerosis

By adhering to the activated endothelium, platelets mediate monocyte activation and recruitment. Initial contact is made between PSGL-1 or GPIIb/IIIa and P- or E-selectin and

triggers platelet rolling and activation. Following platelet activation, PF4 and CCL5 are released from α -granules or platelet-derived microparticles and promote leukocyte recruitment [105]. Integrin activation and CD40L surface expression induce firm platelet - endothelium adhesion, amplifying endothelial cell activation via cytokine and chemokine secretion (IL-8, MCP-1) and adhesion molecule expression (E-Selectin, VCAM-1, ICAM-1). Monocytes are attracted to the inflamed endothelium and bind to platelets thanks to CD40L – platelet CD40, PSGL-1 - P-selectin, Mac-1 - GPIb α /JAM/CD40L/ICAM-2/fibrinogen- α IIb β 3 interactions, leading to Mac-1 and VLA-4 upregulation on monocytes. Consequently, monocytes adhere to the activated endothelium and transmigrate [106-108]. In addition, platelets promote macrophage accumulation and transition into an inflammatory M1-state and decrease their efferocytotic capacity, leading to a higher necrotic core and plaque lesion [101] (**Figure 11**).

4.2.3. Platelets – neutrophils in atherosclerosis

Neutrophils, as well as monocytes, adhere to activated platelets in a PSGL-1 - P-selectin or Mac-1 - GPIb α -dependent manner. Platelet helps neutrophil recruitment at the inflammatory sites and amplifies their activation and vice versa. Activated platelets trigger neutrophil-induced ROS production and NETs release by direct contact or by mediators (PF4 or CCL5) whereas neutrophils activate PARs-induced platelet activation via cathepsin G and elastase secretion. NET formation promotes atherosclerosis as its inhibition reduced atherosclerotic plaque size. Indeed, NETs alter HDL capacity to extrude cholesterol, promote endothelial cell activation and even induce endothelial cell apoptosis (**Figure 11**). In addition, NETs participate in atherothrombosis as they were found in coronary thrombi [109-111].

4.2.4. Platelets – eosinophils in atherosclerosis

Even if eosinophils are not present in stable atherosclerotic plaque, they play a role in the progression of the disease as their depletion reduced atherosclerosis. The potential

mechanism involves endothelium activation by eosinophil degranulation products, major basic protein (MBP) and eosinophil cationic protein (ECP), known to participate in tissue damage and inflammation. Platelets adhering to the endothelium capture eosinophils, which then secrete eosinophil extracellular traps (EETs) (**Figure 11**). As NETs, EETs promote platelet activation and are found in murine and human thrombi [112].

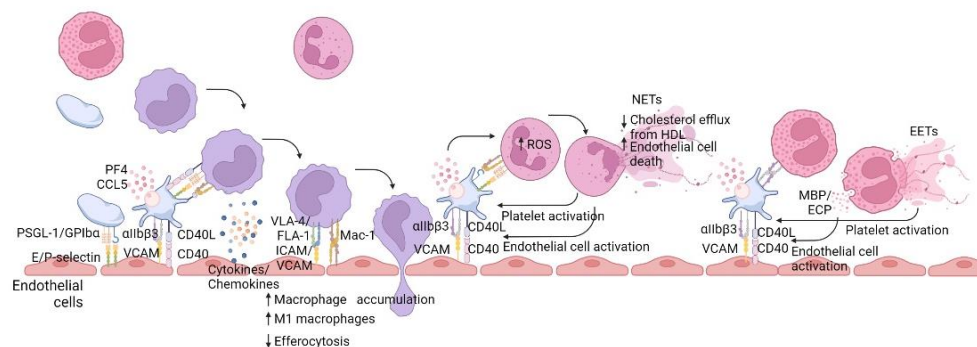


Figure 11. Platelets and leucocytes in atherosclerosis. Platelets adhere to the activated endothelium and recruit monocytes, neutrophils and eosinophils leading to atherosclerosis progression. Contact between platelets and endothelial cells is made between PSGL-1 or GPIIb α and P- or E-selectin and triggers platelet rolling and activation. Firm adhesion occurs after α IIb β 3 - VCAM and CD40L - CD40 interactions. Chemokine and cytokine secretion from activated platelets and endothelial cells enhance leucocyte recruitment and endothelial cell activation. Circulating monocytes bind to platelets via several interactions (CD40L - CD40, PSGL-1 - P-selectin and Mac-1 - GPIIb α /JAM/CD40L/ICAM-2/fibrinogen- α IIb β 3) leading to VLA-4 and Mac-1 upregulation on monocyte surface. Consequently, monocytes firmly adhere to the endothelium and migrate towards the intima, promoting macrophage accumulation, their transition into M1-state and decreasing their efferocytotic capacity. Neutrophils are attracted to the inflamed endothelium and binds to platelets in a PSGL-1 - P-selectin or Mac-1 - GPIIb α -dependent manner. This association triggers neutrophil ROS production, followed by NETs formation, which, in turn, enhance platelet and endothelial cell activation, decrease cholesterol efflux from HDL and increase endothelial cell death. Adhesion of eosinophils to platelets induces eosinophil degranulation and EET formation, leading to an intensification of endothelial cell and platelet activation, respectively. ECP: eosinophil cationic protein; EET: eosinophil extracellular traps; HDL: high-density lipoprotein; ICAM: intercellular adhesion molecule; LDL: low-density lipoprotein; LFA-1: lymphocyte function-associated antigen-1; Mac-1: macrophage-1 antigen; MBP: major basic protein; NETs: neutrophil extracellular traps; oxLDL: oxidized LDL; PSGL-1: P-selectin glycoprotein ligand-1; VCAM: vascular cell adhesion molecule; VLA-4: very late antigen-4.

4.2.5. Platelets – immune cells in atherosclerosis

Immune cells are also attracted to the inflamed area and their interactions with platelets enhance their recruitment. T_H1 lymphocytes, via IFN γ secretion, aggravates atherosclerosis whereas T_H2 lymphocytes and T regulator (Treg) tend to limit the

progression of the disease by decreasing inflammation via IL-10 and TGF β secretion [99].

5. Platelets in inflammation, immunity and tissue regeneration

5.1. Platelets in inflammation

In case of inflammation such as atherosclerosis (see section 4), ischemic stroke or ischemia-reperfusion injury, leukocytes and platelets interact and mediate thrombo-inflammation [113]. As discussed above, an inflammatory state damages or activates endothelial cells allowing platelet and leucocyte adhesion. Platelet CD40L - endothelium CD40 interaction promotes chemokine release that favors neutrophil recruitment and expression of adhesion molecules. Platelet-neutrophil aggregate formation amplifies their activation and NET release leading to thrombus formation [114]. In case of ischemia-reperfusion injury, platelets worsen tissue damage via neutrophils induced-ROS production and matrix metalloproteinase (MMP) secretion. In the brain, the exacerbated inflammation disrupts the blood brain barrier facilitating neutrophil transmigration into the parenchyma. This phenomenon can cause axonal damage via astrocyte and microglial cell activation [115]. In autoimmune diseases such as systemic lupus erythematosus, systemic sclerosis or rheumatoid arthritis, platelets, platelet-derived microparticles and neutrophils interplay and accentuate inflammation and tissue lesion [116].

5.2. Platelets in infection

Beyond its role in hemostasis and inflammation, platelets are involved in immunity. Platelets regulate immune responses either by binding to bacteria or viruses, or by recruiting and modulating the activity of leukocytes.

Platelets bind directly to bacteria via GPIb α , activated α IIb β 3 or TLR2 and 4, or through plasma proteins such as fibrinogen or vWF. By secreting antibacterial molecules, i.e. thrombocidins, kinocidins and β -defensin-1, platelets are able to kill *Bacillus subtilis*, *E. coli*, *Lactococcus lactis* and *S. aureus*. Some pathogen's clearance requires the

contribution of immune cells. A sustained platelet-bacteria bundle presence in the circulation has been shown to favor their detection and death by cytotoxic T lymphocytes (CTLs) or neutrophils [117]. In addition, neutrophils secrete NETs to trap pathogens and kill them [109]. After an infection, platelets, activated through TLR4, are able to bind neutrophils. Platelet-neutrophil aggregates further enhance neutrophil-induced NET secretion [117]. Interestingly, a decrease in platelet-bacteria aggregates impaired NETosis and mice survival [118]. Activation of the classical and alternative complement pathways by platelets also promotes the lysis and elimination of germs [117]. With these functions, platelets play a key role in the development of immunothrombosis during sepsis, a disease characterized by endothelial dysfunction and a hypercoagulable state [119].

Depending on the type of virus and the time after infection, platelets and their releasates either help or decrease viral clearance. Some viruses are internalized by platelets, which limits their propagation and others bind to platelets leading to platelet-neutrophil and -monocyte aggregate formation and innate responses [120]. The inflamed state due to influenza or SARS-CoV-2 infection activates endothelial cells leading to platelet-neutrophil interaction, NETosis and microthrombi formation. Moreover, endothelium damage, caused by a virus infection, induces thrombus formation on the exposed subendothelium in order to seal the breach [121].

5.3. Platelets in cancer

Since long, it has been known that platelets are involved in the pathogenesis of cancer. Indeed, a reduction in the platelet count is associated with fewer metastases [122]. Furthermore, cancer cells trigger platelet activation either by direct association or by the release of thrombin, TxA₂ or ADP. Platelet-cancer cell aggregate formation, through P-selectin, α IIb β 3, GPIb α or PECAM-1, protects the membrane of cancer cells from its destruction under high shear stress and cancer cell recognition by natural killer. The adhesion of activated platelets, “carrying” cancer cells, to endothelial cells allows cancer cell anchoring to the endothelium. Furthermore, platelets release chemokines,

MMPs and pro-angiogenic factors (TGF β , VEGF and PDGF) that contribute to leukocyte recruitment, extracellular matrix destruction for the metastatic nest, angiogenesis and tumoral neovascularization [123].

5.4. Platelets in tissue revascularization and regeneration

Platelet α -granule secretion leads to the release of a plethora of factors that stimulate angiogenesis, and therefore endothelial cell proliferation and migration. The first pro-angiogenic factors to step in are VEGF and basic FGF. Migration of endothelial cells is further enhanced by the chemotactic effect of sphingosine-1-phosphate (S1P) and platelet-derived endothelial growth factor (PD-ECGF) and via MMP-induced extracellular matrix destruction. In the later stages of angiogenesis, smooth muscle cells, fibroblasts, progenitors of endothelial cells and stem cells are recruited to enhance and stabilize the new vessel [124]. Studies revealed that GPVI and CLEC2 receptors, both containing ITAM motifs, are required to maintain vascular integrity during inflammation [125]. Platelets also contain factors that repress angiogenesis to prevent an uncontrolled growth of the vessels. Such type of factors, like TSP-1, are important to impair tumor angiogenesis [126]. Platelet-derived microparticles containing pro-angiogenic factors (VEGF, bFGF, and PDGF) are released at sites of inflammation or endothelial injury. Their role in angiogenesis was highlighted in a model of myocardial infarction where they have been shown to promote revascularization of the ischemic tissue [127]. In addition to vessel regeneration, platelets contribute to cardiac stem cell migration to the myocardium via stromal-derived factor 1 (SDF-1) secretion [128]. SDF-1 is also implicated in hepatocyte and pulmonary capillary endothelial cell proliferation after injury. However, even if platelets seem beneficial in acute inflammation, in case of chronic injury, such as asthma or myocardial ischemia, platelets, by secreting serotonin and TGF- β 1, participate in fibrosis and therefore could be deleterious [124].

II. Key roles of lipids in platelet

Lipids are key elements in platelets as they fulfill significant roles for their functions. Indeed, lipids participate in membrane cell structure, granule secretion, signaling molecule generation, metabolism and post-translational modifications.

1. Lipids in the plasma membrane

The plasma membrane and membrane of organelles, i.e. granules, DTS and mitochondria are made up of a bilayer of PLs and cholesterol. In resting platelets, the plasma membrane is composed of 70% PLs (37% PC, 17% PE, 7% SM, 6% PS, 3% PI, 2% PG/PA) and 30% cholesterol [8]. Granule membrane has the same cholesterol/PLs ratio. However, among PLs, PC is increased, and PS decreased, compared to their level in the plasma membrane. Interestingly, intracellular membrane has less cholesterol and a modified PL composition compared to the plasma membrane. PE is slightly decreased, SM and PS are present in small amounts whereas PC and PI are increased by 2 and 3 fold, respectively. Membrane from platelets-derived microparticles only differs from the plasma membrane in terms of PS content [129].

1.1. Phospholipid structure

PLs and fatty acids can be synthesized *de novo* (see section III.1.) or taken from the plasma. PLs are asymmetrically distributed beyond the two leaflets. PS, PA, PI and PE are mainly in the inner leaflet whereas PC and SM are in the outer leaflet. A PL contains one head group, a glycerol backbone and two fatty acyl chains. Those are linked at the sn-1 position with three possible bounds: ester (acyl), ether (alkyl or plasmayl) or vinyl-ether (alkenyl or plasmalogen). Fatty acyl chains found at the sn-2 position are linked via an ester bond. PLs remodel their fatty acyl chains via deacylation-reacylation and transacylation reactions. Deacylation occurs following PL cleavage by PLA₁ or PLA₂, at sn-1 or sn-2 position, respectively, and results in the formation of a lyso-PL and a fatty acyl chain. Reacylation is the process by which lysophospholipid acyltransferases transfer a fatty acyl-CoA to a lyso-PL, forming a PL with new fatty acyl chains.

Transacylation generates the transfer of one fatty acyl chain from a PL to a lyso-PL [130]. Depending on the head group, the type of fatty acyl chains (length and number of double bond) or the type of linkage, PLs have various properties [131] (**Figure 12**).

1.1.1. Phospholipid head group

PLs can adopt three different conformations: cylindrical, conical and inverted-conical. If the width of the polar head is similar to the one of the tail, PLs, namely PC, PS and PG, are cylindrical and form a flat and quite rigid monolayer. If the tail is wider than the polar head, as for PE and PA, PLs adopt a conical shape and a negative spontaneous curvature. Lyso-PLs and PI have a spontaneous positive curvature due to their wide head when compared to their tail [132] (**Figure 12**).

1.1.2. Phospholipid fatty acyl chain

Membrane stability and fluidity are also affected by the length and degree of unsaturation of fatty acyl tails. Long and saturated acyl chains promote lipid-lipid interaction and render membrane less fluid. In contrast, polyunsaturated lipids decrease membrane rigidity [131]. A recent study in erythrocytes showed that the plasma membrane is also asymmetrical in terms of unsaturation of the fatty acyl chain. The inner leaflet appears two times more unsaturated than the outer leaflet [133]. Interestingly, PE effect on membrane deformability can be overshadowed by increasing oleic acid (18:1) into membrane bilayer as it enhances lipid compaction [134].

1.1.3. Fatty acyl chain linkage

The type of linkage of fatty acyl chains to the glycerol backbone of PL is also known to influence membrane properties. The lack of carbonyl oxygen in ether bound (plasmalogen and plasmalogen) increases the intermolecular hydrogen bond between head groups of PLs, promoting a highly curved membrane. However, the specific vinyl-ether bond (plasmalogen) at the sn-1 position allows close contact between saturated (sn-1) and polyunsaturated (sn-2), mainly C20:4 and C22:6, acyl chains, which induces a tightly packed and less fluid membrane. Furthermore, vinyl-ether bonds are preferentially

oxidized and act as ROS scavengers to protect other lipids from oxidation [135, 136]. Therefore, tight regulation of PL type and fatty acyl chain is required to ensure good membrane functioning.

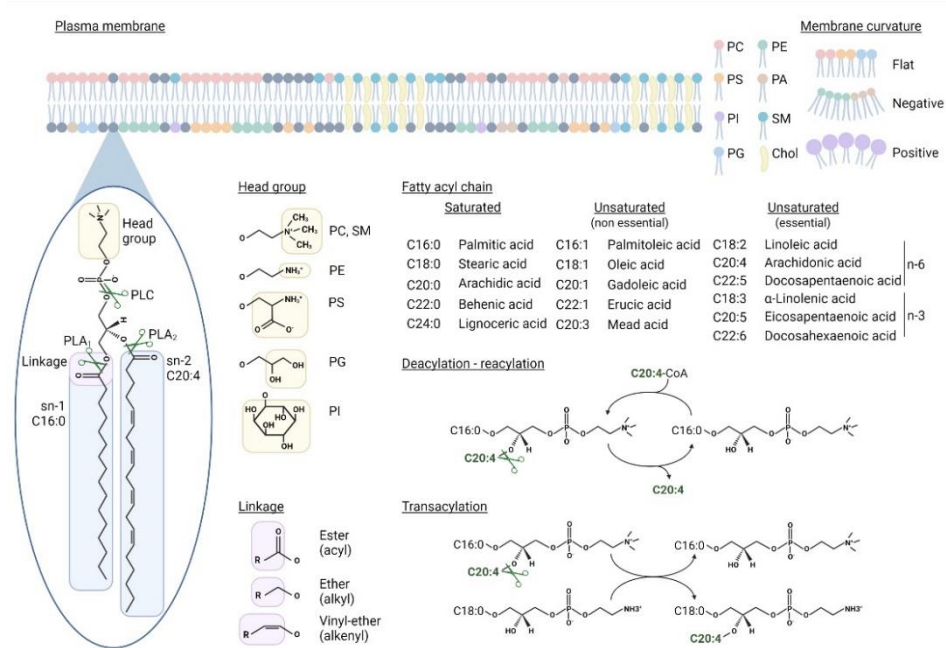


Figure 12. The plasma membrane and phospholipid structure and remodelling. The platelet plasma membrane is composed of cholesterol and a phospholipid bilayer with, in the outer leaflet, PC and SM and, in the inner leaflet PE, PS, PG and PI. Depending on the type of PL, the plasma membrane can be flat or adopt a negative or a positive curvature. A PL is composed of a head group, a glycerol backbone and two fatty acyl chains. Each type of PL has a different head group and link different fatty acyl chains. Saturated/monounsaturated fatty acyl chains are linked both at sn-1 and sn-2 position whereas polyunsaturated are preferentially linked at sn-2 position. Fatty acyl chain linkage at sn-1 position are either ester, ether or vinyl-ether. Remodelling of PL fatty acyl chains via deacylation – reacylation requires 1) PL cleavage by PLA₁ (sn-1) or PLA₂ (sn-2) and 2) addition of a newly formed acyl-CoA. Remodelling by transacylation result in the transfer of a fatty acyl chain from a PL to a lyso-PL. Chol: cholesterol; n-3: omega 3; n-6: omega 6; PA: phosphatidic acid; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PG: phosphatidylglycerol; PI: phosphatidylinositol; PL: phospholipid; PLA₁: phospholipase A₁; PLA₂: phospholipase A₂; PLC: phospholipase C; PS: phosphatidylserine; SM: sphingomyeline.

1.2. Cholesterol in the plasma membrane

The plasma membrane also contains a liquid-ordered domain enriched in SM and cholesterol. These domains, called lipid rafts, are important for granule fusion and signaling events (see section II.2). The platelet plasma membrane composition can be

affected by cholesterol lowering drugs, i.e. statin, which decreases cholesterol/PL ratio and impaired platelet aggregation [137]. Antiplatelet therapy drugs, such as ASA or ticagrelor, an anti-P2Y₁₂ receptor, are able to increase plasma membrane rigidity. A recent lipidomic study showed that ticagrelor effect on the plasma membrane is probably due to an increase in cholesterol and saturated acyl chain (16:0-16:0) – containing plasmalogen PC (O-) in the membrane. Interestingly, polyunsaturated acyl chains, specifically 16:0-20:4, 18:1-20:4 PCO, 16:1-20:4 and 18:2-20:4 PEO, known to increase membrane fluidity, are decreased with ticagrelor [8].

1.3. Membrane lipids in granule secretion

Upon stimulation, granule concentration and localization near OCS favor their secretion. Cholesterol and PE are necessary for the merger. Due to its intrinsic negative curvature, PE presence in the inner leaflet is required for membrane fusion and stability of the fusion pore [138]. Microdomains of cholesterol also help granule fusion either by localizing SNARE proteins, implicated in exocytosis process (see section I.3.3.1), or by stabilizing the negative curvature and therefore the fusion pore [139]. Furthermore, PS externalization is important in granule fusion stabilization [140]. Cholesterol is involved in other vesicle secretion as its depletion has been shown to decrease the release of PS-exposing extracellular vesicles [141].

1.4. Membrane lipids in procoagulant activity

PS asymmetry is maintained by flippase, P4-ATPase, activity which transfers PS from the outer leaflet to the inner leaflet. Upon platelet activation, the increased Ca²⁺ inhibits flippase activity and induces scramblase TMEM16F conformational change. The open state of TMEM16F favors PS transition to the outer leaflet and therefore induces the coagulation cascade (see section I.3.8) [142]. As PS, PE is also externalized and supports coagulation depending on the length and saturation of their fatty acyl chains. Short chain in PE sn-1 position, i.e. 14:0, does not induce coagulation whereas 18:0-20:4 PE and PEP or 14:0- and 18:0-20:4 PS do. Longer chain, i.e. 18:0-22:6 PE reduced

coagulation [143]. Furthermore, oxidation of 20:4 PE at the sn-2 position, generating 12-HETE-PE, does not change its tendency to enhance coagulation [144]. Vesicles secreted after platelet stimulation also participate in thrombin generation as they expose PS at their surface [141]. Interestingly, an impaired transversal mobility during platelet activation, as seen in platelets from patients suffering from Scott syndrome, characterized by an altered PS externalization, is associated with a defect in filopodia formation [145].

2. Lipids in platelet signaling

2.1. Lipid rafts

Lipid rafts form a dynamic microdomain composed of SM, saturated PLs, and cholesterol. During platelet spreading, cholesterol-rich rafts are found mostly at filopodia structure whereas SM-rich rafts are localized at platelet center. SM-rich rafts link the actomyosin system to extracellular fibrin, crosslinked by FXIII, mediating clot retraction [146]. Lipid rafts incorporate proteins participating in granule fusion. Some proteins, such as stomatin, contained in α granule lipid raft, may participate specifically in α granule fusion [147]. Furthermore, lipid rafts integrate platelet receptors ensuring the activation of the downstream signaling cascade. Some, like TP and α IIb β 3, are already present in rafts at resting state and others, such as GPIb/IX/V, GPVI, P2Y12, P2Y1 move into rafts upon stimulation [146]. Signaling event such as ITAM Fc γ -chain phosphorylation by Lyn and Syk requires the translocation of GPVI and GPIb/IX/V into rafts [16]. The presence of PIP₂ into lipid rafts favors PIP₃ production by PI3K into these rafts. PIP₃ allows PLC γ 2 recruitment into the rafts and its activation, which later on, leads to platelet activation (see section I.3.1) [148] (**Figure 13**). Furthermore, partitioning of P2Y12 and P2Y1 into rafts is necessary to induce their signaling pathway [149, 150].

2.2. Plasma membrane-derived lipid mediators

2.2.1. Phosphatidylinositol (4,5) bisphosphate, phosphatidylinositol (3,4,5) trisphosphate and diacylglycerol

PIP₂ is synthesized through the PI cycle. PI synthase generates PI from inositol and CDP-DAG, deriving from PA. Then, PI is phosphorylated twice to form PIP₂. The latter is phosphorylated by PI3K to produce PIP₃ or is cleaved by PLC to give rise to IP₃ and DAG. Via diacylglycerol kinase ϵ (DGK ϵ), DAG is converted back to PA, providing a substrate for the PI cycle [151]. Interestingly, a highly negative membrane curvature favors the activity of DGK ϵ , and therefore PI cycle. This enzyme shows a high preference for DAG containing 1-stearoyl-2-arachidonoyl acyl (18:0-20:4) chain [152]. This could explain why, in resting human or mouse platelets, around 40% of PI contains 18:0-20:4 acyl chain and 20% 16:0-16:0 acyl chain. Interestingly, PIP or PIP₂ do not have 16:0-16:0 fatty acyl chains and 70% of them carry 18:0-20:4 fatty acyl chains. Upon short thrombin or CRP stimulation, 18:0-20:4 PI decreases, 18:0-20:4 PIP₂ barely changed and 18:0-20:4 PIP₃ increases. Interestingly, thrombin is more potent to increase 18:0-20:4 PIP₃ in human platelets whereas in mouse platelets it is CRP stimulation. Of note, stimulation does not change 16:0-16:0 PI content [153].

While PIP₃ is rapidly synthesized upon platelet activation, PIP₂ accumulates later to support actin remodeling, platelet aggregation and clot retraction. PIP₂ is a major actor in actin remodeling by binding to gelsolin, CapZ, WASP/Arp2/3 or cofilin, which favors F-actin polymerization. Furthermore, PIP₂ recruits GEF and GAP of RhoGTPases and participates in platelet activation via their downstream signaling (see section I.3.2.2) [5]. A recent proteomic study discovered new PIP₂ effectors, which pave the way for a better characterization of PIP₂ role in platelet functions [154]. Whether fatty acyl chain composition affects PIP₂ effector binding is still unknown.

PIP₃ is known to bind pleckstrin homology domain of proteins. For example, its interaction with PLC γ 2 or PDK1, participates in signaling events leading to platelet activation. Its role in platelet functions is emphasized by studies showing that PI3K

inhibition or PI3K deletion in mice results in mild platelet aggregation and an altered thrombus growth *in vivo* [7].

DAG, produced by PLC-induced PIP₂ cleavage, is another important signaling molecule that promotes platelet aggregation by activating, for example, PKC and CalDAG-GEF1 (see section I.3.1) (**Figure 13**).

2.2.2. Arachidonic acid and eicosanoids

2.2.2.1. Arachidonic acid and phospholipase A₂

AA comes directly from a dietary source or indirectly, via conversion of dietary linoleic acid (18:2). AA is mostly found esterified to PLs, triglycerides and cholesterol in the plasma. It has been shown that LDL delivers AA-PC and AA-PE to platelets [155]. The small amount of free AA can be converted into arachidonoyl-CoA via acyl-CoA synthetase and incorporated into lysoPL via lysophospholipid acyl-CoA acyltransferase to form AA-containing PL. AA is initially incorporated at the sn-2 position of acyl-lysoPC and to a lesser extent in acyl-lysoPE. Afterward, AA from diacyl-PC is integrated into alkyl and alkenyl-lysoPE and alkyl-lysoPC, via CoA-independent transacylation reaction [156, 157]. AA is predominantly found in PE with an enrichment in plasmalogens [156, 158].

PL cleavage, at the sn-2 position, to generate lysoPL and free AA is mediated by PLA₂. Interestingly, PLA₂ seems to cleave preferentially acyl chains 20:4 > 20:5 > 18:2 = 18:3 = 22:6 >> 18:1 [159]. The remodeling of AA between PLs requires a specific PLA₂, different from the ones involved in AA release response, a Ca²⁺-independent cPLA₂γ [160]. Three types of PLA₂ are present in platelets to mediate AA release: two Ca²⁺-dependent, namely, cPLA₂ and soluble (s)PLA₂ and one Ca²⁺-independent, iPLA₂. cPLA₂ is more sensitive to Ca²⁺ than sPLA₂ as it becomes activated with 1000X less Ca²⁺ (μM vs mM). PLA₂ are activated differentially depending on the specie, the platelet agonist and the concentration used. In human platelets, iPLA₂γ mediates AA release under low thrombin concentration whereas cPLA₂α takes over at high concentration [158, 161]. In murine platelets, cPLA₂ is the more potent to cleave AA-containing PL upon thrombin

stimulation whereas cPLA₂ and iPLA₂γ are both needed upon collagen stimulation [162]. Even if PC and PE are cleaved by PLA₂, our lab and others have highlighted the major role of PL plasmalogens in TxA₂ generation [57, 163-165]. Furthermore, a recent lipidomic study revealed that PI plays a non-negligible role in AA production. Indeed, 18:0-20:4 PI, the second most lipid present in platelet after cholesterol, is downregulated upon stimulation and mediates eicosanoid production [166]. A summary of the current knowledge of the type of PLA₂ and AA-containing PL involved in TxA₂ generation regarding platelet specie and agonist is represented in **Table 1**.

Agonist	Specie	PLA ₂	PL-induced TxA ₂ secretion	References
Thrombin	Human	Low concentration: iPLA ₂ γ High concentration: cPLA ₂ α	PCP and PEP PCP; PEP; PI; PC and PE	[158, 161, 165]
	Mouse	cPLA ₂	PEP	[162] [[57]Unpublished data], our data
collagen	Human	?	PEP	[163]
		?	(CRP) PI	[166]
	Mouse	iPLA ₂ γ	?	[162]
		cPLA ₂ ?	? (CRP) PEP	[167] Our data
U46619	Human	?	PEP	[164]
ADP	Mouse	iPLA ₂ γ	PEP	[162, 167]

Table 1. PL cleavage by PLA₂ leading to TxA₂ secretion upon stimulation in human and murine platelets. Table showing which type of PLA₂ is activated according to the agonist and the specie and which PL is cleaved to generate TxA₂. CRP: collagen-related peptide; PCP: phosphatidylcholine plasmalogen; PEP: phosphatidylethanolamine plasmalogen; PI: phosphatidylinositol; PL: phospholipid; PLA₂: phospholipase A₂.

2.2.2.2. Eicosanoids and platelet functions

AA oxidation by COX or lipoxygenase (LOX) generates eicosanoids. In platelets, PGH₂, derived from COX1 action, is metabolized, by PG synthase, into PGE₂ or PGD₂ or, by

TxA₂ into TxA₂. Besides producing prostanoids, AA can also be oxidized by platelet-type 12-lipoxygenase (12-LOX) to form 12-hydroperoxyeicosatetraenoic acid (12-HPETE). This AA derivative can be further reduced into 12-hydroxyeicosatetraenoic acid (12-HETE). 12-LOX is also responsible for platelet lipoxin A₄ (LXA₄) and B₄ (LXB₄) production from leukotriene A₄ (LTA₄), a molecule produced by neutrophils. This uptake of LTA₄ by platelets also induces LTC₄ generation [168] (**Figure 13**).

PGE₂ exerts either platelet activation via EP₃ receptor or platelet inhibition via EP₂ and EP₄ receptors. EP₃ is a GPCR coupled with G_q or G_i. G_q signaling leads to PLC activation and Ca²⁺ increase whereas G_i decreases cAMP concentration and inhibitory signaling events. In opposition, EP₂ and EP₄ are GPCR coupled with G_s that stimulates cAMP production. At high concentrations (>10 μM), PGE₂ has an inhibitory effect. In human platelets, PGD₂ mediates platelet aggregation inhibition via its receptor, DP₁, coupled to G_s [169] (**Figure 13**).

TxA₂ is an important secondary platelet agonist, participating in platelet activation and thrombus build-up (see sections I.3.4 and I.3.6). PAR₁ triggers TxA₂ formation within 15s whereas 12-HETE reaches maximal level after 120s. This AA derivative has pro and anti-aggregatory effects. It inhibits PLA₂, AA cleavage from the plasma membrane and collagen or TxA₂-induced platelet aggregation. In addition, platelets lacking 12-LOX have a hyperresponsiveness to ADP-induced platelet aggregation that can be reversed by incubating platelets with 12-(S)-HETE [170]. On the contrary, it enhances thrombin-induced platelet aggregation (**Figure 13**). Several studies highlighted a role of 12-HETE in neutrophil recruitment, angiogenesis and atherosclerosis [171].

LXA₄ is an anti-inflammatory molecule as it inhibits leukocyte, and in particular neutrophil, recruitment in inflamed tissue and decreases vascular permeability [172, 173]. Pro-inflammatory mediators are also produced such as LTC₄, which is primarily implicated in eosinophilic pulmonary infection. LTC₄ mediates platelet activation via cysteinyl leukotriene receptor, CysLT₂R, and induces TxA₂ secretion. This prostanoid secretion leads to ICAM and VCAM upregulation and enhances platelet-eosinophil recruitment at pulmonary inflammatory site [174] (**Figure 13**).

2.2.3. Sphingosine-1-phosphate

Another lipid mediator, produced by sphingosine kinase 2 and released by platelets upon activation, is S1P. Generation of S1P participates in thrombin-induced TxA_2 secretion, thrombin- or ADP-induced platelet aggregation and thrombus growth *in vivo* [175]. Furthermore, TxA_2 , by enhancing S1P release, helps monocyte recruitment. In an inflamed state, monocyte recruitment to endothelial cells promotes their activation and adhesion (see section I.4.) [176] (**Figure 13**).

2.2.4. Platelet-activating factor (PAF)

PAF or 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine is a lipid mediator formed by *de novo* synthesis or via PL remodeling. The first step of PL remodeling is lysoPAF synthesis. It occurs via deacylation at the sn-2 position of an alkyl-acyl-PC (x-20:4), mediated by PLA_2 , or via transacylation, in particular from alkenyl-acyl-PE. Acetylation of lysoPAF generates PAF [177]. It's a pro-inflammatory mediator involved in platelet aggregation, leukocyte recruitment and adhesion to the endothelium and vascular permeability. PAF induces platelet aggregation via the binding to its GPCR, PAF receptor (PAFR) [178] (**Figure 13**).

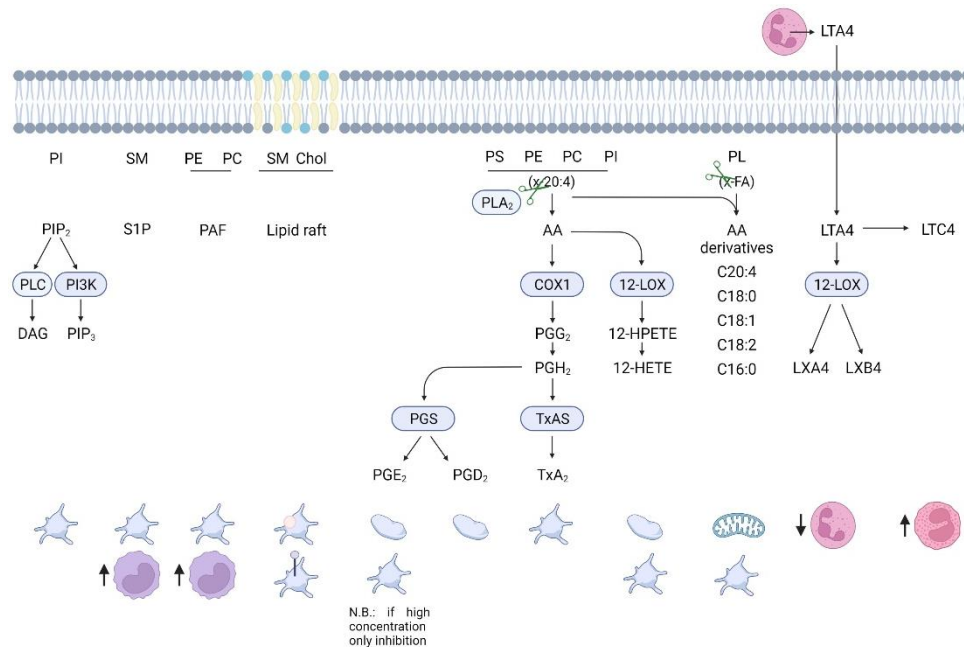


Figure 13. Lipid mediators in platelet signaling and metabolism and leucocyte recruitment. PI derivatives, such as PIP₂, PIP₃ or DAG, and SM derivative, like S1P, contribute to platelet activation. In addition, S1P enhances monocyte recruitment at inflamed areas as PAF does. Lipid rafts, enriched in SM and Chol, participate in granule fusion and platelet receptor downstream signaling cascade leading to platelet activation. The cleavage of 20:4-containing PLs by PLA₂ generates AA, which is either transformed into PGH₂ by COX1 or into 12-HETE by 12-LOX. PGH₂ gives rise to platelet inhibitory (PGE₂ and PGD₂) and prothrombotic (TxA₂) lipid mediators and 12-HETE exerts pro or anti-aggregatory effects depending on the agonist used. Another eicosanoid, LTA₄, is either converted into anti-inflammatory (LXA₄, LXB₄) or pro-inflammatory (LTC₄) lipid mediators. Furthermore, eicosanoids and some fatty acids act as substrates for mitochondrial fatty acid oxidation to generate ATP. 12-HETE: 12-hydroxyeicosatetraenoic acid; 12-HPETE: 12-hydroperoxyeicosatetraenoic acid; 12-LOX: 12-lipoxygenase; AA: arachidonic acid; COX1: cyclooxygenase 1; Chol: cholesterol; DAG: diacylglycerol; LTA₄: leukotriene A₄; LXA₄: lipoxin A₄; LXB₄: lipoxin B₄; PAF: platelet-activating factor; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PGG₂: prostaglandin G₂; PGH₂: prostaglandin H₂; PGS: prostaglandin synthase; PI: phosphatidylinositol; PIP₂: phosphatidylinositol 4,5-bisphosphate; PIP₃: phosphatidylinositol 3,4,5-trisphosphate; PL: phospholipid; PLA₂: phospholipase A₂; PLC: phospholipase C; PS: phosphatidylserine; S1P: sphingosine-1-phosphate; SM: sphingomyelin; TxAS: thromboxane synthase; TxA₂: thromboxane A₂.

3. Lipids in metabolism

3.1. In resting platelets

At basal state, glycolysis generates around ¼ of total ATP synthesis while oxidative phosphorylation accounts for ¾, with approximately 30% coming from endogenous

lipid oxidation [179, 180]. Fatty acid substrates for mitochondrial β -oxidation are generated *de novo* (see section III.1.1) or by PL cleavage. A lipidomic study revealed that palmitoleic acid (C16:1) is the most likely fuel for mitochondria β -oxidation and it does not require PL cleavage by cPLA₂. Other fatty acid fuels, i.e. palmitic acid (C16:0), oleic acid (C18:1), stearic acid (C18:0), linoleic acid (C18:2) and AA (C20:4), require, partly, cPLA₂ action [181]. Long chain fatty acyls have to be converted into their acyl-CoA counterpart by long-chain acyl-CoA synthetase before being transported, via the carnitine palmitoyltransferase (CPT) system, into the mitochondria. CPT system involves, 1) acyl-CoA transformation into acylcarnitine by CPT1, localized at the outer mitochondrial membrane, 2) acylcarnitine transfer into mitochondrial matrix via the carnitine acylcarnitine translocase and 3) acylcarnitine conversion into acyl-CoA by CPTII, located at the inner mitochondrial membrane. The catabolism of one molecule of palmitoyl-CoA gives rise to 8 acetyl-CoA, 7 FADH₂ and 7 NADH + H⁺ molecules. Once acetyl-CoA enters the tricarboxylic acid (TCA) cycle, 3 NADH, 1 FADH₂ and 1 ATP are produced. The mitochondrial respiratory chain complex I and II oxidize NADH and FADH₂, respectively, which leads to ATP production [182]. At resting state, this ATP synthesis, accounting for 50% of platelet mitochondrial function, is mainly used by flippase to maintain PL asymmetry (see section II.1.4) and by Ca²⁺-ATPase pumps to maintain Ca²⁺ concentration at a low level [183]. The latter is achieved by the plasma membrane Ca²⁺-ATPase (PMCA), which extrudes Ca²⁺ from cytosol towards extracellular medium and the sarco(endo)plasmic reticulum Ca²⁺-ATPase (SERCA), which uptakes Ca²⁺ into DTS [184].

3.2. In activated platelets

During activation, glucose and glutamine uptake, glycogen breakdown and fatty acid cleavage increase energetic substrate availability, which is important to respond to the enhanced ATP demand required to support platelet activation [180, 185, 186]. Interestingly, PA accumulation, PI and AA breakdown depend more on ATP than granule secretion or platelet aggregation [187]. PL breakdown is required for thrombin-

induced increase in oxygen consumption rate (OCR). Thrombin-induced PL cleavage by cPLA₂ generates mainly eicosanoids, C20:4, C18:0; C18:1, C18:2 and C16:0 fatty acyl chains, which fuel mitochondria β -oxidation [181] (**Figure 13**). Even if inhibiting fatty acid oxidation leads to an inhibition of thrombin-induced an increase in OCR, it is not sufficient to impair platelet aggregation [180]. Indeed, glycolysis is increased during platelet activation and can probably compensate for the lack of fatty acid oxidation. Inhibition of both glycolysis and fatty acid oxidation are required to drastically impair platelet functions [180, 188].

4. Lipids as substrates for post-translational modifications

Fatty acyl chains can act as donor groups for protein modifications. Even if very short acyl chains (from malonyl-CoA (C3:0) to succinyl-CoA C4:0) transfer to lysine residues of proteins has been characterized in cells, their role in platelets is still unknown [189]. Nevertheless, myristoylation (C14:0) and palmitoylation (C16:0) have been found and studied in platelets.

4.1. Myristoylation

N-myristoylation is a process mediated by N-myristoyl transferase and characterized by myristoyl-CoA addition to the N-terminal glycine of proteins. In platelets, this process occurs rarely and up to now, few proteins are known to carry this modification. Recently, it has been shown that myristoylated SR2 peptide linked to G α 13 impaired talin activation and subsequently integrin activation [190, 191]. Platelet functions are also impacted as a reduction in platelet adhesion and aggregate size were observed [192].

4.2. Palmitoylation

S-palmitoylation refers to the transfer of palmitoyl-CoA, by palmitoyl acyltransferases (PATs), to cysteine residues. The removal of s-acyl modifications occurs thanks to acyl protein thioesterase (APT) 1. The platelet palmitoylome identifies more than 200

protein candidates, with 1/3 involved in signal transduction, 1/5 in vesicle and small molecule transport and 1/10 in metabolism [193]. Palmitoylation has been shown to partition proteins into lipid rafts, which triggers some signaling events important for platelet activation (see section II.2.1.), and to regulate granule secretion, platelet aggregation and thrombi accumulation *in vivo* [146, 194, 195].

III. *De novo* lipogenesis and acetyl-CoA carboxylase

De novo lipogenesis (DNL) refers to the conversion of non-lipid based substrates, such as carbohydrates, into lipids (**Figure 14**).

1. *De novo* lipogenesis in health and diseases

1.1. *De novo* fatty acid synthesis

Platelets have all of the enzymes required for fatty acid synthesis [196, 197]. Three are implicated in this process: 1) ATP citrate lyase (ACL), which produces acetyl-CoA from citrate, 2) acetyl-CoA carboxylase (ACC), which carboxylates acetyl-CoA into malonyl-CoA and finally 3) fatty acid synthase (FAS) that generates acyl-CoA from malonyl-CoA (up to C16:0). Citrate is provided by glucose (glycolysis) or glutamine (catabolism) before being converted to acetyl-CoA in the cytoplasm. In addition to citrate, carnitine and acetate are also substrates for acetyl-CoA. Carboxylation of acetyl-CoA into malonyl-CoA is the rate-limiting step of lipogenesis. FAS catalyzes palmitate production from acetyl-CoA and malonyl-CoA. The reaction requires NADPH and includes the sequential formation of acyl intermediates [198]. Elongases and desaturases form more complex fatty acids. Elongases convert acyl-CoA (CX:0) into longer saturated acyl-CoA (CX+2:0) by addition of malonyl-CoA and desaturases induce double bond formation into acyl-CoA (CX:0+1) (**Figure 14**). Some polyunsaturated fatty acids (PUFAs) can only be synthesized from precursors found in the diet, i.e. omega6-linoleic acid (C18:2) and omega3- α -linolenic acid (C18:3). As for *de novo* fatty acid synthesis, elongases and desaturases are responsible for the formation of longer and more PUFA such as

omega6-AA or omega3-docosahexaenoic acid (C22:6) [199]. Fatty acyl chains are then esterified into PL, DAG or TAG [131].

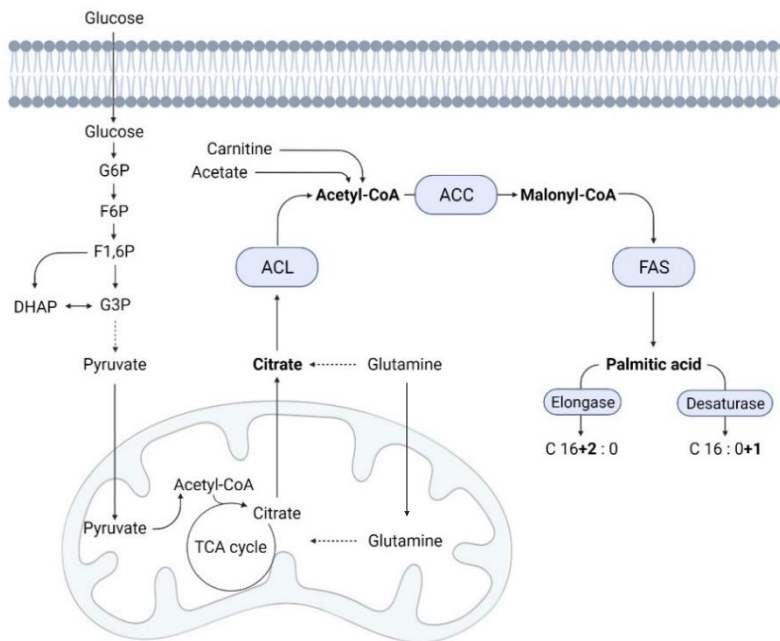


Figure 14. De novo lipogenesis (DNL). Non lipid-based substrates are converted into palmitic acid via 1) citrate production, either into the mitochondria via glucose or glutamine catabolism, or, into the cytoplasm via glutamine catabolism, 2) citrate conversion into acetyl-CoA by ACL, 3) carboxylation of acetyl-coA into malonyl-CoA by ACC, 4) elongation of malonyl-CoA by addition of acetyl-CoA up to C16:0 (palmitic acid) by FAS. Elongases and desaturases form longer and unsaturated fatty acid, respectively. ACC: acetyl-CoA carboxylase; ACL: ATP-citrate lyase; DHAP: dihydroxyacetone phosphate; F1,6P: fructose-1,6-bisphosphate; F6P: fructose-6-phosphate; FAS: fatty acid synthase; G3P: glyceraldehyde-3-phosphate; G6P: glucose-6-phosphate.

1.2. De novo synthesis of phospholipids

Platelets can also synthesize PL *de novo* [200]. Glycolysis and DNL are required for PL formation. Two intermediate products of glycolysis are used as PL backbone: glyceraldehyde-3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP). The addition of two acyl-CoAs to G3P induces PA formation which is the precursor for all PLs, i.e. PC, PE, PS, PI, and DAG. Conversion of acyl-DHAP into alkyl-DHAP allows alkyl-PA formation, the precursor for alkyl-PC and -PE. The latter can be converted into

alkenyl-PE, which can be transformed into alkenyl-PC [131] (**Figure 15**). Afterwards, deacylation-reacylation and transacylation reactions remodel fatty acyl chains of PL. The diversity of PL is important to ensure proper membrane function (see section II.1.1).

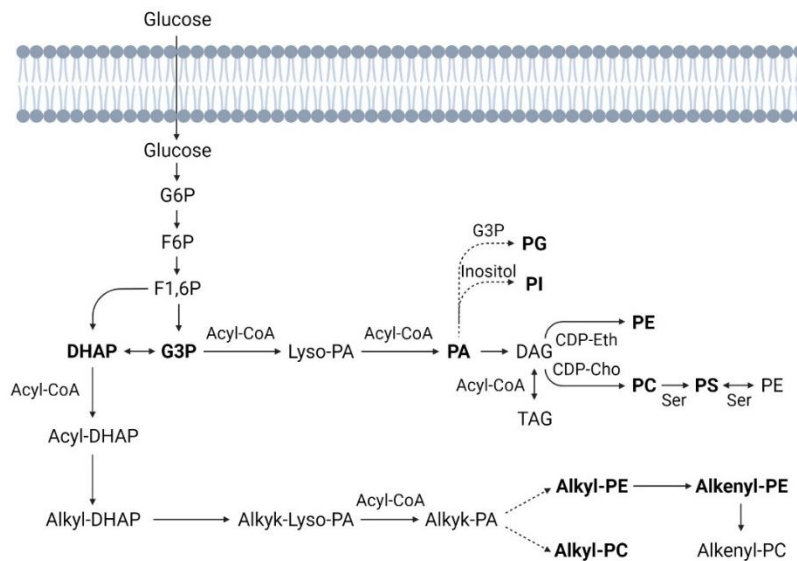


Figure 15. De novo synthesis of phospholipids. PL production consists in the addition of two acyl-CoAs to DHAP or G3P, products of glycolysis, forming PA. The addition of G3P or inositol to PA gives rise to PG and PI, respectively. Conversion of PA into DAG allows PE and PC formation, which can turn into PS. Conversion of acyl-DHAP into alkyl-DHAP allows alkyl-PA formation, the precursor for alkyl-PC and -PE. The latter can be converted into alkenyl-PE, which can be transformed into alkenyl-PC. CDP-Cho: cytidine diphosphate-choline; CDP-Eth: cytidine diphosphate-ethanolamine; DAG: diacylglycerol; DHAP: dihydroxyacetone phosphate; F1,6P: fructose-1,6-bisphosphate; F6P: fructose-6-phosphate; G3P: glyceraldehyde-3-phosphate; G6P: glucose-6-phosphate; PA: phosphatidic acid; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PG: phosphatidylglycerol; PI: phosphatidylinositol; PL: phospholipid; PS: phosphatidylserine; Ser: serine; TAG: triacylglycerol.

1.3. De novo lipogenesis in diseases

1.3.1. DNL and nonalcoholic fatty liver disease (NAFLD)

NAFLD is a disease characterized by TAG accumulation in the liver. These lipids come from excessive fatty acid intake, insulin resistance-induced increased lipid influx in the liver and hepatic DNL. Increased DNL accounts for ¼ of fat accumulation in patients suffering from NAFLD [201, 202]. Therefore, targeting DNL has become one therapeutic

strategy to limit NAFLD. Inhibitors of FAS, ACC, stearoyl-CoA desaturase (SCD) and diacylglycerol acyltransferase (DGAT) have been or are being tested [203]. Two years ago, a liver-specific allosteric ACC inhibitor, GS-0976 or Firsocostat, has been used in a phase II trial. Results showed a significant decrease (29%) in liver fat content in nonalcoholic steatohepatitis (NASH) patients [204].

1.3.2. Type 2 diabetes

Type 2 diabetes patients are characterized with abnormal hepatic fatty acid accumulation and insulin resistance. As for NAFLD, DNL participates in hepatic lipid deposition in insulin-resistant patients. The dysregulated DNL, in turn, exacerbates insulin resistance. As such, ACC inhibition by antisense oligonucleotide decreases hepatic steatosis and insulin resistance. Therefore, orally administered ACC inhibitors seemed to be good candidates for type 2 diabetic patients [205, 206]. Interestingly, clinical trial of a non-selective ACC1/ACC2 inhibitor, PF-05175157, ended early because of an unexpected platelet count decrease [207].

1.3.3. Cancer

Most cancer cells increase DNL to support the energy demands for proliferation and migration. The common lipids produced by DNL are saturated fatty acids, which are known to induce tight membrane packing and reduce membrane fluidity (see section II.1.1). Consistent with that, cancer cells with an active DNL have a less fluid membrane, conferring them resistance to chemotherapy drug uptake [208]. Studies in breast and prostate cancer cells have shown the detrimental impact of silencing ACC1 on growth and cell survival. Therefore, the use of pharmacological ACC inhibitors for highly lipogenic cancer cells could be beneficial [209].

2. Acetyl-CoA carboxylase

2.1. Structure, reaction and isoforms

ACC contains three domains required for its catalytic activity: a biotin carboxylase (BC), a biotin carboxyl carrier protein (BCCP), and a carboxyl transferase (CT) domain. An interaction domain (BT), which mediates the association between BC and CT domains and a non-catalytic central domain region (CD) are also present. It can be found as a homodimer (low activity) or polymers (high activity) [210].

Its reaction, i.e. the carboxylation of acetyl-CoA into malonyl-CoA in the presence of ATP and bicarbonate, takes place in two steps. First, BC domain carboxylates the biotin protein bound to the BCCP domain, in an ATP-dependent manner. Secondly, the newly formed carboxybiotin moves towards the CT site, which allows the transfer of the carboxy group from the biotin to acetyl-CoA, generating malonyl-CoA [211] (**Figure 16**). Our DNA encodes for two different ACC isoforms: ACC1 and ACC2. Both catalyze the same reaction but differ in terms of tissue and cellular localization and role. ACC1 (265 kDa) is mainly expressed in lipogenic tissues such as the liver or adipocytes and ACC2 (280 kDa) in oxidative organs such as the heart, skeletal muscles and also the liver [212]. The molecular weight variation is due to the additional 114 amino acids found in the N-terminal sequence of ACC2. This extra sequence contains a hydrophobic part with a mitochondrial targeting motif [213]. Studies of ACC1 or ACC2 knockout (KO) mice have revealed roles discrepancy between the two enzymes [212, 214]. Malonyl-CoA produced by ACC1 acts mainly as a substrate for lipogenesis whereas malonyl-CoA from ACC2 inhibits CPT1 and therefore controls fatty acid oxidation. In murine and human platelets, ACC1 is the only isoform detected [57, 197, 215].

2.2. Regulation

Several transcription factors regulate ACC level. Sterol regulatory element binding protein 1c (SREBP1c) and carbohydrate response element binding protein (ChREBP) are activated via an increase in insulin and glucose, respectively, and enhance ACC1

expression. Peroxisome-proliferator-activated receptors (PPARs) are also known to increase ACC transcription [202].

ACC activity is regulated by allosteric modulators and phosphorylation. ACC activity is increased by citrate, which enhances its polymerization, and glutamate and insulin, which induces ACC dephosphorylation by protein phosphatase 2A (PP2A) and further ACC polymerization [216, 217]. Palmitoyl-CoA binding to the BC domain reduces catalytic ACC activity. If its concentration is 10 times higher than citrate, palmitoyl-CoA prevents citrate-induced ACC polymerization. Two kinases are known to phosphorylate ACC: AMP-activated protein kinase (AMPK), on residues Ser79, Ser1200 and Ser1215 (ACC1), and protein kinase A (PKA), on residues Ser77 and Ser1200 (ACC1) [210]. Only, AMPK-mediates Ser79/Ser212 phosphorylation inhibits ACC1/ACC2 activity [218, 219]. This inhibition seems to be mediated by a disruption of the BC dimerization. Moreover, breast cancer type 1 susceptibility protein (BRCA1) binding to the phosphorylated Ser1262 residue prevents Ser79 dephosphorylation, reinforcing ACC inhibition. Once phosphorylated, citrate can barely induce ACC activity [210] (**Figure 16**).

Several pharmacological compounds have already been developed in order to modulate the activity of ACC. Some inhibit the enzyme thanks to a structure related to an acyl-CoA (TOFA) [220] while others bind to a specific catalytic domain, i.e. either BC domain (Soraphen A) or CT domain (CP640.186) [221]. A crystal structure study revealed that CP640.186 binds at the interface between the two monomers of CT dimer and prevents the binding of the biotin substrate to inhibit ACC activity. Of note, CP640.186 does not need a conformational change of the CT domain to interact with it [222].

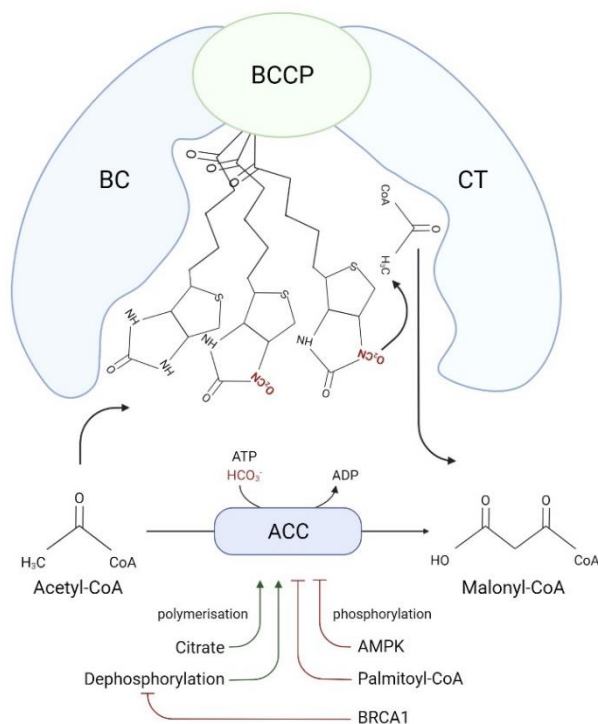


Figure 16. ACC reaction and regulation. First, BC domain of ACC carboxylates the biotin protein bound to the BCCP domain, in an ATP-dependent manner. Then, the carboxybiotin is heading to the CT site. There, the carboxy group is transferred from the biotin to acetyl-CoA, generating malonyl-CoA. ACC is inhibited via the binding of palmitoyl-CoA or by phosphorylation by AMPK on residue Ser79. BRCA1 interaction with ACC prevents Ser79 dephosphorylation, reinforcing ACC inhibition. In addition to be activated by dephosphorylation, ACC activity is enhanced by citrate, which induces its polymerization. ACC: acetyl-CoA carboxylase; AMPK: AMP-activated protein kinase; BC: biotin carboxylase; BCCP: biotin carboxyl carrier protein; BRCA1: breast cancer type 1 susceptibility protein; CT: carboxyl transferase.

2.3. Role of acetyl-CoA carboxylase in phospholipid composition

Through its role in DNL, ACC is a regulator of membrane PL composition. As such, lipidomic analyses of endothelial or cancer cells treated with a pharmacological ACC inhibitor, soraphen A or TOFA, revealed that long-term ACC inhibition modulates PL composition and not cholesterol concentration. More precisely, ACC inhibition is associated with a reduction in saturated or monounsaturated acyl chains and an

increase in PUFA chains (more than three unsaturations) mainly in PC [223-226]. Interestingly, ACC inhibition increases membrane lateral and transversal mobility probably due to the increment in PUFA content [224]. However, even if it's known that PUFA decreases membrane rigidity (see section II.1.1), ACC1 inhibition renders plasma membrane less fluid and more rigid [223, 225, 226]. In CD34⁺cell-derived megakaryocytes, ACC inhibition reduces saturated PC content. The effect on polyunsaturated fatty acids is disparate as some decrease (C38:3, C38:4 and C38:5) and some increase (C34:3 and C36:4). This change in fatty acyl chain composition lead to an underdeveloped DMS [207]. Our previous work showed that platelets with an active ACC have a decrease in PC and an increase in PEP content. Interestingly, the polyunsaturated fatty acyl chain 20:4 follows the same trend, i.e. AA-containing PC is decreased whereas AA-containing PEP is increased. No particular effect on platelet morphology was observed [57]. In a hyperlipidemic environment such as atherosclerosis, platelet ACC is more phosphorylated/inhibited, which is associated with a downregulation of C14:0-containing TAG content [227]. So far, the specific contribution of platelet ACC1 on platelet lipid content is not known.

3. Role of acetyl-CoA carboxylase in platelets and thrombosis: our previous work

A recent study highlighted the role of human ACC in megakaryocyte maturation and platelet production but the impact on platelet functions was not investigated [207]. A few years ago, we started to decipher the role of ACC activity on platelet functions [57]. The impact of an active ACC on platelet activation and thrombus formation was assessed thanks to ACC1-S79A and ACC2-S212A knock-in (ACC DKI) mice. Mutations render the residues insensitive to AMPK-induced ACC phosphorylation/inhibition. Accordingly, hepatic DNL was increased and fatty acid oxidation decreased in ACC DKI mice [219]. The lack of AMPK-induced ACC phosphorylation was also validated in ACC DKI platelets. These mutations do not impact platelet bioenergetics as seen by

comparable values of mitochondrial OCR. However, lipidomic analyses revealed that ACC DKI platelets have an increase in AA-containing PEP content. Platelet functions analyses revealed that this change in lipid content is associated with an enhancement of TxA₂ secretion followed by dense granule release upon collagen stimulation. As a result, thrombus formation, on collagen-coated surface, and thrombus growth, *in vivo*, after ferric chloride (FeCl₃)-induced carotid injury, were increased [57] (**Figure 17**). The impact of platelet-specific ACC inhibition or deletion on platelet functions is still unknown.

Interestingly, we observed elevated levels of phosphorylated ACC in platelets from coronary artery disease (CAD) patients. Thrombin generation, evaluated by D-dimers, thrombin anti-thrombin complex (TATc) and fragment 1.2 (F1.2) formation, is increased in CAD patients. Even if short thrombin stimulation induces ACC phosphorylation, the increased ACC phosphorylation in CAD patients is not due to thrombin generation but rather due to oxLDL [227, 228]. The impact of sustained ACC inhibition or deletion on thrombus formation in an atherosclerotic environment is still unknown.

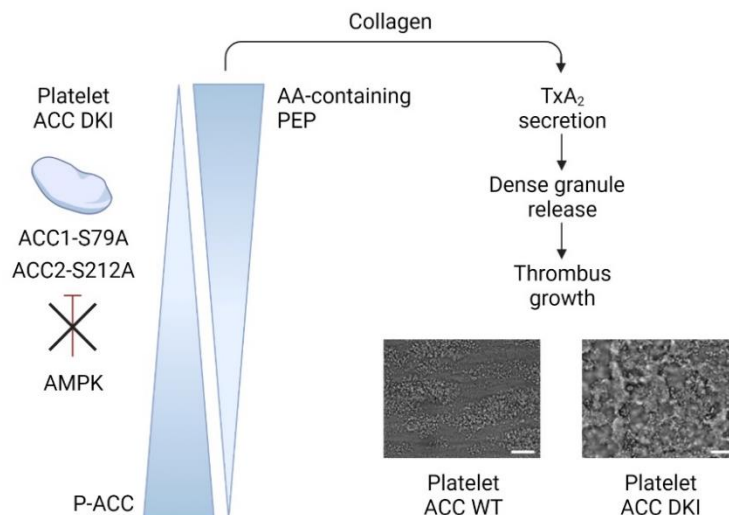


Figure 17. AMPK-ACC signaling potentiates thrombus growth. Upon collagen stimulation, the increased AA-containing PEP level observed in ACC DKI platelets leads to more TxA₂ generation. Once secreted, it enhances dense granule release. Both TxA₂ and ADP (from dense granules) amplify thrombus formation *ex vivo*, in flow chamber model and *in vivo*, in FeCl₃-induced thrombosis model. AA: arachidonic acid; ACC: acetyl-CoA carboxylase; AMPK: AMP-activated protein kinase; DKI: double knock-in; P-ACC: ACC phosphorylation; PEP: phosphatidylethanolamine plasmalogen; S79/212A: Serine79/212Alanine; TxA₂: thromboxane A₂; WT: wild-type.

IV. Protein acetylation and acetyl-CoA carboxylase

1. Regulation of acetylation

Acetylation refers to the addition of an acetyl group, provided by acetyl-CoA, to lysine residues of proteins. It occurs non-enzymatically or enzymatically via acetyllysine transferases (KATs) when acetyl-CoA level is high. The removal of acetyl group is mediated by two groups of lysine deacetylases (KDACs): Zn²⁺-dependent lysine deacetylase, called histone deacetylase (HDAC) or NAD⁺-dependent lysine deacetylase, called sirtuin (SIRT) [229] (**Figure 18**). In cells, at least 20 KATs and 18 KDACs have been identified. They are located in the cytoplasm, the nucleus or mitochondria. In platelets, three KATs have been described so far: p300, p300/CBP-associated factor (PCAF) and alpha tubulin acetyltransferase 1 (α TAT1). Upon stimulation, p300 activity is increased and dephosphorylated on its Ser89 residue [230, 231]. Among the 11 HDACs and 7 SIRTs

present in mammalian cells, only HDAC4, HDAC6, SIRT1, SIRT2, SIRT3 and SIRT5 have been found at protein level in platelets [197, 232-235].

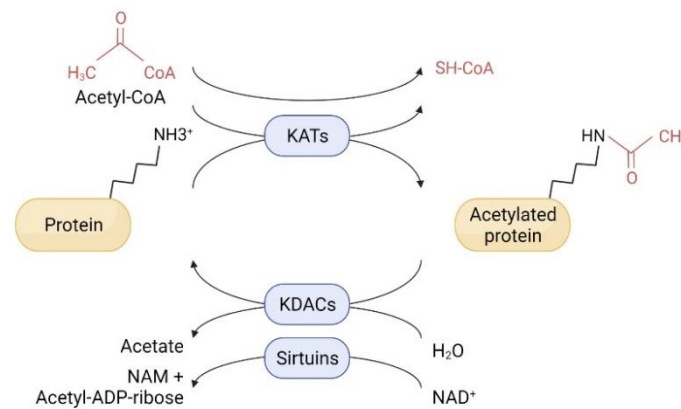


Figure 18. Regulation of protein acetylation. Protein acetylation occurs non enzymatically or enzymatically by KATs. Protein deacetylation is mediated by KDACs or sirtuins. KATs: acetyllysine transferases; KDACs: lysine deacetylases; NAD: nicotinamide adenine dinucleotide; NAM: nicotinamide.

2. Linking protein acetylation and acetyl-CoA carboxylase

Cytosolic acetyl-CoA pool is the driver of histone acetylation. Indeed, blocking citrate conversion into acetyl-CoA by siRNA against ACL decreases histone acetylation [236], whereas repressing ACC1 expression, and so far acetyl-CoA carboxylation, leads to an increase in histone acetylation either in yeast, plant or cells [237-239]. In addition, liver-specific ACC KO mice showed an enhancement in acetylation of non-histone proteins. More precisely, they showed that ACC deletion results in an increase in extra-mitochondrial protein acetylation and a decrease in mitochondrial protein acetylation [240].

3. Protein acetylation in platelets: focus on α -tubulin

3.1. Acetylation in platelets

Platelet proteomics analysis identified 273 acetylatable proteins with 2/3 of them involved in platelet metabolism or cytoskeleton. Upon stimulation, some proteins become more acetylated while others are deacetylated. Consequently, it has been

shown that blocking acetylation or deacetylation impairs platelet functions. Upon CRP stimulation, actin, cortactin and filamin are acetylated in a p300-dependent manner. Inhibition of p300-induced protein acetylation altered F-actin polymerization, α IIb β 3 activation, platelet spreading and aggregation [230]. Furthermore, enhancing SIRT1 deacetylase activity decreases PAFR expression on platelet surface leading to an impaired PAF or AA-induced platelet aggregation and pulmonary thrombus formation [241]. On the other hand, increasing protein acetylation also alters platelet functions. Indeed, enhancing Akt or α -tubulin acetylation level, via SIRT2 and HDAC6 inhibition, respectively, is associated with an impaired platelet aggregation [232, 235]. Furthermore, SIRT2, and not SIRT1 or 5, is involved in the regulation of collagen-induced platelet aggregation [234, 235, 241]. In addition to control platelet functions, acetylation plays a role in apoptosis. Indeed, via p53 acetylation, sirtuins inhibition prevents p53 degradation, allows its translocation into the mitochondria, Bax activation and later on calpain-induced platelet apoptosis [233].

3.2. α -tubulin acetylation in platelets

α -tubulin in association with β -tubulin polymerize to form MTs. Their association at platelet circumference forms the marginal band, which maintains resting platelets in their discoid shape. Upon stimulation, a huge MT reorganization takes place, which involves MTs condensation towards the center of platelets, depolymerization, fragmentation and repolymerization to generate a new MT coil [242-244]. The marginal band is acetylated at basal state and becomes deacetylated during platelet activation [232]. Therefore, stable and dynamics MTs are characterized by elevated or low acetylated α -tubulin level, respectively [242-244]. In platelets, α TAT1 is, in part, responsible for α -tubulin acetylation at Lys40 residue and HDAC6 is the only KDAC mediating its deacetylation upon stimulation [231, 232, 235]. Altering α -tubulin acetylation level in platelets barely affects their structure. Indeed, increasing α -tubulin acetylation, via HDAC6 inhibition by tubacin, slightly altered the marginal band whereas its deacetylation, induced by α TAT1 deletion, has no impact [231, 232]. Moreover, α -

tubulin deacetylation during platelet activation is not required to allow platelet shape change as platelet morphology and actin structure are not affected by HDAC6 deletion. However, preventing α -tubulin deacetylation affects platelet spreading kinetics and aggregation [231, 245]. Interestingly, tubacin treatment only impairs CRP-induced platelet aggregation but not α granule secretion or α IIb β 3 activation. The signaling cascade involved includes PAK, LIMK1, MEK, ERK1/2 and Akt [232]. A summary of the impact of protein acetylation and deacetylation on platelet functions is represented in **Table 2**.

Protein modification	Enzyme	Protein	Platelet function	References
Acetylation	p300	Actin, cortactin, filamin	F-actin polymerization α IIb β 3 activation Platelet spreading Platelet aggregation	[230]
	α TAT	α -tubulin	Platelet aggregation Platelet spreading kinetics	[231]
Deacetylation	SIRT1	?	PAF or AA-induced platelet aggregation Pulmonary thrombus formation	[241]
	HDAC6	α -tubulin	Platelet aggregation Platelet spreading kinetics	[232, 245]
	SIRT2	Akt	Platelet aggregation	[235]
	Sirtuins	p53	Platelet apoptosis	[233]

Table 2. Impact of protein acetylation and deacetylation on human and murine platelet functions. Enzyme in red: what happens on platelet functions if inhibition, enzyme in green: what happens on platelet functions if activation (in red: a decrease, in green: an increase).

Aim

I. Evaluating the impact of ACC inhibition or invalidation on platelet lipid content and functions.

Lipids fulfill significant roles for platelet functions, as they ensure membrane composition and structure, granule secretion, signaling molecule generation, ATP production and post-translational modifications. ACC is the first rate-limiting enzyme regulating lipogenesis [211]. Preventing AMPK-induced ACC phosphorylation/inhibition renders basal ACC active and increases AA-containing PEP content. Upon collagen stimulation, this higher pool of AA-PEP participates in the increased TxA₂ generation, dense granule secretion and thrombus growth [57]. Therefore, we hypothesized that ACC inhibition or deletion could be a protective mechanism against thrombus formation.

To investigate this, we used two different approaches, one using human platelets treated with a pharmacological ACC inhibitor, CP640.186, before thrombin stimulation and one using a platelet-specific ACC1 KO mouse model. Our main objectives were

- 1) to evaluate the impact of ACC inhibition/deletion on platelet lipid content, bioenergetics and platelet functions
- 2) to assess the role of ACC1 deletion, *in vivo*, on hemostasis and arterial thrombus formation.

Results of the impact of pharmacological ACC inhibition by CP640.186 on human platelet functions are described in the manuscript “Acetyl-CoA Carboxylase Inhibitor CP640.186 Increases Tubulin Acetylation and Impairs Thrombin-Induced Platelet Aggregation” published in International Journal of Molecular Sciences (DOI: 10.3390/ijms222313129).

II. Impact of platelet ACC phosphorylation on thrombus growth in an atherosclerotic mice model

We demonstrated that patients at risk of thrombotic events, i.e. CAD patients, have an increase in platelet ACC phosphorylation level. This was confirmed in an atherosclerotic mouse model, the ApoE^{-/-} x SRBI^{flx/flx} mouse model. Moreover, platelet ACC phosphorylation level increases with the severity of atherosclerosis [227]. Given the impact of an active ACC on thrombus growth, we postulated that platelet ACC phosphorylation/inhibition could be a protective mechanism to attenuate platelet activation in an atherogenic environment. To address this assumption, we generated an atherosclerotic mouse model (ApoE^{-/-} x SRBI^{flx/flx}) expressing either ACC WT or ACC DKI isoforms in their hematopoietic stem cells. Our main objectives were

- 1) to assess atherosclerosis development and
- 2) to evaluate FeCl₃-induced thrombus formation.

Results

PART1: Acetyl-CoA Carboxylase Inhibitor CP640.186 Increases Tubulin Acetylation and Impairs Thrombin-Induced Platelet Aggregation

This chapter is dedicated to the work we published in the International Journal of Molecular Sciences in 2021. The aim of the paper was to assess the impact of pharmacological ACC inhibition (2 hours, 60 μ M CP640.186) on platelet lipid content, α -tubulin acetylation level and functions. Then, we deciphered the signaling pathway involved in the alteration of platelet aggregation.

In this article, we reported that:

- 1) Lipogenesis occurs in human platelet and is inhibited by pharmacological ACC inhibitor (CP640.186).
- 2) Two hours of ACC inhibition does not drastically change platelet lipid content or mitochondrial function but is sufficient to increase α -tubulin acetylation level.
- 3) Two hours of ACC inhibition recapitulates the impact of an increased α -tubulin acetylation level on platelet functions, i.e. a decrease in platelet aggregation without any impact on granule secretion or α IIb β 3 activation.
- 4) Two hours of ACC inhibition does not alter MT or actin cytoskeleton.
- 5) Downstream signaling cascade involved Rac1-PAK2 pathway and PAK effectors (NOX2, ERK).

**Acetyl-CoA Carboxylase Inhibitor CP640.186 Increases
Tubulin Acetylation and Impairs Thrombin-Induced
Platelet Aggregation**

Marie Octave¹, Laurence Piroton¹, Audrey Ginion¹, Valentine Robaux¹, Sophie Lepropre¹, Jérôme Ambroise², Caroline Bouzin³, Bruno Guigas⁴, Martin Giera⁵, Marc Foretz⁶, Luc Bertrand¹, Christophe Beauloye^{1,7} and Sandrine Horman^{1,*}

¹Pôle de Recherche Cardiovasculaire (CARD), Institut de Recherche Expérimentale et Clinique (IREC), Université Catholique de Louvain (UCLouvain), 1200 Brussels, Belgium; marie.octave@uclouvain.be (M.O.); laurence.piroton@uclouvain.be (L.P.); audrey.ginion@uclouvain.be (A.G.); valentine.roboux@uclouvain.be (V.R.); sophie.lepropre@gmail.com (S.L.); luc.bertrand@uclouvain.be (L.B.); christophe.beauloye@uclouvain.be (C.B.)

²Centre de Technologies Moléculaires Appliquées, Institut de Recherche Expérimentale et Clinique (IREC), Université Catholique de Louvain (UCLouvain), 1200 Brussels, Belgium; jerome.ambroise@uclouvain.be

³IREC Imaging Platform, Institut de Recherche Expérimentale et Clinique (IREC), Université Catholique de Louvain (UCLouvain), 1200 Brussels, Belgium; caroline.bouzin@uclouvain.be

⁴Department of Parasitology, Leiden University Medical Center, 2333 ZA Leiden, The Netherlands; B.G.A.Guigas@lumc.nl

⁵Department of Molecular Cell Biology, Leiden University Medical Center, 2333 ZA Leiden, The Netherlands; m.a.giera@lumc.nl

⁶CNRS, INSERM, Institut Cochin, Université de Paris, F-75014 Paris, France; marc.foretz@inserm.fr

⁷Division of Cardiology, Cliniques Universitaires Saint-Luc, 1200 Brussels, Belgium

*Correspondence: Sandrine.horman@uclouvain.be; Tel.: +32-2-764-55-66

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Abstract

Acetyl-CoA carboxylase (ACC) is the first enzyme regulating de novo lipid synthesis via the carboxylation of acetyl-CoA into malonyl-CoA. The inhibition of its activity decreases lipogenesis and, in parallel, increases the acetyl-CoA content, which serves as a substrate for protein acetylation. Several findings support a role for acetylation signaling in coordinating signaling systems that drive platelet cytoskeletal changes and aggregation. Therefore, we investigated the impact of ACC inhibition on tubulin acetylation and platelet functions. Human platelets were incubated 2 h with CP640.186, a pharmacological ACC inhibitor, prior to thrombin stimulation. We have herein demonstrated that CP640.186 treatment does not affect overall platelet lipid content, yet it is associated with increased tubulin acetylation levels, both at the basal state and after thrombin stimulation. This resulted in impaired platelet aggregation. Similar results were obtained using human platelets that were pretreated with tubacin, an inhibitor of tubulin deacetylase HDAC6. In addition, both ACC and HDAC6 inhibitions block key platelet cytoskeleton signaling events, including Rac1 GTPase activation and the phosphorylation of its downstream effector, p21-activated kinase 2 (PAK2). However, neither CP640.186 nor tubacin affects thrombin-induced actin cytoskeleton remodeling, while ACC inhibition results in decreased thrombin-induced reactive oxygen species (ROS) production and extracellular signal-regulated kinase (ERK) phosphorylation. We conclude that when using washed human platelets, ACC inhibition limits tubulin deacetylation upon thrombin stimulation, which in turn impairs platelet aggregation. The mechanism involves a downregulation of the Rac1/PAK2 pathway, being independent of actin cytoskeleton.

Keywords: acetyl-CoA carboxylase; platelet; platelet aggregation; acetylation; tubulin; actin cytoskeleton; Rac1 signaling

1. Introduction

Acetyl-CoA carboxylase (ACC) catalyzes the first step of de novo lipogenesis (DNL) by carboxylating acetyl-CoA into malonyl-CoA [246]. There are two ACC isoforms, ACC1 and ACC2, which are differentially distributed amongst cells and tissues [247]. ACC1 provides a pool of cytosolic malonyl-CoA, which serves as a substrate for fatty acid synthase and chain elongation systems. ACC2 produces malonyl-CoA near the mitochondrial membrane where it acts as an allosteric inhibitor of carnitine palmitoyltransferase 1 (CPT1); this enzyme is responsible for transporting long-chain fatty acids into mitochondria [211-213]. ACC activity is acutely controlled by both allosteric modulators and the phosphorylation of several serine residues [211, 248]. We previously demonstrated that ACC1 is the predominant isoform in murine and human platelets [57]. ACC1 promotes thrombosis through platelet phospholipid production, which is required for the synthesis of arachidonic acid (AA), a key mediator of platelet activation [57]. More recently, the presence of ACC in DNL has also been implicated in megakaryocyte (MK) maturation and platelet production [207]. ACC's critical role in megakaryocytes (MKs) and platelets raises the question of a potential therapeutic usefulness of prescribing pharmacological ACC inhibitors for a plethora of diseases characterized by DNL dysregulation [204, 205, 208, 209], including Type 2 diabetes, cancer, or non-alcoholic fatty liver disease [202, 208].

ACC inhibition not only decreases lipogenesis, but concomitantly results in elevated cytoplasmic levels of acetyl-CoA, which serves as a substrate for protein acetylation [237, 239]. The latter process is a posttranslational modification that is regulated by lysine acetyltransferases (KATs) and histone deacetylases and sirtuins (HDACs, SIRTs) [249]. In platelets, protein acetylation on lysine residues is catalyzed by KATs p300 and α -TAT [230, 231]. Deacetylation is induced by lysine deacetylases HDAC6 and SIRT2 [232, 235]. Among acetylatable proteins, as identified in platelets using proteomic studies [230], tubulin is recognized to play a crucial role in regulating platelet formation and functions [232, 250-252]. MKs and platelets express at least six isoforms of α - and β -

tubulin. In resting platelets, stable microtubules that form the marginal band are composed of α - and β -tubulin heterodimers, which are highly acetylated (on Lys40 residue of α -tubulin) so as to preserve the discoid shape [242-244]. Upon agonist stimulation, α -tubulin is massively deacetylated by HDAC6, which results in the condensation of microtubules towards the platelets' center, where they undergo depolymerization [232]. This process is followed by their fragmentation and re-polymerization in order to generate a new microtubule coil [24-26]. According to several studies, this deacetylation process coordinates cytoskeletal and signaling events upon platelet activation, which in turn promote platelet spreading and aggregation [232, 245].

In this study, we focused on the impact of the pharmacological ACC inhibitor, CP640.186, on the acetylation status of tubulin in human platelets. In addition, we examined the effect of ACC inhibition on platelet aggregation and secretion, as well as on cytoskeleton signaling and organization. We have herein demonstrated for the first time that ACC inhibition limits tubulin deacetylation upon thrombin stimulation, thereby impairing platelet aggregation. The molecular mechanism involves a downregulation of the Rac1/PAK2 pathway, while being independent of actin cytoskeleton. Similar results were obtained with tubacin, an inhibitor of HDAC6. Moreover, we have detected a decrease in thrombin-induced reactive oxygen species (ROS) production and extracellular signal-regulated kinase (ERK) phosphorylation in CP640.186-treated platelets. These findings suggest that ACC inhibitors may possibly alter platelet functions through a mechanism that is similar, at least to some extent, to that of HDAC inhibitors, which are well recognized to result in thrombocytopenia and hemostasis defects [253, 254].

2. Results

2.1. *Short-term ACC Inhibition Using CP640.186 Does Not Reduce Lipid Content in Human Platelets and Preserves Mitochondrial Function*

We first investigated whether short-term pharmacological ACC inhibition would be able to reduce DNL in human platelets. Platelets were incubated with [1-¹⁴C]-acetic acid and treated for 2 h with DMSO or CP640.186 (60 μM), the latter being an isozyme-nonspecific inhibitor of mammalian ACC. As a result, fatty acid synthesis was drastically reduced in CP640.186-treated platelets (**Figure 1a**). To evaluate the consequences of CP640.186 treatment on overall platelet lipid content, we undertook a quantitative lipidomic analysis of dimethyl sulfoxide (DMSO)- and CP640.186-treated platelets. Overall, 672 lipids were detected, including different phospholipids (phosphatidylethanolamine [PE], phosphatidylethanolamine plasmalogen [PEP], plasmalogen phosphatidylethanolamine [PEO], phosphatidylcholine [PC], lysophosphatidylethanolamine [LPE], lysophosphatidylcholine [LPC]), sphingomyelin [SM], cholesteryl esters [CE], free fatty acids [FFA], diacylglycerol [DAG], and triacylglycerol [TAG]) (**Figure 1b**). Paradoxically, a slight increase in 63 lipids belonging to TAG, LPC, and LPE lipid classes was observed following CP640.186 treatment (**Figure 1b; Table S1**). However, lipid variations were low, consisting of a maximum 1.25-fold change, which indicates that a 2-h CP640.186 treatment does not drastically modify overall platelet lipid composition.

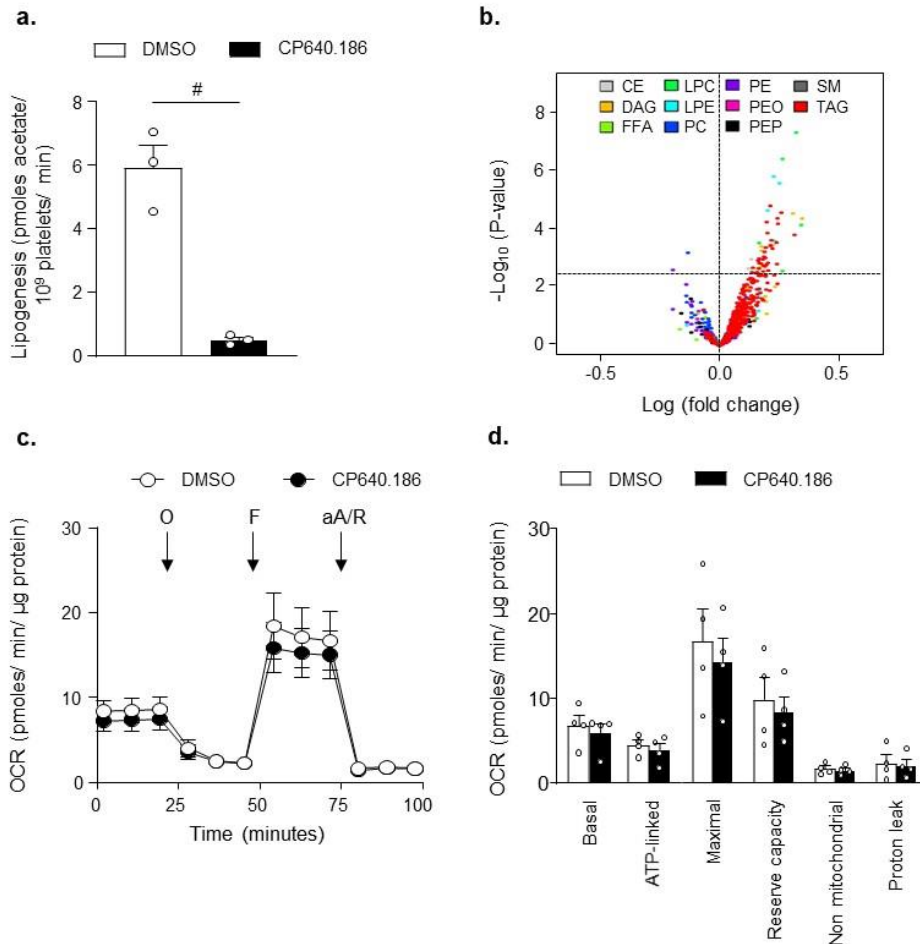


Figure 1. ACC inhibition induced by CP640.186 does not reduce overall lipid content and pre-serves mitochondrial function. **(a)** Washed human platelets were incubated with [1 - ¹⁴C]--acetic acid for 2 h in the presence of dimethyl sulfoxide (DMSO) or CP640.186 (60 μM). Radioactivity incorporation into lipid fractions was determined. Data were expressed as means ± SEM (n = 3); # p-value ≤ 0.0001 as compared to DMSO condition. Data underwent unpaired t-test. **(b)** Washed human platelets were preincubated with DMSO or CP640.186 (60 μM) before lipid extraction. Volcano plot representation of 672 detected lipid species. Lipids above the horizontal dotted line were up (right)-- or down (left)--regulated with significant adjusted p-values ≤ 0.05. Log fold-changes and adjusted p-values were calculated from the multivariate regression model. CE = cholesterol ester; DAG = diacylglycerol; FFA = free fatty acid; LPC = lysophosphatidylcholine; LPE = lysophosphatidylethanolamine; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PEO = plasmalogen phosphatidylethanolamine; PEP = plasmalogen phosphatidylethanolamine; SM = sphingomyelin; TAG = triacylglycerol. **(c,d)** Oxygen consumption rate (OCR) was measured in washed human platelets preincubated for 2 h with DMSO or CP640.186 before bio-energetic measurements. OCR was assessed under basal condition and after treatment with 1 μM oligomycin (O), 0.45 μM carbonyl cyanide p-trifluoromethoxyphenylhydrazone (F), and a mix of 1μM antimycin A (aA) and 1μM rotenone (R). **(c)** OCR profile is shown. **(d)** Mitochondrial function was evaluated by calculating basal, ATP-linked, maximum, non-mitochondrial OCR, re-serve capacity, and proton leak. Results were expressed as means ± SEM (n = 4). The data were analyzed using unpaired t--test.

Next, we sought to investigate whether pharmacological ACC inhibition within platelets actually affected platelet bioenergetics, which was assessed by measuring mitochondrial function. As illustrated in Figure 1c,d, extracellular flux analysis revealed that the platelets' basal oxygen consumption rate (OCR) was similar between DMSO- and CP640.186-treated platelets, and it was comparable to data published in the literature, as well [30]. The oxidative phosphorylation component was further assessed by injecting oligomycin, which is a complex V inhibitor. This led to an expected decrease in OCR. ATP-linked respiration was similar under both conditions. An injection of carbonyl cyanide p-trifluoromethoxyphenylhydrazone, a proton-ionophore, induced maximal OCR. DMSO and CP640.186-treated platelets displayed a similar maximal OCR and reserve capacity. Finally, antimycin A and rotenone, which are complex III/I inhibitors, totally inhibited mitochondrial-induced OCR. The non-mitochondrial OCR and proton leak remained unchanged (**Figure 1c,d**). These data confirm that, in platelets, ACC1 does not play a major role in mitochondrial respiration.

2.2. CP640.186 Increases α -Tubulin Acetylation Level and Impairs Platelet Aggregation

To examine the impact of CP640.186 on platelet α -tubulin Lys 40 acetylation, we performed western blot analysis of α -tubulin deacetylation upon thrombin stimulation. As seen in **Figure 2a**, platelet pretreatment using CP640.186 increased α -tubulin acetylation levels at basal state and after thrombin stimulation. Next, we investigated the consequences of ACC inhibition on platelet functions. CP640.186 inhibited platelet aggregation in response to low thrombin doses (**Figure 2b**). A similar effect was observed using tubacin, an HDAC6 inhibitor causing a drastic increase in α -tubulin acetylation (**Figures S1a and 2c**). Impaired platelet aggregation was unlikely accounted for altered platelet secretion processes, given that neither CP640.186 nor tubacin decreased P-selectin surface exposure or ATP secretion upon thrombin stimulation (**Figures 2d,e and S1b,c**). Moreover, ACC inhibition did not affect thrombin-induced

platelet α IIb β 3 activation, as measured by the binding of PAC-1 to platelets (**Figure 2f**). A comparable result was obtained with tubacin (**Figure S1d**).

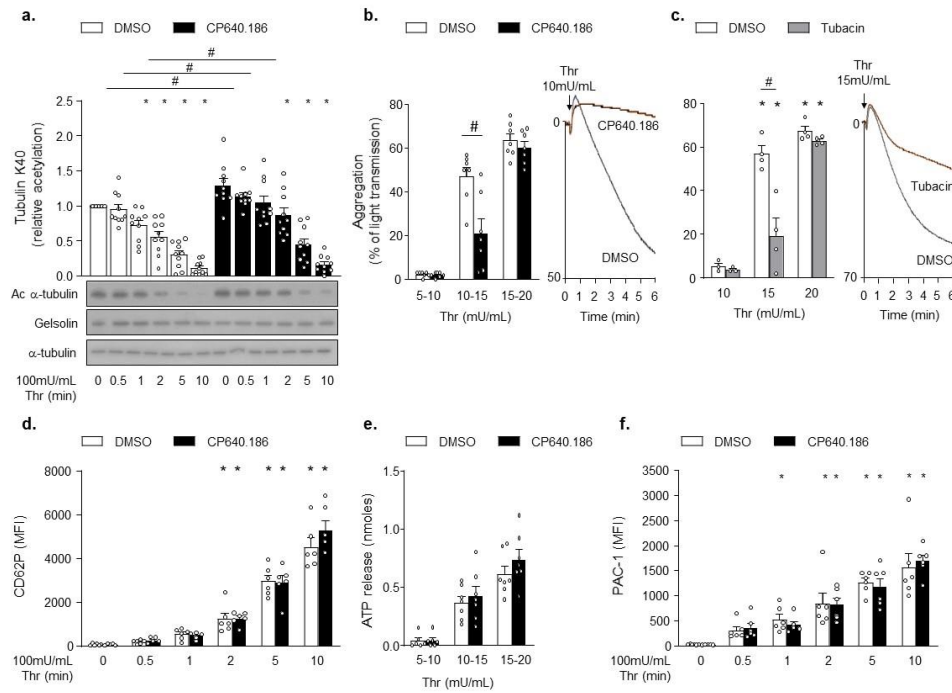


Figure 2. CP640.186-induced ACC inhibition increases tubulin acetylation levels and impairs platelet aggregation. (**a,b,d,f**) Washed human platelets were pre-incubated for 2 h with dimethyl sulfoxide (DMSO) or CP640.186 (60 μ M) or (**c**) for 1 h with DMSO or tubacin (10 μ M), before being stimulated with thrombin (Thr). (**a**) Platelets were stimulated with 100mU/mL Thr at different time points. Whole platelet lysates were subjected to western blot analysis and probed with acetyl α -tubulin (Ac α -tubulin), α -tubulin, or gelsolin antibodies. Data were expressed as means $\pm$ SEM (n = 10); * p-value $\leq$ 0.05 in relation to unstimulated conditions. # p-value $\leq$ 0.05 in relation to DMSO conditions. Data were analyzed using 2--way ANOVA (analysis of variance). (**b,c**) Aggregation was analyzed by turbidimetry (Chrono-Log) in platelets stimulated with various Thr concentrations. Profile is shown on the right. Data were expressed as means $\pm$ SEM (n = 7). # p-value $\leq$ 0.0001 in relation to DMSO conditions. Data were analyzed using 2--way ANOVA. (**d**) P-selectin (CD62P) exposure was analyzed, by flow cytometry, in platelets stimulated with 100mU/mL Thr at various time points. Data were expressed as means $\pm$ SEM (n = 6). * p-value $\leq$ 0.05 in relation to unstimulated conditions. Data were analyzed using 2--way ANOVA. (**e**) Dense granule secretion was assessed following addition of luciferase-luciferin reagent in platelets stimulated with various Thr concentrations. Data were expressed as means $\pm$ SEM (n = 7). Data were analyzed using 2--way ANOVA. (**f**) Platelets were stimulated with 100mU/mL Thr at various time points, while α IIb β 3 activation (PAC-1) was detected by flow cytometry. Data were expressed as means $\pm$ SEM (n = 6). * p-value $\leq$ 0.05 in relation to unstimulated condition. Data were analyzed using 2--way ANOVA.

2.3. *CP640.186 Does Not Affect Platelet Microtubule Dynamics*

Given the impact of α -tubulin acetylation on microtubule dynamics in resting and collagen-related peptide (CRP)-activated platelets [232], we postulated that an altered microtubule structure could explain the effect of CP640.186 on thrombin-induced platelet aggregation. To assess the impact of CP640.186-induced tubulin acetylation on microtubules, washed human platelets were treated with or without the ACC inhibitor; then, they were fixed, plated onto fibrinogen-coated coverslips, and incubated with specific antibodies targeting both α -tubulin and acetylated α -tubulin (K40) (**Figure 3a**). In DMSO-treated platelets, the marginal band was observed as a continuous ring structure, which was not altered by CP640.186 pre-incubation (**Figure 3a**; white arrow). Next, we examined tubulin condensation in platelets plated onto fibrinogen-coated coverslips for 10 min, with or without thrombin, then fixed and stained as described above. In DMSO- and CP640.186-treated platelets, the sole activation by fibrinogen induced the coiling of the microtubule ring, in parallel of α -tubulin deacetylation (**Figure 3b**). Thrombin-induced platelet activation further promoted the microtubules' remodeling by inducing their depolymerization and formation of a new flat and de-acetylated ring at platelet periphery, in addition to the small one observed in the platelet center (**Figure 3c**, white arrow). The degree of platelet α -tubulin condensation did not differ between DMSO- and CP640.186-treated platelets (**Figure 3d,f**), indicating that ACC inhibition and the subsequent increase in α -tubulin acetylation did not result in obvious tubulin cytoskeleton changes in resting and thrombin-stimulated platelets. These conclusions were supported by results showing that tubacin, which drastically increases tubulin acetylation, did not alter the dynamics of tubulin organization either in resting or thrombin-activated platelets (**Figure S2**).

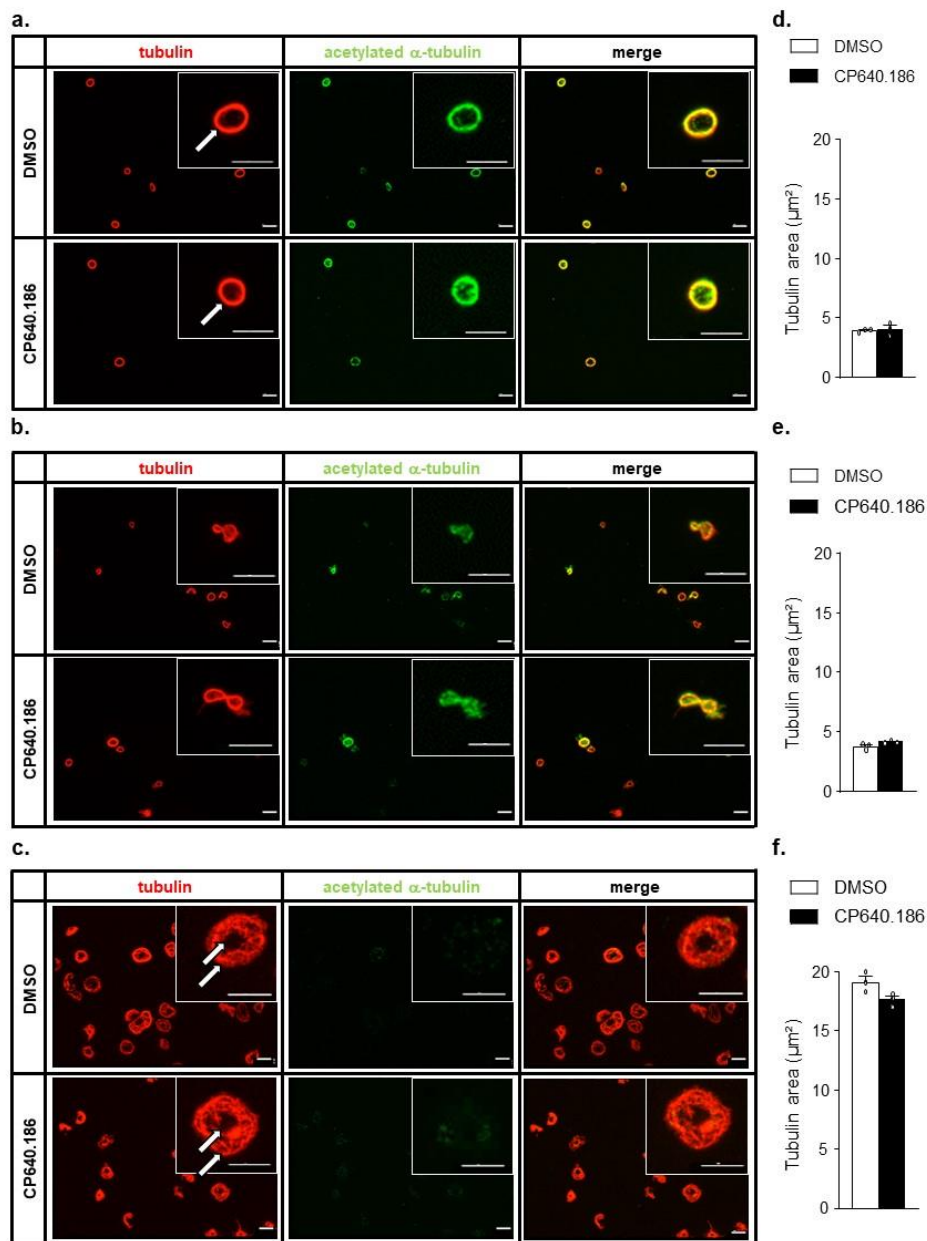


Figure 3. CP640.186-induced ACC inhibition does not affect microtubule dynamics. (a–f) Washed human platelets were pre-incubated for 2 h with dimethyl sulfoxide (DMSO) or CP640.186 (60 μM) before being added to fibrinogen-coated coverslips for 10 min. (a) Unstimulated platelets were fixed directly using 1% PFA, before being added to coverslips. (b) Platelets were added to coverslips before being fixed with 1% PFA. (c) Platelets were stimulated with 100mU/mL thrombin and then added to coverslips before being fixed with 1% PFA. (a–c) Platelets were stained using tubulin (red) or acetyl α -tubulin (green) antibodies. Representative pictures are shown. Microtubule rings are indicated by white arrows. Scale bar: 5 μm . (d–f) Quantification of tubulin area. Data were expressed as means $\pm$ SEM ($n = 3$). Data were compared using unpaired t-test.

2.4. CP640.186 Reduces the Activation of Rac1-PAK2 Pathway in Response to Thrombin, without Affecting Actin Cytoskeleton Remodeling

Next, we hypothesized that the increased acetylation state of platelet α -tubulin could regulate specific signaling pathways activated downstream of protease-activated receptor 1 (PAR1) or PAR4, and that this mechanism could contribute to the partial disruption of platelet aggregation induced by ACC or HDAC6 inhibition. Aslan et al. previously reported that HDAC6 influences signaling upstream of the Rho GTPase Rac and its effector, p21-activated kinase (PAK) [232]. Both proteins likely appear to be critical players in platelet activation downstream of PAR receptors [47]. Importantly, Rac1 and PAK2 have previously been implicated in regulating actin polymerization in platelets, MKs, [47, 255] and other cell types [256-258]. We sought to better understand whether this pathway's downregulation could contribute to the reduced aggregation as observed in CP640.186-treated platelets. To address this issue, we investigated the CP640.186 effect on Rac1 activation, PAK2 phosphorylation, and actin polymerization in thrombin-stimulated platelets. Thrombin caused a rapid and sustained activation of Rac1, with maximal activity reached within 30 s (**Figure 4a**). Thrombin-induced Rac1 activity was reduced in CP640.186-treated platelets (**Figure 4a**), as was PAK2 phosphorylation (**Figure 4b**). Comparable results were obtained with tubacin (**Figure 4c,d**). Since the Rac1-PAK pathway tightly regulates actin cytoskeletal polymerization, we investigated the CP640.186 effect on the phosphorylation state of cofilin, which represents a downstream cytoskeletal target that is involved in actin turnover regulation. In platelets, a rapid first phase of cofilin de-phosphorylation/activation is detected, which is then followed by a second phase consisting of LIM domain kinase 1 (LIMK1)-mediated slow cofilin re-phosphorylation/inactivation [45, 54]. As seen in **Figure 4e**, CP640.186 pre-incubation did not influence the cofilin de-phosphorylation/phosphorylation cycle in response to thrombin. Accordingly, thrombin-induced F-actin formation was not altered in CP640.186-treated platelets (**Figure 4f**). To confirm that actin cytoskeleton was not

impacted by ACC inhibition, nor by the subsequent alteration of thrombin-induced Rac1-PAK2 signaling, we additionally examined filopodia, lamellipodia, and stress fiber formation within platelets plated onto fibrinogen-coated coverslips and stimulated with thrombin for 15 min. Under thrombin stimulation, most platelets were fully spread and actually formed stress fibers. CP640.186 treatment did not change the proportion of platelets containing filopodia, lamellipodia, or stress fibers (**Figure S3**). Again, the impact of tubacine on cofilin phosphorylation and actin polymerization/organization was comparable to that of CP640.186 (**Figure S4**). This observation clearly supports that, under these experimental conditions, the inhibitory effect of CP640.186 on platelet aggregation is most likely due to the increased α -tubulin acetylation level without being caused by altered actin cytoskeleton remodeling.

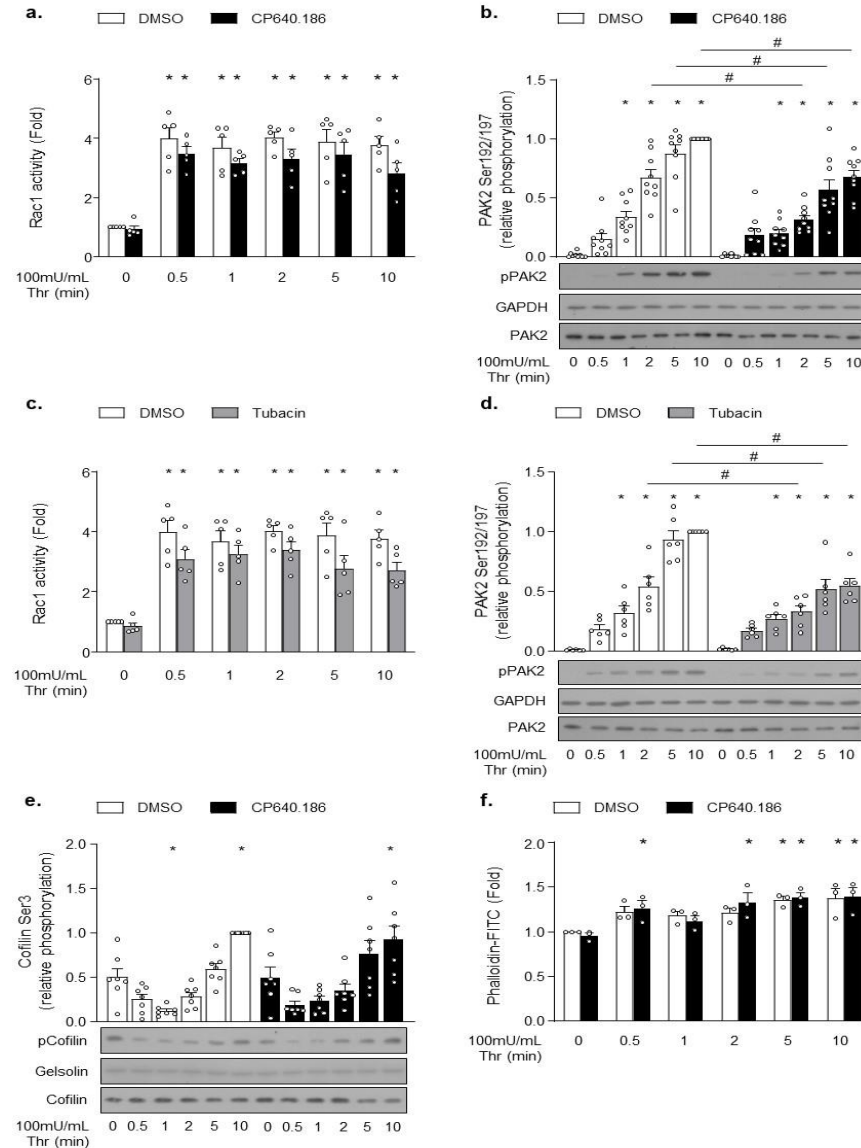


Figure 4. CP640.186-induced ACC inhibition reduces the Rac1-PAK2 pathway activation without impacting actin polymerization upon thrombin stimulation. (a,b,e,f) Washed human platelets were pre-incubated for 2 h with dimethyl sulfoxide (DMSO) or CP640.186 (60 μ M) or (c,d) for 1 h with DMSO or tubacin (10 μ M), before being stimulated with thrombin (100 mU/mL) at different time points. (a,c) Rac1 activity was assessed by means of G-LISA in whole platelet lysates. Data were expressed as means $\pm$ SEM (n = 5). * p-value $\leq$ 0.01 in relation to unstimulated conditions. Data were analyzed using 2-way ANOVA. (b,d,e) Whole platelet lysates were subjected to western blot and probed using phosphoPAK, PAK2, GAPDH, phosphoCofilin, Cofilin, or gelsolin antibodies. Data were expressed as means $\pm$ SEM (at least n = 7). * p-value $\leq$ 0.05 in relation to unstimulated condition. # p-value $\leq$ 0.05 in relation to DMSO conditions. Data were analyzed using 2-way ANOVA (analysis of variance). (f) Platelets were stained with FITC-conjugated phalloidin (10 μ M) for 1 h. F-actin content was analyzed by means of flow cytometry. Data were expressed as means $\pm$ SEM (n = 3). * p-value $\leq$ 0.05 in relation to unstimulated conditions. Data were analyzed using 2-way ANOVA.

2.5. CP640.186 Alters Thrombin-Induced ROS Production and ERK Phosphorylation

Rac1 and PAK likely affect downstream signaling events that are unrelated to actin cytoskeleton. For instance, Rac1 is an integral part of ROS generation by NADPH oxidase isoforms NOX1 and NOX2 [50, 259]. To test whether ACC inhibition did alter ROS production, platelets were incubated with 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) probe. Consistent with Rac1's role in NOX2 activation, CP640.186-treated platelets exhibited a significant decrease in basal and thrombin-induced ROS production (**Figure 5a**). Similar results were obtained in tubacin-treated platelets (**Figure S5a**). PAK also displays non-cytoskeletal substrates, as demonstrated by the mitogen-activated protein kinase (MAPK) activation through phosphorylation and activation of MEK proteins [46, 47]. Accordingly, the downstream MEK-driven phosphorylation of ERK was reduced in CP640.186-treated platelets following thrombin stimulation (**Figure 5b**). However, under these experimental conditions, we were unable to reveal any tubacin impact on ERK phosphorylation (**Figure S5b**).

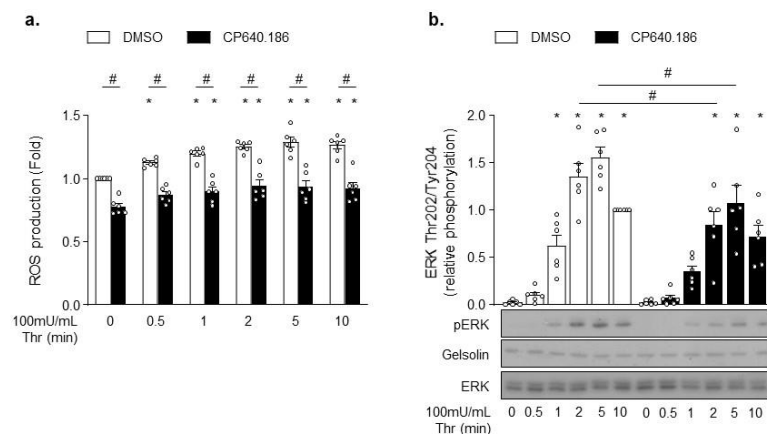


Figure 5. CP640.186 decreases thrombin-induced reactive oxygen species (ROS) production and ERK phosphorylation. **(a,b)** Washed human platelets were pre-incubated for 2 h with dimethyl sulfoxide (DMSO) or CP640.186 (60 μ M) before being either stimulated or not with thrombin (100 mU/mL) at different time points. **(a)** Reactive oxygen species (ROS) were detected by means of flow cytometry using H₂DCFDA (10 μ M) probe. Data were expressed as means $\pm$ SEM (n = 6). * p-value $\leq$ 0.05 in relation to unstimulated conditions. # p-value $\leq$ 0.05 in relation to DMSO conditions. Data were compared using 2-way ANOVA (analysis of variance). **(b)** Whole platelet lysates were subjected to western blot and probed with phosphoERK, ERK, or gelsolin antibodies. Data were expressed as means $\pm$ SEM (n = 6). * p-value $\leq$ 0.05 in relation to unstimulated conditions. # p-value $\leq$ 0.05 in relation to DMSO conditions. Data were analyzed using 2-way ANOVA.

3. Discussion

ACC is a key enzyme of lipid synthesis and oxidation pathways, rendering it an attractive target for various metabolic diseases [204, 205, 208, 209]. Over the last years, several ACC inhibitors have entered various stages of clinical testing or have already been approved for clinical use [260]. Moreover, this new class of therapeutics has been stated to share unexpected clinical implications, including the potential to lower platelet counts in humans and nonhuman primates [207]. Regarding platelet functions, the potential impact of these molecules remains elusive. In this study, we examined in vitro the impact of ACC inhibition on platelet activation, while investigating the underlying mechanisms. Using washed human platelets, we have demonstrated for the first time that a short-term incubation with CP640.186, which is a nonselective, reversible, and ATP-noncompetitive inhibitor of ACC1/2 [261], increases α -tubulin acetylation levels in both resting states and under thrombin-stimulated conditions. This was associated with impaired platelet aggregation. The molecular mechanism connects α -tubulin acetylation to the down-regulation of Rac1-PAK2 signaling and decreased ROS production.

The first publications reporting a beneficial impact of ACC inhibition on various metabolic diseases originated from studies investigating the long-chain fatty acid analog, 5-(tetradecyloxy)-2-furoic acid (TOFA) [262]. However, the similarity of TOFA to various long-chain fatty acids raised the issue as to the specificity of this drug with respect to ACC, and this in relation to the inhibition of other long-chain fatty acyl-CoA-utilizing enzymes [261, 263]. CP640.186 binds ACC within the carboxyltransfer domain, and the agent has been identified by Pfizer researchers through high-throughput screening [261]. Its binding site appears to be distinct from that found on other enzymes that catalyze carboxyltransfer reactions, including pyruvate carboxylase and propionyl-CoA carboxylase, which renders this drug highly specific [222]. This compound was shown to inhibit DNL in hepatic cells [261]. In human platelets, CP640.186 treatment for a maximum of 2 h was associated with DNL decreases without

drastically affecting overall platelet lipid composition. Several lipid species among TAG, LPC, and LPE classes were even found to be increased. Similar paradoxical findings have been reported in neuroblastoma cells in which TOFA-induced DNL inhibition resulted in an elevation of long chain fatty acids (tetracosanoic acid [C24:0]) and of some sphingomyelins, as well [46]. Regarding fatty acid utilization to produce ATP, our results clearly showed that mitochondrial respiration is not affected by pharmacological ACC inhibition.

In an earlier study, a lower ACC1 expression in yeast was demonstrated to be associated with the hyperacetylation of several proteins, including histones and cytoplasmic proteins that are involved in metabolism [237]. More recently, the impact of ACC inhibition on protein acetylation was found to be linked to cancer cells' metastatic capacity [239]. The present study is the first to connect ACC inhibition and protein acetylation within human platelets. The results clearly showed CP640.186, similarly to HDAC6-specific inhibitor tubacin, to be able to limit thrombin-induced deacetylation of tubulin, thereby inhibiting platelet aggregation. The acetylation of α -tubulin has been recognized to play a key role in microtubule organization and stability [232, 264, 265]. However, following the immobilization of CP640.186-treated platelets on fibrinogen-coated coverslips, we were unable to identify any treatment impact on the marginal band of resting platelets, nor on their ability to condense upon fibrinogen and thrombin stimulation. Platelet spreading and morphology were also preserved. These results are supported by similar observations reported in tubacin-treated human platelets or in platelets isolated from HDAC6 knockout mice [231, 245]. In contrast, Aslan et al. did not reach the same conclusions. Indeed, based on the visualization of tubacin-treated human platelets using super-resolution structured illumination microscopy, these authors described a disrupted marginal band at resting state, along with a reduced ability to condense upon CRP stimulation [232].

Additionally, CP640.186 and tubacin were shown to reduce PAK2 phosphorylation downstream of Rac1 GTPase activation, although the molecular link between microtubule acetylation and Rac1 is still poorly understood. In neurons, it has been

proposed that p120 catenin associates with and stabilizes microtubules, which promotes Rac1 activation and neurite outgrowth [266]. Interestingly, p120-catenin is present in platelets and likely influences Rho-GTPases activity and key signaling molecules involved in platelet aggregation, including E-cadherin [267]. The potential contribution of p120-catenin to the mechanism by which CP640.186 regulates Rac1 in platelets warrants further investigations. Once activated, Rac1 is able to modulate different enzymatic activities. For instance, Rac1 plays a crucial role in activating Nox family NADPH oxidase enzymes dedicated to ROS production, including superoxide anion radical or hydrogen peroxide. NOX2 is activated in a Rac1-dependent manner. In the GTP-bound form, Rac1 directly binds to the oxidase activator p67 (PHOX), which in turn interacts with NOX2, leading to superoxide anion production [50]. Consistent with the CP640.186 effect on Rac1 activity, ROS generation was significantly decreased in response to thrombin stimulation.

PAK mediates the phosphorylation of a plethora of proteins involved in actin cytoskeleton, such as LIMK1, merlin, or myosin light chain kinase (MLCK) [257]. In platelets, the Rac1-PAK signaling cascade has been shown to regulate actin polymerization via LIMK1-cofilin phosphorylation and lamellipodia formation [46, 47, 268]. However, we did not observe any differences in thrombin-induced cofilin (de)phosphorylation, shape alterations, or F-actin content between DMSO- and CP640.186-treated platelets. Accordingly, platelets from HDAC6 KO mice displayed normal platelet shape changes, which let us assume that acetylated α -tubulin is not implicated in the regulation of actin cytoskeleton [231, 245]. Interestingly, PAK is able to activate substrates other than proteins regulating cytoskeleton organization. For instance, ERK is rapidly phosphorylated in activated platelets. This phosphorylation notably depends on PAK [46, 47, 257], and it has been shown to play a role in platelet aggregation and thrombus formation in a laser-injured subcutaneous arteriole model [269, 270]. Mechanistically, ERK activation has been reported to constitute a key signaling event designed to expose on platelet surface adhesion molecules that are necessary for platelet tethering. The latter constitutes the first step of platelet

aggregation [269]. The impaired thrombin-induced aggregation of CP640.186-treated platelets possibly results from a similar mechanism. Moreover, our data are in line with studies showing that tubacin-induced increases in α -tubulin acetylation levels were associated with a decrease in CRP-induced ERK phosphorylation [232, 271]. We failed to detect any significant decrease in ERK phosphorylation in thrombin-stimulated, tubacin-incubated platelets. This observation suggests that ACC and HDAC6 inhibitions likely exhibit different downstream effects. A limitation of our study is that we only correlate α -tubulin acetylation with the inhibition of platelet aggregation and Rac1/PAK2 signaling. We cannot exclude that other acetylated proteins may contribute to the effects of both CP640.186 and tubacin.

In summary, our data demonstrate that pharmacological ACC inhibition is able to impair thrombin-induced platelet aggregation, an effect which is associated with an increase in α -tubulin acetylation levels. The underlying mechanisms involve a downregulation of the Rac1-PAK2 signaling pathway, which is associated with a decrease in ROS production and ERK phosphorylation. Our work adds momentum to the emerging notion that precautions would be needed for studies using ACC inhibitors in Type 2 diabetes, cancer, or nonalcoholic steatohepatitis. Reducing platelet activation may likewise exert positive effects, notably in some inflammatory or thrombotic disorders. Future research investigating in vivo the impact of DNL inhibitors on platelet functions are now needed, given that new molecules are currently entering clinical development.

4. Materials and Methods

4.1. CP640.186 Alters Thrombin-Induced ROS Production and ERK Phosphorylation

CP640.186 (#17691) was purchased from Cayman Chemical (Ann Arbor, Michigan, United States of America [USA]). Thrombin from bovine plasma (#T6634), apyrase (#A6132), fibrinogen (#F4129), fluorescein isothiocyanate (FITC)-conjugated phalloidin (#P5282), sigmacote (#SL2), Dulbecco's Modified Eagle's Medium (DMEM, #D5030), prostaglandin E1 (PGE1) (#P5515), and horse radish peroxidase (HRP)- conjugated anti-rabbit (#A0545) antibodies (Abs) were purchased from Sigma-Aldrich (Overijse, Belgium). [1-¹⁴C]-Acetic Acid was obtained from Perkin Elmer (#NEC084H001MC) (Zaventem, Belgium). S-MONOVETTE CPDA tube (#11610001) was from Sarstedt (Etten-Leur, The Netherlands). Eptifibatide was obtained from GlaxoSmithKline (Wavre, Belgium). Bovine serum albumin (BSA) (#3854.4) was purchased from Roth (Keerbergen, Belgium). Fatty acid-free BSA (#126575) was from Millipore (Overijse, Belgium). Anti-tubulin antibody (#62204) and H₂DCFDA probe (#D-399) were purchased from Thermo-Fisher Scientific (Waltham, MA, USA). Complete, Mini Protease Inhibitor Cocktail (#11836153001) was obtained from Roche (Brussels, Belgium). Luciferin-luciferase reagent (#CH395) was from Stago BNL (Mont-Saint-Guibert, Belgium), and Cell Tak (#354240) was from Corning (Lasne, Belgium). Oligomycin, FCCP, antimycin-A, and rotenone were from Agilent (Mito Stress Test Kit, #103015-100) (Santa Clara, CA, USA). Rac1 G-LISA kit (#BK128) was from Cytoskeleton, Inc, (Denver, CO, USA). Anti-phospho-cofilin (#3313), anti-cofilin (#5175), anti-phospho-ERK (Thr202/Tyr204) (#9101), anti-ERK (#9102), anti-phospho-PAK (Ser199/204; Ser 192/197) (#2605), anti-PAK2 (#2615), anti-acetyl- α -tubulin (#5335), anti-GAPDH (#2118), anti-rabbit IgG HRP-linked (#7074), and anti-gelsolin (#12953) antibodies were obtained from Cell Signaling (Danvers, MA, USA). Phycoerythrin-conjugated anti-CD62P (#555524), FITC-conjugated anti-active α IIb β 3 (PAC-1) (#340507), HRP-conjugated anti-mouse (#554002), donkey anti-mouse IgG coupled with alexa fluor 594 (#A-21203), and donkey anti-rabbit IgG

coupled with alexa fluor 488 (#A-21206) antibodies were purchased from BD Bioscience (San Jose, CA, USA).

4.2. *Human Platelets Preparation*

Venous blood was taken from human healthy volunteers (without drug intake one week before blood sampling) having provided their consent in citrated tubes (1/10; citrate, phosphate, dextrose, adenine). Platelet rich plasma (PRP) was obtained by centrifugation at 330 x g for 20 min. After adding eptifibatide (4 µg/mL) and apyrase (1 U/mL), platelets were pelleted by a centrifugation at 800x g for 10 min. Platelets were washed once with a modified Tyrode's buffer (135 mM NaCl, 12 mM NaHCO₃, 3 mM KCl, 0.3 mM Na₂HPO₄, 1 mM MgCl₂, 5 mM D-glucose, 10 mM Hepes, and 0.35% BSA, pH 7.4, 37°C) containing eptifibatide (4 µg/mL) and apyrase (1 U/mL) at 1000x g for 10 min. Platelets were re-suspended in modified Tyrode's buffer to a density of 2.5 x 10⁵/µL (unless said otherwise). Washed platelets were incubated 2 h at 37°C with CP640.186 before platelet function assays.

4.3. *Lipogenesis Measurement*

Platelets were re-suspended in a modified Gaintner buffer (4.92 mM KH₂PO₄, 40 mM NaH₂PO₄.H₂O, 102.7 mM NaCl, 40 mM NaOH, and 5.5 mM D-glucose, pH 6.5, 37°C) at a density of 6.5 x 10⁵/µL. Washed platelets (6.5 x 10⁸) were incubated for 2 h at 37°C in a solution including 1mL of modified Gaintner buffer, 4.15 mM acetate, and 4.415 µCi/µmol [1-¹⁴C]-acid acetic. To stop the reaction, samples were centrifuged at 2500x g for 10 min at 4°C. After two washings, lipids were extracted with methanol/chloroform (1:2 v/v). The addition of NaCl 0.9% enabled the formation of hydrophilic (upper) and hydrophobic (lower) parts. The hydrophobic part was washed three times (1000x g, 10 min, 25°C) before being added to scintillation vials. Lipids were concentrated via nitrogen flow. Radioactivity was measured using a scintillation counter (Tri-Carb 2800TR, PerkinElmer).

4.4. *Lipidomics Analysis*

The platelets were re-suspended in a modified Tyrode's buffer with 0.35% free fatty acid BSA at a density of $10 \times 10^5/\mu\text{L}$. Washed platelets (1.5×10^9) were centrifuged at $11,700 \times g$ for 15 sec before being frozen. Lipid extraction and a lipidomics analysis were carried out, as previously described [57].

4.5. *Extracellular Flux Analysis of Mitochondrial Respiration*

PRP was centrifuged at 800g for 10 min in the presence of PGE1 (100 ng/mL) and apyrase (1 U/mL). Platelet pellets were washed in modified Tyrode's buffer without BSA containing apyrase (1 U/mL) and PGE1 (100 ng/mL). Washed platelets were re-suspended in modified DMEM's medium (DMEM, 143mM NaCl, 0.5% phenol red, 5 mM D-glucose, 1mM sodium pyruvate, and 4 mM L-glutamine, pH 7.35) at a density of $2 \times 10^6/\mu\text{L}$ and incubated for 30 min at 37°C with CP640.186. The 96-well plate was coated with 150 $\mu\text{g/mL}$ "cell tak" solution (diluted in 0.1 M NaHCO_3 , pH 8.0) for 20 min at room temperature (RT) before being washed with distilled water. Overall, 15×10^6 platelets were added to the 96-well plate, and their adhesion was induced by two centrifugations at RT ($148 \times g$ for 1 min and $213 \times g$ for 1 min). The 96-well plate was incubated for 1 h 15 min in a CO_2 -free incubator before the assay. After basal oxygen consumption rate (OCR) measurement, 1 μM oligomycin, 0.45 μM FCCP, and 1 μM rotenone/antimycin A mix were sequentially injected into the wells every 12 min. OCR was measured every 4 min using Seahorse extracellular flux XFp (Biosciences). Mitochondrial functional parameters were assessed via basal OCR, which was estimated by subtracting rotenone/antimycin A OCR from basal OCR, ATP-linked OCR, calculated by subtracting OCR after oligomycin from baseline, maximal OCR, reserve capacity, evaluated by subtracting basal OCR from OCR after FCCP, non-mitochondrial OCR, obtained after rotenone/antimycin A injection and proton leak, calculated by subtracting rotenone/antimycin A OCR from OCR after oligomycin.

4.6. *Western Blotting*

Washed human platelets (5×10^7) were stimulated with 100 mU/mL thrombin for the indicated time points in the presence of 2 mM Ca^{2+} before being lysed in modified blue Laemmli's buffer (50mM Tris pH 6.8, 10% [w/v] glycerol, 2% [w/v] SDS, 0.5mM EDTA, 0.01% [w/v] bromophenol blue, and 25mM DTT). Whole platelet lysates were subjected to western blot. All antibodies were diluted in 5% BSA. The dilution for primary antibodies were (1/1000) except for anti-acetyl- α -tubulin (1/4000), anti-tubulin (1/10,000), anti-phosphocofilin (1/10,000), anti-cofilin (1/20,000), anti-phospho-ERK (1/100,000), anti-ERK (1/2000), anti-gelsolin (1/50,000), and anti-GAPDH (1/100,000). Secondary HRP-conjugated anti-rabbit antibodies from Cell Signaling were diluted (1/5000), as were HRP-conjugated anti-rabbit and anti-mouse antibodies from Sigma (1/20,000). Band intensities were normalized to loading controls on the same gel. The respective total protein analyses were performed on different gels. Image J was applied to quantify all bands.

4.7. *Platelet Aggregation and ATP Secretion*

Aggregation and dense granules secretion were assessed using an aggregometer (Chrono-log 700, STAGO). Washed platelets (52.5×10^6) were constantly stirred at 1200 rpm, 37°C. Light transmission was recorded after CaCl_2 (2 mM) addition and thrombin stimulation. To analyze ATP secretion, a luciferase/luciferin mix was added, which was followed by ATP standard.

4.8. *Flow Cytometry Analysis*

For CD62P exposure and $\alpha\text{IIb}\beta_3$ activation, PGE1 (100 ng/mL) and apyrase (1 U/mL) were added to PRP before being centrifuged at 800x g for 10 min. Platelets were washed in a modified Tyrode's buffer with 1.5% BSA. Washed platelets (6.25×10^6) were incubated with anti-CD62P and PAC-1 antibodies (1/14.5) for 20 min at RT. Platelets were stimulated with thrombin 100 mU/mL at the indicated time points at 37°C before being fixed with paraformaldehyde (PFA) 4%. For ROS production, washed platelets

(6.75×10^6) were resuspended in a modified Tyrode's buffer without BSA and incubated with $10 \mu\text{M}$ H_2DCFDA probe for 30 min at 37°C . Platelets were washed two times with a modified Tyrode's buffer without BSA containing apyrase (1 U/mL). Re-suspended platelets were stimulated with 100 mU/mL thrombin at different time points. The reaction was stopped by diluting platelets 14X with the modified Tyrode's buffer without BSA. For F-actin content, washed platelets (6.25×10^6) were re-suspended in the modified Tyrode's buffer with 1.5% BSA. Re-suspended platelets were stimulated with 100 mU/mL thrombin at different time points at 37°C before being fixed with 4% PFA. Platelets were permeabilized with 0.1% Triton and stained with FITC-conjugated phalloidin ($10 \mu\text{M}$) for 1 h at RT. Platelets were washed two times with phosphate buffer saline (PBS). All samples were analyzed using BD Canto II flow cytometer, and 10,000 events were recorded. As necessary, appropriate compensation controls were included.

4.9. Platelet Spreading and Immunofluorescence Staining

Washed platelets were re-suspended at a density of $3 \times 10^4/\mu\text{L}$. Coverslips were coated with fibrinogen ($100 \mu\text{g/mL}$) overnight at 4°C , blocked with PBS-BSA (5 mg/mL) for 1 h at RT and then washed with PBS. Platelets (6×10^6) were either stimulated or not with 100 mU/mL thrombin in the presence of 2 mM CaCl_2 and were immediately placed on coverslips for 15 min (actin) or 10 min (tubulin) at RT. Unstimulated platelets were fixed directly with 1% PFA and then added to coverslips. Non-adherent platelets were removed using a PBS wash. For actin visualization, adherent platelets were fixed with 1% PFA for 30 min at RT, permeabilized with 0.1% Triton, and stained with FITC-conjugated phalloidin (1:300) for 45 min at RT. To visualize the microtubule cytoskeleton, adherent platelets were blocked 30 min at RT before being incubated with anti-tubulin (1/800) and anti-acetyl- α -tubulin (1/100) antibodies for 1 h at RT. Coverslips were washed with PBS; anti-rabbit IgG coupled with alexa fluor 488 (1/500) and anti-mouse IgG coupled with alexa fluor 594 (1/1000) antibodies were added for 1 h at RT. Platelets were visualized using a Zeiss ($\times 100$ oil immersion) fluorescence

microscope (Carl Zeiss) equipped with ApoTome (AxioImager). The area (actin) covered by adherent platelets were quantified by means of Visiopharm software. Tubulin area was quantified using Image J.

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Institutional Review Board Statement: Human studies were authorized by the 'Commission d'Ethique Biomédicale Hospitalo-Facultaire de l'Université catholique de Louvain'.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Lipidomics data have been provided in Supplementary Materials.

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Conflicts of Interest: The authors declare no conflict of interest.

6. Abbreviations

aA	Antimycin A	SM	Sphingomyelin
AA	Arachidonic acid	SIRT	Sirtuin
ACC	Acetyl-CoA carboxylase	TAG	Triacylglycerol
CE	Cholesteryl ester	Thr	Thrombin
CPT1	Carnitine palmitoyltransferase 1	TOFA	5-(tetradecyloxy)-2-furoic acid
CRP	Collagen-related peptide		
DAG	Diacylglycerol		
DMSO	Dimethyl sulfoxide		
DNL	<i>De novo</i> lipogenesis		
ERK	Extracellular signal-regulated kinase		
F	carbonyl cyanide p-trifluoromethoxyphenylhydrazone		
FFA	Free fatty acid		
FITC	Fluorescein isothiocyanate		
H ₂ DCFDA	2',7'-dichlorodihydrofluorescein diacetate		
HDAC	Histone deacetylase		
HRP	Horse radish peroxidase		
KAT	Lysine acetyltransferase		
LIMK1	LIM domain kinase 1		
LPC	Lysophosphatidylcholine		
LPE	Lysophosphatidylethanolamine		
MAPK	Mitogen-activated protein kinase		
MK	Megakaryocyte		
MLCK	Myosin light chain kinase		
NOX	NADPH oxidase		
O	Oligomycin		
OCR	Oxygen consumption rate		
PAK	p21-activated kinase		
PAR	Protease-activated receptor		
PBS	Phosphate buffer saline		
PC	Phosphatidylcholine		
PE	Phosphatidylethanolamine		
PEO	Plasmanyl phosphatidylethanolamine		
PEP	Phosphatidylethanolamine plasmalogen		
PFA	Paraformaldehyde		
PGE1	Prostaglandin E1		
R	Rotenone		
ROS	Reactive oxygen species		

7. Supplementary materials

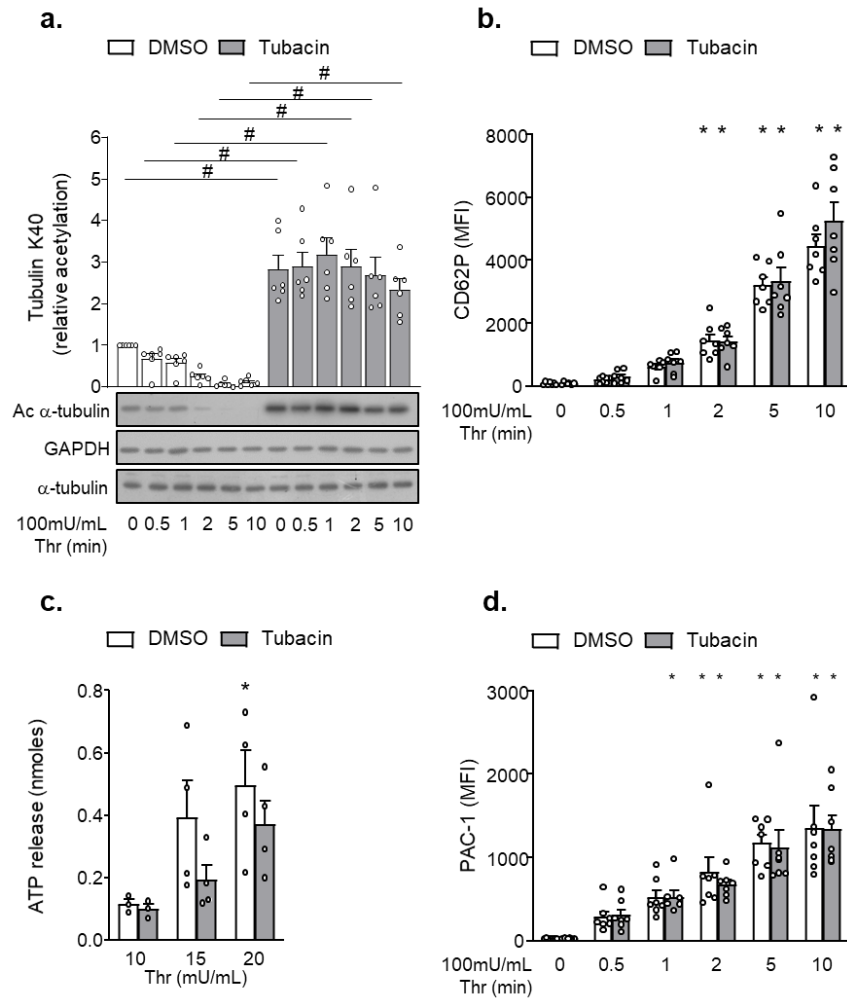


Figure S1. HDAC6 inhibition induced by tubacin increases a-tubulin acetylation level with no impact on granules secretion or α IIb β 3 activation upon thrombin stimulation.

(a-e) Washed human platelets were preincubated for 1 hour with DMSO or tubacin (10 μ M) before being stimulated with thrombin (Thr). (a) Platelets were stimulated with 100mU/mL Thr at different time points. Whole platelet lysates were subjected to western blot and probed with acetyl a-tubulin (Ac α -tubulin), a-tubulin or gelsolin antibodies. Data are expressed as means $\pm$ SEM (n = 6). # p-value $\leq$ 0.0001 relative to DMSO condition. Data were compared using 2-way ANOVA. (b) P-selectin (CD62P) exposure was analyzed by flow cytometry in platelets stimulated with 100mU/mL Thr at different time points. Data are expressed as means $\pm$ SEM (n = 7). * p-value $\leq$ 0.05 relative to unstimulated condition. Data underwent 2-way ANOVA. (d) Dense granules secretion was assessed via addition of luciferase-luciferin reagent in platelets stimulated with different Thr concentrations. Data are expressed as means $\pm$ SEM (n = 4). Data were compared using 2-way ANOVA. (e) Platelets were stimulated with 100mU/mL thrombin (Thr) at different time points and α IIb β 3 activation (PAC-1) was detected by flow cytometry. Data are expressed as means $\pm$ SEM (n = 7). * p-value $\leq$ 0.05 relative to unstimulated condition. Data were compared using 2-way ANOVA.

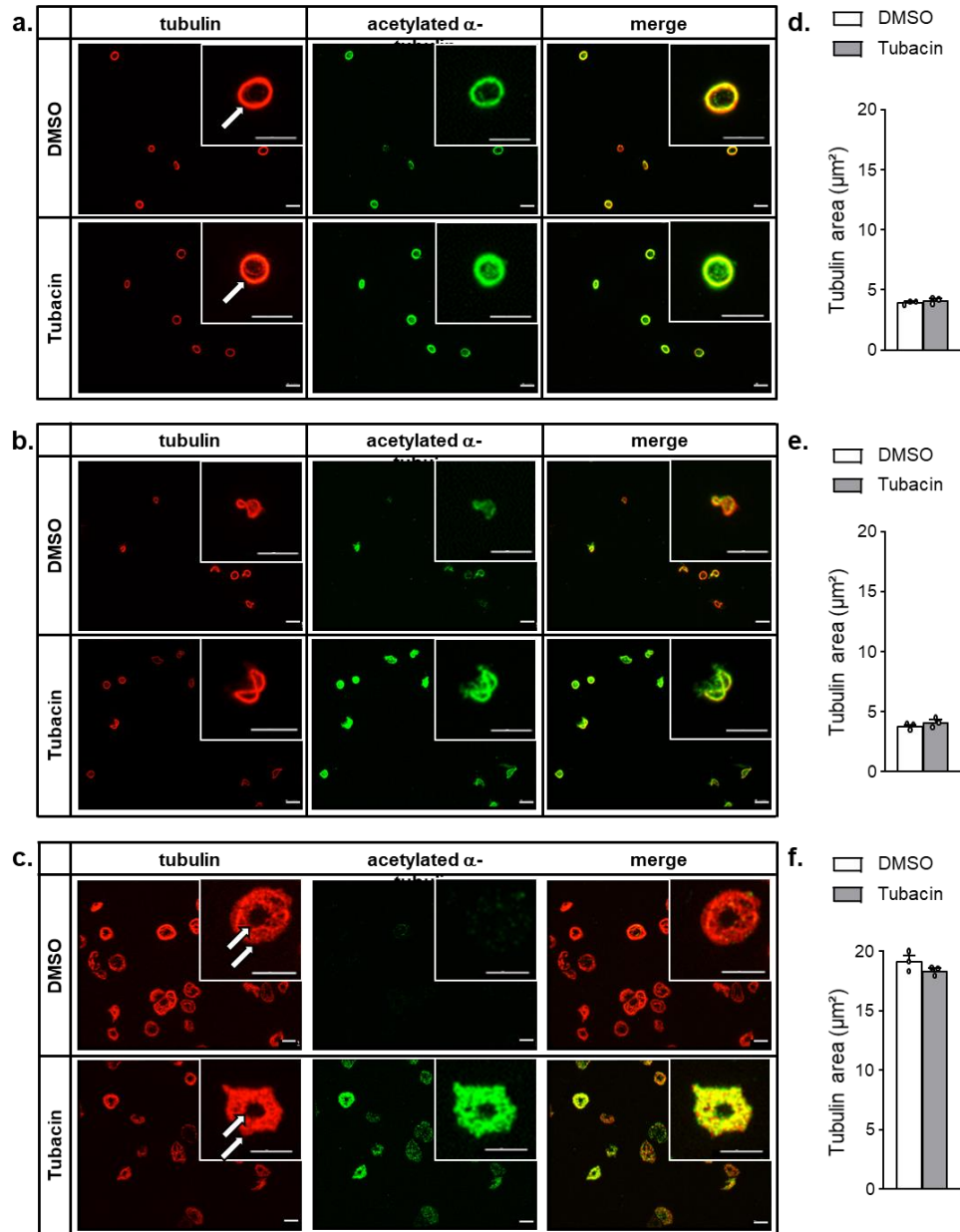


Figure S2. HDAC6 inhibition induced by tubacin does not affect microtubules organization.

(a-f) Washed human platelets were preincubated for 1 hour with DMSO or tubacin (10 μM) before being added to fibrinogen-coated coverslips for 10 min. (a) Unstimulated platelets were fixed directly with 1% PFA and then added to coverslips. (b) Platelets were added to coverslips before being fixed with 1% PFA. (c) Platelets were stimulated with 100mU/mL thrombin and then added to coverslips before being fixed with 1% PFA. (a-c) Platelets were stained with tubulin (red) or acetyl α -tubulin (green) antibodies. Representative pictures are shown. Microtubule rings are indicated by white arrows. Scale bar, 5 μm . (d-f) Quantification of tubulin area. Data are expressed as means $\pm$ SEM (n = 3). Data were compared using unpaired t-test.

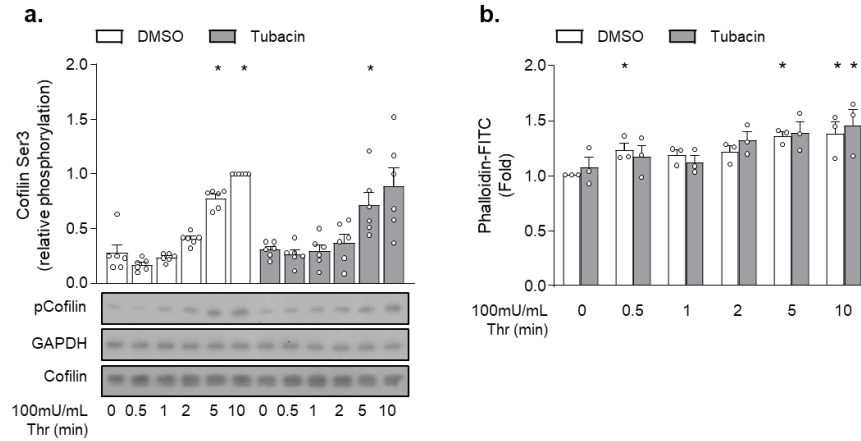


Figure S3. HDAC6 inhibition induced by tubacin reduces the activation of Rac1-PAK2 pathway in response to thrombin but does not affect actin polymerization.

(a-d) Washed human platelets were preincubated for 1 hour with DMSO or tubacin (10 μ M) before being stimulated with thrombin (Thr) (100mU/mL) at different time points. (a) Whole platelet lysates were subjected to western blot and probed with phosphoCofilin, Cofilin or GAPDH antibodies. Data are expressed as means $\pm$ SEM (n = 6). * p-value $\leq$ 0.05 relative to unstimulated condition. Data were analyzed using 2-way ANOVA. (b) Platelets were stained with FITC-conjugated phalloidin (10 μ M) for 1 hour. F-actin content was analyzed by flow cytometry. Data are expressed as means $\pm$ SEM (n = 3). * p-value $\leq$ 0.05 relative to unstimulated condition. Data were analyzed using 2-way ANOVA.

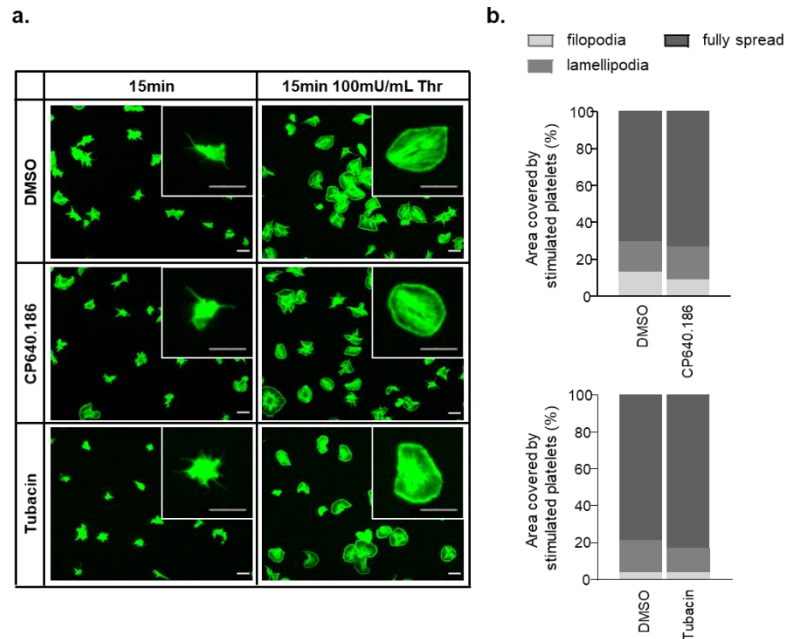


Figure S4. ACC or HDAC6 inhibition does not impact actin cytoskeleton remodeling.

(a-b) Washed human platelets were preincubated for 2 h with DMSO or CP640.186 (60 μ M) or 1 h with tubacin (10 μ M) (a; left panel) without thrombin (Thr) or (a; right panel) after Thr stimulation (100mU/mL) for 15 minutes. Platelets were stained with phalloidin-FITC for 45 min. (a) Representative pictures are shown. Scale bar, 5 μ m. (b) Quantification of area covered by stimulated platelets expressing filopodia, lamellipodia or being fully spread. Data underwent 2-way ANOVA.

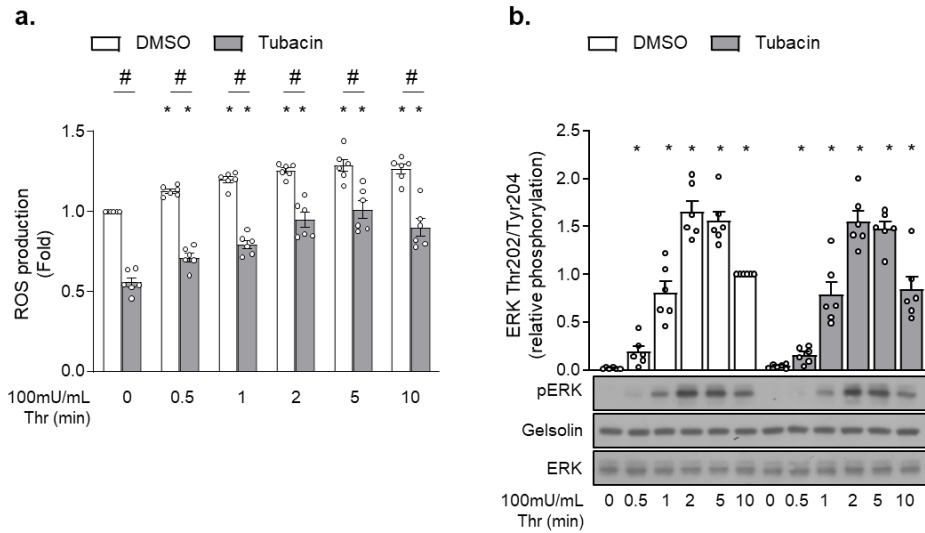


Figure S5. HDAC6 inhibition induced by tubacine decreases thrombin-induced reactive oxygen species (ROS) production but does not impact ERK phosphorylation.

(a-b) Washed human platelets were preincubated for 1 h with DMSO or tubacin (10 μ M) before being stimulated or not with thrombin (100mU/mL) at different time points. (a) Reactive oxygen species (ROS) were detected by flow cytometry using H₂DCFDA (10 μ M) probe. Data are expressed as means $\pm$ SEM (n = 6). * p-value $\leq$ 0.05 relative to unstimulated condition. # p-value $\leq$ 0.05 relative to DMSO condition. Data underwent 2-way ANOVA. (b) Whole platelet lysates were subjected to western blot and probed with phosphoERK, ERK or gelsolin antibodies. Data are expressed as means $\pm$ SEM (n = 6). * p-value $\leq$ 0.05 relative to unstimulated condition. Data underwent 2-way ANOVA.

PART2: Deletion of platelet ACC1 alters phosphatidylethanolamine plasmalogen level and limits platelet activation and thrombosis

The aim of this chapter was to evaluate the impact of platelet-specific ACC1 deletion on platelet lipid content, platelet functions and thrombus formation *ex vivo*.

In this article, we reported that:

- 1) Platelet-specific ACC1 deletion reduces platelet count and increases mean platelet volume
- 2) Platelet-specific ACC1 deletion results in higher polyunsaturated fatty acyl chain-containing phospholipid level compared to the low saturated fatty acyl chain level.
- 3) Platelet-specific ACC1 deletion results in a decrease in PEP content and especially, AA-containing PEP
- 4) ACC1 pKO platelets exhibit a slight decrease in thrombin-induced oxygen consumption rate and a reduction in ECAR, reflecting an altered glycolysis
- 5) ACC1 pKO platelets have a reduced thrombin and CRP-induced TxA₂ generation and dense granule secretion.
- 6) Platelet-specific ACC1 deletion results in a decreased thrombus formation over a collagen-coated surface under flow conditions.

Deletion of platelet ACC1 alters phosphatidylethanolamine plasmalogen level and limits platelet activation and thrombosis

Marie Octave¹, Laurence Piroton¹, Alice Marino¹, Z. Nagy², Martin Giera³, Jérôme Ambroise⁴, Marijke Kuijpers^{5,6}, Constance Baaten⁵, Johan W M Heemskerk⁵, Julien De Poortere¹, Audrey Ginion¹, Valentine Robaux¹, Davide Brusa⁷, Guillaume Courtoy⁸, Caroline Bouzin⁸, Bruno Guigas⁹, Luc Bertrand¹, Y. Senis¹⁰, Christophe Beauloye^{1,11}, Sandrine Horman^{1,*}

¹Pôle de Recherche Cardiovasculaire (CARD), Institut de Recherche Expérimentale et Clinique (IREC), Université catholique de Louvain (UCLouvain), Brussels, Belgium

²Institute of Experimental Biomedicine, University Hospital and Rudolf Virchow Center, University of Würzburg, Würzburg, Germany

³Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, The Netherlands

⁴Centre de Technologies Moléculaires Appliquées, Institut de Recherche Expérimentale et Clinique, UCLouvain, Brussels, Belgium

⁵Department of Biochemistry, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University, Maastricht, The Netherlands

⁶Thrombosis Expertise Centre, Heart and Vascular Centre, Maastricht University Medical Centre, Maastricht, the Netherlands

⁷IREC Flow Cytometry Platform, IREC, UCLouvain, Brussels, Belgium

⁸IREC Imaging Platform, IREC, UCLouvain, Brussels, Belgium

⁹Department of Parasitology, Leiden University Medical Center, Leiden, The Netherlands

¹⁰Institut National de la Santé et de la Recherche Médicale (INSERM), Etablissement Français du Sang Grand Est, Unité Mixte de Recherche (UMR) S1255, Fédération de Médecine Translationnelle de Strasbourg, Université de Strasbourg, Strasbourg, France

¹¹Division of Cardiology, Cliniques Universitaires Saint-Luc, Brussels, Belgium

*Corresponding author: Prof. Sandrine Horman, PhD; CARD; IREC; UCLouvain ; 55, Avenue Hippocrate, B-1200, Brussels, Belgium; sandrine.horman@uclouvain.be; +32 2 764 55 66

Abstract

We recently discovered that systemic activation of acetyl-CoA carboxylase (ACC) increases the platelet content of arachidonic acid (AA)-containing phosphatidylethanolamine plasmalogen (PEP) and influences platelet reactivity to collagen. We thus hypothesized that platelet ACC was a key enzyme in regulating platelet lipid levels and functions. Using platelet-specific ACC1 knockout mice (ACC1 pKO), we reveal that this enzyme is a tight regulator of saturated and polyunsaturated fatty acyl chain levels contained in platelet phospholipids. In particular, ACC1 pKO platelets have a decrease in AA-containing PEP content. These arachidonylated plasmalogens represent a main source of thromboxane and other eicosanoids, respectively involved in platelet activation and bioenergetics. Accordingly, we demonstrate that ACC1 deletion impairs mitochondrial respiratory capacity, thromboxane generation and dense granule secretion in activated platelets. This results in a reduced thrombus formation on collagen-coated surfaces under flow conditions. Collectively, our data demonstrate that ACC1 is essential to maintain specific phospholipid pools required for platelet energy and reactivity and might therefore constitute a novel potential target for pharmacological anti-thrombotic applications.

Keywords: acetyl-CoA carboxylase, collagen-related peptide, granule, phospholipid, platelet, thrombin, thromboxane, thrombus

1. Introduction

Blood platelets are essential for mediating hemostasis in response to injury [272]. Most of their functionalities largely depend on the plasma membrane composition which plays a pivotal role in membrane fluidity and curvature, platelet shape change, and generation of bioactive signaling molecules [131, 140, 181, 273, 274]. As such, analysis of the lipidome is a valuable tool to better understand platelet function and consequently, several lipidomic studies have been undertaken, on either resting or stimulated platelets, to characterize the global platelet lipid profile [8, 150, 165, 166, 181, 275]. Phospholipids are the dominant category of lipids in platelets and include phosphatidylcholine (PC; 37%), phosphatidylethanolamine (PE; 17%), phosphatidylserine (PS; 6%) and phosphatidylinositol (PI; 2%) [8]. These lipids are not only defined by their head groups but also by the length and saturation of their two fatty acyl chains [131]. Watanabe et al previously reported that major saturated fatty acids on platelet plasma membrane are palmitic acid (PAL, 16:0) and stearic acid (SA, 18:0), while oleic acid (OA, 18:1) and arachidonic acid (AA, 20:4) represent the dominant unsaturated fatty acids [276]. Interestingly, AA is particularly present in PE plasmalogen (PEP), a PE subclass characterized by a vinyl-ether bound at the sn-1 position of the glycerol backbone [131]. These arachidonylated plasmalogens are known to be selectively hydrolyzed by phospholipase A₂ (PLA₂) upon platelet agonist stimulation, which leads to AA release and lysophospholipid species formation [156, 158, 159]. Oxidation of AA by the cyclooxygenase and lipoxygenase pathways then yields key lipid mediators such as thromboxane A₂ (TxA₂) and other eicosanoids, respectively involved in platelet activation and bioenergetics [57, 166, 181].

Platelets have the ability to synthesize phospholipids and fatty acyls *de novo* [200, 277]. Acetyl-CoA carboxylase (ACC) is a key enzyme regulating lipid metabolism since it catalyzes the production of malonyl-CoA, the rate limiting substrate for *de novo* lipogenesis (DNL) [246]. There are two isoforms, ACC1 and ACC2, that differ in tissue distribution [247]. ACC1 is predominant in lipogenic tissue and produces malonyl-CoA

as a substrate for the fatty acid synthase (FAS) enzyme and chain elongation system. Malonyl-CoA generated through the action of ACC2, acts as an inhibitor of carnitine palmitoyltransferase 1 (CPT1) and thus regulates lipid β -oxidation in oxidative tissue [211-213]. ACC1 is the main isoform expressed in platelets [57, 197, 215].

Although DNL is described as a moderate contributor to intracellularly stored and circulating lipids, shifting lipid acquisition from lipid uptake toward DNL can dramatically change membrane lipid saturation and properties in various cells including endothelial cells [223] and cancer cells [224, 225]. In platelets, our research group has previously demonstrated that sustained ACC activation modifies specifically AA-containing PEP content and influences platelet reactivity to collagen and thrombosis through increased TxA_2 generation [57]. More recently, ACC has also been implicated in megakaryocyte (MK) demarcation membrane formation, a process required for platelet biogenesis [207]. However, whether platelets produced from ACC-deficient MKs are dysfunctional or less activatable is not known. The objective of the present work was to determine the impact of ACC1 invalidation on platelet lipid content and functions. By using platelet-specific ACC1 knockout mice, we demonstrate that this enzyme is a tight regulator of platelet PEP lipid class and overall levels of saturated and unsaturated fatty acyl chains contained in phospholipids, especially AA-containing PEP. Ablation of ACC1 strongly affects the intrinsic functions of platelets which results in reduced thrombus formation. Our data support a mechanism in which ACC1 deletion impaired glycolysis, TxA_2 generation and dense granule secretion in activated platelets. DNL or ACC1 inhibition may therefore represent a novel strategy to reduce platelet activation in thrombotic disorders.

2. Results

2.1. Platelet-specific ACC1 knockout mice display reduced platelet count and increased α IIb β 3 expression.

Platelet-specific ACC1 knockout (GPIb α -Cre^{+/-KI} x ACC1^{flx/flx}; ACC1 pKO) mice, generated by crossing GPIb α -Cre^{+/-KI} males with homozygous floxed ACC1 (ACC1^{flx/flx}) females, have an efficient loss of ACC1 protein in platelets, with no compensatory ACC2 upregulation (**Fig. 1a**). ACC1 was similarly expressed in leukocytes isolated from ACC1 pKO and their wild-type littermates (GPIb α -Cre^{+/-} X ACC1^{flx/flx}, ACC1 WT) (**Fig. 1b**), confirming that the GPIb α -Cre deleter mouse enables highly specific ablation of the ACC1 floxed gene in the MK lineage. Liver extract was used as a positive control for ACC2 detection (**Fig. 1a, b**). ACC1 deficiency was associated with a reduction of platelet count by ~30% and an increase in platelet volume by ~30% in ACC1 pKO compared with ACC1 WT mice (**Fig. 1c, d**). No major differences were found in white blood cell, lymphocyte, granulocyte or red blood cell counts, and hemoglobin and hematocrit levels, between both genotypes (**Supplementary Table 1**). Flow cytometry measurement of major platelet surface receptors revealed a significant increase in α IIb β 3 expression in ACC1 pKO compared to ACC1 WT platelets, likely reflecting the increase in platelet volume. However, the surface expression of GPIb α and GPVI was similar between both genotypes (**Fig. 1e**). When using GPIb α -Cre^{+/-KI} mice as an alternative control, we observed that GPIb α expression was significantly reduced in platelets from these mice, and this was associated with an increase in platelet volume, as previously described [278] (**Fig. 1d, e**). The comparison between ACC1 pKO and GPIb α -Cre^{+/-KI} mice still highlights the difference in platelet volume, integrin α IIb β 3 surface expression and the reduction in platelet count, although to a lower extent (**Fig. 1c-e**). To determine whether the lower platelet count in ACC1 pKO mice resulted from an increased platelet turnover, the *in vivo* lifespan of ACC1 pKO platelets was evaluated by flow cytometry

after injection of Alexa488-GPIIb β antibodies into mice. No significant differences were observed between ACC1 WT and ACC1 pKO animals (**Fig. 1f**).

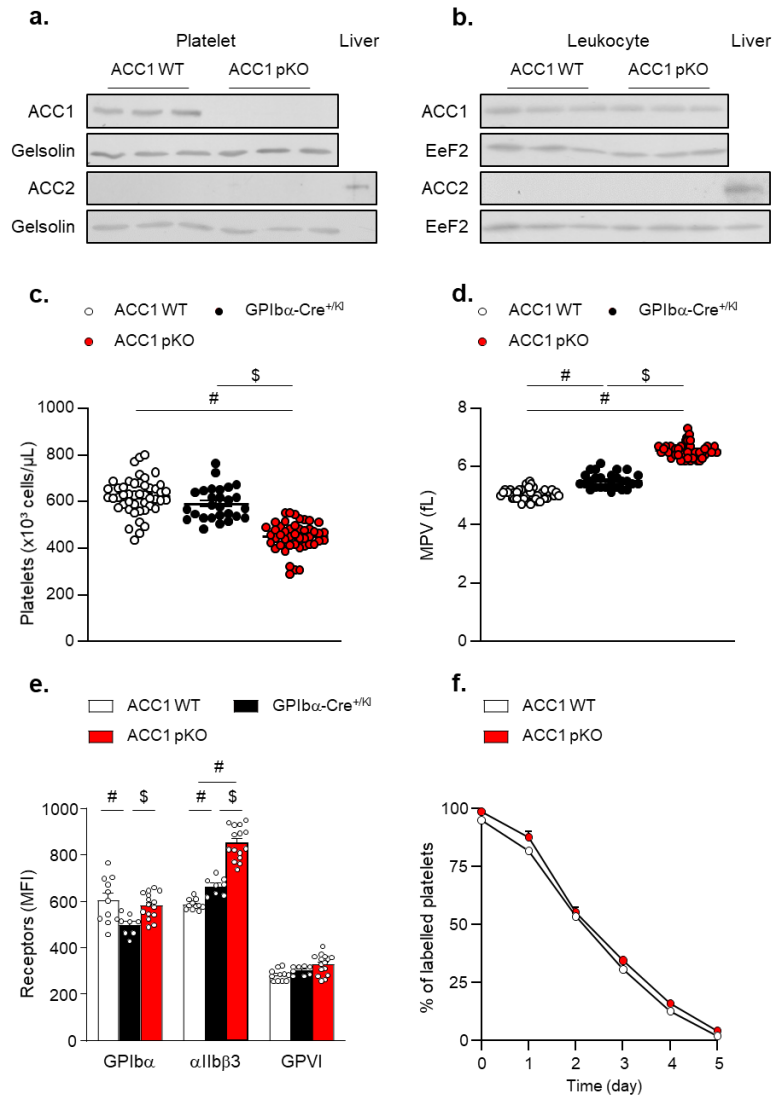


Figure1. Platelet parameters of ACC1 WT, GPIIb α -Cre^{+/-} and ACC1 pKO. (a-b) Washed murine platelets (a) or leukocytes (b) were lysed and subjected to western blotting for ACC1 and ACC2 expression analysis. Liver extract was used as positive control for ACC2. Gelsolin and EeF2 served as loading control for platelets and leukocytes/liver, respectively. (c-d) Platelet counts and mean platelet volumes. Data are expressed as means $\pm$ SEM (n = at least 27). # p-value $\leq$ 0.0001 relative to ACC1 WT mice, \$ p-value $\leq$ 0.0001 relative to GPIIb α -Cre^{+/-} mice. Data underwent one-way ANOVA. (e) Platelet surface receptor expression of GPIIb α , α IIb β 3 and GPVI were measured by flow cytometry. Data are expressed as median fluorescence intensity (MFI) $\pm$ SEM (n = at least 8). # p-value $\leq$ 0.05 relative to ACC1 WT mice. \$ p-value $\leq$ 0.01 relative to GPIIb α -Cre^{+/-} mice. Data underwent 2-way ANOVA. (f) GPIIb β -X488 Ab was injected (IV) into ACC1 WT and ACC1 pKO mice to visualize platelet lifespan. Blood was taken every day during 5 days and % of positive platelets was analyzed by flow cytometry. Data are expressed as means $\pm$ SEM (n = at least 4). Data underwent unpaired t-test.

2.2. *ACC1 deficiency results in a decrease in AA-containing PEP content.*

To analyze platelet lipid content, we performed an untargeted lipidomic analysis of resting platelets from ACC1 WT and ACC1 pKO mice using the Lipidizer platform. The analysis identified 390 lipid species originating from 14 different lipid classes including cholesterol ester (CE), ceramide (CER), dihydroceramide (DCER), hydroxyceramide (HCER), diacylglycerol (DAG), free fatty acid (FFA), lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), PC, PE, plasmalogen PE (PEO), PEP, sphingomyelin (SM), and triacylglycerol (TAG). A total of 127 lipid species, representing 33% of the analyzed platelet lipidome, showed a differential regulation between both genotypes (**Fig. 2a, Supplementary Table 2**). Class enrichment analysis indicated that ACC deficiency resulted in enrichment of downregulated lipids among PEP and PEO lipid classes, while an upregulation among CER and PC lipid classes was observed (**Fig. 2b**). To assess whether the difference between both experimental groups was due to specific subspecies, we next focused on fatty acid chain length. In agreement with previous data [57, 223, 225], analysis of fatty acyl chain length, at the sn-2 position, indicated that ACC1 deficiency was associated with a decrease in PAL (C16:0) contained in the four phospholipid classes (**Fig. 2c**). Moreover, when compared to PAL-containing phospholipids, long polyunsaturated fatty acids (PUFA) including AA (C20:4), eicosapentaenoic acid (EPA, C20:5) and docosahexaenoic acid (DHA, C22:6) were increased (**Fig. 2c**). Interestingly, ACC1 pKO platelets displayed a significant downregulation of all AA-containing PEP and one AA-PEO, compared to AA-PE and -PC (**Fig. 2d**). Taken together, our results demonstrate that ACC1 influences the level of saturated and polyunsaturated fatty acyl chains in platelet phospholipids. Of particular interest, the regulation of arachidonylated plasmalogens is susceptible to affect the platelet response to agonists through modulation of mitochondrial respiratory capacity and/or TxA₂ generation.

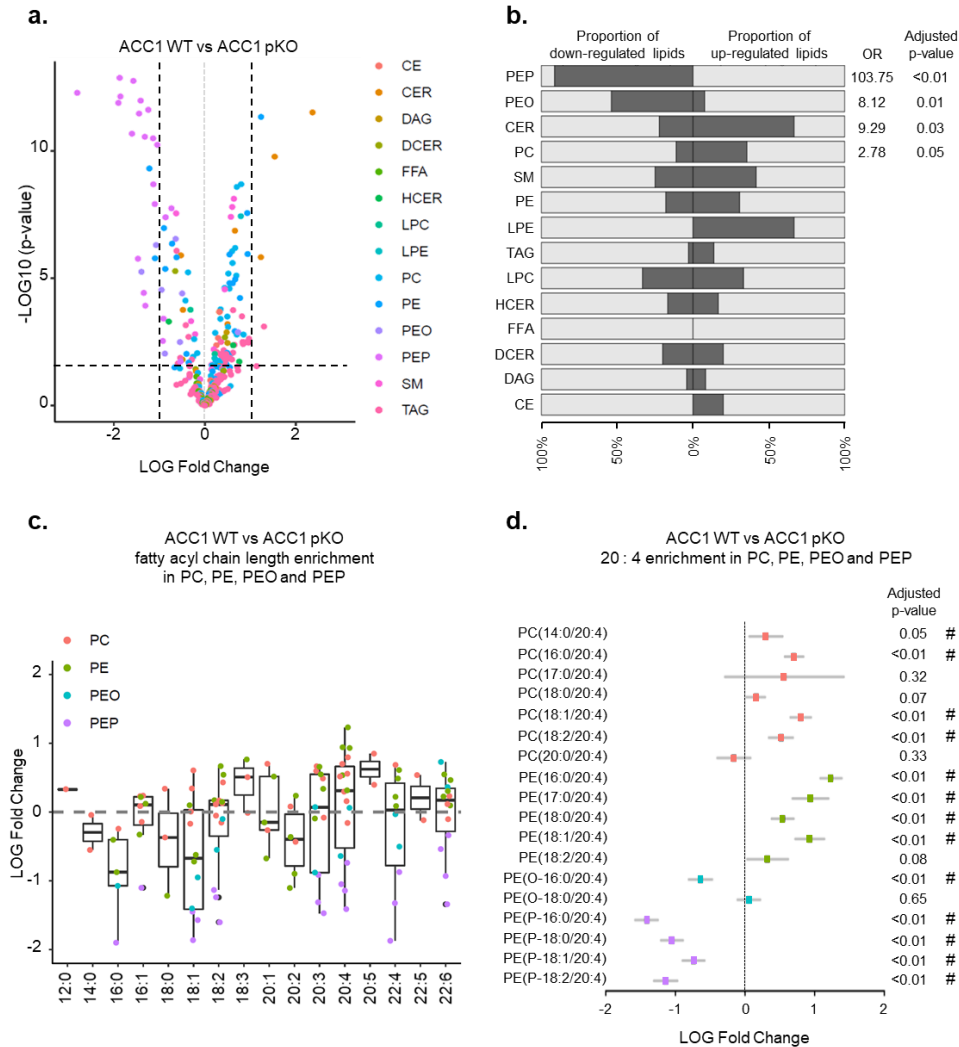


Figure 2. Platelet-specific ACC1 deletion decreases AA-containing phosphatidylethanolamine plasmalogen content. Untargeted lipid extraction were performed from washed murine platelets. **(a)** Volcano plot representation of 390 lipid detected species. Lipids above the horizontal dotted line were up (right)- or down (left)-regulated with significant adjusted p-values ≤ 0.05 ($n=6$). Log fold-changes and adjusted p-values were calculated from the multivariate regression model. **(b)** The percentage (dark gray bar) of down- or upregulated lipid species relative to the whole lipid class was calculated in ACC1 pKO relative to ACC1 WT platelets. * adjusted p-value ≤ 0.05 . **(c)** Fatty acyl chain length from phospholipids (PC, PE, PEO and PEP) are arranged in ascending order. **(d)** Comparison of AA-containing PC, PE, PEO and PEP between ACC1 WT and ACC1 pKO mice. The data are presented as log fold change (squares) relative to ACC1 WT platelets. Statistical significance was based on false discovery rate p-values (adjusted p-values). Multivariate regression analysis was performed. * adjusted p-value ≤ 0.05 between ACC1 WT mice and ACC1 pKO mice. AA = arachidonic acid; CE = cholesterol ester; CER = ceramide; DAG = diacylglycerol; DCE = dihydroceramide; FFA = free fatty acid; HCE = hydroxyceramide; LPC = lysophosphatidylcholine; LPE = lysophosphatidylethanolamine; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PEO = plasmalogen phosphatidylethanolamine; PEP = phosphatidylethanolamine plasmalogen; SM = sphingomyelin; TAG = triacylglycerol.

2.3. *ACC1 pKO platelets exhibit defective mitochondrial respiratory capacity and glycolysis upon thrombin stimulation.*

Since acyl-CoA, and in particular AA (C20:4) and eicosanoids are substrates for platelet mitochondrial β -oxidation [166, 181], we evaluated the impact of ACC1 deficiency on energy metabolism. Basal oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured followed by programmed sequential injection of oligomycin (ATP synthase inhibitor), carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), an uncoupler that disrupts the mitochondrial membrane potential, antimycin A (complex III inhibitor), and rotenone (complex I inhibitor) to evaluate mitochondrial bioenergetics. Under non-stimulated condition, we found no significant difference in basal or maximal OCR between ACC1 WT and ACC1 pKO platelets. ATP turnover demonstrated equimolecular oxygen use for ATP production by oxidative phosphorylation in both genotypes (**Fig. 3a, b**). Thrombin stimulation increases mitochondrial respiration in platelets, which has been shown to be a result of increased mitochondrial lipid oxidation [180, 181]. Consistent with these observations, thrombin increased mitochondrial respiration in ACC1 WT platelets. However, a reduced OCR stimulation was observed in ACC1 pKO platelets (**Fig. 3a, b**). Alternatively, a compensatory increase in glycolysis could offset impaired mitochondrial metabolism to sustain normal levels of ATP [180]. However, ACC1 deletion reduced ECAR, at basal state or after thrombin stimulation (**Fig. 3c, d**), indicating that ACC1 pKO platelets are not able to compensate for a loss of oxidative phosphorylation. Next, we added FCCP to determine the maximal OCR that the cells can sustain, and the reserve capacity, which represents the theoretical extra capacity of the electron transport chains to drive oxidative phosphorylation under situations of energetic stress. Maximal OCR and reserve capacity were similar in both genotypes, in basal conditions. As previously reported [57, 180, 181], thrombin stimulation induced a drop in maximal OCR and abolished reserve capacity in ACC1 WT platelets (**Fig. 3a, b**), which is likely the result of a modest inhibition of complex II linked substrates oxidation

[180, 181]. We show that the inhibitory impact of thrombin on reserve capacity is reduced in ACC1 pKO platelets (**Fig. 3a, b**). When rotenone and antimycin A were added to inhibit electron transport, the OCR falls to equally low level for both ACC1 WT and pKO platelets. Finally, proton leak was unchanged in all groups (**Fig. 3a, b**). Taken together, these findings indicate defective mitochondrial respiratory capacity following thrombin stimulation in ACC1 pKO platelets, which could possibly result from the reduced availability of endogenous substrates. The reduction of thrombin-induced OCR was not compensated by increased ECAR, reinforcing the conclusion that ACC1 pKO platelets are energetically impaired under the tested conditions.

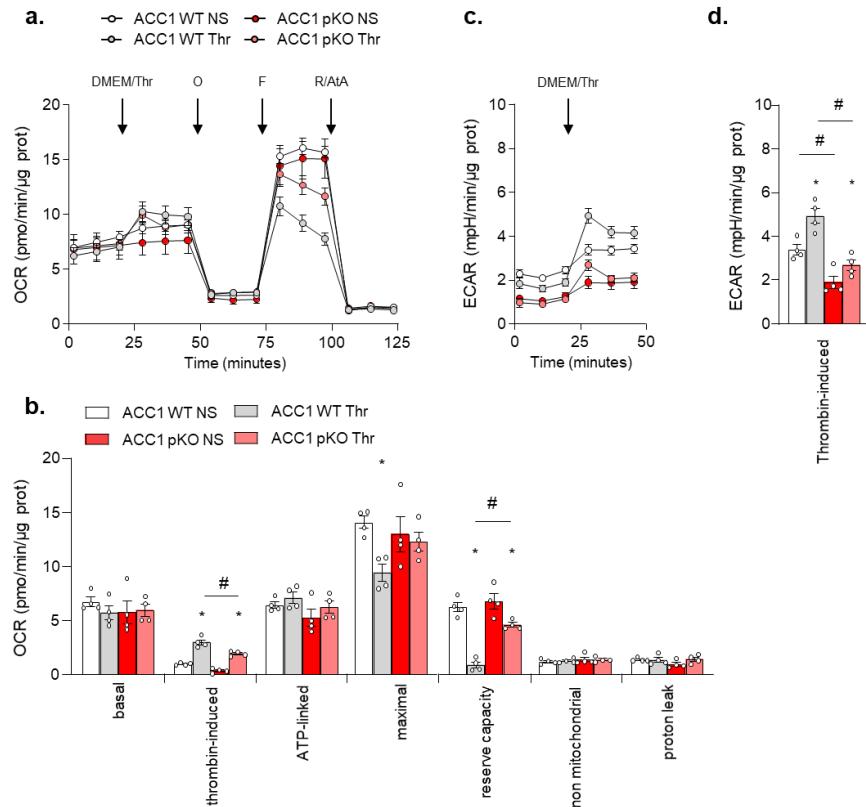


Figure 3. ACC1 pKO platelets exhibit defective mitochondrial respiratory capacity and glycolysis upon thrombin stimulation. (a-d) Oxygen consumption rate (OCR) (a; c) and extracellular acidification rate (ECAR) (b; d) were measured in washed murine platelets. OCR et ECAR are assessed under basal or 1 U/mL thrombin (Thr)-stimulated conditions and after treatment with 1 μM oligomycin (O), 0.4 μM carbonyl cyanide p-trifluoromethoxyphenylhydrazone (F), and a mix of 1 μM antimycin A (atA) / rotenone (R). (a; c) OCR and ECAR profiles are shown as means ± SEM (n=4). (b) Mitochondrial function was evaluated by calculating basal, ATP-linked, maximal, non mitochondrial OCR, reserve capacity and proton leak. Results are expressed as means ± SEM (n = 4). * p-value ≤ 0.05 relative to unstimulated condition. # p-value ≤ 0.05 relative to ACC1 WT mice. Data underwent 2-way ANOVA. (d) Quantification of basal and thrombin-induced ECAR. Results are expressed as means ± SEM (n = 4). Data underwent 2-way ANOVA.

2.4. Reduced content of arachidonylated PEP limits TxA₂ generation and dense granule release in ACC1 pKO platelets.

We next evaluated the impact of ACC1 deficiency on TxA₂ production, measured by TxB₂ detection. Lack of platelet ACC1 significantly reduced thrombin and CRP-induced TxA₂ generation (Fig. 4a). Since TxA₂ is a secondary platelet agonist implicated in αIIbβ3 activation and dense granule secretion [279], we further assessed the effect of thrombin and CRP on platelet reactivity. First, we investigated αIIbβ3 inside-out

activation by analyzing platelet aggregation. Platelets from ACC1 pKO mice had a reduced thrombin and CRP-induced platelet aggregation when compared to ACC1 WT platelets (**Fig. 4b, c**). Impaired aggregation was associated with less $\alpha\text{IIb}\beta 3$ activation, detected by JON/A Abs (**Fig. 4d**). Next, we analyzed platelet granule secretion. P-selectin surface exposure was similar between ACC1 pKO and WT platelets (**Fig. 4e**), indicating that the ACC1 pKO phenotype does not seem associated with a change in α -granule secretion, at least for those containing P-selectin. Regarding dense granule secretion, a significant decrease in ATP secretion was detected after thrombin or CRP stimulation in ACC1 pKO platelets compared with WT (**Fig. 4f, g**). Importantly, similar effects on aggregation and dense granule release can be reached by comparing platelets from $\text{GPIIb}\alpha\text{-Cre}^{+/Kl}$ and ACC1 pKO mice (**Supplementary Fig. 1a-d**). Taken together, these data emphasize the central role of ACC1 in the regulation of platelet reactivity through the synthesis of AA-containing PEP, which serves as a source for both endogenous energy substrates and TxA_2 .

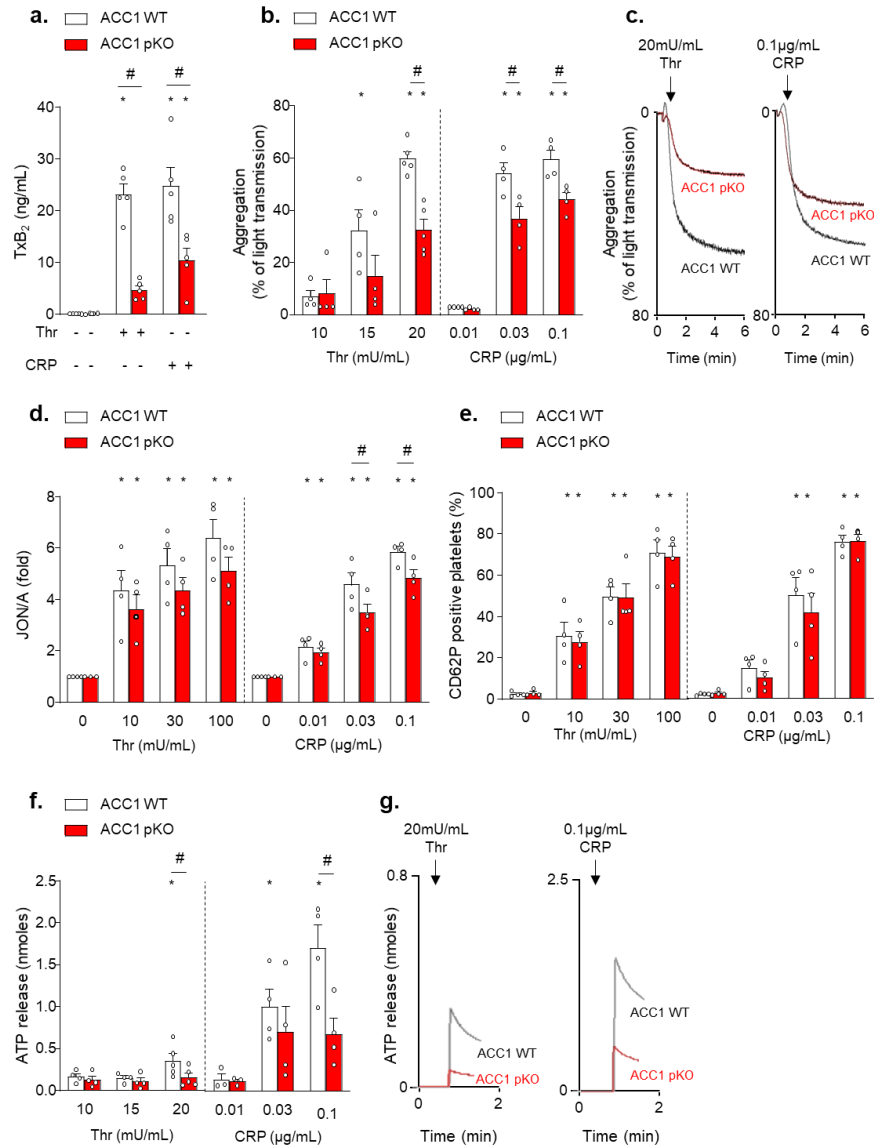


Figure 4. Reduced level of AA-containing PEP limits TxA_2 generation and dense granule release in ACC1 pKO platelets. (a-g) Washed murine platelets were stimulated with various thrombin (Thr) or collagen-related peptide (CRP) concentrations. (a) Platelets were stimulated with 100mU/mL Thr or 0.3μg/mL CRP for 5min and TxB_2 was measured in the supernatant using ELISA kit. Data are expressed as means $\pm$ SEM (n=4). * p-value $\leq$ 0.05 relative to unstimulated condition. # p-value $\leq$ 0.05 relative to ACC1 WT mice. Data underwent unpaired 2-way ANOVA. (b-c) Aggregation was analyzed by turbidimetry (Chrono-Log). (c) Profiles are shown. Data are expressed as means $\pm$ SEM (n= at least 3). * p-value $\leq$ 0.05 relative to the first agonist concentration. # p-value $\leq$ 0.05 relative to ACC1 WT mice. Data underwent 2-way ANOVA. (d-e) $\alpha\text{IIb}\beta_3$ activation (JON/A) (d) and p-selectin (CD62P) exposure (e) were detected by flow cytometry. Data are expressed as means fold change $\pm$ SEM (n = 4). * p-value $\leq$ 0.05 relative to unstimulated condition. # p-value $\leq$ 0.05 relative to ACC1 WT mice. Data underwent 2-way ANOVA. (f-g) Dense granules secretion was assessed via addition of Luciferase-luciferin reagent (Chrono-log). (g) Profiles are shown. Data are expressed as means $\pm$ SEM (n=4). * p-value $\leq$ 0.05 relative to the first agonist concentration. # p-value $\leq$ 0.05 relative to ACC1 WT mice. Data underwent 2-way ANOVA.

2.5. Platelet-specific ACC1 deletion reduces thrombus formation on collagen-coated surface in flow conditions.

To assess platelet functions in dynamic conditions, we perfused anti-coagulated blood over collagen I- and III-coated surfaces, at a shear rate of 1000 s^{-1} , and analyzed thrombus formation. Brightfield images were captured to evaluate platelet adhesion and thrombus buildup. Platelet adhesion was quantified by the conventional analysis of platelet surface area coverage (SAC%). Thrombus signature was composed of thrombus multilayer (SAC%), and thrombus contraction score [280, 281]. Both the surface area covered by adherent platelets and buildup of multilayered platelet thrombi were decreased with ACC1 pKO blood compared to ACC1 WT or GPIIb α -Cre^{+/KI} blood (**Fig. 5a-d, Supplementary Fig. 2a-d**). This effect likely results from the reduced level of secreted TxA₂ and ADP, seen with ACC1 pKO platelets, as both of them are secondary agonists playing a central role in the formation of platelet aggregates on collagen [57, 91, 282]. Platelet activation was simultaneously monitored by perfusing fluorescent anti-P-selectin Ab as a marker of α -granule secretion, JON/A Ab to measure α IIb β 3 activation, and annexin V to analyze PS externalization, reflecting platelet procoagulant activity. The thrombi in the flow chamber were stained for the three platelet activation markers confirming that platelets were indeed activated (**Fig. 5a, Supplementary Fig. 2a**). The quantification demonstrated lower level of platelet activation markers with ACC1 pKO perfused blood (**Fig. 5e-g, Supplementary Fig. 2e-g**), confirming the loss-of-function phenotype of ACC1 pKO mice, even under conditions mimicking those in a blood vessel in which thrombus formation occurs under flow conditions.

a.

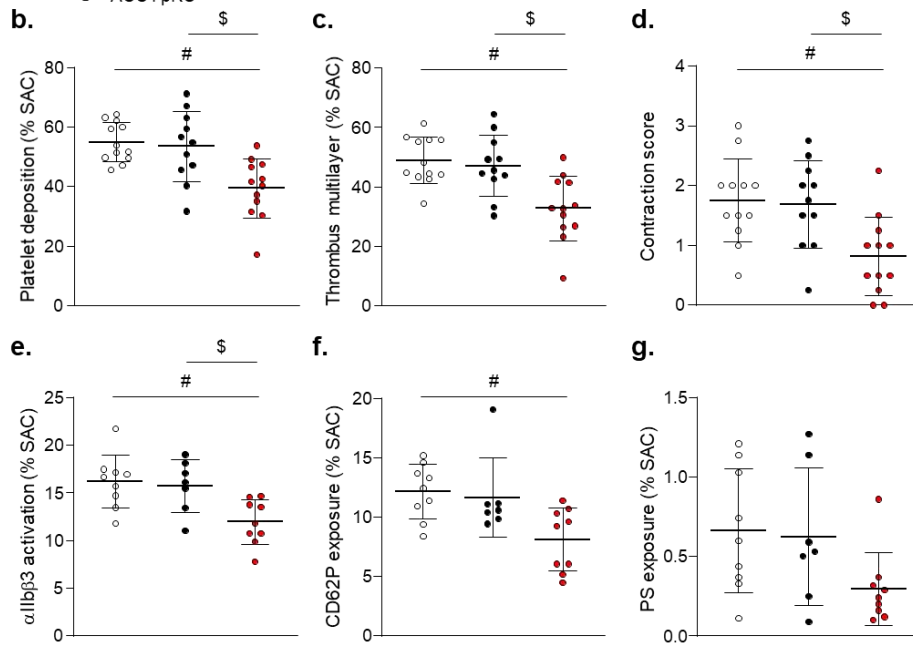
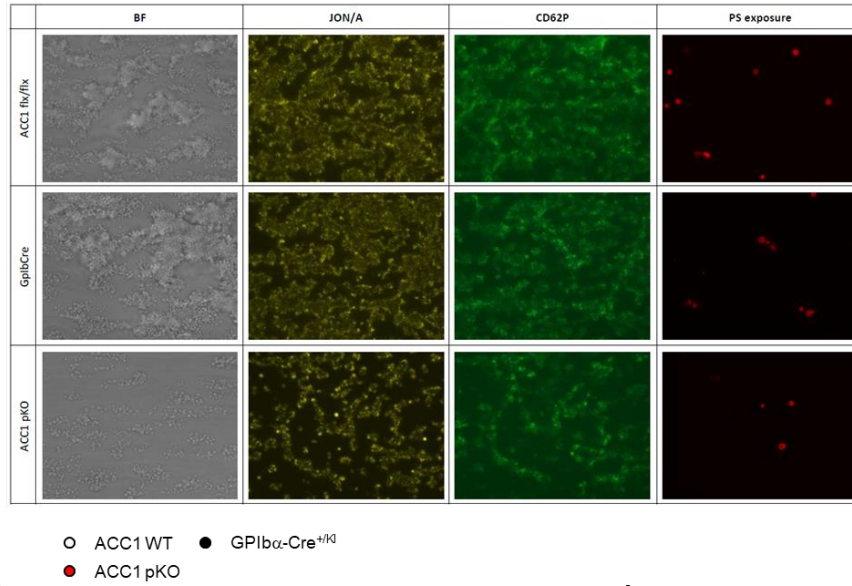


Figure 5. Platelet-specific ACC1 deletion decreases thrombus formation *ex vivo*. (a-g) Whole blood from ACC1 WT, GpiIbCre^{+/Kl} and ACC1 pKO mice was perfused over collagen I-coated surface (50 μ g/mL) at a shear rate of 1000 s⁻¹. (a) Representative images of multilayered platelet thrombi and JON/A, CD62P and PS exposure staining. (b-d) Platelet deposition, thrombus formation and contraction score were assessed on brightfield images. (e-g) α IIb β 3 activation was evaluated by JON/A Ab (e), p-selectin expression by staining with CD62P Ab (f) and phosphatidylserine (PS) exposure by Annexin V (g). The results are expressed as means $\pm$ SD (at least 4 mice/group). # p-value $\leq$ 0.05 relative to ACC1 WT mice. \$ p-value $\leq$ 0.05 relative to GpiIbCre^{+/Kl} mice. Data underwent one-way ANOVA.

3. Discussion

Lipid metabolism in platelets has gained attention in recent years. We previously demonstrated that ACC, when active, promotes platelet activation and thrombosis, based on its ability to stimulate endogenous lipid synthesis [57]. However, the impact of specific platelet ACC deletion on platelet lipid levels and functions has not been investigated so far. We thus generated platelet-specific ACC1 knockout mice by using the GPIIb α -Cre deleter mouse strain [278]. This model allows MK/platelet-specific gene invalidation, while ACC1 expression in leukocytes is preserved, confirming the lineage-specific gene ablation provided by the GPIIb α -Cre mouse [278]. Analysis of hematological parameters shows that the absence of ACC1 is associated with an increase in MPV. This result may explain, on one hand, the increase in surface expression of α Ib β 3 in ACC1 pKO platelets as fibrinogen receptor level is known to correlate with MPV [278, 283, 284] and, on the other hand, the modest decrease in GPIIb α expression observed in GPIIb α -Cre^{+/-} mice as a reduced Gplb α is associated with an increased MPV [285, 286]. Interestingly, ACC1 deficiency also leads to a significant reduction in platelet count. This is in agreement with a recent report demonstrating the inhibitory impact of pharmacological ACC inhibitors on platelet biogenesis in humans and monkeys [207]. The proposed mechanism involves an inhibition of DNL-dependent fully saturated PC species synthesis, resulting in an impaired MK demarcation membrane formation [207]. Surprisingly, in this study, the authors failed to highlight a default of platelet production in rodents. Our findings, however, indicate that the platelet count is reduced in ACC1 pKO mice and does not result from an increased platelet turnover. The reason for this discrepancy is not clear but the possibility that a higher dose of ACC inhibitors or a longer duration of administration in rodents could reach comparable effects to those observed in monkeys and humans, cannot be excluded.

A very important finding in our study is that the specific genetic ACC1 invalidation in platelets affects the levels of saturated and polyunsaturated fatty acyl chains contained

in phospholipids, with a decrease of PAL, and an increase in PUFA mainly in PC and PE classes. Similar observations were reported in endothelial and cancer cells where ACC inhibitors reduced saturated and increased polyunsaturated fatty acyl chains, which reduced membrane fluidity and altered the migratory capacity of cells [223-225]. In platelets, a modification of phospholipid composition in the plasma membrane can also fluidize or rigidify the lipid bilayer and influence platelet shape, volume, and response to drugs [8]. Some specific lipid species with high biological functionality can also be impacted, especially plasmalogens such as PEP, which play a crucial role in platelet plasma membrane-derived lipid signaling molecules [57, 158, 162]. Our study highlights that ACC1 deficiency is associated with a decrease in AA-containing PEP, limiting the production of biologically active mediators such as TxA₂ and eicosanoids. The mechanism by which ACC1 deletion decreases AA-containing PEP levels is not clear. As the CoA-independent transacylase is the enzyme responsible for the mobilization of AA from diacyl phospholipids such as PC to ether-phospholipids, especially PEP [156, 157], it can be postulated that ACC1 deficiency would result in an alteration of its activity, but this remains to be investigated in further studies.

Importantly, our data reinforce the link between ACC1, AA-containing PEP level, TxA₂ generation and dense granule secretion in activated platelets. We also show that ACC1 deletion in platelets causes a reduced platelet aggregation and thrombus formation under flow, a result that contrasts with our previous study showing that sustained ACC1 activation may augment thrombosis through mechanisms that are independent of α IIb β 3 [57]. A plausible hypothesis to explain the default in platelet aggregation might be the reduction of glycolysis upon agonist stimulation. Indeed, Ravi et al. previously reported that inhibition of glycolysis is sufficient to inhibit platelet aggregation [180]. It is not fully understood why glycolysis is impaired upon ACC1 invalidation. Interestingly, lower ACC1 expression and ACC1 inhibition have been associated with the hyperacetylation of several proteins, including histones and cytoplasmic proteins involved in metabolism, in yeast, liver or cancer cells [237-239]. We recently demonstrated, using washed human platelets, that pharmacological ACC inhibition

limits α -tubulin deacetylation upon thrombin stimulation, which in turn impairs platelet aggregation [277]. It would be worth analyzing the impact of ACC1 invalidation on acetylation level of cytoskeletal or glycolytic enzymes in platelets, and how it influences their activity and/or energy metabolism.

In conclusion, we report here the essential roles of ACC1 and *de novo* lipogenesis in platelets. Our work clearly demonstrates that this enzyme is crucial for specific phospholipid pools required, not only for platelet biogenesis, but also for platelet energy and reactivity. Further studies are needed to investigate potential clinical applications based on the use of DNL inhibitors in the field of thrombotic disorders.

4. Material and methods

4.1. Chemicals and reagents.

Collagen-related peptide (CRP) was obtained from Cambcol laboratories. Thrombin from bovine plasma (#T6634), apyrase (#A6132), acetylsalicylic acid (ASA) (#A5376), acid citrate-dextrose solution (ACD) (#C3821), Dulbecco's Modified Eagle's Medium (DMEM, #D5030), histopaque-1077 (10771), histopaque-1119 (11191) and horse radish peroxidase (HRP)-conjugated anti-rabbit (#A0545) antibodies (Abs) were purchased from Sigma. Horm type I collagen (#10345787) was from Nycomed. Human type III collagen (1230-01S) was purchased from SouthernBiotech. Bovine serum albumin (BSA) (#3854.4) was purchased from Roth. Fatty acid-free BSA (#126575) was from Millipore. Luciferin-luciferase reagent (#CH395) was from Stago BNL, and Cell Tak (#354240) was from Corning. Oligomycin, FCCP, antimycin A and rotenone were from Agilent (Mito Stress Test Kit, #103015-100). Anti-ACC1 (#4190), anti-ACC2 (#8578), anti-rabbit IgG HRP-linked (#7074) and anti-gelsolin (#12953) Abs were obtained from Cell Signaling. Anti-eEF2 (#PA5-17794), anti-P2Y12 recombinant (#702516), halt protease and phosphatase inhibitor cocktail 100X and carboxyfluorescein succinimidyl ester (CFSE) (#C34554) were purchased from ThermoFisher Scientific. TxA₂ ELISA kit (#ADI-900-002) came from Enzo Life Science. U46619 (#1932) was purchased from Tocris. Anti-PAR-3 (#sc-393127) was from Santa Cruz. Phycoerythrin (PE) -conjugated anti-active α IIb β 3 (JON/A) (#MO23-2), PE-conjugated rat IgG (#P190-2), PE-conjugated anti-CD42b (#MO40-2), PE-conjugated anti- α IIb β 3 (#MO25-2) and Fluorescein isothiocyanate (FITC)-conjugated anti-GPVI (#MO11-1) Abs were purchased from Emfret analytics. FITC-conjugated anti-CD62P (#553744), FITC-conjugated rat IgG1 λ (#553995), PE-conjugated rat IgG2b (#556925), PE-conjugated rat IgG2a (#553930) and FITC-conjugated rat IgG2a (#553929) were from BD pharmingen. Anti- protease-activated receptor-4 (PAR-4) (#APR-034) Ab was obtained from Alomone Labs. Ketamine (Nimatek) was purchased from Dechra. Xylazine (Rompun) was obtained from Bayer. Domitor and antisedan were purchased from Orion pharma.

4.2. *Animals.*

ACC1^{flx/flx} mice purchased from Jackson laboratory, and GPIIb α -Cre^{+/Kl} mice generated as described previously [278] served as controls. All mice were in C57BL/6 background. Platelet-specific ACC1 knockout mice (ACC1 pKO) were generated by crossing a GpIb α -Cre^{+/Kl} male with a homozygous floxed ACC1 (ACC1^{flx/flx}) female. Deletion of ACC1 was confirmed by western blot. Eight- to sixteen-week-old male mice were used and fed with a normal chow diet. Animal procedures and protocols were approved by local authorities (Comité d'éthique facultaire pour l'expérimentation animale, 2016/UCL/MD/027 and 2021/UCL/MD/012) and performed in accordance with the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH Publication, revised 2011).

4.3. *Blood collection and platelet preparation.*

Mice were anesthetized with a mix of ketamine:xylazine (150mg/Kg:20mg/Kg). Blood was taken from the retro-orbital plexus and collected in a mix of ACD:apyrase (1/6:1U/mL). It was centrifuged at 800g for 5 seconds, followed by 5 minutes at 100g. A mix of ACD:apyrase (2/3:1U/mL) was added to the obtained platelet-rich plasma (PRP) before centrifugation at 400g for 5 minutes. Platelets pellet was resuspended to a density of $2.5 \times 10^5/\mu\text{L}$ (unless stated otherwise) in modified Tyrode's buffer (135mM NaCl, 12mM NaHCO₃, 3mM KCl, 0.3mM Na₂HPO₄, 1mM MgCl₂, 5mM D-glucose, 10mM Hepes, 0.35 % BSA, pH 7.4, 37 °C). Hematological parameters were measured using Cell-dyn Emerald (Abbott).

4.4. *Untargeted lipidomics.*

Platelets (2×10^8) were resuspended in modified Tyrode's buffer with 0.35% free fatty acid BSA. A dry platelet pellet was obtained by centrifugation at 11,700g for 15 seconds. Platelet pellets were kept at -80°C before lipid extraction. Lipid extraction and lipidomics analysis were carried out as previously described [57].

4.5. *Extracellular flux analysis of mitochondrial respiration.*

Platelets were resuspended in modified DMEM's medium (DMEM, 143mM NaCl, 0.5% phenol red, 5mM D-glucose, 1mM sodium pyruvate, 4mM L-glutamine, pH 7.35). Platelets (30×10^6) were added to 96-well plate coated with 150 μ g/mL Cell Tak (diluted in 0.1M NaHCO₃, pH 8.0). Platelet adhesion was induced by two centrifugations at RT (148 x g for 1min and 213 x g for 1min). Platelets were incubated for 1h at 37°C in a CO₂-free incubator before the assay. OCR and ECAR were measured every 4 minutes with Seahorse extracellular flux XFp (Biosciences). After basal OCR measurement, 1U/mL thrombin or DMEM, 1 μ M oligomycin, 0.4 μ M FCCP and 1 μ M rotenone/antimycin A (R/AtA) mix were sequentially injected into wells every 12 minutes to allow assessment of mitochondrial functional parameters. Basal OCR was evaluated by subtracting R/AtA OCR from basal OCR and thrombin-induced OCR by subtracting the baseline from OCR after thrombin injection. ATP-linked OCR was calculated by subtracting OCR after oligomycin from the baseline. Reserve capacity was evaluated by subtracting the baseline from OCR after FCCP. Non mitochondrial OCR was obtained after R/AtA injection and proton leak by subtracting R/AtA OCR from OCR after oligomycin.

4.6. *TxA₂ assay.*

TxA₂ was evaluated by enzyme-linked immunosorbent assay (ELISA). Platelets (18×10^6) were stimulated with 100mU/mL thrombin or 0.3 μ g/mL CRP for 5 minutes at 37°C. Platelet supernatants were collected after centrifugation at 11,700 g for 15 seconds and then frozen in liquid nitrogen. Before TxA₂ analysis, supernatants were diluted 20X for thrombin- and CRP-stimulated conditions. TxA₂ analysis was performed according to the manufacturer's instructions.

4.7. *Leukocyte isolation.*

Mouse tibia and femur were flushed in modified Hank's Balanced Salt Solution's (HBSS) buffer (137mM NaCl, 5.4mM KCl, 0.44mM KH₂PO₄, 5.5mM glucose, 0.4mM NaH₂PO₄,

3.3mM Na₂CO₃, 19.8mM citrate, pH 7.4) to isolate bone marrow cells. Cells were filtered and washed twice (centrifugation of 230g for 6 minutes). Cells pellets were resuspended in 3mL modified HBSS's buffer and added to a Falcon containing 3mL histopaque-1077 and 3mL histopaque-1119 (at the bottom). White blood cells were separated from the rest of the bone marrow cells thanks to a centrifugation of 900g for 30 minutes. The upper phase, containing leukocytes with a minority of neutrophils, was washed with 15mL of modified HBSS's buffer (354g, 7minutes). Cells were resuspended in 1mL modified HBSS's buffer and centrifuged at 230g for 7 minutes. Cell pellets were frozen in liquid nitrogen. White blood cell concentration was evaluated with Cell-dyn Emerald.

4.8. *Western blot analysis.*

Liver, leukocytes and platelets were lysed in triton-lysis buffer (50mM Tris pH 7.5, 0.27M sucrose, 1% triton, 1mM EGTA, 1mM EDTA, 25mM dithiothreitol (DTT), halt protease and phosphatase inhibitor cocktail 1X). Protein concentration was evaluated thanks to the Bradford protein assay. Protein extracts were diluted 4X in modified blue Laemmli's buffer (50mM Tris pH 6.8, 10% (w/v) glycerol, 2% (w/v) SDS, 0.5mM EDTA, 0.01% (w/v) bromophenol blue, 25mM DTT) to obtain a concentration of 5/20µg/mL, 10µg/mL and 15µg/mL for liver (ACC1/ACC2), white blood cells and platelets respectively. Lysates were subjected to western blot for ACC1 and ACC2. All Abs were diluted in 5% BSA. The dilution for primary Abs were (1/1,000) except for anti-gelsolin (1/50,000) and anti-eEF2 (1/4,000). Secondary HRP-conjugated anti-rabbit Abs from Cell Signaling were diluted 1/5,000 and HRP-conjugated anti-rabbit Abs from Sigma were diluted 1/20,000.

4.9. *Platelet aggregation and ATP secretion.*

An aggregometer (Chrono-log 700, STAGO) was used to assess aggregation and dense granules secretion. Light transmission, reflecting platelet aggregation, was recorded under constant stirring (1200 rpm) at 37°C, after CaCl₂ (2mM) addition and agonist

stimulation. ATP secretion was evaluated by adding Luciferase/Luciferin reagent and comparing the luminescence generated by platelet ATP release with an ATP standard.

4.10. Flow cytometry analysis.

For mouse platelet receptors GPIIb/IIIa, α IIb β 3 and GPVI, platelets were resuspended in Tyrod's modified buffer at a concentration of $50 \times 10^3/\mu\text{L}$. Platelets (1×10^6) were incubated with either PE-anti-CD42b, PE-anti- α IIb β 3, FITC-anti-GPVI or their isotype control Abs (1/5) for 15 minutes at RT. The reaction was stopped by adding 400 μL PBS-BSA 0.1%-azide 0.1%. For CD62P exposure and α IIb β 3 activation, platelets were resuspended in modified Tyrod's buffer with 1.5% BSA. Platelets (3.75×10^6) were incubated with anti-CD62P and JON/A Abs (1/14.5) for 15 minutes at RT. Platelets were stimulated with thrombin or CRP at the indicated concentration, at 37°C, for 8 minutes or 5 minutes, respectively, before being fixed with paraformaldehyde (PFA) 4%. For platelet clearance, mice were anesthetized with a mix of ketamine:domitor (75mg/Kg:1.5mg/Kg). X488-anti-GpIb β Ab diluted in sterile PBS was injected (IV, 0.5g/g) into mice. Mice were woken up at 37°C with an IP injection of antisedan (1.5mg/Kg). Platelet staining was analyzed from 1 hour up to 5 days after Ab injection. Blood (5 μL) was collected from mice tails into modified Tyrod's buffer with a mix of ACD:apyrase (1/6:1U/mL). Platelets were isolated via centrifugation at 125g for 5 minutes and stained with PE-anti- α IIb β 3 (1/100) for 40 minutes at room temperature (RT). Samples were diluted with modified Tyrod's buffer with a mix of ACD:apyrase (1/3:1U/mL) and centrifuged at 800g for 5 minutes. Platelet pellet was resuspended into 300 μL of modified Tyrod's buffer. All samples were analyzed by BD Canto II flow cytometer, and 10,000 events were recorded. When necessary, appropriate compensation controls were included.

4.11. Thrombus formation in a flow chamber system.

Experiment was carried out as previously described [57]. Briefly, blood was taken from the retro-orbital plexus into 48 mM D-Phenylalanyl-prolyl-arginyl Chloromethyl Ketone,

5 U/mL heparin and 40 U/mL fragmin. 500 μ L of anti-coagulated blood was perfused over type I collagen- (50 μ g/mL) or type III collagen (100 μ g/mL)-coated coverslips mounted on a transparent flow chamber (50 mm depth, 3mm width and 20 mm length) at a shear rate of 1000 s⁻¹. After 3.5 minutes, platelets were stained with FITC-anti-P-selectin Ab (1:20), PE-JON/A Ab (1:20) and Alexa Fluor 647-annexin A5 (1:200). Brightfield phase-contrast and fluorescence images were recorded by a nonconfocal 2-camera system. Surface coverage was analyzed by ImagePro software (Media Cybernetics) [280].

4.12. Lipidomic statistical analysis.

Lipid species concentrations were analyzed using R.4.2.0 according to the following bioinformatics pipeline. The lipid species concentration matrix and the associated metadata were imported into a SummarizedExperiment object from the SummarizedExperiment v.1.26.1 Bioconductor package. Lipid associated with more than 80 percent of missing data were filtrated out. Data were normalized with the MS total useful signal (MSTUS) method, and a log2 transformation was applied on normalized concentrations. The Limma v.3.52.0 Bioconductor package was used to build a regression model for each lipid species. Fold-change estimates and corresponding P-values were derived from regression models for each lipid species. To control for multiple testing, all P-values were further adjusted for the Benjamini-Hochberg false discovery rate (FDR), and FDR $\leq$ 0.05 was considered as significant. Considering that the lipid species belong to 14 lipid classes (CE, CER, DAG, DCER, FFA, HCER, LPC, LPE, PC, PE, PEO, PEP, SM, TAG), a lipid set enrichment analysis was conducted by using the fgsea v.1.22.0 Bioconductor package.

4.13. Statistical analysis.

Statistical parameters including sample size, mean, standard error of the mean (SEM) and statistical significance are described in figure legends. P-values $\leq$ 0.05 were

considered significant. Statistical analyses were made in GraphPad Prism 9.1.2 (GraphPad Software, San Diego, CA).

5. Supplementary material

Hematological parameters	ACC1 WT (mean $\pm$ SD; n = 46)	GPIIb α Cre ^{+/Kl} (mean $\pm$ SD; n = 27)	ACC1 pKO (mean $\pm$ SD; n = 45)	p-value
WBC (x10 ³ cells/ μ L)	3.923 $\pm$ 1.233	4.200 $\pm$ 1.062	3.459 $\pm$ 1.112 ^{\$}	\$0.0242
Lym (x10 ³ cells/ μ L)	3.436 $\pm$ 1.136	3.724 $\pm$ 0.9902	2.99 $\pm$ 0.9684 ^{\$}	\$0.0121
Mid (x10 ³ cells/ μ L)	0.384 $\pm$ 0.2454	0.3668 $\pm$ 0.1515	0.3704 $\pm$ 0.1892	ns
Gra (x10 ³ cells/ μ L)	0.1026 $\pm$ 0.05912	0.1081 $\pm$ 0.02731	0.0982 $\pm$ 0.047	ns
RBC (x10 ⁶ cells/ μ L)	7.199 $\pm$ 0.4282	6.986 $\pm$ 0.2413	6.967 $\pm$ 0.4946 [#]	#0.0245
HGB (g/L)	10.82 $\pm$ 0.6592	10.65 $\pm$ 0.4291	10.36 $\pm$ 0.8291 [#]	#0.0050
HCT (%)	30.97 $\pm$ 1.435	30.55 $\pm$ 0.9023	30.32 $\pm$ 1.933	ns
MCV (fL)	43.31 $\pm$ 1.062	43.74 $\pm$ 0.8345	43.69 $\pm$ 1.066	ns
MHC (pg)	15.07 $\pm$ 0.4088	15.24 $\pm$ 0.6141	14.89 $\pm$ 0.5162 ^{\$}	\$0.0135
MHCH (g/dL)	34.8 $\pm$ 1.061	34.86 $\pm$ 1.084	34.1 $\pm$ 1.397 ^{#, \$}	#0.0179; \$0.0292
RDW (%)	15.36 $\pm$ 0.6513	14.96 $\pm$ 0.3641 [#]	15.5 $\pm$ 0.5431 ^{\$}	#0.0094; \$0.0292
PLT (x10 ³ cells/ μ L)	623.7 $\pm$ 76.57	591.6 $\pm$ 69.73	451 $\pm$ 61.82 ^{#, \$}	#, \$ <0.0001
MPV (fL)	5.038 $\pm$ 0.1776	5.519 $\pm$ 0.2617 [#]	6.55 $\pm$ 0.2429 ^{#, \$}	#, \$ <0.0001

Table 1. Hematological parameters of ACC1 WT, GPIIb α -Cre^{+/Kl} and ACC1 pKO mice.

Hematological parameters were analysed with Cell-dyn Emerald. Data are expressed as means $\pm$ SD (n= at least 27). # p-value relative to ACC1 WT mice. \$ p-value relative to GPIIb α -Cre^{+/Kl} mice. Data underwent one-way ANOVA. WBC = white blood cell; Lym = lymphocyte; Mid = mid-range cell; Gra = granulocyte; RBC = red blood cell; HGB = Hemoglobin; HCT = haematocrit; MCV = mean cell volume; MHC = mean cell haemoglobin; MHCH = mean cell haemoglobin concentration; RDW = red cell distribution width; PLT = platelet; MPV = mean platelet volume.

lipid	logFC	adj.P.Val	lipid	logFC	adj.P.Val
PE.P.16.0.22.4.	-1.87493024023121	0.00000000000344920890742527	CER.24.1.	0.316303276421401	0.00119929652592932
PE.P.18.0.18.1.	-1.57392833305881	0.00000000000344920890742527	CE.18.3.	0.324483784929532	0.00119929652592932
PE.P.18.1.16.0.	-2.80840343878214	0.0000000000676393292217914	PC.18.2.20.3.	0.493561852036883	0.00177663363431343
PE.P.16.0.18.1.	-1.85867471681958	0.0000000000704488139332784	TAG54.3.FA18.2.	0.815867469532812	0.00178520881095433
PE.P.16.0.20.4.	-1.41327876314829	0.0000000000082353404966782	PC.18.0.18.0.	0.341074098481556	0.00213112574654436
PE.P.18.0.16.0.	-1.90292622545349	0.00000000000841032412894	SM.26.0.	-0.303814691215269	0.00257274640725491
PE.P.18.0.18.2.	-1.24470393793793	0.000000000134667641868104	HCEP.24.0.	-0.794376600969585	0.00265000938072238
CER.22.1.	2.36753155249634	0.000000000147691257583784	PE.16.0.20.3.	0.65875697018101	0.00590536511265343
PE.P.18.1.18.1.	-1.44808084353272	0.000000000147691257583784	CER.16.0.	0.498066596455264	0.00329014129909331
PE.16.0.20.4.	1.23485234415914	0.00000000017903426571321	TAG50.0.FA18.0.	-0.420680036833431	0.00356181004857632
PE.P.16.0.18.2.	-1.60420459895242	0.000000000743770591936625	PC.18.2.20.1.	0.700019197586006	0.00745459774289769
PE.P.18.0.22.4.	-1.321670264655105	0.000000000901041695218125	PE.O.18.0.22.6.	0.729968693937742	0.00636230433087424
PE.P.18.2.20.4.	-1.13936415297105	0.000000000957835241453329	TAG54.6.FA18.2.	1.30026713100151	0.00397096410100532
PE.P.18.0.20.4.	-1.04735017833093	0.00000000159499893006703	PC.16.0.18.3.	0.509639615757465	0.00406269168734555
CER.20.0.	1.53568563955685	0.00000000435467891373497	DAG.16.0.18.0.	0.477348583675687	0.00639814053351402
PE.18.0.18.0.	-1.22121586339679	0.0000000011853234583958	PC.16.0.16.1.	0.22662039793642	0.00660817711378056
PC.18.1.20.4.	0.797812748244783	0.000000044220775682339	SM.22.0.	-0.21549065477146	0.00735910653321054
PE.P.18.1.18.2.	-1.13340951243925	0.000000044220775682339	PC.16.0.20.5.	0.849417251686928	0.00745459774289769
PE.16.0.20.4.	0.702294700558444	0.0000000529681822868361	PE.P.18.2.22.6.	-0.925436775505609	0.0124090731521852
SM.22.1.	0.638267450856569	0.000000147078289350383	TAG50.0.FA16.0.	-0.358983226126769	0.00904861463747979
PE.P.18.1.16.1.	-1.1019097628832	0.00000022532666237796	PE.O.16.0.18.2.	-0.548496412966616	0.0131307729590038
SM.18.0.	0.601346069157795	0.000000280061321996847	DCER.22.0.	0.440538512126979	0.00932939051787164
PE.P.18.1.20.4.	-0.738561650160882	0.000000301452301469059	CE.18.2.	0.292621915743016	0.00985345525986834
PE.18.1.20.4.	0.931258328538187	0.000000438776684045462	TAG54.5.FA18.2.	0.970162043677796	0.0103865100900173
SM.24.0.	-0.63199022834544	0.000000438776684045462	PC.16.0.16.0.	-0.235092301383505	0.0106513508899766
LPE.16.0.	0.790649074930108	0.000000545528408837572	PE.O.16.0.20.3.	-0.879972798001727	0.0318142276310397
SM.16.0.	0.571525578104449	0.00000058908697688462	TAG54.7.FA18.2.	0.957097132036337	0.0135175027932458
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PE.18.0.20.2.	-0.900632393006136	0.00000143455086349267	TAG50.2.FA18.1.	0.353292363702717	0.0131307729590038
CER.22.0.	0.661207709134967	0.00000176438323199717	TAG54.4.FA18.2.	0.830204662130765	0.0131307729590038
PE.O.16.0.20.4.	-0.644131176747164	0.0000035702152486542	TAG52.4.FA18.2.	0.897986219713285	0.0132781883329901
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PC.18.1.20.3.	0.666101678895718	0.00000736851385227996	TAG52.4.FA16.0.	0.844411733186737	0.0145385962138988
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PC.14.0.18.1.	0.607589640360818	0.0000222404250188152	TAG54.4.FA18.1.	0.618521332435456	0.0301060763905502
PE.O.16.0.18.1.	-1.39577321768516	0.0000461400118173489	PE.18.2.20.1.	0.522086564483462	0.0354422492248947
PE.16.0.16.0.	-0.871687188643726	0.0000371981365353811	PE.18.1.22.4.	0.490092964526495	0.0302640929435146
DCER.24.0.	-0.652027290738506	0.000042988080586421	TAG44.0.FA14.0.	0.338487951953327	0.0323886279973845
PC.16.0.18.0.	-0.371036177284731	0.0000447336573424528	DAG.18.1.22.6.	-0.493401906240654	0.0480123954822155
PC.18.2.20.4.	0.517237345002926	0.0000518645464338397	PC.18.0.18.1.	-0.167414522250886	0.0308140758494955
PC.16.0.22.4.	0.692109973548185	0.0000607956942773649	PE.18.0.16.0.	-0.404462508527292	0.0323378796846877
PE.18.1.18.2.	0.667258975687909	0.000084005688895731	TAG56.6.FA20.4.	-0.542134824774418	0.0430661586770124
PE.16.0.18.2.	0.542820398886691	0.000114676032426164	LPC.16.0.	0.216124047248552	0.0323886279973845
PE.16.0.22.4.	0.613037151130374	0.000114676032426164	TAG49.1.FA18.1.	0.52206751168797	0.0437113308713862
PE.P.16.0.22.6.	-1.34493313574372	0.000252526165764445	TAG52.2.FA18.2.	0.465752415985074	0.0323886279973845
PE.O.18.0.18.1.	-0.953002188486378	0.000194801182501587	TAG54.3.FA18.0.	0.455390152534385	0.037477907998335
PC.18.1.18.2.	0.432423095701925	0.000172168880883597	PC.16.0.20.1.	-0.257981115856248	0.0394525074762321
PC.16.0.22.5.	0.540979230268771	0.000180699009618296	TAG52.3.FA18.1.	0.541762131059743	0.043145719122025
SM.18.1.	0.435679646055815	0.000189894319227519	LPE.18.0.	0.213810204562281	0.0436536578265587
PE.18.0.18.3.	0.771812125591548	0.000386512959888325	TAG52.3.FA16.0.	0.594460690840936	0.0480123954822155
PE.O.16.0.22.4.	-0.49924517107176	0.00025936697848212	TAG49.0.FA16.0.	-0.28911610391043	0.0482126735484333
PE.P.18.0.20.3.	-1.31022168024846	0.000750774381101315			
PC.16.0.20.2.	-0.427068294742364	0.000481247705394052			
TAG47.0.FA17.0.	0.6198192050222	0.00105426704803128			
PE.P.18.1.20.3.	-0.910757634248965	0.00210820692502403			
PC.18.1.18.1.	0.344260573468068	0.00102058446933892			
LPC.18.0.	-0.317200614667126	0.00104420258783042			
CER.26.0.	-0.483462079406124	0.00104420258783042			

Table 2. Complete list of significantly modified lipids analyzed in ACC1 WT versus ACC1 pKO platelet.

Adj. P.Val: adjusted p-value; CE = cholesterol ester; CER = ceramide; DAG = diacylglycerol; DCE = dihydroceramide; FFA = free fatty acid; HCE = hydroxyceramide; LPC = lysophosphatidylcholine; LPE = lysophosphatidylethanolamine; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PEO = plasmalogen phosphatidylethanolamine; PEP = phosphatidylethanolamine plasmalogen; SM = sphingomyelin; TAG = triacylglycerol.

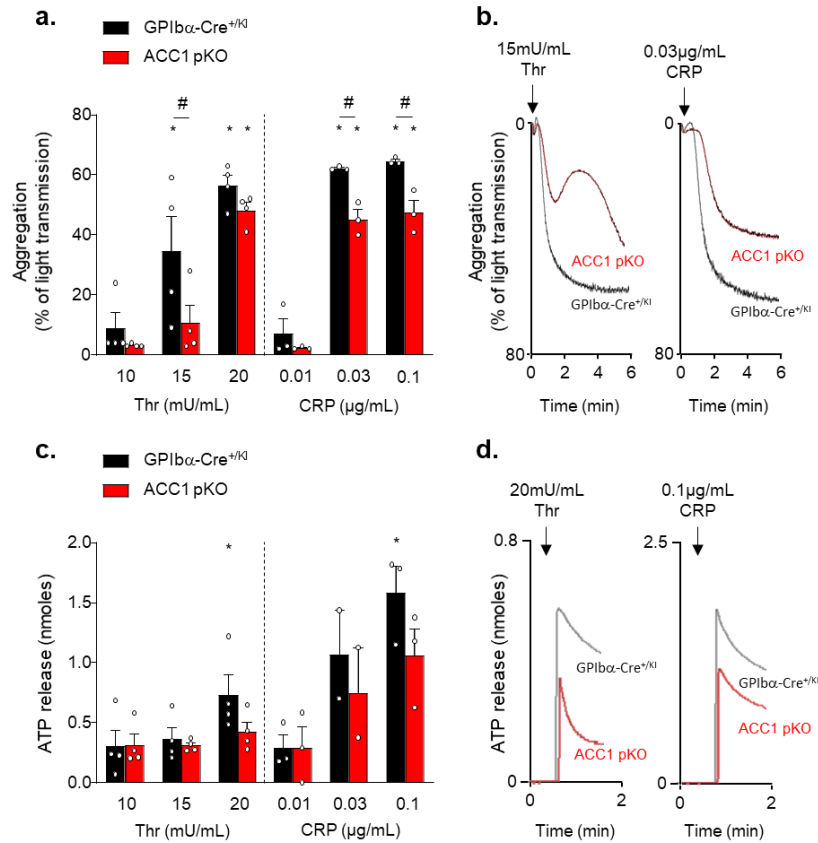
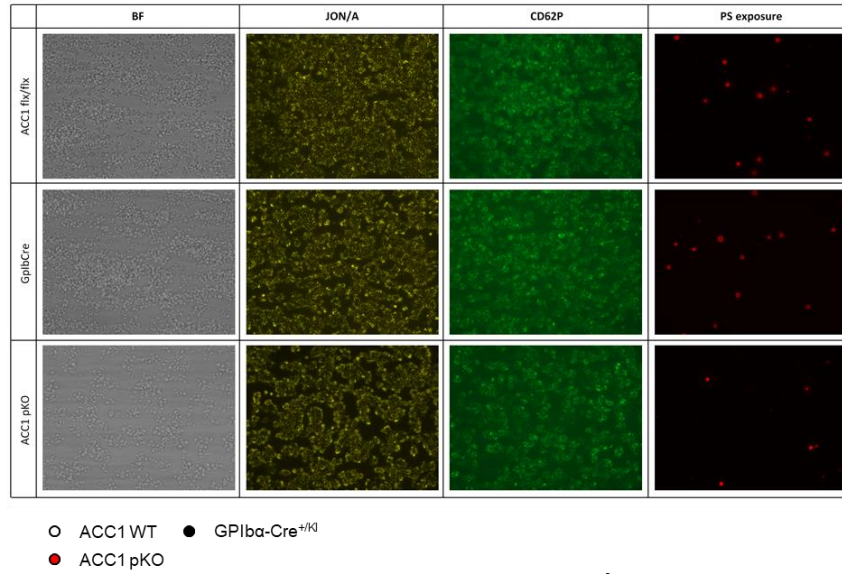
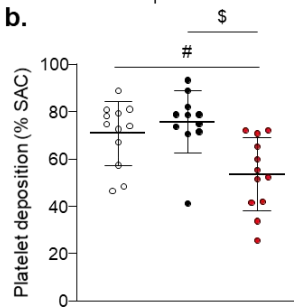


Figure S1. Platelet-specific ACC1 deletion decreases thrombin and CRP-induced platelet aggregation. (a-d) Washed murine platelets were stimulated with various thrombin (Thr) or collagen-related peptide (CRP) concentrations. (a-b) Aggregation was analysed by turbidimetry (Chrono-Log). (b) Profiles are shown. Data are expressed as means ± SEM (n = at least 3). * p-value ≤ 0.05 relative to the first agonist concentration. # p-value ≤ 0.05 relative to GPIIb/IIIa-Cre^{+/Kl} mice. Data underwent 2-way ANOVA. (c-d) Dense granules secretion was assessed via addition of luciferase-luciferin reagent (Chrono-log). (d) Profiles are shown. Data are expressed as means ± SEM (n = at least 3). * p-value ≤ 0.05 relative to the first agonist concentration. Data underwent 2-way ANOVA.

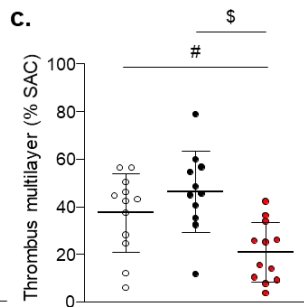
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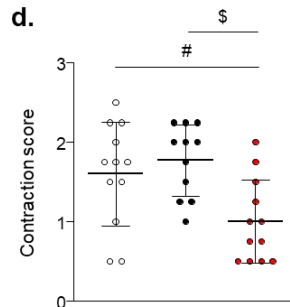
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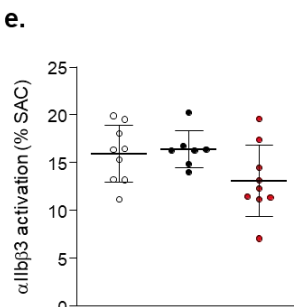
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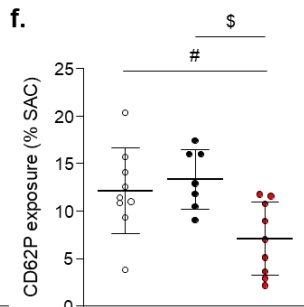
d.



e.



f.



g.

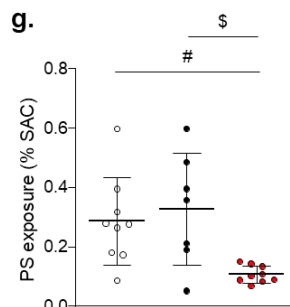


Figure S2. Platelet-specific ACC1 deletion decreases thrombus formation *ex vivo*. (a-g) Whole blood from ACC1 WT, GpiIb-Cre^{+/-} and ACC1 pKO mice was perfused over collagen III-coated surface (100 µg/mL) at a shear rate of 1000 s⁻¹. (a) Representative images of multilayered platelet thrombi and JON/A, CD62P and PS exposure staining. (b-d) Platelet deposition, thrombus formation and contraction score were assessed on brightfield images. (e-g) αIIbβ3 activation was evaluated by JON/A Ab (e), p-selectin expression by staining with CD62P Ab (f) and phosphatidylserine (PS) exposure by Annexin V (g). The results are expressed as means ± SD (at least 4 mice/group). # p-value ≤ 0.05 relative to ACC1 WT mice. \$ p-value ≤ 0.05 relative to GpiIb-Cre^{+/-} mice. Data underwent one-way ANOVA.

PART3: Role of platelet acetyl-CoA carboxylase phosphorylation on thrombus formation in atherosclerosis

The aim of this chapter was to evaluate the impact of platelet ACC phosphorylation on thrombus formation *in vivo* in an atherosclerotic environment.

In this article, we reported that:

- 1) Platelet ACC phosphorylation is increased in ApoE^{-/-} x SRBI^{flx/flx} mice transplanted with ACC WT bone marrow fed with a high-chol diet for four weeks.
- 2) Platelet ACC phosphorylation does not alter atherosclerosis formation in ApoE^{-/-} x SRBI^{flx/flx} transplanted mice.
- 3) Platelet ACC phosphorylation does not alter ferric chloride-induced thrombosis in ApoE^{-/-} x SRBI^{flx/flx} transplanted mice.

Role of platelet acetyl-CoA carboxylase phosphorylation on thrombus formation in atherosclerosis

Marie Octave¹, Laurence Piroton¹, Alice Marino¹, Valentine Robaux¹, Audrey Ginion¹, Caroline Bouzin², Guillaume Courtoy², Gregory R. Steinberg³, Bruce E. Kemp^{4,5}, Luc Bertrand¹, Christophe Beauloye^{1,6}, Sandrine Horman¹

¹Pôle de Recherche Cardiovasculaire (CARD), Institut de Recherche Expérimentale et Clinique (IREC), Université catholique de Louvain (UCLouvain), Brussels, Belgium

²IREC Imaging Platform, IREC, UCLouvain, Brussels, Belgium

³Division of Endocrinology and Metabolism, Departments of Medicine and Biochemistry and Biomedical Sciences, McMaster University, Hamilton, ON, Canada

⁴St. Vincent's Institute of Medical Research and Department of Medicine, University of Melbourne, Fitzroy, VIC, Australia

⁵Mary MacKillop Institute for Health Research, Australian Catholic University, Fitzroy, VIC, Australia

⁶Division of Cardiology, Cliniques Universitaires Saint-Luc, Brussels, Belgium

Abstract

Acetyl-CoA carboxylase (ACC) is a rate-limiting enzyme in *de novo* lipogenesis (DNL) by carboxylating acetyl-CoA into malonyl-CoA. Its phosphorylation on serine 79 residue by AMP-activated protein kinase (AMPK) induces its inhibition. The lack of ACC phosphorylation/inhibition, as seen in ACC double knockin (DKI) mice, leads to an increased thrombus growth *in vivo*, while pharmacological ACC inhibition decreases platelet aggregation. We recently demonstrated that circulating platelet ACC phosphorylation level is associated with the severity of atherosclerotic plaque burden either in high-risk coronary artery disease (CAD) patients or in atherosclerotic mice. Therefore, we hypothesized that the higher level of ACC phosphorylation observed in acute coronary syndrome patients could be a counter-regulatory mechanism to limit thrombosis. To investigate this, we performed a bone marrow transplant (BMT) from ACC WT or ACC DKI mice to ApoE^{-/-} x SRBI^{flx/flx} mice, fed with a hypercholesterolemic diet. Our work demonstrate that phosphorylation of ACC does not influence atherosclerosis development or thrombus growth in atherosclerotic mice, at least in a model based on FeCl₃-induced carotid artery injury.

Keywords: acetyl-CoA carboxylase, atherosclerosis, platelet, thrombus.

1. Introduction

In 2019, World Health Organization (WHO) estimated that 32% of deaths were due to cardiovascular diseases. Heart attack, mainly due to atherothrombosis, and stroke both account for 85% of these deaths [287]. Atherothrombosis is characterized by thrombus formation after rupture or erosion of an atherosclerotic plaque. When it obstructs coronary arteries, thrombosis leads to myocardial infarction [288]. Over the past decades, the Framingham risk score, based on sex, age, blood pressure, hyperlipidemia, smoking and diabetes, has been used to predict the occurrence of coronary artery disease (CAD) [289]. However, this score has limitations as some people with low or middle risk score experienced CAD. Therefore, several studies have tried to identify more efficient biomarkers to detect people with a higher risk of developing an acute coronary syndrome (ACS) [290]. Recently, we demonstrated that an increase in circulating platelet acetyl-CoA carboxylase (ACC) phosphorylation level is associated with the severity of atherosclerotic plaque burden either in high-risk coronary artery disease (CAD) patients or in ApoE^{-/-} x SRBI^{flx/flx} mice fed 12 weeks with a western type, hypercholesterolemic (high-chol), diet [227].

ACC is the rate-limiting enzyme of *de novo* lipogenesis (DNL). It carboxylates acetyl-CoA into malonyl-CoA, the precursor for fatty acids synthesis [246]. Its phosphorylation on serine 79 residue by AMP-activated protein kinase (AMPK) induces its inhibition [211]. Using a mouse model expressing mutant ACC isoforms that can not be phosphorylated by AMPK, i.e. ACC double knock-in (ACC DKI) mice, we demonstrated that lack of ACC phosphorylation/inhibition leads to an increased thrombus growth *in vivo* [57]. Moreover, pharmacological ACC inhibition decreases thrombin-induced platelet aggregation [277]. However, the impact of ACC phosphorylation on thrombus formation in an atherogenic environment remains unknown.

In this study, we explored the role of ACC phosphorylation on thrombus formation in ApoE^{-/-} x SRBI^{flx/flx} mice irradiated and transplanted with ACC WT or ACC DKI bone marrow (BM) and fed with a high-chol diet. We reported that bone marrow transplant

(BMT) of ACC WT hematopoietic stem cells in atherosclerotic mice fed with a high-cholesterol diet show an increased level of ACC phosphorylation in circulating platelets, while platelet ACC is not phosphorylated after ACC DKI BMT. The development of atherosclerotic lesions into artery branch sites from the aortic arch and along the aorta does not differ between ApoE^{-/-} x SRBI^{flx/flx} mice transplanted with ACC WT or ACC DKI BM. Comparable results were obtained when atherosclerotic plaque formation was analyzed in the aortic root of mice. Finally, thrombus growth was not different between the two types of atherosclerotic mice, at least in a model based on FeCl₃-induced carotid artery injury.

2. Results

2.1. ACC phosphorylation is increased in ApoE^{-/-} x SRBI^{flx/flx} mice transplanted with ACC WT bone marrow fed with a high-chol diet for four weeks.

First, we analyzed platelet ACC phosphorylation levels in our atherosclerotic mice, i.e. ApoE^{-/-} x SRBI^{flx/flx} mice irradiated, transplanted with ACC WT or ACC DKI BM and fed with a chow or a high-chol diet for four weeks. In agreement with our previous data [227], we show that platelets from ApoE^{-/-} x SRBI^{flx/flx} mice transplanted with ACC WT hematopoietic stem cells, and fed with a high-chol diet, have an enhancement in ACC phosphorylation level (**Figure 1a**). As expected, ApoE^{-/-} x SRBI^{flx/flx} mice receiving ACC DKI BM showed no phosphorylation of ACC, whatever the diet (**Figure 1a**).

2.2. Platelet ACC phosphorylation does not alter atherosclerosis formation in ApoE^{-/-} x SRBI^{flx/flx} transplanted mice.

Next, we investigated whether platelet ACC phosphorylation could affect atherosclerosis. Aorta was isolated from the two types of atherosclerotic mice, then fixed and stained with Oil red O to visualize lipid-rich lesion areas. As observed in **Figure 1b**, a four-week high-chol diet is sufficient to induce atherosclerotic lesions into artery branch sites from the aortic arch and along the aorta. However, no difference in lipid deposition was observed between mice with ACC WT or ACC DKI BM (**Figure 1b-c**). These results were confirmed after analysis of atherosclerotic plaque formation in the aortic root of mice fed with a high-chol diet for six weeks (**Figure 1d-e**).

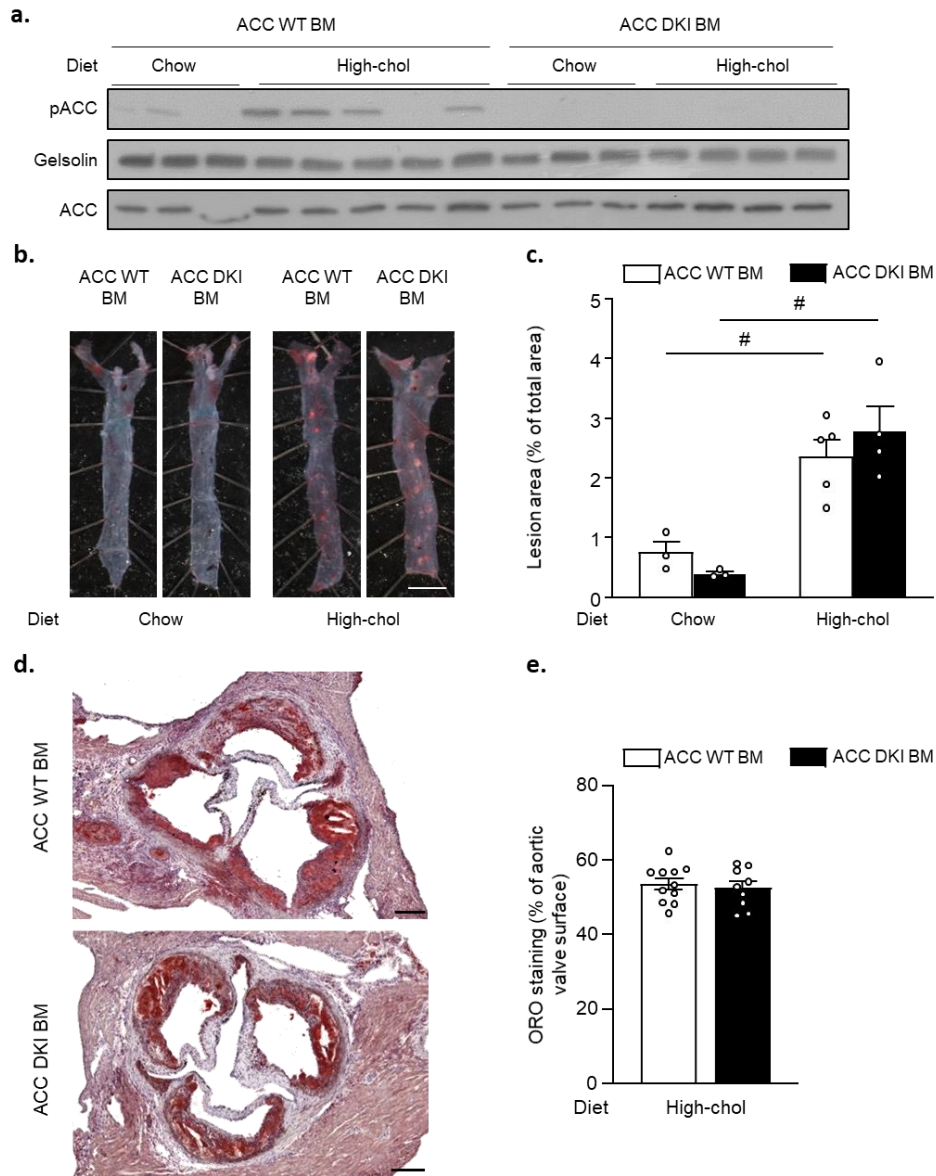


Figure 1. Platelet ACC phosphorylation and lipid deposition in *ApoE*^{-/-} x *SRBI*^{flx/flx} mice transplanted with ACC WT or ACC DKI bone marrow (BM). (a-e) *ApoE*^{-/-} x *SRBI*^{flx/flx} mice were irradiated and transplanted with ACC WT or ACC DKI BM. After four weeks, mice were fed with either a chow or a high-cholesterol (high-chol) diet for four weeks (a-c) or six weeks (d-e). (a) Washed murine platelets were lysed and subjected to western blotting for phosphoACC, gelsolin or ACC expression analysis. Gelsolin served as loading control for platelets. (b-c) Lipid deposition in the aorta was stained with Oil Red O. Representative pictures (b) and their quantifications (c) are shown. Scale bar; 2.5mm. Data are expressed as means $\pm$ SEM (n= at least 3). # p-value $\leq$ 0.001 relative to chow fed mice. Data underwent 2-way ANOVA. (d-e) Cross-sections of aortic roots from mice fed with high-chol diet were stained with Oil Red O. Representative pictures (d) and their quantifications (e) are shown. Scale bar; 200 μ m. Data are expressed as means $\pm$ SEM (n= at least 9). Data underwent unpaired t-test.

2.3. Platelet ACC phosphorylation does not alter ferric chloride-induced thrombosis in ApoE^{-/-} x SRBI^{flx/flx} transplanted mice.

To assess the impact of platelet ACC phosphorylation on thrombus formation in an atherosclerotic environment, we applied a 10% FeCl₃ solution on a carotid artery of ApoE^{-/-} x SRBI^{flx/flx} transplanted mice fed with a high-chol diet for eleven weeks (**Figure 2a-c**). FeCl₃ application induced thrombus formation in ApoE^{-/-} x SRBI^{flx/flx}-transplanted mice within 12 minutes (**Figure 2a-c**). The area occupied by thrombus gradually increased over time, and tended to be increased in mice with ACC DKI BM, compared to mice with ACC WT BM (**Figure 2b**), while the time necessary to reach the thrombus maximal size was not different in both types of mice (**Figure 2c**).

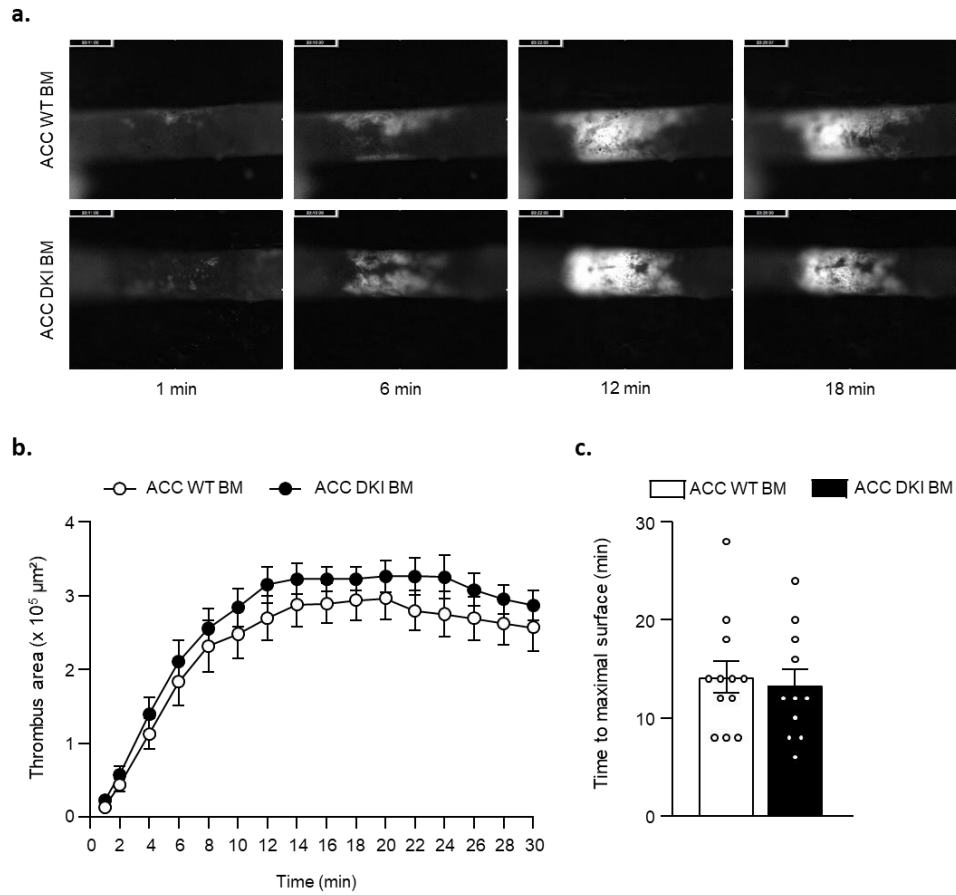


Figure 2. Thrombus formation in $\text{ApoE}^{-/-} \times \text{SRBI}^{\text{flx/flx}}$ mice transplanted with ACC WT or ACC DKI bone marrow (BM). (a-c) $\text{ApoE}^{-/-} \times \text{SRBI}^{\text{flx/flx}}$ mice were irradiated and transplanted with ACC WT or ACC DKI BM. After four weeks, mice were fed with a high-cholesterol diet for eleven weeks. Then, mice were subjected to *in vivo* FeCl_3 -induced thrombosis in carotid artery (10% FeCl_3 , 10 min). Thrombus formation was visualized by analyzing exogenous carboxyfluorescein succinimidyl ester (CFSE)-stained platelet accumulation by intravital microscopy. (a) Representative pictures of thrombus formation at 1, 6, 12 and 18 min after FeCl_3 application on a carotid artery. (b) Quantification of thrombus formation by measuring its area over time. Data are expressed as means $\pm$ SEM ($n = \text{at least } 11$). Data underwent unpaired t-test. (c) Analyses of the time when the thrombus reaches its maximal size. Data are expressed as means $\pm$ SEM ($n = \text{at least } 11$). Data underwent unpaired t-test.

3. Discussion

We recently reported that platelet ACC phosphorylation level is a potential risk stratification biomarker of CAD [227]. We also showed that modulating ACC activity in a non-atherogenic environment alters platelet aggregation and thrombus growth [57, 277]. Here, we investigated the impact of platelet ACC phosphorylation on thrombus formation in atherosclerotic mice. Using ApoE^{-/-} x SRBI^{flx/flx} mice irradiated, transplanted with ACC WT or ACC DKI BM and fed with a high-chol diet, we confirm that ACC phosphorylation is increased in ACC WT platelets of atherosclerotic mice. However, it does not influence atherosclerosis development and barely impacts thrombosis in a model of FeCl₃-induced carotid artery injury.

It is recognized that macrophage-derived ApoE protects these cells from lipid accumulation and foam cells formation by inducing cholesterol efflux. This effect limit the development of atherosclerotic lesions [291, 292]. Unsurprisingly, ApoE^{-/-} mice transplanted with ApoE WT BM and fed with a western type diet for four weeks display complete protection against aortic atherosclerosis [292]. However, atherosclerotic lesions were observed after four weeks of high-chol diet in ApoE^{-/-} x SRBI^{flx/flx} mice transplanted with ACC WT or ACC DKI BM. This discrepancy can be explained by the fact that SRBI^{flx/flx} mice are hypomorphic and develop atherosclerosis when fed with a western type diet [293]. Lowering SRBI expression in ApoE^{-/-} mice further enhances atherosclerosis progression, compared to SRBI^{-/-} or ApoE^{-/-} mice [294, 295]. Moreover, the protective effect of macrophage-derived ApoE on atherosclerotic lesions in ApoE^{-/-} mice is reduced when these mice are fed with a western type diet for a longer period (15 weeks) [296]. This data suggest a contribution of non-hematopoietic cells-derived ApoE in atherosclerosis development.

Platelets are important in atherosclerosis progression. They recruit monocytes to the injured endothelium [100, 101]. While increasing platelet activation enhances atherosclerotic lesions, blocking platelet adhesion to the endothelium decreases plaque formation [102-104]. Here, we confirm that AMPK-ACC signaling is activated in

circulating platelets of atherosclerotic mice [227]. However, we previously showed that increased ACC phosphorylation, in atherosclerotic mice or in high-risk CAD patients, was not related to platelet activation, but was associated with altered metabolic phenotypes, especially triglyceride downregulation [227]. Although we did not evaluate platelet lipid content or reactivity after BMT experiments, we show here that the phosphorylation status of platelet ACC does not influence lipid deposition along the aorta and at the aortic root.

As we demonstrated that ACC DKI mice had a prothrombotic phenotype [57], we hypothesized that a higher ACC phosphorylation level in platelets could be a counter-regulatory mechanism for thrombosis in a proatherogenic environment. However, we do not see any differences in thrombus formation between ApoE^{-/-} x SRBI^{flx/flx} mice with ACC WT or ACC DKI BM, at least in a model based on FeCl₃-induced carotid artery injury. Of note, this model has several limitations notably about the underlying mechanisms by which FeCl₃ triggers thrombosis. It's commonly admitted that FeCl₃ induces oxidative stress that leads to lipid peroxidation and endothelial cell damages [297]. However, some studies suggest that red blood cells initiate thrombosis by binding to Fe³⁺ at the endothelium and then recruit platelets [298]. The potential implication of other blood components still needs further investigations. Moreover, thrombus stability differs depending on the mouse strain and the concentration of FeCl₃ used [298].

The complex regulation of ACC activity should also be taken into account. Indeed, allosteric binding of palmitoyl-CoA inhibit ACC activity [210]. Since platelets from CAD patients can uptake triglycerides (TAG) from the plasma [227], it is plausible that C16:0 fatty acyl chains from TAG could act as ACC inhibitors and attenuate the impact of platelet ACC DKI on plaque development or thrombosis. Therefore, BMT experiments using platelet-specific ACC1 KO mice could help us to investigate whether ACC inactivation is a counter-regulatory mechanism limiting thrombosis in a proatherogenic environment.

4. Materiel and methods

4.1. Reagents

Apyrase (#A6132), acid citrate-dextrose solution (ACD) (#C3821), Oil Red O (#O0625) and horse radish peroxidase (HRP)-conjugated anti-rabbit (#A0545) antibodies (Abs) were purchased from Sigma. Bovine serum albumin (BSA) (#3854.4) and D-saccharose (#9286.1) were purchased from Roth. Fatty acid-free BSA (#126575) was from Millipore. Anti-ACC (#3676), anti-phospho-ACC (#11818) anti-rabbit IgG HRP-linked (#7074) and anti-gelsolin (#12953) Abs were obtained from Cell Signaling. Carboxyfluorescein succinimidyl ester (CFSE) (#C34554) and Red Blood cell Lysis buffer 1X (#00-4333-57) were purchased from ThermoFisher Scientific. α MEM 1X + Glutamax medium (#32561-029) was from Gibco. Iso-pentane was purchased from vWR (#103616V). Ketamine (Nimatek) was purchased from Dechra. Xylazine (Rompun) was obtained from Bayer. Tissue-Tek® O.C.T. compound was from Sakura (#4583).

4.2. Mice

ApoE^{-/-} x SRBI^{flx/flx} mice were kindly received from Philippe Lesnik [293]. ACC1/2 DKI mice were described previously [219]. Fourteen- to twenty-seven-week-old male and female mice were studied. Animal procedures and protocols were approved by local authorities (Comité d'éthique facultaire pour l'expérimentation animale, 2016/UCL/MD/027 and 2021/UCL/MD/012) and performed in accordance with the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH Publication, revised 2011).

4.3. Bone marrow transfer

Six- to eight-week-old ApoE^{-/-} x SRBI^{flx/flx} mice (recipient mice) were irradiated (2 x 25min; 10 Gy) and transplanted with ACC WT or ACC DKI bone marrow (BM; donor mice). BM cells were flushed from mice tibia and femur in α MEM 1X + Glutamax + Pen/Strep (1%) medium. Cells were filtered and centrifuged at 300g for 7 minutes. Cells

pellet was resuspended into red blood cells lysis buffer for 5 minutes before being washed twice with PBS (300g; 7 minutes). Finally, cells were resuspended in PBS endotoxin free solution at 10×10^6 cell/mL. BM cells (2×10^6) were injected, via the retro-orbital plexus, into recipient mice, previously anesthetized with a mix of ketamine: domitor (75mg/Kg:1.5mg/Kg). Mice were woke up at 37°C with an IP injection of antisedan (1.5mg/Kg) and kept in ventilated cages. After four weeks, mice were fed with either a chow or a hypercholesterolemic (high-chol), i.e. western type diet (A04 + 1.25% cholesterol, 16% cocoa butter, U8220 version 151, Safe, Augy, France) for four weeks, unless says otherwise.

4.4. Murine platelets preparation

Mice were anesthetized with a mix of ketamine:xylazine (150mg/Kg:20mg/Kg). Blood was taken from retro-orbital plexus, collected in a mix of ACD : apyrase (1/6 : 1U/mL) and centrifuged at 800g for 5 seconds, followed by 5 minutes at 100g. Platelet-rich plasma (PRP) was isolated and mix with ACD : apyrase (2/3 : 1U/mL) before a centrifugation at 400g for 5 minutes. Platelets pellet was resuspended to a density of $2.5 \times 10^5/\mu\text{L}$ (unless say otherwise) in modified Tyrode's buffer (135mM NaCl, 12mM NaHCO_3 , 3mM KCl, 0.3mM Na_2HPO_4 , 1mM MgCl_2 , 5mM D-glucose, 10mM Hepes, 0.35 % BSA, pH 7.4, 37 °C). Cell-dyn Emerald (Abbott) was used to verify platelet count.

4.5. Western blotting

Platelets were lysed with modified blue Laemmli's buffer 1X (50mM Tris pH 6.8, 10% (w/v) glycerol, 2% (w/v) SDS, 0.5mM EDTA, 0.01% (w/v) bromophenol blue, 25mM DTT). Lysates were subjected to western blot for phospho-ACC, ACC and gelsolin. All Abs were diluted in 5% BSA. The dilution for primary Abs were (1/1,000) except for anti-gelsolin (1/50,000). Secondary HRP-conjugated anti-rabbit Abs from Cell Signaling were diluted (1/5,000), HRP-conjugated anti-rabbit Abs from Sigma were diluted (1/20,000).

4.6. Lipids staining in the aorta

Aorta was isolated and external fat removed before being fixed with 4% paraformaldehyde (PFA) solution for 20 minutes. Washed aorta was stained with 0.2% Oil Red O for 20 minutes and then washed again. Aorta was open before taking pictures using a camera (Canon EOS M50 with EF-M 32mm f/1.4 STM lens) on a microscope (Olympus SZ61; KL 1500). Lipids deposition were quantified using Image J software.

4.7. Lipids staining in aortic valves

Mice were fed with high-chol diet for six weeks. Hearts were harvested and fixed in 4% PFA solution at 4°C, overnight. After three PBS washes, hearts were cut into three parts, i.e. the base, the middle and the apex, and put in 30% sucrose solution at 4°C, overnight. Sucrose solution was replaced by OCT:30% sucrose (1:1) solution for one night at 4°C. Pieces of the heart were embedded into OCT:30% sucrose solution and frozen with liquid nitrogen. Cross-sections of the aortic valve were stained with Oil Red O for 15 minutes, washed and counterstained with hematoxylin for 2 minutes. Images were taken using Axio Scan.Z1 slide scanner (Zeiss) and analyzed by QuPath software.

4.8. Ferric-chloride induced thrombosis

Mice were fed for eleven weeks with a high-chol diet before the experiment. PRP was incubated 2 minutes with CFSE (4.3µg/mL) before being centrifuged at 958g for 10 minutes. Platelet pellet was resuspended in sterile 0.9% NaCl and injected in the jugular vein of anesthetized (150 mg/Kg ketamine : 10 mg/Kg xylazine) mice with the corresponding genotype. A carotid artery of the anesthetized mice was injured by local application of 10% ferric chloride (FeCl₃) solution for 10 minutes. Fluorescence was recorded by Zeiss fluorescence microscope (Carl Zeiss). Images were captured using screenshot software (50 images/second) and exported every 2 minutes for quantification. Thrombus formation was quantified using Image J software.

General discussion and perspectives

I. The contribution of acetyl-CoA carboxylase in the regulation of platelet functions: *de novo* lipogenesis and/or protein acetylation?

Inhibition of ACC1 activity results, on one hand, in a modification of PL composition and, on the other hand, in an increase in protein acetylation. Both processes regulate platelet functions as modifying acetylation level by targeting HDAC6 impaired platelet aggregation, and increasing platelet AA-containing PEP content enhanced thrombus growth [57, 232]. The change in protein acetylation occurs faster than the modification of fatty acyl chains-containing PLs as inhibiting ACC for two hours is sufficient to increase α -tubulin acetylation but does not alter significantly platelet PL content [277]. Accordingly, in endothelial cells, a short-time treatment (4 hours) with an ACC inhibitor does not affect significantly PL composition [223]. Therefore, the regulation of protein acetylation level seems to be a way by which a short-term ACC inhibition controls platelet aggregation *in vitro*.

As it has been shown in Src-transformed 3T3 and endothelial cells, that 24 hours treatment with a pharmacological ACC inhibitor is sufficient to modify PL composition, it is not surprising that platelet-specific ACC1 deletion alters PL levels [223, 225]. As ACC1 is implicated in DNL, we could expect a global decrease in lipid content after its deletion. However, we only observed a downregulation of PEP and PEO lipid classes. Furthermore, it decreases saturated fatty acyl chains and increases polyunsaturated fatty acyl chains in PC and PE. These data are also observed in cells treated with pharmacological ACC inhibitor and result in a less fluid and more rigid membrane [223-226]. Interestingly, omega-3 PUFA are known to exert cardioprotective effect via antithrombotic property. Indeed, mice fed six weeks with a DHA/EPA-enriched diet show a decrease in laser-induced cremaster arteriole thrombosis and fibrin formation

[299]. The decrease in AA-containing PEP and the modifications in PL composition observed in ACC1 pKO platelets are therefore probably responsible for the decrease in TxA₂ generation and dense granule secretion, with subsequent decreased aggregation and thrombus formation *ex vivo*. Moreover, the assumed membrane rigidity might alter filopodia formation which is also known to stabilize platelet aggregates [73, 74]. Furthermore, the alteration of ACC interactome in ACC1 pKO mice could participate in the difference observed between our *in vitro* data using a pharmacological ACC inhibitor and our results obtained with ACC1 pKO mice. However, little is known about the proteins associated with ACC and it is therefore hard to speculate about proteins that would be directly impacted by ACC1 deletion in platelets [300].

We cannot leave out an impact of protein acetylation in ACC1 pKO platelets. In line with that statement, we observed an increase in α -tubulin acetylation level in platelets from ACC1 pKO mice (data not shown). Moreover, it has previously been reported that inhibiting ACC modifies protein and histone acetylation [237, 240]. Interestingly, an increase in acetylated enzyme involved in glycogen breakdown, glycolysis and fatty acid oxidation were observed in mice lacking hepatic ACC1/2 [240]. It would be worth analyzing the impact of platelet-specific ACC1 deletion on protein level and acetylation level in platelets and MKs.

II. Acetyl-CoA carboxylase in the regulation of phospholipid content

Intriguingly, ACC inhibition increases some PUFA-containing PC and PE, while they have a dietary source [223-226]. Moreover, blocking DNL by inhibiting FAS enzyme also leads to an increase in PUFA-containing PC [301]. All together, these data raise the question about the source of these increased PLs in platelets. It is recognized that circulating LDL, HDL and VLDL can transfer PLs to platelet without lipoprotein internalization [155, 302]. However, *in vitro* studies showed an increase in some PUFA-PC and PE even in the absence of external sources of lipids, suggesting the participation of an endogenous regulation [223-226, 301]. A possible mechanism could be an increase of PA generation

(= PL precursor) from glucose catabolism (G3P and DHAP) in combination with PL remodeling, i.e. deacylation and reacylation cycle or transacylation. Lipidomic analyses of plasma and MKs of platelet-specific ACC1 KO mice might tell us if platelets acquire this modified PL content from plasma/MKs or if there are PL modifications occurring only in platelets.

III. The involvement of acetyl-CoA carboxylase in platelet activation in an atherogenic environment

Hyperlipidemic environment results in a prothrombotic phenotype in murine and human platelets [303, 304]. Because we showed that active ACC increases thrombus growth [57], while ACC inhibition/invalidation impairs platelet aggregation [277], we postulated that the higher ACC phosphorylation level seen in CAD patients could be a protective mechanism to attenuate platelet activation in an atherogenic environment. However, we did not observe any difference in thrombus formation between ApoE^{-/-} x SRBI^{flx/flx} with a high level of platelet ACC phosphorylation (ACC WT BM) and ApoE^{-/-} x SRBI^{flx/flx} with platelets without ACC phosphorylation (ACC DKI BM). One possible explanation could be that the hyperlipidemic environment hides ACC DKI-induced thrombus growth increase effect by enhancing platelet reactivity in both genotypes. It is also possible that ACC DKI platelets, lacking AMPK-induced ACC phosphorylation, can be allosterically inhibited by the increase in acyl-CoAs. As platelets from CAD patients uptake TAG from the circulation [227], we can postulate that platelets from our atherosclerotic mouse models have a higher TAG content acting as a reservoir of fatty acyl chains that, once cleaved, can inhibit ACC activity. Deciphering more precisely the role of platelet ACC1 on atherosclerosis and platelet aggregation requires further investigations. For example, we could study thrombus formation of platelets lacking ACC1 submitted to a proatherogenic environment. A BMT of ACC1^{flx/flx} mice or ACC1 pKO mice into ApoE^{-/-} x SRBI^{flx/flx} mice has to be performed, followed by the analysis of FeCl₃-induced thrombosis. Several ACC inhibitors have also been developed and can be used *in vivo*. As a dysregulated DNL is associated with pathologies such as NAFLD, type

II diabetes or cancer [202, 208], ACC inhibitors were first designed as a potential treatment for these diseases [204, 205, 208, 209, 221]. To the best of our knowledge, the impact of ACC inhibitors has not been investigated in the context of atherosclerosis. In the case of NASH, a specific liver-targeted small-molecule ACC1/2 inhibitor shows a reduced liver TAG, while these lipids are increased in plasma [204, 305]. The latter has been confirmed in mice lacking liver ACC1/2 [305]. Interestingly, another study revealed that PF-05175157 ACC inhibitor decreases platelet count but still within the normal range [207]. Thus, it is possible that ACC inhibitors, by increasing plasma TAG, enhance platelet reactivity and therefore promote atherosclerosis development. In the contrary, it could also attenuate its progression by decreasing platelet count, macrophage-induced atherosclerotic lesions [306] and reduce platelet reactivity [277]. It could be worth analyzing the impact of clinically used ACC inhibitors on thrombus growth.

IV. Lysine deacetylases in platelet activation in an atherogenic environment

As a short-term ACC inhibition alters platelet aggregation via an increase in α -tubulin acetylation level [277], HDAC or SIRT inhibitors could also be interesting targets as potential treatment for prothrombotic diseases like atherosclerosis. Unsurprisingly, the effect of HDAC inhibitors on endothelial dysfunction and atherosclerosis progression was already nicely reviewed in 2020. Depending on the type of HDACs targeted, inhibitors have pro- or anti-atherogenic properties [307]. Interestingly, specific HDAC6 inhibitor, tubacin, prevents atherosclerosis development in atherosclerotic mice [308]. However, the authors did not assess the impact of tubacin on thrombosis in their model. Therefore, it would be worth analyzing the impact of HDAC6 inhibition on thrombus formation in atherosclerotic mice.

V. Acetyl-CoA carboxylase in the regulation of anti- and pro-inflammatory lipid mediators

Sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to infection. It is characterized by vasodilatation, hyperpermeability and a procoagulant state leading to more thrombin generation [309, 310]. Moreover, an alteration of anti- and pro-inflammatory lipid mediators occurs during sepsis. The increase in AA release from PLs seems to promote inflammation as an enhancement of prostaglandins, TxA₂, LTB₄ or 12-HETE molecules occurs during sepsis. However, this could be counterbalanced by resolvins as their generation from either eicosapentaenoic acid (EPA, C20:5), docosahexaenoic acid (DHA, C22:6) or docosapentaenoic acid (DPA, C22:5) is also observed [311]. As thrombin increases platelet ACC phosphorylation level [228], we postulated that platelets from septic shock patients could have a higher level of ACC phosphorylation. This is now being evaluated by our group in the PLACCSEPS (Prospective evaluation of PLatelets Acetyl-CoA Carboxylase phosphorylation state in SEPTic Shock patients) clinical study (NCT04107402). Moreover, since we recently showed that platelet ACC1 is involved in the regulation of PL composition, its inhibition could modulate platelet inflammatory lipid mediators synthesis and subsequent endothelial cell and/or platelet activation. This hypothesis is currently tested in the lab in ACC WT and ACC DKI submitted to a sublethal dose of lipopolysaccharide (LPS) (10mg/Kg).

VI. Polymorphisms in acetyl-CoA carboxylase 1 gene

ACACA, the gene encoding for ACC1 is located, in humans, on chromosome 17. Some single nucleotide polymorphisms (SNPs) of this gene are associated with the overall survival of patients suffering from hepatocellular carcinoma (HCC), a disease linked with an increased hepatic DNL [312-314]. Given the role of ACC in lipogenesis, studies between ACACA SNPs and fatty acid content were investigated. They revealed that some SNPs are associated with a change in short-chain fatty acid (C8:0, C10:0 and

C12:0) and middle and long-chain fatty acid (C14:0, C16:0, C18:0) composition in bovine milk [315-317]. Another study showed an association with hypertriglyceridemia in patients taking some antipsychotic drugs [318]. So far, no research has been done regarding the impact of ACACA SNPs on platelet functions.

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Annexes



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Article

Acetyl-CoA Carboxylase Inhibitor CP640.186 Increases Tubulin Acetylation and Impairs Thrombin-Induced Platelet Aggregation

Marie Octave ¹, Laurence Piroton ¹, Audrey Ginion ¹, Valentine Robaux ¹, Sophie Lepropre ¹, Jérôme Ambroise ², Caroline Bouzin ³, Bruno Guigas ⁴, Martin Giera ⁵, Marc Foretz ⁶, Luc Bertrand ¹, Christophe Beauloye ^{1,7} and Sandrine Horman ^{1,*}



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- ¹ Pôle de Recherche Cardiovasculaire (CARD), Institut de Recherche Expérimentale et Clinique (IREC), Université Catholique de Louvain (UCLouvain), 1200 Brussels, Belgium; marie.octave@uclouvain.be (M.O.); laurence.piroton@uclouvain.be (L.P.); audrey.ginion@uclouvain.be (A.G.); valentine.roboux@uclouvain.be (V.R.); sophie.lepropre@gmail.com (S.L.); luc.bertrand@uclouvain.be (L.B.); christophe.beauloye@uclouvain.be (C.B.)
- ² Centre de Technologies Moléculaires Appliquées, Institut de Recherche Expérimentale et Clinique (IREC), Université Catholique de Louvain (UCLouvain), 1200 Brussels, Belgium; jerome.ambroise@uclouvain.be
- ³ IREC Imaging Platform, Institut de Recherche Expérimentale et Clinique (IREC), Université Catholique de Louvain (UCLouvain), 1200 Brussels, Belgium; caroline.bouzin@uclouvain.be
- ⁴ Department of Parasitology, Leiden University Medical Center, 2333 ZA Leiden, The Netherlands; B.G.A.Guigas@lumc.nl
- ⁵ Department of Molecular Cell Biology, Leiden University Medical Center, 2333 ZA Leiden, The Netherlands; m.a.giera@lumc.nl
- ⁶ CNRS, INSERM, Institut Cochin, Université de Paris, F-75014 Paris, France; marc.foretz@inserm.fr
- ⁷ Division of Cardiology, Cliniques Universitaires Saint-Luc, 1200 Brussels, Belgium
- * Correspondence: Sandrine.horman@uclouvain.be; Tel.: +32-2-764-55-66

Abstract: Acetyl-CoA carboxylase (ACC) is the first enzyme regulating de novo lipid synthesis via the carboxylation of acetyl-CoA into malonyl-CoA. The inhibition of its activity decreases lipogenesis and, in parallel, increases the acetyl-CoA content, which serves as a substrate for protein acetylation. Several findings support a role for acetylation signaling in coordinating signaling systems that drive platelet cytoskeletal changes and aggregation. Therefore, we investigated the impact of ACC inhibition on tubulin acetylation and platelet functions. Human platelets were incubated 2 h with CP640.186, a pharmacological ACC inhibitor, prior to thrombin stimulation. We have herein demonstrated that CP640.186 treatment does not affect overall platelet lipid content, yet it is associated with increased tubulin acetylation levels, both at the basal state and after thrombin stimulation. This resulted in impaired platelet aggregation. Similar results were obtained using human platelets that were pretreated with tubacin, an inhibitor of tubulin deacetylase HDAC6. In addition, both ACC and HDAC6 inhibitions block key platelet cytoskeleton signaling events, including Rac1 GTPase activation and the phosphorylation of its downstream effector, p21-activated kinase 2 (PAK2). However, neither CP640.186 nor tubacin affects thrombin-induced actin cytoskeleton remodeling, while ACC inhibition results in decreased thrombin-induced reactive oxygen species (ROS) production and extracellular signal-regulated kinase (ERK) phosphorylation. We conclude that when using washed human platelets, ACC inhibition limits tubulin deacetylation upon thrombin stimulation, which in turn impairs platelet aggregation. The mechanism involves a downregulation of the Rac1/PAK2 pathway, being independent of actin cytoskeleton.

Keywords: acetyl-CoA carboxylase; platelet; platelet aggregation; acetylation; tubulin; actin cytoskeleton; Rac1 signaling

1. Introduction

Acetyl-CoA carboxylase (ACC) catalyzes the first step of de novo lipogenesis (DNL) by carboxylating acetyl-CoA into malonyl-CoA [1]. There are two ACC isoforms, ACC1 and ACC2, which are differentially distributed amongst cells and tissues [2]. ACC1 provides a pool of cytosolic malonyl-CoA, which serves as a substrate for fatty acid synthase and chain elongation systems. ACC2 produces malonyl-CoA near the mitochondrial membrane where it acts as an allosteric inhibitor of carnitine palmitoyltransferase 1 (CPT1); this enzyme is responsible for transporting long-chain fatty acids into mitochondria [3–5]. ACC activity is acutely controlled by both allosteric modulators and the phosphorylation of several serine residues [5,6]. We previously demonstrated that ACC1 is the predominant isoform in murine and human platelets [7]. ACC1 promotes thrombosis through platelet phospholipid production, which is required for the synthesis of arachidonic acid (AA), a key mediator of platelet activation [7]. More recently, the presence of ACC in DNL has also been implicated in megakaryocyte (MK) maturation and platelet production [8]. ACC's critical role in megakaryocytes (MKs) and platelets raises the question of a potential therapeutic usefulness of prescribing pharmacological ACC inhibitors for a plethora of diseases characterized by DNL dysregulation [9–12], including Type 2 diabetes, cancer, or non-alcoholic fatty liver disease [10,13].

ACC inhibition not only decreases lipogenesis, but concomitantly results in elevated cytoplasmic levels of acetyl-CoA, which serves as a substrate for protein acetylation [14,15]. The latter process is a posttranslational modification that is regulated by lysine acetyltransferases (KATs) and histone deacetylases and sirtuins (HDACs, SIRT2) [16]. In platelets, protein acetylation on lysine residues is catalyzed by KATs P300 and α -TAT [17,18]. Deacetylation is induced by lysine deacetylases HDAC6 and SIRT2 [19,20]. Among acetylatable proteins, as identified in platelets using proteomic studies [17], tubulin is recognized to play a crucial role in regulating platelet formation and functions [19,21–23]. MKs and platelets express at least six isoforms of α and β -tubulin. In resting platelets, stable microtubules that form the marginal band are composed of α - and β -tubulin heterodimers, which are highly acetylated (on Lys40 residue of α -tubulin) so as to preserve the discoid shape [24–26]. Upon agonist stimulation, α -tubulin is massively deacetylated by HDAC6, which results in the condensation of microtubules towards the platelets' center, where they undergo de-polymerization [19]. This process is followed by their fragmentation and re-polymerization in order to generate a new microtubule coil [24–26]. According to several studies, this deacetylation process coordinates cytoskeletal and signaling events upon platelet activation, which in turn promote platelet spreading and aggregation [19,27].

In this study, we focused on the impact of the pharmacological ACC inhibitor, CP640,186, on the acetylation status of tubulin in human platelets. In addition, we examined the effect of ACC inhibition on platelet aggregation and secretion, as well as on cytoskeleton signaling and organization. We have herein demonstrated for the first time that ACC inhibition limits tubulin deacetylation upon thrombin stimulation, thereby impairing platelet aggregation. The molecular mechanism involves a downregulation of the Rac1/PAK2 pathway, while being independent of actin cytoskeleton. Similar results were obtained with tubacin, an inhibitor of HDAC6. Moreover, we have detected a decrease in thrombin-induced reactive oxygen species (ROS) production and extracellular signal-regulated kinase (ERK) phosphorylation in CP640,186-treated platelets. These findings suggest that ACC inhibitors may possibly alter platelet functions through a mechanism that is similar, at least to some extent, to that of HDAC inhibitors, which are well recognized to result in thrombocytopenia and hemostasis defects [28,29].

2. Results

2.1. Short-term ACC Inhibition Using CP640,186 Does Not Reduce Lipid Content in Human Platelets and Preserves Mitochondrial Function

We first investigated whether short-term pharmacological ACC inhibition would be able to reduce DNL in human platelets. Platelets were incubated with [$1\text{-}^{14}\text{C}$]-acetic

acid and treated for 2 h with DMSO or CP640.186 (60 μ M), the latter being an isozyme-nonselective inhibitor of mammalian ACC. As a result, fatty acid synthesis was drastically reduced in CP640.186-treated platelets (Figure 1a). To evaluate the consequences of CP640.186 treatment on overall platelet lipid content, we undertook a quantitative lipidomic analysis of dimethyl sulfoxide (DMSO)- and CP640.186-treated platelets. Overall, 672 lipids were detected, including different phospholipids (phosphatidylethanolamine [PE], phosphatidylethanolamine plasmalogen [PEP], plasmalogen phosphatidylethanolamine [PEO], phosphatidylcholine [PC], lysophosphatidylethanolamine [LPE], lysophosphatidylcholine [LPC]), sphingomyelin [SM], cholesteryl esters [CE], free fatty acids [FFA], diacylglycerol [DAG], and triacylglycerol [TAG]) (Figure 1b). Paradoxically, a slight increase in 63 lipids belonging to TAG, LPC, and LPE lipid classes was observed following CP640.186 treatment (Figure 1b; Table S1). However, lipid variations were low, consisting of a maximum 1.25-fold change, which indicates that a 2-h CP640.186 treatment does not drastically modify overall platelet lipid composition.

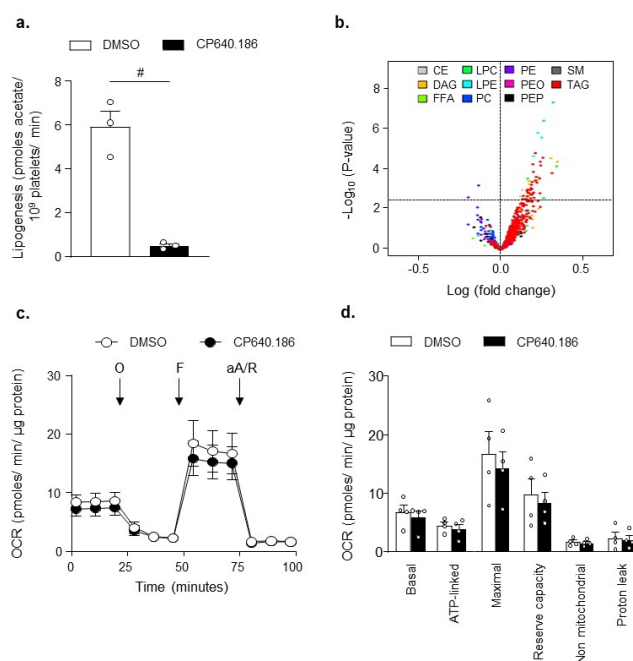


Figure 1. ACC inhibition induced by CP640.186 does not reduce overall lipid content and preserves mitochondrial function. (a) Washed human platelets were incubated with [1 – ¹⁴C]–acetic acid for 2 h in the presence of dimethyl sulfoxide (DMSO) or CP640.186 (60 μ M). Radioactivity incorporation into lipid fractions was determined. Data were expressed as means $\pm$ SEM ($n = 3$); # p -value ≤ 0.0001 as compared to DMSO condition. Data underwent unpaired t-test. (b) Washed

human platelets were preincubated with DMSO or CP640.186 (60 μ M) before lipid extraction. Volcano plot representation of 672 detected lipid species. Lipids above the horizontal dotted line were up (right)– or down (left)–regulated with significant adjusted p -values ≤ 0.05 . Log fold-changes and adjusted p -values were calculated from the multivariate regression model. CE = cholesterol ester; DAG = diacylglycerol; FFA = free fatty acid; LPC = lysophosphatidylcholine; LPE = lysophosphatidylethanolamine; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PEO = plasmalogen phosphatidylethanolamine; PEP = plasmalogen phosphatidylethanolamine; SM = sphingomyelin; TAG = triacylglycerol. (c,d) Oxygen consumption rate (OCR) was measured in washed human platelets preincubated for 2 h with DMSO or CP640.186 before bioenergetic measurements. OCR was assessed under basal condition and after treatment with 1 μ M oligomycin (O), 0.45 μ M carbonyl cyanide p -trifluoromethoxyphenylhydrazone (F), and a mix of 1 μ M antimycin A (aA) and 1 μ M rotenone (R). (e) OCR profile is shown. (d) Mitochondrial function was evaluated by calculating basal, ATP-linked, maximum, non-mitochondrial OCR, reserve capacity, and proton leak. Results were expressed as means $\pm$ SEM ($n = 4$). The data were analyzed using unpaired t -test.

Next, we sought to investigate whether pharmacological ACC inhibition within platelets actually affected platelet bioenergetics, which was assessed by measuring mitochondrial function. As illustrated in Figure 1c,d, extracellular flux analysis revealed that the platelets' basal oxygen consumption rate (OCR) was similar between DMSO- and CP640.186-treated platelets, and it was comparable to data published in the literature, as well [30]. The oxidative phosphorylation component was further assessed by injecting oligomycin, which is a complex V inhibitor. This led to an expected decrease in OCR. ATP-linked respiration was similar under both conditions. An injection of carbonyl cyanide p -trifluoromethoxyphenylhydrazone, a proton-ionophore, induced maximal OCR. DMSO- and CP640.186-treated platelets displayed a similar maximal OCR and reserve capacity. Finally, antimycin A and rotenone, which are complex III/I inhibitors, totally inhibited mitochondrial-induced OCR. The non-mitochondrial OCR and proton leak remained unchanged (Figure 1c,d). These data confirm that, in platelets, ACC1 does not play a major role in mitochondrial respiration.

2.2. CP640.186 Increases α -Tubulin Acetylation Level and Impairs Platelet Aggregation

To examine the impact of CP640.186 on platelet α -tubulin Lys 40 acetylation, we performed western blot analysis of α -tubulin deacetylation upon thrombin stimulation. As seen in Figure 2a, platelet pretreatment using CP640.186 increased α -tubulin acetylation levels at basal state and after thrombin stimulation. Next, we investigated the consequences of ACC inhibition on platelet functions. CP640.186 inhibited platelet aggregation in response to low thrombin doses (Figure 2b). A similar effect was observed using tubacin, an HDAC6 inhibitor causing a drastic increase in α -tubulin acetylation (Figures S1a and 2c). Impaired platelet aggregation was unlikely accounted for altered platelet secretion processes, given that neither CP640.186 nor tubacin decreased P-selectin surface exposure or ATP secretion upon thrombin stimulation (Figures 2d,e and S1b,c). Moreover, ACC inhibition did not affect thrombin-induced platelet α IIb β 3 activation, as measured by the binding of PAC-1 to platelets (Figure 2f). A comparable result was obtained with tubacin (Figure S1d).

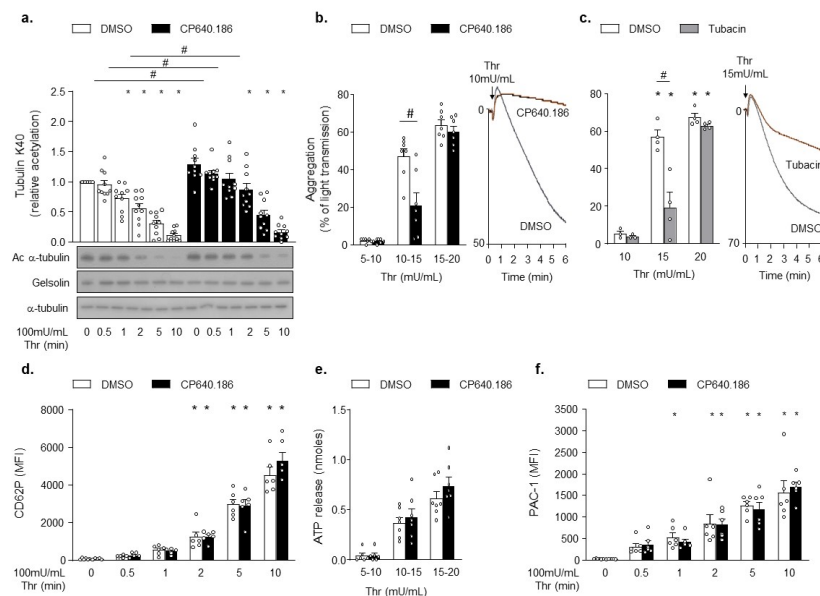


Figure 2. CP640.186-induced ACC inhibition increases tubulin acetylation levels and impairs platelet aggregation. (a,b,d,f) Washed human platelets were pre-incubated for 2 h with dimethyl sulfoxide (DMSO) or CP640.186 (60 μ M) or (c) for 1 h with DMSO or tubacin (10 μ M), before being stimulated with thrombin (Thr). (a) Platelets were stimulated with 100mU/mL Thr at different time points. Whole platelet lysates were subjected to western blot analysis and probed with acetyl α -tubulin (Ac α -tubulin), α -tubulin, or gelsolin antibodies. Data were expressed as means $\pm$ SEM ($n = 10$); * p -value ≤ 0.05 in relation to unstimulated conditions. # p -value ≤ 0.05 in relation to DMSO conditions. Data were analyzed using 2-way ANOVA (analysis of variance). (b,c) Aggregation was analyzed by turbidimetry (Chrono-Log) in platelets stimulated with various Thr concentrations. Profile is shown on the right. Data were expressed as means $\pm$ SEM ($n = 7$). # p -value ≤ 0.0001 in relation to DMSO conditions. Data were analyzed using 2-way ANOVA. (d) P-selectin (CD62P) exposure was analyzed, by flow cytometry, in platelets stimulated with 100mU/mL Thr at various time points. Data were expressed as means $\pm$ SEM ($n = 6$). * p -value ≤ 0.05 in relation to unstimulated conditions. Data were analyzed using 2-way ANOVA. (e) Dense granule secretion was assessed following addition of luciferase-luciferin reagent in platelets stimulated with various Thr concentrations. Data were expressed as means $\pm$ SEM ($n = 7$). Data were analyzed using 2-way ANOVA. (f) Platelets were stimulated with 100mU/mL Thr at various time points, while α Ib β 3 activation (PAC-1) was detected by flow cytometry. Data were expressed as means $\pm$ SEM ($n = 6$). * p -value ≤ 0.05 in relation to unstimulated condition. Data were analyzed using 2-way ANOVA.

2.3. CP640.186 Does Not Affect Platelet Microtubule Dynamics

Given the impact of α -tubulin acetylation on microtubule dynamics in resting and collagen-related peptide (CRP)-activated platelets [19], we postulated that an altered microtubule structure could explain the effect of CP640.186 on thrombin-induced platelet aggregation. To assess the impact of CP640.186-induced tubulin acetylation on microtubules, washed human platelets were treated with or without the ACC inhibitor; then, they were fixed, plated onto fibrinogen-coated coverslips, and incubated with specific antibodies targeting both α -tubulin and acetylated α -tubulin (K40) (Figure 3a). In DMSO-

treated platelets, the marginal band was observed as a continuous ring structure, which was not altered by CP640.186 pre-incubation (Figure 3a; white arrow). Next, we examined tubulin condensation in platelets plated onto fibrinogen-coated coverslips for 10 min, with or without thrombin, then fixed and stained as described above. In DMSO- and CP640.186-treated platelets, the sole activation by fibrinogen induced the coiling of the microtubule ring, in parallel of α -tubulin deacetylation (Figure 3b). Thrombin-induced platelet activation further promoted the microtubules' remodeling by inducing their depolymerization and formation of a new flat and de-acetylated ring at platelet periphery, in addition to the small one observed in the platelet center (Figure 3c, white arrow). The degree of platelet α -tubulin condensation did not differ between DMSO- and CP640.186-treated platelets (Figure 3d,f), indicating that ACC inhibition and the subsequent increase in α -tubulin acetylation did not result in obvious tubulin cytoskeleton changes in resting and thrombin-stimulated platelets. These conclusions were supported by results showing that tubacin, which drastically increases tubulin acetylation, did not alter the dynamics of tubulin organization either in resting or thrombin-activated platelets (Figure S2).

2.4. CP640.186 Reduces the Activation of Rac1-PAK2 Pathway in Response to Thrombin, without Affecting Actin Cytoskeleton Remodeling

Next, we hypothesized that the increased acetylation state of platelet α -tubulin could regulate specific signaling pathways activated downstream of protease-activated receptor 1 (PAR1) or PAR4, and that this mechanism could contribute to the partial disruption of platelet aggregation induced by ACC or HDAC6 inhibition. Aslan et al. previously reported that HDAC6 influences signaling upstream of the Rho GTPase Rac and its effector, p21-activated kinase (PAK) [19]. Both proteins likely appear to be critical players in platelet activation downstream of PAR receptors [31]. Importantly, Rac1 and PAK2 have previously been implicated in regulating actin polymerization in platelets, MKs, [31,32] and other cell types [33–35]. We sought to better understand whether this pathway's downregulation could contribute to the reduced aggregation as observed in CP640.186-treated platelets. To address this issue, we investigated the CP640.186 effect on Rac1 activation, PAK2 phosphorylation, and actin polymerization in thrombin-stimulated platelets. Thrombin caused a rapid and sustained activation of Rac1, with maximal activity reached within 30 s (Figure 4a). Thrombin-induced Rac1 activity was reduced in CP640.186-treated platelets (Figure 4a), as was PAK2 phosphorylation (Figure 4b). Comparable results were obtained with tubacin (Figure 4c,d). Since the Rac1-PAK pathway tightly regulates actin cytoskeletal polymerization, we investigated the CP640.186 effect on the phosphorylation state of cofilin, which represents a downstream cytoskeletal target that is involved in actin turnover regulation. In platelets, a rapid first phase of cofilin de-phosphorylation/activation is detected, which is then followed by a second phase consisting of LIM domain kinase 1 (LIMK1)-mediated slow cofilin re-phosphorylation/inactivation [36,37]. As seen in Figure 4e, CP640.186 pre-incubation did not influence the cofilin de-phosphorylation/phosphorylation cycle in response to thrombin. Accordingly, thrombin-induced F-actin formation was not altered in CP640.186-treated platelets (Figure 4f). To confirm that actin cytoskeleton was not impacted by ACC inhibition, nor by the subsequent alteration of thrombin-induced Rac1-PAK2 signaling, we additionally examined filopodia, lamellipodia, and stress fiber formation within platelets plated onto fibrinogen-coated coverslips and stimulated with thrombin for 15 min. Under thrombin stimulation, most platelets were fully spread and actually formed stress fibers. CP640.186 treatment did not change the proportion of platelets containing filopodia, lamellipodia, or stress fibers (Figure S3). Again, the impact of tubacin on cofilin phosphorylation and actin polymerization/organization was comparable to that of CP640.186 (Figure S4). This observation clearly supports that, under these experimental conditions, the inhibitory effect of CP640.186 on platelet aggregation is most likely due to the increased α -tubulin acetylation level without being caused by altered actin cytoskeleton remodeling.

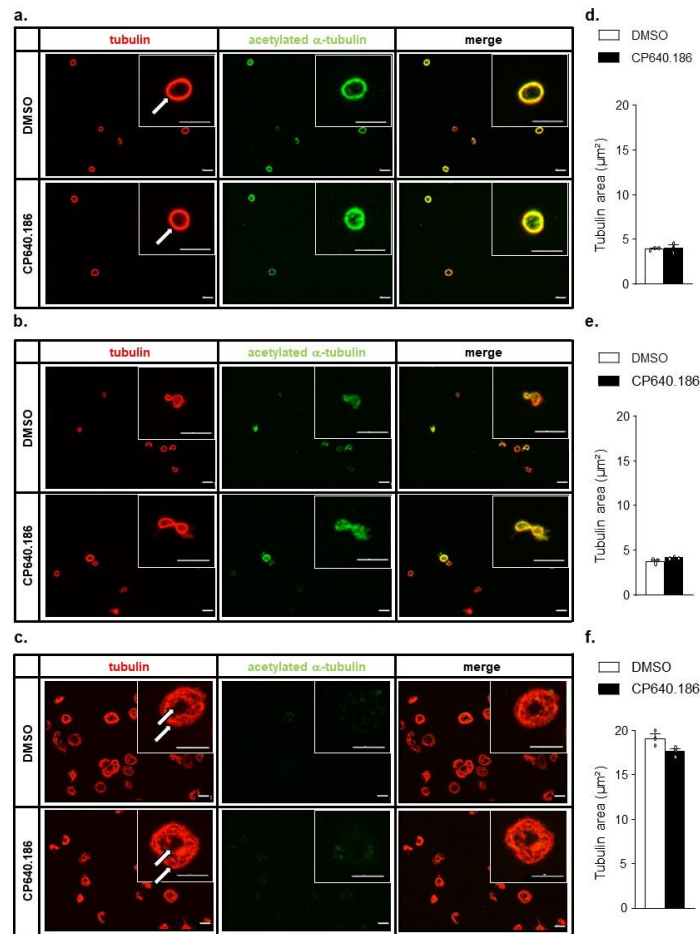


Figure 3. CP640.186-induced ACC inhibition does not affect microtubule dynamics. (a–f) Washed human platelets were pre-incubated for 2 h with dimethyl sulfoxide (DMSO) or CP640.186 (60 μM) before being added to fibrinogen-coated coverslips for 10 min. (a) Unstimulated platelets were fixed directly using 1% PFA, before being added to coverslips. (b) Platelets were added to coverslips before being fixed with 1% PFA. (c) Platelets were stimulated with 100mU/mL thrombin and then added to coverslips before being fixed with 1% PFA. (a–c) Platelets were stained using tubulin (red) or acetyl α -tubulin (green) antibodies. Representative pictures are shown. Microtubule rings are indicated by white arrows. Scale bar: 5 μm . (d–f) Quantification of tubulin area. Data were expressed as means $\pm$ SEM ($n = 3$). Data were compared using unpaired t -test.

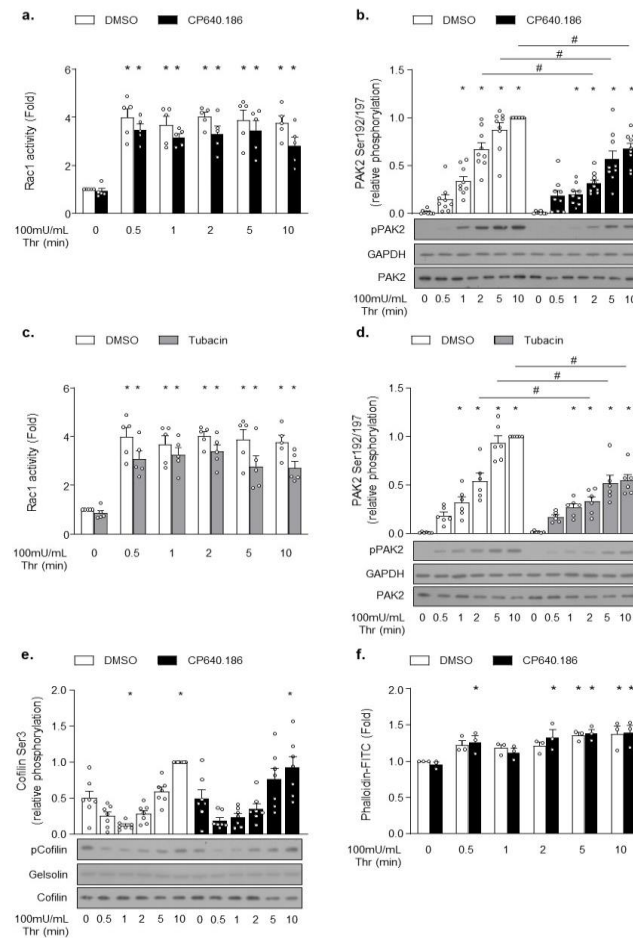


Figure 4. CP640.186-induced ACC inhibition reduces the Rac1–PAK2 pathway activation without impacting actin polymerization upon thrombin stimulation. (**a,b,e,f**) Washed human platelets were pre-incubated for 2 h with dimethyl sulfoxide (DMSO) or CP640.186 (60 μ M) or (**c,d**) for 1 h with DMSO or tubacin (10 μ M), before being stimulated with thrombin (100 mU/mL) at different time points. (**a,c**) Rac1 activity was assessed by means of G–LISA in whole platelet lysates. Data were expressed as means $\pm$ SEM ($n = 5$). * p -value ≤ 0.01 in relation to unstimulated conditions. Data were analyzed using 2-way ANOVA. (**b,d,e**) Whole platelet lysates were subjected to western blot and probed using phosphoPAK, PAK2, GAPDH,

phosphoCofilin, Cofilin, or gelsolin antibodies. Data were expressed as means $\pm$ SEM (at least $n = 7$). * p -value ≤ 0.05 in relation to unstimulated condition. # p -value ≤ 0.05 in relation to DMSO conditions. Data were analyzed using 2-way ANOVA (analysis of variance). (f) Platelets were stained with FITC-conjugated phalloidin (10 μ M) for 1 h. F-actin content was analyzed by means of flow cytometry. Data were expressed as means $\pm$ SEM ($n = 3$). * p -value ≤ 0.05 in relation to unstimulated conditions. Data were analyzed using 2-way ANOVA.

2.5. CP640.186 Alters Thrombin-Induced ROS Production and ERK Phosphorylation

Rac1 and PAK likely affect downstream signaling events that are unrelated to actin cytoskeleton. For instance, Rac1 is an integral part of ROS generation by NADPH oxidase isoforms NOX1 and NOX2 [38,39]. To test whether ACC inhibition did alter ROS production, platelets were incubated with 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) probe. Consistent with Rac1's role in NOX2 activation, CP640.186-treated platelets exhibited a significant decrease in basal and thrombin-induced ROS production (Figure 5a). Similar results were obtained in tubacin-treated platelets (Figure S5a). PAK also displays non-cytoskeletal substrates, as demonstrated by the mitogen-activated protein kinase (MAPK) activation through phosphorylation and activation of MEK proteins [31,40]. Accordingly, the downstream MEK-driven phosphorylation of ERK was reduced in CP640.186-treated platelets following thrombin stimulation (Figure 5b). However, under these experimental conditions, we were unable to reveal any tubacin impact on ERK phosphorylation (Figure S5b).

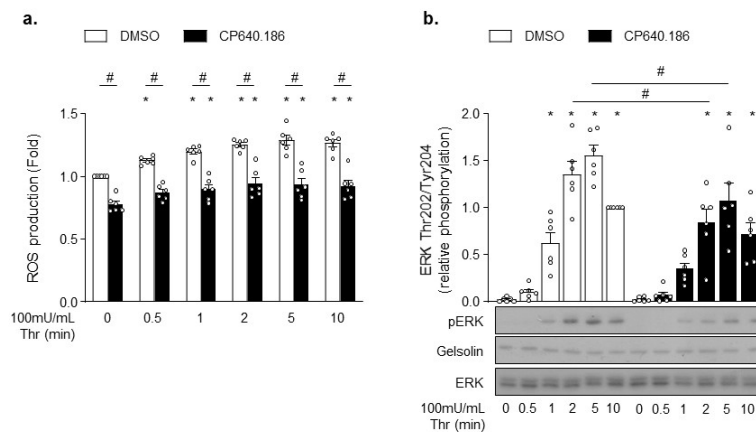


Figure 5. CP640.186 decreases thrombin-induced reactive oxygen species (ROS) production and ERK phosphorylation. (a,b) Washed human platelets were pre-incubated for 2 h with dimethyl sulfoxide (DMSO) or CP640.186 (60 μ M) before being either stimulated or not with thrombin (100 mU/mL) at different time points. (a) Reactive oxygen species (ROS) were detected by means of flow cytometry using H₂DCFDA (10 μ M) probe. Data were expressed as means $\pm$ SEM ($n = 6$). * p -value ≤ 0.05 in relation to unstimulated conditions. # p -value ≤ 0.05 in relation to DMSO conditions. Data were compared using 2-way ANOVA (analysis of variance). (b) Whole platelet lysates were subjected to western blot and probed with phosphoERK, ERK, or gelsolin antibodies. Data were expressed as means $\pm$ SEM ($n = 6$). * p -value ≤ 0.05 in relation to unstimulated conditions. # p -value ≤ 0.05 in relation to DMSO conditions. Data were analyzed using 2-way ANOVA.

3. Discussion

ACC is a key enzyme of lipid synthesis and oxidation pathways, rendering it an attractive target for various metabolic diseases [9–12]. Over the last years, several ACC inhibitors have entered various stages of clinical testing or have already been approved for clinical use [41]. Moreover, this new class of therapeutics has been stated to share unexpected clinical implications, including the potential to lower platelet counts in humans and nonhuman primates [8]. Regarding platelet functions, the potential impact of these molecules remains elusive. In this study, we examined *in vitro* the impact of ACC inhibition on platelet activation, while investigating the underlying mechanisms. Using washed human platelets, we have demonstrated for the first time that a short-term incubation with CP640.186, which is a nonselective, reversible, and ATP-noncompetitive inhibitor of ACC1/2 [42], increases α -tubulin acetylation levels in both resting states and under thrombin-stimulated conditions. This was associated with impaired platelet aggregation. The molecular mechanism connects α -tubulin acetylation to the down-regulation of Rac1-PAK2 signaling and decreased ROS production.

The first publications reporting a beneficial impact of ACC inhibition on various metabolic diseases originated from studies investigating the long-chain fatty acid analog, 5-(tetradecyloxy)-2-furoic acid (TOFA) [43]. However, the similarity of TOFA to various long-chain fatty acids raised the issue as to the specificity of this drug with respect to ACC, and this in relation to the inhibition of other long-chain fatty acyl-CoA-utilizing enzymes [42,44]. CP640.186 binds ACC within the carboxyltransfer domain, and the agent has been identified by Pfizer researchers through high-throughput screening [42]. Its binding site appears to be distinct from that found on other enzymes that catalyze carboxyltransfer reactions, including pyruvate carboxylase and propionyl-CoA carboxylase, which renders this drug highly specific [45]. This compound was shown to inhibit DNL in hepatic cells [42]. In human platelets, CP640.186 treatment for a maximum of 2 h was associated with DNL decreases without drastically affecting overall platelet lipid composition. Several lipid species among TAG, LPC, and LPE classes were even found to be increased. Similar paradoxical findings have been reported in neuroblastoma cells in which TOFA-induced DNL inhibition resulted in an elevation of long chain fatty acids (tetracosanoic acid [C24:0]) and of some sphingomyelins, as well [46]. Regarding fatty acid utilization to produce ATP, our results clearly showed that mitochondrial respiration is not affected by pharmacological ACC inhibition.

In an earlier study, a lower ACC1 expression in yeast was demonstrated to be associated with the hyperacetylation of several proteins, including histones and cytoplasmic proteins that are involved in metabolism [15]. More recently, the impact of ACC inhibition on protein acetylation was found to be linked to cancer cells' metastatic capacity [14]. The present study is the first to connect ACC inhibition and protein acetylation within human platelets. The results clearly showed CP640.186, similarly to HDAC6-specific inhibitor tubacin, to be able to limit thrombin-induced deacetylation of tubulin, thereby inhibiting platelet aggregation. The acetylation of α -tubulin has been recognized to play a key role in microtubule organization and stability [19,47,48]. However, following the immobilization of CP640.186-treated platelets on fibrinogen-coated coverslips, we were unable to identify any treatment impact on the marginal band of resting platelets, nor on their ability to condense upon fibrinogen and thrombin stimulation. Platelet spreading and morphology were also preserved. These results are supported by similar observations reported in tubacin-treated human platelets or in platelets isolated from HDAC6 knockout mice [18,27]. In contrast, Aslan et al. did not reach the same conclusions. Indeed, based on the visualization of tubacin-treated human platelets using super-resolution structured illumination microscopy, these authors described a disrupted marginal band at resting state, along with a reduced ability to condense upon CRP stimulation [19].

Additionally, CP640.186 and tubacin were shown to reduce PAK2 phosphorylation downstream of Rac1 GTPase activation, although the molecular link between microtubule acetylation and Rac1 is still poorly understood. In neurons, it has been proposed that

p120 catenin associates with and stabilizes microtubules, which promotes Rac1 activation and neurite outgrowth [49]. Interestingly, p120-catenin is present in platelets and likely influences Rho-GTPases activity and key signaling molecules involved in platelet aggregation, including E-cadherin [50]. The potential contribution of p120-catenin to the mechanism by which CP640.186 regulates Rac1 in platelets warrants further investigations. Once activated, Rac1 is able to modulate different enzymatic activities. For instance, Rac1 plays a crucial role in activating Nox family NADPH oxidase enzymes dedicated to ROS production, including superoxide anion radical or hydrogen peroxide. NOX2 is activated in a Rac1-dependent manner. In the GTP-bound form, Rac1 directly binds to the oxidase activator p67 (PHOX), which in turn interacts with NOX2, leading to superoxide anion production [39]. Consistent with the CP640.186 effect on Rac1 activity, ROS generation was significantly decreased in response to thrombin stimulation.

PAK mediates the phosphorylation of a plethora of proteins involved in actin cytoskeleton, such as LIMK1, merlin, or myosin light chain kinase (MLCK) [34]. In platelets, the Rac1-PAK signaling cascade has been shown to regulate actin polymerization via LIMK1-cofilin phosphorylation and lamellipodia formation [31,40,51]. However, we did not observe any differences in thrombin-induced cofilin (de)phosphorylation, shape alterations, or F-actin content between DMSO- and CP640.186-treated platelets. Accordingly, platelets from HDAC6 KO mice displayed normal platelet shape changes, which let us assume that acetylated α -tubulin is not implicated in the regulation of actin cytoskeleton [18,27]. Interestingly, PAK is able to activate substrates other than proteins regulating cytoskeleton organization. For instance, ERK is rapidly phosphorylated in activated platelets. This phosphorylation notably depends on PAK [31,34,40], and it has been shown to play a role in platelet aggregation and thrombus formation in a laser-injured subcutaneous arteriole model [52,53]. Mechanistically, ERK activation has been reported to constitute a key signaling event designed to expose on platelet surface adhesion molecules that are necessary for platelet tethering. The latter constitutes the first step of platelet aggregation [52]. The impaired thrombin-induced aggregation of CP640.186-treated platelets possibly results from a similar mechanism. Moreover, our data are in line with studies showing that tubacin-induced increases in α -tubulin acetylation levels were associated with a decrease in CRP-induced ERK phosphorylation [19,54]. We failed to detect any significant decrease in ERK phosphorylation in thrombin-stimulated, tubacin-incubated platelets. This observation suggests that ACC and HDAC6 inhibitions likely exhibit different downstream effects. A limitation of our study is that we only correlate α -tubulin acetylation with the inhibition of platelet aggregation and Rac1/PAK2 signaling. We cannot exclude that other acetylated proteins may contribute to the effects of both CP640.186 and tubacin.

In summary, our data demonstrate that pharmacological ACC inhibition is able to impair thrombin-induced platelet aggregation, an effect which is associated with an increase in α -tubulin acetylation levels. The underlying mechanisms involve a downregulation of the Rac1-PAK2 signaling pathway, which is associated with a decrease in ROS production and ERK phosphorylation. Our work adds momentum to the emerging notion that precautions would be needed for studies using ACC inhibitors in Type 2 diabetes, cancer, or nonalcoholic steatohepatitis. Reducing platelet activation may likewise exert positive effects, notably in some inflammatory or thrombotic disorders. Future research investigating *in vivo* the impact of DNL inhibitors on platelet functions are now needed, given that new molecules are currently entering clinical development.

4. Materials and Methods

4.1. Reagents

CP640.186 (#17691) was purchased from Cayman Chemical (Ann Arbor, Michigan, United States of America [USA]). Thrombin from bovine plasma (#T6634), apyrase (#A6132), fibrinogen (#F4129), fluorescein isothiocyanate (FITC)—conjugated phalloidin (#P5282), sigmacote (#SL2), Dulbecco's Modified Eagle's Medium (DMEM, #D5030), prostaglandin E1 (PGE1) (#P5515), and horse radish peroxidase (HRP)-conjugated anti-rabbit (#A0545) an-

tibodies (Abs) were purchased from Sigma-Aldrich (Overijse, Belgium). [$1\text{-}^{14}\text{C}$]-Acetic Acid was obtained from Perkin Elmer (#NEC084H001MC) (Zaventem, Belgium). S-MONOVETTE CPDA tube (#11610001) was from Sarstedt (Etten-Leur, The Netherlands). Eptifibatide was obtained from GlaxoSmithKline (Wavre, Belgium). Bovine serum albumin (BSA) (#3854.4) was purchased from Roth (Keerbergen, Belgium). Fatty acid-free BSA (#126575) was from Millipore (Overijse, Belgium). Anti-tubulin antibody (#62204) and H_2DCFDA probe (#D-399) were purchased from Thermo-Fisher Scientific (Waltham, MA, USA). Complete, Mini Protease Inhibitor Cocktail (#11836153001) was obtained from Roche (Brussels, Belgium). Luciferin-luciferase reagent (#CH395) was from Stago BNL (Mont-Saint-Guibert, Belgium), and Cell Tak (#354240) was from Corning (Lasne, Belgium). Oligomycin, FCCP, antimycin-A, and rotenone were from Agilent (Mito Stress Test Kit, #103015-100) (Santa Clara, CA, USA). Rac1 G-LISA kit (#BK128) was from Cytoskeleton, Inc. (Denver, CO, USA). Anti-phospho-cofilin (#3313), anti-cofilin (#5175), anti-phospho-ERK (Thr202/Tyr204) (#9101), anti-ERK (#9102), anti-phospho-PAK (Ser199/204; Ser 192/197) (#2605), anti-PAK2 (#2615), anti-acetyl- α -tubulin (#5335), anti-GAPDH (#2118), anti-rabbit IgG HRP-linked (#7074), and anti-gelsolin (#12953) antibodies were obtained from Cell Signaling (Danvers, MA, USA). Phycoerythrin-conjugated anti-CD62P (#555524), FITC-conjugated anti-active $\alpha\text{IIb}\beta_3$ (PAC-1) (#340507), HRP-conjugated anti-mouse (#554002), donkey anti-mouse IgG coupled with alexa fluor 594 (#A-21203), and donkey anti-rabbit IgG coupled with alexa fluor 488 (#A-21206) antibodies were purchased from BD Bioscience (San Jose, CA, USA).

4.2. Human Platelets Preparation

Venous blood was taken from human healthy volunteers (without drug intake one week before blood sampling) having provided their consent in citrated tubes (1/10; citrate, phosphate, dextrose, adenine). Platelet rich plasma (PRP) was obtained by centrifugation at $330\times g$ for 20 min. After adding eptifibatide ($4\text{ }\mu\text{g/mL}$) and apyrase (1 U/mL), platelets were pelleted by a centrifugation at $800\times g$ for 10 min. Platelets were washed once with a modified Tyrode's buffer (135 mM NaCl, 12 mM NaHCO_3 , 3 mM KCl, 0.3 mM Na_2HPO_4 , 1 mM MgCl_2 , 5 mM D-glucose, 10 mM Hepes, and 0.35% BSA, pH 7.4, 37°C) containing eptifibatide ($4\text{ }\mu\text{g/mL}$) and apyrase (1 U/mL) at $1000\times g$ for 10 min. Platelets were re-suspended in modified Tyrode's buffer to a density of $2.5\times 10^5/\mu\text{L}$ (unless said otherwise). Washed platelets were incubated 2 h at 37°C with CP640.186 before platelet function assays.

4.3. Lipogenesis Measurement

Platelets were re-suspended in a modified Gaintner buffer (4.92 mM KH_2PO_4 , 40 mM $\text{NaH}_2\text{PO}_4\cdot\text{H}_2\text{O}$, 102.7 mM NaCl, 40 mM NaOH, and 5.5 mM D-glucose, pH 6.5, 37°C) at a density of $6.5\times 10^5/\mu\text{L}$. Washed platelets (6.5×10^8) were incubated for 2 h at 37°C in a solution including 1 mL of modified Gaintner buffer, 4.15 mM acetate, and $4.415\text{ }\mu\text{Ci}/\mu\text{mol}$ [$1\text{-}^{14}\text{C}$]-acid acetic. To stop the reaction, samples were centrifuged at $2500\times g$ for 10 min at 4°C . After two washings, lipids were extracted with methanol/chloroform (1:2 v/v). The addition of NaCl 0.9% enabled the formation of hydrophilic (upper) and hydrophobic (lower) parts. The hydrophobic part was washed three times ($1000\times g$, 10 min, 25°C) before being added to scintillation vials. Lipids were concentrated via nitrogen flow. Radioactivity was measured using a scintillation counter (Tri-Carb 2800TR, PerkinElmer).

4.4. Lipidomics Analysis

The platelets were re-suspended in a modified Tyrode's buffer with 0.35% free fatty acid BSA at a density of $10\times 10^5/\mu\text{L}$. Washed platelets (1.5×10^9) were centrifuged at $11,700\times g$ for 15 sec before being frozen. Lipid extraction and a lipidomics analysis were carried out, as previously described [7].

4.5. Extracellular Flux Analysis of Mitochondrial Respiration

PRP was centrifuged at $800 \times g$ for 10 min in the presence of PGE1 (100 ng/mL) and apyrase (1 U/mL). Platelet pellets were washed in modified Tyrode's buffer without BSA containing apyrase (1 U/mL) and PGE1 (100 ng/mL). Washed platelets were re-suspended in modified DMEM's medium (DMEM, 143mM NaCl, 0.5% phenol red, 5 mM D-glucose, 1mM sodium pyruvate, and 4 mM L-glutamine, pH 7.35) at a density of 2×10^6 /μL and incubated for 30 min at 37 °C with CP640.186. The 96-well plate was coated with 150 μg/mL "cell tak" solution (diluted in 0.1 M NaHCO₃, pH 8.0) for 20 min at room temperature (RT) before being washed with distilled water. Overall, 15×10^6 platelets were added to the 96-well plate, and their adhesion was induced by two centrifugations at RT ($148 \times g$ for 1 min and $213 \times g$ for 1 min). The 96-well plate was incubated for 1 h 15 min in a CO₂-free incubator before the assay. After basal oxygen consumption rate (OCR) measurement, 1 μM oligomycin, 0.45 μM FCCP, and 1 μM rotenone/antimycin A mix were sequentially injected into the wells every 12 min. OCR was measured every 4 min using Seahorse extracellular flux XFp (Biosciences). Mitochondrial functional parameters were assessed via basal OCR, which was estimated by subtracting rotenone/antimycin A OCR from basal OCR, ATP-linked OCR, calculated by subtracting OCR after oligomycin from baseline, maximal OCR, reserve capacity, evaluated by subtracting basal OCR from OCR after FCCP, non-mitochondrial OCR, obtained after rotenone/antimycin A injection and proton leak, calculated by subtracting rotenone/antimycin A OCR from OCR after oligomycin.

4.6. Western Blotting

Washed human platelets (5×10^7) were stimulated with 100 mU/mL thrombin for the indicated time points in the presence of 2 mM Ca²⁺ before being lysed in modified blue Laemmli's buffer (50 mM Tris pH 6.8, 10% [w/v] glycerol, 2% [w/v] SDS, 0.5mM EDTA, 0.01% [w/v] bromophenol blue, and 25mM DTT). Whole platelet lysates were subjected to western blot. All antibodies were diluted in 5% BSA. The dilution for primary antibodies were (1/1000) except for anti-acetyl-α-tubulin (1/4000), anti-tubulin (1/10,000), anti-phosphocofilin (1/10,000), anti-cofilin (1/20,000), anti-phospho-ERK (1/100,000), anti-ERK (1/2000), anti-gelsolin (1/50,000), and anti-GAPDH (1/100,000). Secondary HRP-conjugated anti-rabbit antibodies from Cell Signaling were diluted (1/5000), as were HRP-conjugated anti-rabbit and anti-mouse antibodies from Sigma (1/20,000). Band intensities were normalized to loading controls on the same gel. The respective total protein analyses were performed on different gels. Image J was applied to quantify all bands.

4.7. Platelet Aggregation and ATP Secretion

Aggregation and dense granules secretion were assessed using an aggregometer (Chrono-log 700, STAGO). Washed platelets (52.5×10^6) were constantly stirred at 1200 rpm, 37 °C. Light transmission was recorded after CaCl₂ (2 mM) addition and thrombin stimulation. To analyze ATP secretion, a luciferase/luciferin mix was added, which was followed by ATP standard.

4.8. Flow Cytometry Analysis

For CD62P exposure and αIIbβ3 activation, PGE1 (100 ng/mL) and apyrase (1 U/mL) were added to PRP before being centrifuged at $800 \times g$ for 10 min. Platelets were washed in a modified Tyrode's buffer with 1.5% BSA. Washed platelets (6.25×10^6) were incubated with anti-CD62P and PAC-1 antibodies (1/14.5) for 20 min at RT. Platelets were stimulated with thrombin 100 mU/mL at the indicated time points at 37 °C before being fixed with paraformaldehyde (PFA) 4%. For ROS production, washed platelets (6.75×10^6) were re-suspended in a modified Tyrode's buffer without BSA and incubated with 10 μM H₂DCFDA probe for 30 min at 37 °C. Platelets were washed two times with a modified Tyrode's buffer without BSA containing apyrase (1 U/mL). Re-suspended platelets were stimulated with 100 mU/mL thrombin at different time points. The reaction was stopped by diluting platelets 14X with the modified Tyrode's buffer without BSA. For F-actin content, washed

platelets (6.25×10^6) were re-suspended in the modified Tyrode's buffer with 1.5% BSA. Re-suspended platelets were stimulated with 100 mU/mL thrombin at different time points at 37 °C before being fixed with 4% PFA. Platelets were permeabilized with 0.1% Triton and stained with FITC-conjugated phalloidin (10 μ M) for 1 h at RT. Platelets were washed two times with phosphate buffer saline (PBS). All samples were analyzed using BD Canto II flow cytometer, and 10,000 events were recorded. As necessary, appropriate compensation controls were included.

4.9. Platelet Spreading and Immunofluorescence Staining

Washed platelets were re-suspended at a density of $3 \times 10^4/\mu$ L. Coverslips were coated with fibrinogen (100 μ g/mL) overnight at 4 °C, blocked with PBS-BSA (5 mg/mL) for 1 h at RT and then washed with PBS. Platelets (6×10^6) were either stimulated or not with 100 mU/mL thrombin in the presence of 2 mM CaCl_2 and were immediately placed on coverslips for 15 min (actin) or 10 min (tubulin) at RT. Unstimulated platelets were fixed directly with 1% PFA and then added to coverslips. Non-adherent platelets were removed using a PBS wash. For actin visualization, adherent platelets were fixed with 1% PFA for 30 min at RT, permeabilized with 0.1% Triton, and stained with FITC-conjugated phalloidin (1:300) for 45 min at RT. To visualize the microtubule cytoskeleton, adherent platelets were blocked 30 min at RT before being incubated with anti-tubulin (1/800) and anti-acetyl- α -tubulin (1/100) antibodies for 1 h at RT. Coverslips were washed with PBS; anti-rabbit IgG coupled with alexa fluor 488 (1/500) and anti-mouse IgG coupled with alexa fluor 594 (1/1000) antibodies were added for 1 h at RT. Platelets were visualized using a Zeiss (x 100 oil immersion) fluorescence microscope (Carl Zeiss) equipped with ApoTome (AxioImager). The area (actin) covered by adherent platelets were quantified by means of Visiopharm software. Tubulin area was quantified using Image J.

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Abbreviations

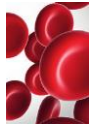
aA	Antimycin A
AA	Arachidonic acid
ACC	Acetyl-CoA carboxylase
CE	Cholesteryl ester
CPT1	Carnitine palmitoyltransferase 1
CRP	Collagen-related peptide
DAG	Diacylglycerol
DMSO	Dimethyl sulfoxide
DNL	<i>De novo</i> lipogenesis
ERK	Extracellular signal-regulated kinase
F	carbonyl cyanide p-trifluoromethoxyphenylhydrazone
FFA	Free fatty acid
FITC	Fluorescein isothiocyanate
H ₂ DCFDA	2',7'-dichlorodihydrofluorescein diacetate
HDAC	Histone deacetylase
HRP	Horse radish peroxidase
KAT	Lysine acetyltransferase
LIMK1	LIM domain kinase 1
LPC	Lysophosphatidylcholine
LPE	Lysophosphatidylethanolamine
MAPK	Mitogen-activated protein kinase
MK	Megakaryocyte
MLCK	Myosin light chain kinase
NOX	NADPH oxidase
O	Oligomycin
OCR	Oxygen consumption rate
PAK	p21-activated kinase
PAR	Protease-activated receptor
PBS	Phosphate buffer saline
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PEO	Plasmanyl phosphatidylethanolamine
PEP	Phosphatidylethanolamine plasmalogen
PFA	Paraformaldehyde
PGE1	Prostaglandin E1
R	Rotenone
ROS	Reactive oxygen species
SIRT	Sirtuin
SM	Sphingomyelin
TAG	Triacylglycerol
Thr	Thrombin
TOFA	5-(tetradecyloxy)-2-furoic acid

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PLATELETS AND THROMBOPOIESIS

AMPK-ACC signaling modulates platelet phospholipids and potentiates thrombus formation

Sophie Lepropre,^{1,*} Shakeel Kautbally,^{1,*} Marie Octave,¹ Audrey Ginion,¹ Marie-Blanche Onselaer,^{1,2} Gregory R. Steinberg,³ Bruce E. Kemp,^{4,5} Alexandre Hego,⁶ Odile Wéra,⁶ Sanne Brouns,⁷ Frauke Swieringa,⁷ Martin Giera,⁸ Victor M. Darley-Usmar,⁹ Jérôme Ambroise,¹⁰ Bruno Guigas,^{11,12} Johan Heemskerk,⁷ Luc Bertrand,¹ Cécile Ury,⁶ Christophe Beauloye,^{1,13,†} and Sandrine Horman^{1-†}

¹Pôle de Recherche Cardiovasculaire, Institut de Recherche Expérimentale et Clinique, Université catholique de Louvain, Brussels, Belgium; ²Institute of Cardiovascular Sciences, College of Medical and Dental Sciences, University of Birmingham, Birmingham, United Kingdom; ³Division of Endocrinology and Metabolism, Departments of Medicine and Biochemistry and Biomedical Sciences, McMaster University, Hamilton, ON, Canada; ⁴St. Vincent's Institute of Medical Research and Department of Medicine, University of Melbourne, Fitzroy, VIC, Australia; ⁵Mary MacKillop Institute for Health Research, Australian Catholic University, Fitzroy, VIC, Australia; ⁶Laboratory of Thrombosis and Hemostasis and Valvular Heart Disease, Grappe Interdisciplinaire de Génoprotéomique Appliquée-Cardiovascular Sciences, Department of Cardiology, Université de Liège, CHU Sart-Tilman, Liège, Belgium; ⁷Department of Biochemistry, Cardiovascular Research Institute Maastricht, University of Maastricht, Maastricht, The Netherlands; ⁸Center for Proteomics and Metabolomics, Leiden University Medical Center, Leiden, The Netherlands; ⁹Department of Pathology, University of Alabama at Birmingham Mitochondrial Medicine Laboratory, Center for Free Radical Biology, University of Alabama at Birmingham, Birmingham, AL; ¹⁰Centre de Technologies Moléculaires Appliquées, Institut de Recherche Expérimentale et Clinique, Université catholique de Louvain, Brussels, Belgium; ¹¹Department of Parasitology and ¹²Department of Cell and Chemical Biology, Leiden University Medical Center, Leiden, The Netherlands; and ¹³Division of Cardiology, Cliniques Universitaires Saint-Luc, Université catholique de Louvain, Brussels, Belgium

KEY POINTS

- AMPK-ACC signaling in platelets is a key mechanism regulating primary hemostasis and arterial thrombosis.
- AMPK-ACC signaling controls collagen-induced TXA₂ generation and dense granule release by modulating platelet phospholipid content.

AMP-activated protein kinase (AMPK) α1 is activated in platelets on thrombin or collagen stimulation, and as a consequence, phosphorylates and inhibits acetyl-CoA carboxylase (ACC). Because ACC is crucial for the synthesis of fatty acids, which are essential for platelet activation, we hypothesized that this enzyme plays a central regulatory role in platelet function. To investigate this, we used a double knock-in (DKI) mouse model in which the AMPK phosphorylation sites Ser79 on ACC1 and Ser212 on ACC2 were mutated to prevent AMPK signaling to ACC. Suppression of ACC phosphorylation promoted injury-induced arterial thrombosis in vivo and enhanced thrombus growth ex vivo on collagen-coated surfaces under flow. After collagen stimulation, loss of AMPK-ACC signaling was associated with amplified thromboxane generation and dense granule secretion. ACC DKI platelets had increased arachidonic acid-containing phosphatidylethanolamine plasmalogen lipids. In conclusion, AMPK-ACC signaling is coupled to the control of thrombosis by specifically modulating thromboxane and granule release in response to collagen. It appears to achieve this by increasing platelet phospholipid content required for the generation of arachidonic acid, a key mediator of platelet activation. (Blood. 2018;132(11):1180-1192)

Introduction

A growing body of evidence shows that lipids are essential in regulating platelet functions. Indeed, the platelet exhibits a complex array of more than 5000 distinct lipid species, with more than 700 responding to thrombin activation.¹ Given this increased focus on the role of lipid species in platelet function, it is imperative to understand the molecular basis of their regulation during platelet activation. Acetyl-CoA carboxylase (ACC) is a good candidate because of its established role as a central regulator of fatty acid metabolism.² ACC catalyzes acetyl-CoA carboxylation to form malonyl-CoA. Its 2 isoforms, ACC1 and ACC2, have distinct cellular distributions.³ ACC1 is present in the cytosol and synthesizes malonyl-CoA for de novo lipogenesis,⁴ whereas ACC2 is localized on the outer mitochondrial membrane and generates malonyl-CoA, which inhibits fatty acid transport

into mitochondria for oxidation.^{5,6} ACC is a bona fide substrate of AMP-activated protein kinase (AMPK), and its phosphorylation is typically used as a marker of AMPK activation in cells and tissues, including platelets.⁷⁻⁹ AMPK phosphorylates ACC1/2 on serine residues (Ser79/212), leading to suppression of ACC activity.^{10,11} We have previously reported that AMPKα1 is activated in platelets upon thrombin stimulation, and increases the phosphorylation of myosin regulatory light chains, cofilin, and vasodilator-stimulated phosphoprotein.⁷ These cytoskeletal proteins are critical for triggering platelet shape change and the centralization of secretory granules during platelet activation.¹² However, the effect of AMPK-mediated ACC phosphorylation on platelet function has never been investigated.

Fatty acids fulfill at least 3 main roles: structural, signaling, and energy storage. Phospholipids (PL) are the major structural lipids

in platelets. Upon platelet activation, reorganization of the plasma membrane PL facilitates shape change and filopodia and lamellipodia formation,¹³ as well as granule secretion¹⁴ or microvesicle formation. In addition to this structural role, PL provide substrates for phospholipases A (PLA) or C (PLC) to generate bioactive species, including phosphatidylinositides, 1,2-diacylglycerol (DAG), inositol 1,4,5-trisphosphate, and eicosanoids/prostaglandins.¹ These secondary mediators are crucial for the tight regulation of platelet activation.^{15–17}

Finally, lipids contribute to platelet energy metabolism. In the basal state, platelets are more oxidative than glycolytic,^{18,19} and lipid oxidation contributes to at least one-third of the total oxygen consumed by mitochondria.^{18,20,21} Mitochondrial lipid oxidation increases on thrombin stimulation to cope with the energetic demands of platelet activation,^{20,22} a process that is supported by the enhanced availability of eicosanoids released from the membrane PL through the action of Ca^{2+} -dependent cytosolic phospholipase A_2 (cPLA₂).¹

Clearly, the signaling needed to coordinate these diverse functions of PL metabolism is important to define. In the current study, we hypothesized that ACC controls key aspects of platelet function using mice with alanine knock-in mutations in both ACC1 (at Ser79) and ACC2 (at Ser212; ACC double knock-in [ACC DKI] mice). These mice harbor functional ACC and AMPK, but express a mutant form of ACC that can no longer be inhibited by AMPK phosphorylation, resulting in a persistent active form of the enzyme.²

Here, we report that blood from ACC DKI mice have increased thrombus formation on collagen-coated surfaces or after vascular injury. The underlying mechanisms involve elevated levels of arachidonic acid (AA)-containing phosphatidylethanolamine plasmalogen (PEP) lipids in ACC DKI platelets compared with wild-type (WT), increasing thromboxane A_2 (TXA₂) generation and granule secretion after platelet stimulation with collagen. Although mitochondrial fatty acid oxidation is important in thrombin-dependent platelet activation, we found that AMPK-ACC signaling had no effect on platelet bioenergetics.

These findings highlight a novel metabolic regulatory pathway in platelets that influences thrombus formation by modulating the content of specific PL, which generates key mediators of platelet activation.

Methods

Mice

ACC1/2 DKI mice have been described previously.² WT mice served as controls. Eight- to 16-week-old male mice were studied. Animal procedures and protocols were approved by local authorities (Comité d'éthique facultaire pour l'expérimentation animale, 2012/UCL/MD/003 and 2016/UCL/MD/027) and performed in accordance with the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Platelet preparation

Mice were bled under ketamine and xylazine anesthesia from the retro-orbital plexus. Blood was collected in 1/6

citrate-dextrose solution with apyrase 1 U/mL. Platelet-rich plasma was obtained by centrifugation at 800g for 5 seconds, followed by 5 minutes at 100g. It was washed by adding 2 volumes of citrate-dextrose with apyrase 1 U/mL. The platelets were pelleted by centrifugation at 400g for 5 minutes and resuspended to a density of $2.5 \times 10^9/\mu\text{L}$ (unless stated otherwise) in modified Tyrode's buffer. Platelets were counted with Cell-Dyn Emerald. They were stimulated with agonists in the presence of 2 mM CaCl_2 .

Flow chamber assay

Blood was collected from the retro-orbital plexus into 48 μM D-Phenylalanyl-prolyl-arginyl Chloromethyl Ketone, 5 U/mL heparin, and 40 U/mL fragmin. Samples of 400 μL were flowed over type I collagen-coated (100 $\mu\text{g}/\text{mL}$), von Willebrand factor (VWF)-binding protein (12.5 $\mu\text{g}/\text{mL}$) and laminin (50 $\mu\text{g}/\text{mL}$)-coated or VWF-binding protein (12.5 $\mu\text{g}/\text{mL}$), laminin (50 $\mu\text{g}/\text{mL}$), and rhodocytin (250 $\mu\text{g}/\text{mL}$)-coated coverslips mounted on a transparent, parallel plate flow chamber (50 μm depth, 3 mm width, and 20 mm length), at a shear rate of 1000 s^{-1} for 3.5 minutes, as described.²³ Alternatively, samples were preincubated with 20 μM ticagrelor for 10 minutes or the corresponding vehicle (dimethyl sulfoxide) and flowed over type I collagen-coated coverslips, as described earlier. Activated platelets in thrombi were poststained with fluorescein isothiocyanate-labeled anti-P-selectin antibody (Ab; 1:40), PE-labeled JON/A Ab against the active conformation of $\alpha\text{IIb}\beta_3$ (1:20), or Alexa Fluor 647-annexin A5 (1:200), all diluted in Tyrode's buffer. Labeling was undertaken for 2 minutes (stasis), after which unbound Abs were removed by perfusion with Tyrode's buffer. Brightfield phase-contrast and fluorescence images were recorded by a nonconfocal 2-camera system. Surface coverage was analyzed by ImagePro software (Media Cybernetics).²⁴

Ferric chloride-induced thrombosis

Carotid arteries were injured in anesthetized mice by topical application of 10% ferric chloride (FeCl_3) for 5 minutes, as described previously.²⁵ Briefly, exogenous carboxyfluorescein succinimidyl ester-labeled platelets were injected into the jugular vein of anesthetized mice, and fluorescence was recorded by BX61WI microscope (Olympus). Digital images were captured every 2 minutes for a total of 24 minutes.

Untargeted lipidomics

Lipidomics analysis was carried out on the commercial Lipidzyzer platform, according to the manufacturer's instructions (Sciex).²⁶ The amount of platelets needed to obtain consistent results was evaluated before application of the commercial platform. Lipid analysis was performed in flow-injection mode, separating lipid classes by differential mobility spectroscopy,²⁶ followed by tandem mass spectrometry of lipid species with QTrap 5500 in multiple reaction monitoring mode. Lipid species were identified and quantified on the basis of characteristic mass spectrometric transitions. Commercial Lipidzyzer software automatically calculated lipid species concentrations. All samples were analyzed in a randomized fashion. Control plasma samples, as well as fortified plasma samples, were assessed daily as quality controls. Relative standard deviations of quality control samples were below 15% for all lipid classes, except for sphingomyelin, for which a relative standard deviation of 25% was noted.

A detailed description of the reagents and the methods is provided in supplemental Methods, available on the Blood Web site.

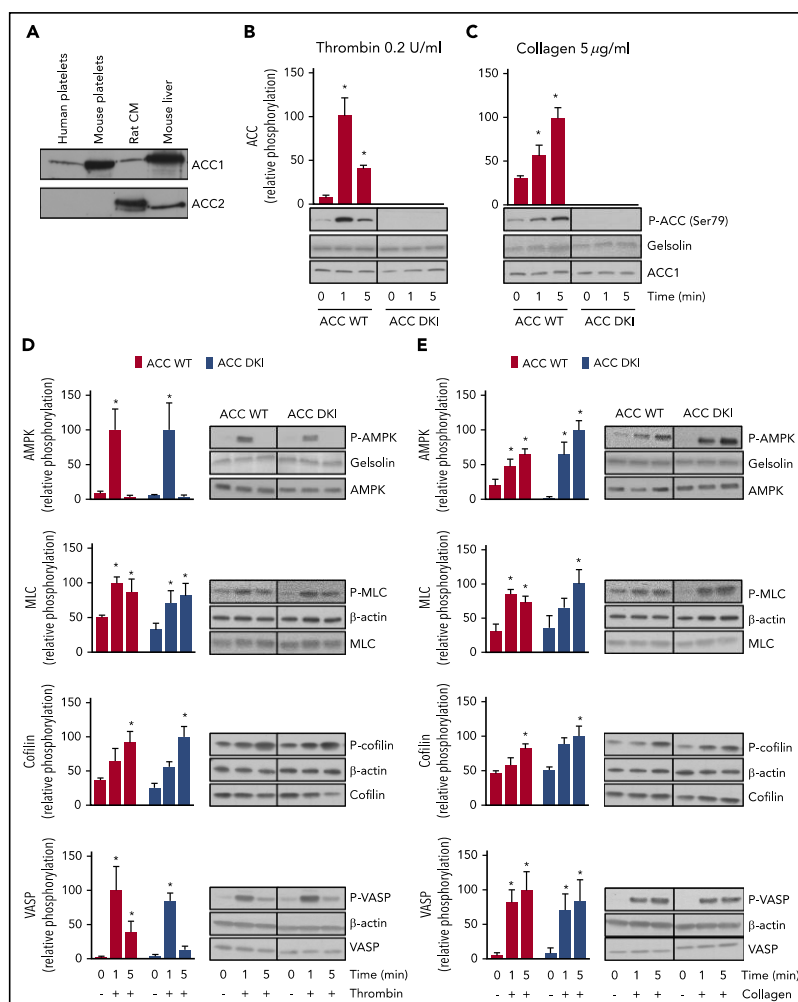


Figure 1. Lack of AMPK-ACC phosphorylation does not affect AMPK signaling or phosphorylation of cytoskeletal proteins. (A) Washed murine and human platelets were lysed and subjected to Western blotting for ACC1 or ACC2 isoform expression analysis. Isolated rat cardiomyocytes (CM) and mouse liver extracts served as positive controls for the detection of ACC2 and ACC1, respectively. (B-E) ACC WT and ACC DKI platelets were stimulated with 0.2 U/ml thrombin (B,D) or 5 µg/ml collagen (C,E) for the indicated times. Whole-platelet lysates were subjected to Western blotting and probed with Ser79 phosphorylated ACC Ab (B-C), Thr172 phosphorylated AMPK, Ser19 phosphorylated myosin light chain (MLC), Ser3 phosphorylated cofilin and Thr278 phosphorylated vasodilator-stimulated phosphoprotein (VASP) Abs (D-E). Gelsolin and β -actin were used as loading controls. Quantification and representative Western blotting are systematically shown. The solid lines on the Western blots indicate that samples were run on the same gel.

Results

Lack of AMPK-ACC phosphorylation does not affect AMPK signaling or cytoskeletal protein phosphorylation

ACC WT and ACC DKI mice have comparable erythrocyte, leukocyte, and platelet counts in whole blood (supplemental Table 1). Expression levels of the major platelet surface receptors, α IIb β 3, GPIIb α , the collagen receptor GPVI, and the protease-activated receptor-3 and protease-activated receptor-4, were equivalent in ACC WT and ACC DKI platelets (supplemental Figure 1A-B).

ACC1, but not ACC2, was detected in both human and murine platelets (Figure 1A). Consistent with previous observations,⁷ 0.2 U/mL thrombin (Figure 1B) or 5 μ g/mL collagen (Figure 1C) induced phosphorylation on Ser79 of ACC in platelets from WT mice with maximal effects after 1 and 5 minutes, respectively. As expected, no Ser79 ACC phosphorylation was detected in either basal or thrombin- or collagen-treated ACC DKI platelets (Figure 1B-C). ACC1 mutation did not influence AMPK activation, reflected by Thr172 phosphorylation (Figure 1D-E). In addition, thrombin- or collagen-induced phosphorylation of known AMPK cytoskeletal targets, myosin light chain, cofilin, and vasodilator-stimulated phosphoprotein were similar between ACC DKI and ACC WT platelets (Figure 1D-E), as well as platelet-spreading and lamellipodia/filopodia formation after platelet immobilization on fibrinogen-coated coverslips (supplemental Figure 1C-H). These data confirm that although ACC phosphorylation was impaired, AMPK signaling was unaltered in ACC DKI platelets.

ACC DKI mice display enhanced primary hemostasis and thrombosis

The effect of AMPK-mediated ACC phosphorylation on hemostasis and thrombosis was evaluated *in vivo*. No spontaneous bleeding or thrombotic event was observed in 91 ACC DKI young mice (up to 4 months) or 19 ACC DKI older mice (at least 9 months old). However, median tail bleeding time was significantly shorter in ACC DKI mice than in their WT counterparts (ACC DKI: 82 ± 42 seconds vs ACC WT: 188 ± 51 seconds; $P < .05$; Figure 2A). We then investigated the role of ACC phosphorylation on arterial thrombosis *in vivo*, in the collagen-dependent carotid artery thrombosis model, in response to a 10% FeCl₃ application. Arterial thrombus formation was monitored in real time by intravital fluorescence microscopy (Figure 2B-C). The rate of thrombus growth was significantly increased in ACC DKI mice compared with the WT animals. The fold change increase in thrombus growth during a 20-minute period after arterial injury was 62.4 ± 17.4 for ACC DKI mice vs 31.3 ± 8.2 for ACC WT mice relative to baseline (Figure 2B). These data demonstrate that AMPK-ACC signaling is a key mechanism regulating primary hemostasis and arterial thrombosis *in vivo*.

Lack of AMPK-ACC signaling favors thrombus formation during perfusion on collagen in flow conditions

We next examined thrombus formation *ex vivo*, using a flow chamber system. Whole blood from ACC WT and DKI mice was

perfused at an intermediate shear rate of 1000 s^{-1} , on 3 different coated surfaces containing collagen, laminin, or VWF-binding protein in the absence or presence of rhodocytin.²⁴ Brightfield images were captured to assess overall platelet adhesion and thrombus formation. Platelet activation was simultaneously monitored by infusing fluorescently labeled anti-CD62P Ab as a marker of α -granule secretion, JON/A Ab to measure α IIb β 3 activation, and annexin V to analyze phosphatidylserine externalization, reflecting platelet procoagulant activity.

On collagen-coated coverslips, the surface area covered by adherent platelets and their activation state (Figure 2D-F) were similar between the 2 genotypes. However, buildup of multilayered platelet thrombi was increased with ACC DKI blood compared with WT (Figure 2G), indicating that AMPK-ACC signaling affects mechanisms involved in the secondary formation of platelet aggregates on collagen. Of note, no differences in platelet adhesion and activation processes were observed between ACC DKI and WT blood perfused on laminin and VWF-binding protein in the absence or presence of rhodocytin (data not shown).

ACC DKI platelets display increased dense granule secretion and thromboxane generation on collagen stimulation

To further decipher the mechanisms responsible for the gain-of-function phenotype of ACC DKI mice on thrombus formation, we studied the effect of a lack of AMPK-mediated ACC phosphorylation on washed platelet reactivity. First, we investigated the effect of thrombin or collagen on α IIb β 3 inside-out activation by analyzing platelet aggregation. ACC DKI platelets aggregated normally at low and high concentrations of thrombin or collagen (Figure 3A; supplemental Figure 2A). Accordingly, thrombin- or collagen-induced activation of α IIb β 3, detected by JON/A Ab, was normal (Figure 3B). Integrin α IIb β 3-mediated clot retraction consistently showed no differences between ACC WT and ACC DKI platelets after thrombin stimulation (supplemental Figure 2B-C).

In addition to α IIb β 3 activation, TXA₂ generation and ADP released from dense granules are important players in collagen-induced platelet aggregate formation under flow^{27,28} and in thrombus formation *in vivo*.^{29,30} Interestingly, the lack of AMPK-ACC signaling amplified dense granule release, specifically in response to collagen. Indeed, a significant 30% increase of ATP (Figure 3C-D) and serotonin secretion (Figure 3E) was detected after collagen stimulation in ACC DKI platelets compared with WT, but not in response to thrombin (Figure 3C,E). To further conclude that this increase was related to secretion rather than to augmented packaging of dense granules, we measured total ADP, ATP, and serotonin content in platelet extracts and found no difference between genotypes (Figure 3F-G). Regarding α -granule secretion, P-selectin surface exposure was similar between ACC WT and ACC DKI platelets (supplemental Figure 3A). However, platelet factor 4 (PF4) secretion was potentiated on collagen stimulation in ACC DKI platelets (supplemental Figure 3B), although total PF4 levels in α -granules

Figure 1 (continued) but were not contiguous. The results are expressed as means $\pm$ standard error of the mean (SEM; at least 3 experiments for each condition). *Values statistically different from respective untreated platelets; $P \leq .05$. Analysis was performed by 2-way analysis of variance (ANOVA). See also supplemental Figure 1 and supplemental Table 1.

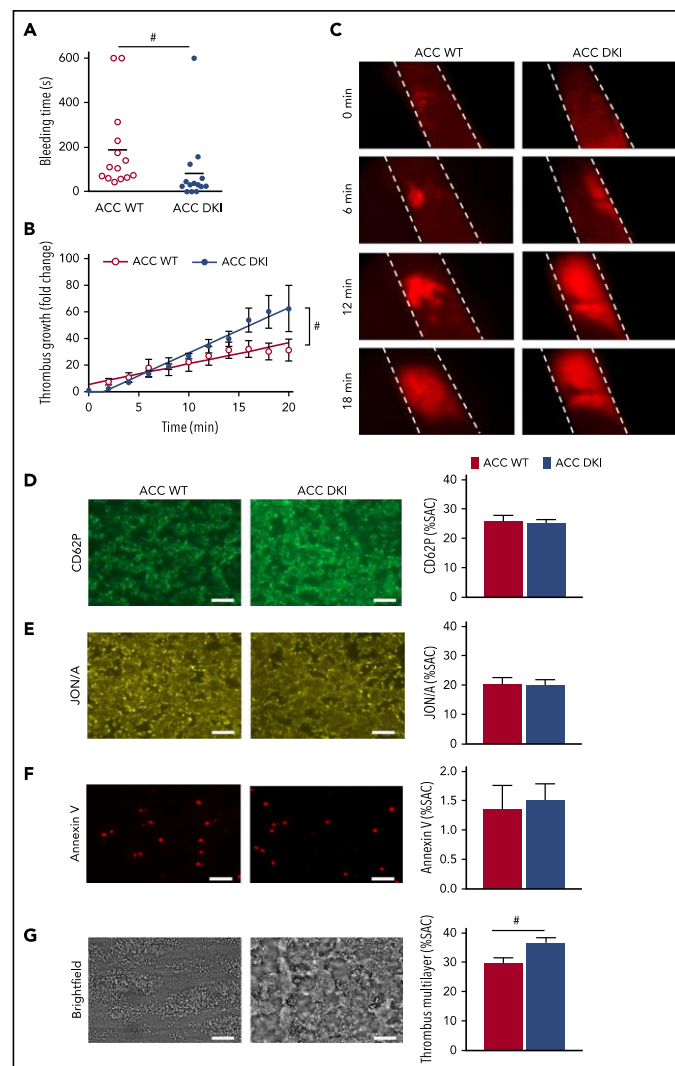


Figure 2.

showed no change (supplemental Figure 3C), indicating that the ACC DKI phenotype seems also associated with increased α -granule secretion, at least those α -granules containing PF4.

Lack of AMPK-ACC signaling potentiated TXA₂ generation in response to collagen (Figure 3H), independent of any change in basal cyclooxygenase 1 expression or activity (supplemental Figure 4A-B). To evaluate the connection between ACC DKI-related increased TXA₂ generation and dense granule secretion, collagen-stimulated ACC DKI platelets were pretreated with 1 mM aspirin before assessment of serotonin secretion. As expected, cyclooxygenase 1 inhibition by aspirin almost completely abolished collagen-induced TXA₂ generation (Figure 3H). In this condition, collagen failed to further enhance serotonin secretion in ACC DKI compared with WT platelets (Figure 3I), demonstrating that the increase in dense granule secretion observed in ACC DKI platelets depends on TXA₂ synthesis. The increased TXA₂ generation in ACC DKI platelets did not result from the enhanced dense granule release induced by collagen, as ticagrelor, a P2Y₁₂ inhibitor, preserved TXA₂ potentiation in ACC DKI platelets (Figure 3J). More interestingly, whole-blood flow perfusion experiments over type I collagen showed that ticagrelor completely abolished the increased thrombus buildup in ACC DKI blood compared with WT, demonstrating the key implication of dense granule secretion in the enhanced thrombus formation (Figure 4A-B).

In addition to thrombin and collagen, ACC WT and ACC DKI platelets were also treated with increasing concentrations of ADP, U46619, rhodocytin, an agonist of the C-type lectin such as receptor-2, or collagen-related peptide, a specific GPVI agonist. For all agonists and concentrations tested, ACC DKI platelets aggregated normally (supplemental Figure 5A-B), and there was no difference in ATP released from dense granules (supplemental Figure 5C). These data reinforce the conclusion that the effect of AMPK-ACC signaling on TXA₂ generation and dense granule secretion is specific to platelet response to collagen. Moreover, these results reveal that collagen increases TXA₂ generation in ACC DKI platelets, most likely through a mechanism involving the α 2b1 integrin, which is compatible with the enhancing role of this integrin in GPVI-dependent thrombus stability via TXA₂ production.³¹

Taken together, these results indicate that the absence of AMPK-ACC signaling in collagen-stimulated platelets elicits increased TXA₂ generation and, subsequently, enhanced granule secretion. These events might contribute to exacerbated thrombus growth via a mechanism that is independent of an altered α Ib β 3 activation.

Lack of AMPK-ACC signaling does not affect lipid oxidation, but results in an increased AA-containing PL pool

To test whether persistent activation of ACC in platelets may affect platelet bioenergetics, we measured the thrombin- or collagen-dependent increase in mitochondrial function in ACC WT and ACC DKI platelets. As illustrated in Figure 5A-F, extracellular flux analysis showed that platelet basal oxygen consumption rate (OCR) was similar between ACC WT and DKI mice, and similar to reported studies in the literature.^{1,20} As expected, thrombin stimulated OCR (Figure 5A-B), which has been shown to be a result of increased mitochondrial lipid oxidation.^{1,20} The oxidative phosphorylation component was further assessed by injecting oligomycin, a complex V inhibitor, which led to an expected decrease in OCR. ATP-linked respiration was not different in ACC DKI platelets compared with WT platelets. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, a proton-ionophore, then induced maximal OCR and a corresponding decrease in maximal respiration and reserve capacity on thrombin stimulation, as a result of the mobilization of endogenous metabolic substrates to cope with increased energetic demands. This occurred to a similar extent in platelets from both genotypes (Figure 5A-B). Finally, antimycin A and rotenone, complex III/II inhibitors, totally inhibited mitochondrial-induced OCR. Proton leak was unchanged in all groups (Figure 5A-B). It has not been previously reported that collagen also stimulates mitochondrial function using this approach. As shown in Figure 5C-D, collagen stimulates OCR with similar characteristics to thrombin. Taken together, these data indicate that in platelets, AMPK-ACC signaling is not playing a major role in mitochondrial respiration, both basally and in the presence of thrombin or collagen. The comparable impact of Etomoxir, a carnitine palmitoyltransferase-1 inhibitor, on the ACC WT and ACC DKI platelet mitochondrial bioenergetics further supports the conclusion that platelets from both genotypes similarly rely on fatty acids to produce ATP (Figure 5E-F).

To investigate whether the gain-of-function of ACC DKI platelets relies on a modified lipid content, we undertook a quantitative lipidomic analysis of resting and stimulated platelets. Isolated ACC DKI and ACC WT platelets were either left unstimulated or activated with 25 μ g/mL collagen, 0.3 U/mL thrombin, or a mix of both. The lipids classes analyzed included different PL (PE, PEP, plasmalogen phosphatidylethanolamine, phosphatidylcholine [PC], lysophosphatidylethanolamine, lysophosphatidylcholine), sphingomyelin, cholesteryl esters, free fatty acids, DAG, and triglycerides.³²

In agreement with previous data,³³ class enrichment analysis indicated that collagen significantly upregulated lipid

Figure 2. ACC DKI mice display enhanced primary hemostasis and thrombosis. (A) Tail bleeding times of ACC WT and ACC DKI mice in saline at 37°C. Individual values are plotted on the graph (n = 14 in each group). Bars indicate means. $\#P \leq .05$. Data were analyzed by the Mann-Whitney U test. (B-C) ACC WT and ACC DKI mice were subjected to in vivo FeCl₃-induced thrombosis of carotid arteries (10% FeCl₃, 5 min). Thrombus formation was monitored by analyzing exogenous carboxyfluorescein succinimidyl ester-labeled platelet accumulation by intravital microscopy and recording videos (fluorescence) of microscopic images every 2 minutes. (D) Thrombus growth kinetics was evaluated by dividing the area of the thrombus at time (t) by the area of the same thrombus at time 0, defined as the time at which the thrombus first reaches 100 μ m. Thrombus growth is expressed as means $\pm$ SEM per group at different times with fitted regression lines (at least 6 mice/group). Slopes are statistically different on the basis of significant interaction ($\#P \leq .05$) between slope and group in a linear model, with group, time, and their interaction as covariates. (E) Representative fluorescence microscopy images at 0, 6, 12, and 18 minutes after FeCl₃ application (original magnification $\times 10$). (D-G) Whole blood from ACC WT and ACC DKI mice was perfused over collagen-coated surfaces (100 μ g/mL) at a shear rate of 1000 s⁻¹. Exposure of P-selectin was evaluated by staining with CD62P Ab (D), α Ib β 3 integrin activation by JON/A Ab (E) and phosphatidylserine externalization by Annexin V (F). Thrombus formation was assessed on brightfield images taken 3.5 minutes after initial blood perfusion (G). Representative images appear on the left. Scale bars represent 20 μ m. Histograms indicate quantification of surface area covered (SAC) by P-selectin (D), activated α Ib β 3 (E), Annexin V (F)-positive platelets or multilayered platelet thrombi (G). The results are expressed as means $\pm$ SEM (at least 4 mice/group). $\#P \leq .05$. Data were analyzed by the Mann-Whitney U test.

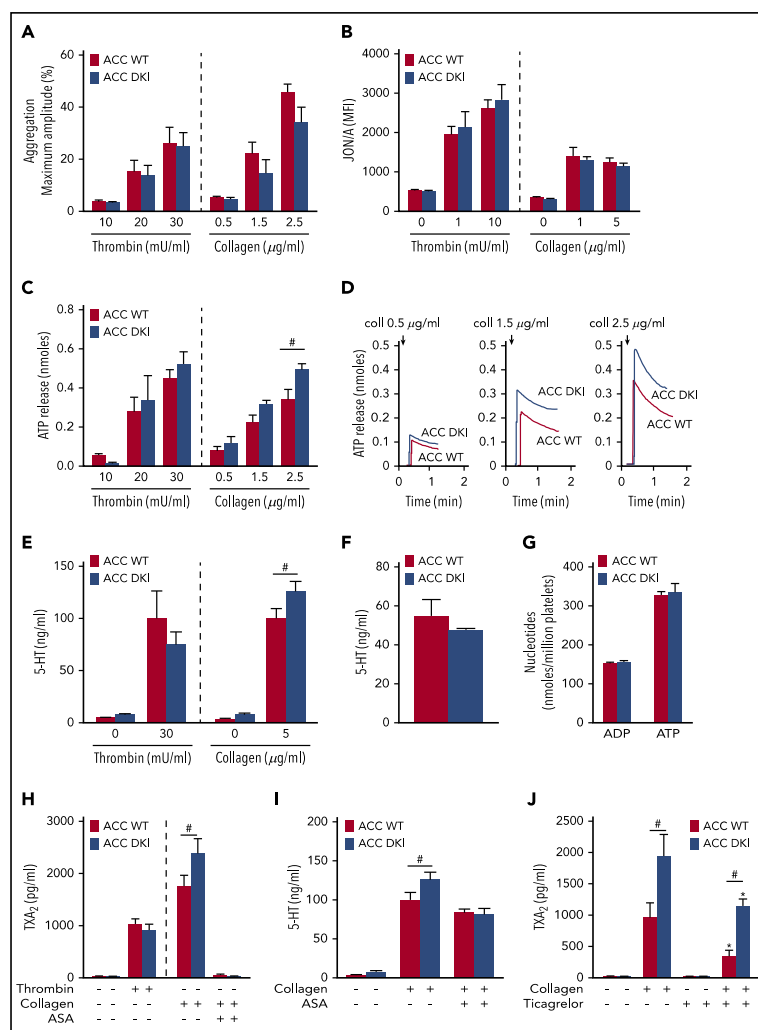


Figure 3. ACC DKI platelets display increased dense granule secretion and thromboxane generation on collagen stimulation. (A) Washed platelets were stimulated with thrombin or collagen at the indicated concentrations, and light transmission was measured (Chrono-Log). Aggregation is expressed as the maximal percentage of light transmitted. The dashed line represents separate analyses. The results are expressed as means $\pm$ SEM (at least 4 experiments for each condition). (B) α IIb β 3 activation (binding of JON/A) was analyzed by flow cytometry in washed ACC WT and DKI platelets stimulated with thrombin for 8 minutes or collagen for 30 minutes at the indicated concentrations. The dashed line represents separate analyses. The results are expressed as mean fluorescence intensity (MFI) $\pm$ SEM (at least 4 experiments for each condition). (C-D) Washed

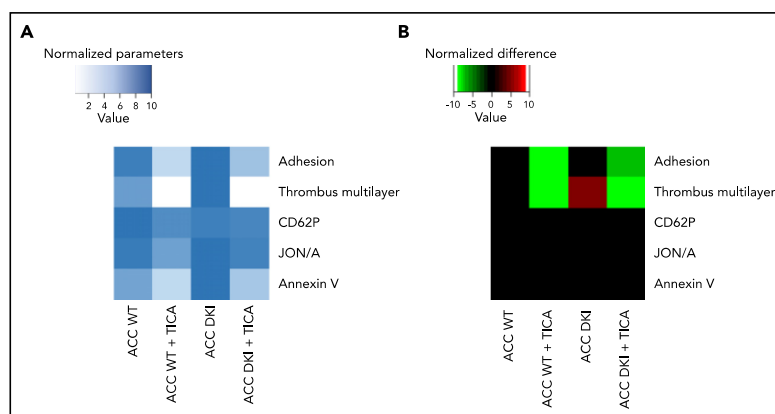


Figure 4. Increased thrombus formation in ACC DKI mice is prevented by P2Y12 inhibition. (A-B) Whole-blood flow perfusion experiments were performed with collagen type I as platelet-adhesive substrate. Where indicated, autocrine effects were blocked by preincubation with 20 μ M ticagrelor (TICA) or vehicle (dimethyl sulfoxide) for 10 minutes. Microscopic images were analyzed for indicated parameters, and per parameter normalized on scale from 0 to 10.²⁴ (A) Heat map of normalized parameters. (B) Subtraction heat map of normalized differences compared with WT mice, filtered for changes with $P \leq .05$.

species among free fatty acids (31%), lysophosphatidylcholine (57%), lysophosphatidylethanolamine (38%), and DAG (21%) lipid classes, in both ACC WT and ACC DKI platelets (Figure 6A; supplemental Table 2). Enrichment of free fatty acids and DAG was also observed in response to thrombin alone (supplemental Figure 6A) and to a combination of thrombin and collagen (supplemental Figure 6B). More important, loss of AMPK-ACC signaling resulted in enrichment of upregulated lipid species among PEP lipid classes (28%), independent of the basal or simulated condition (Figure 6B; supplemental Table 3). Indeed, an increase in lipid species among PEP was strongly associated with lack of ACC phosphorylation (odds ratio, 48 [CI, 14-217]; Figure 6B). ACC DKI also displayed an increment of downregulated lipid species among cholesteryl esters (38%) and PC (14%; Figure 6B). Given that ACC DKI platelets show increased TXA₂ generation, we specifically focused our data analysis

on AA-containing PEP and PC, the 2 PL classes particularly containing differentially regulated lipids. Relevant PEP species with side-chains 16:0/20:4 and 18:1/20:4 were significantly increased in ACC DKI platelets (Figure 6C). We showed that they, respectively, contributed 18.9% and 7.3% to the total reservoir of AA-containing PL (Figure 6D). In contrast, PC species with side-chains 18:2/20:4 and 14:0/20:4 were decreased (Figure 6C) and marginally accounted for 0.8% and 0.4%, respectively, of all AA-containing PL (Figure 6D). Taken together, upregulated AA-containing PEP in ACC DKI platelets clearly supports the enhanced TXA₂ formation observed in this condition. Of note, the AMPK-ACC signaling did not influence platelet palmitate uptake (supplemental Figure 7A). Moreover, collagen-induced TXA₂ production was not modulated by extracellular fatty acid availability in ACC WT or ACC DKI platelets (supplemental Figure 7B).

Figure 3 (continued) ACC WT and DKI platelets were stimulated with thrombin or collagen at the indicated concentrations in the presence of Luciferase-Luciferin reagent, and ATP release was measured in a Lumi-aggregometer. (C) The dashed line represents separate analyses. The results are expressed as mean amount of ATP released (nmoles) $\pm$ SEM (at least 4 experiments for each condition). $\#P \leq .05$ between ACC WT and DKI platelets. The data underwent 2-way ANOVA. (D) Representative traces of ATP secretion after 0.5 μ g/mL, 1.5 μ g/mL, and 2.5 μ g/mL collagen stimulation. (E) Washed platelets ($30 \times 10^5/\mu$ L) were stimulated with 30 mU/mL thrombin or 5 μ g/mL collagen for 5 minutes, and serotonin (5-HT) was measured in the supernatant by ELISA kit. The dashed line represents separate analyses. The results are normalized to ACC WT-stimulated platelets and are expressed as means $\pm$ SEM (at least 3 experiments for each condition). $\#P \leq .05$ between ACC WT and DKI platelets. The data underwent 2-way ANOVA. (F) Washed platelets ($7.5 \times 10^5/\mu$ L) were centrifuged, the pellet was lysed, and serotonin (5-HT) was assayed in the lysate. The results are expressed as means $\pm$ SEM ($n = 3$). (G) Washed platelets were centrifuged, the pellet was lysed, and ADP and ATP content assayed in the lysate by reverse-phase high-performance liquid chromatography. Results are expressed as means $\pm$ SEM ($n = 3$). (H) Washed platelets were stimulated with 100 mU/mL thrombin alone or preincubated or not for 45 minutes with 1 mM aspirin (ASA), and stimulated with 5 μ g/mL collagen. TXA₂ was measured in the supernatant by ELISA. The dashed line represents separate analyses. The results are expressed as means $\pm$ SEM (at least 3 experiments for each condition). $\#P \leq .05$ between ACC WT and DKI platelets. The data underwent 2-way ANOVA. (I) Washed platelets were preincubated or not for 45 minutes with 1 mM ASA and stimulated with 5 μ g/mL collagen. Serotonin (5-HT) was measured in the supernatant by ELISA. The results are expressed as means $\pm$ SEM (at least 3 experiments for each condition). $\#P \leq .05$ between ACC WT and DKI platelets. The data were assessed by 2-way ANOVA. (J) Washed platelets were preincubated with 30 μ M ticagrelor or the corresponding vehicle (dimethyl sulfoxide) for 30 minutes and stimulated with 5 μ g/mL collagen for 5 minutes. TXA₂ was measured in the supernatant by ELISA. The results are expressed as means $\pm$ SEM ($n = 4$). $*P \leq .05$ relative to respective untreated platelets, $\#P \leq .05$ between ACC WT and ACC DKI platelets. The data underwent 2-way ANOVA. See also supplemental Figures 1-5.

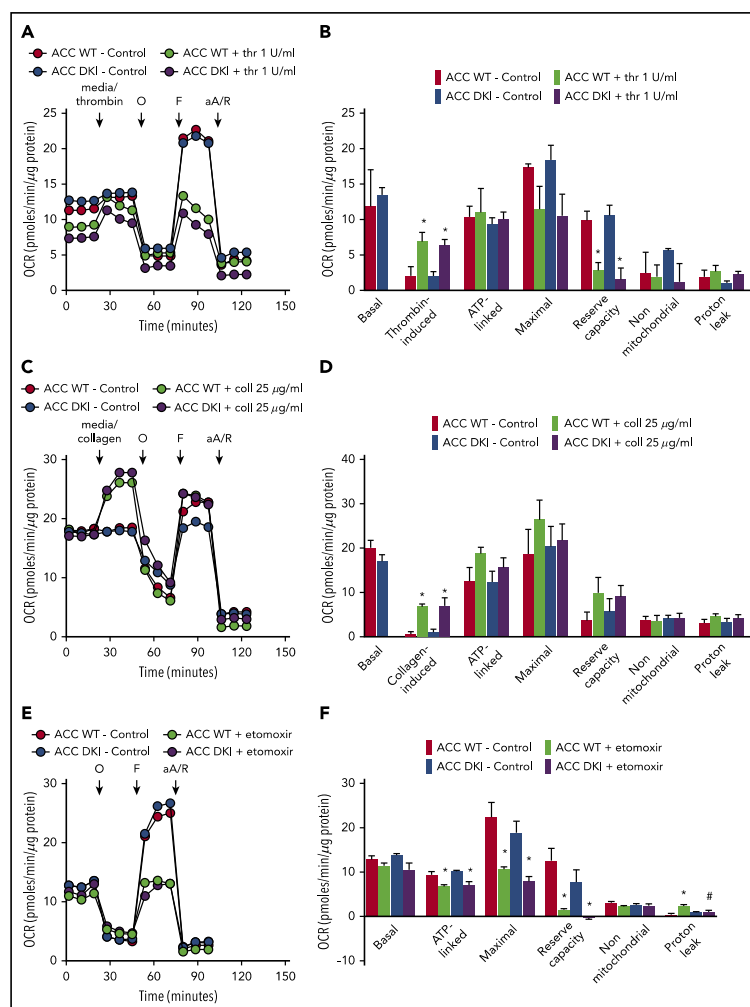


Figure 5. Lack of ACC phosphorylation does not affect oxidative metabolism. (A-F) Oxygen consumption rate (OCR) was measured in washed platelets pretreated or not with Etomoxir (25 µM) (E-F) for 1 hour before bioenergetic measurements. OCR was assessed under basal conditions, after injection of media alone or 1 U/ml thrombin (A, B) or 25 µg/ml collagen (C, D) or followed by treatment with 1 µM oligomycin (O), 0.1 µM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (F), and a mix of 1 µM antimycin A (aA) and 1 µM rotenone (R). (A, C, E) Representative OCR profiles with thrombin (A) and collagen stimulation (C) or Etomoxir treatment (E). (B, D) Mitochondrial function was assessed by calculating basal, thrombin-induced (B) or collagen-induced (D), ATP-linked, maximal, nonmitochondrial, reserve capacity and proton leak OCR. In addition, bioenergetics measurements were assessed in Etomoxir-treated platelets (F). The results are expressed as means $\pm$ SEM (at least $n = 3$). * $P \leq .05$ relative to respective untreated platelets, # $P \leq .05$ between ACC WT and ACC DKI platelets. The data underwent 2-way ANOVA.

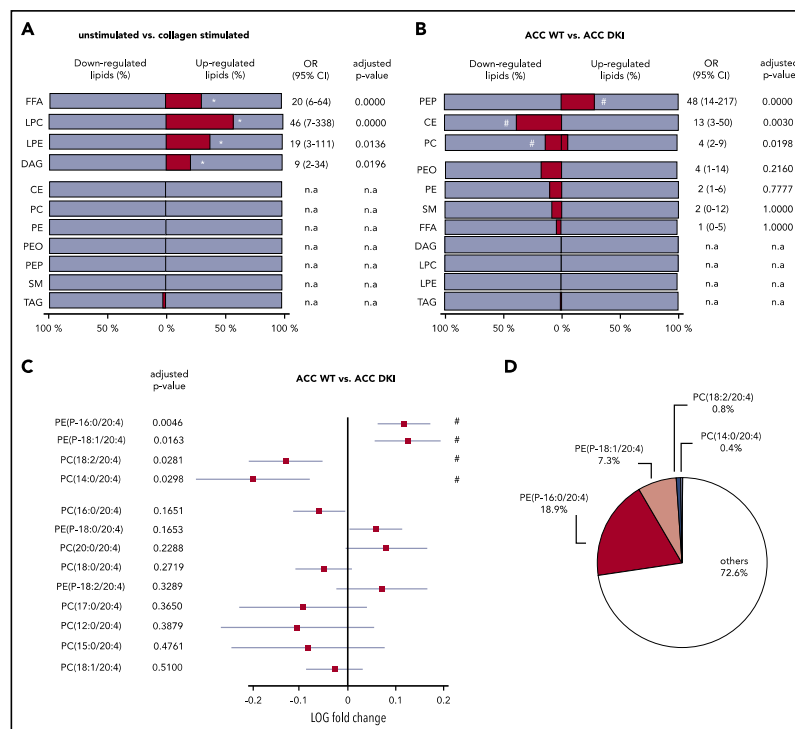


Figure 6. Lack of AMPK-ACC signaling results in an increased AA-containing PL pool. (A,B) The percentage (dark gray bar) of down- or upregulated lipid species relative to the whole lipid class was calculated in collagen-stimulated platelets relative to unstimulated platelets (A) or ACC DKI relative to ACC WT platelets (B). The different lipid classes are arranged from top down in order of increasing adjusted *P* values. (A) *Adjusted *P* ≤ .05 between collagen-stimulated platelets and unstimulated platelets (*n* = 3). (B) #*P* ≤ .05 between ACC DKI and WT platelets (*n* = 3). Multivariate regression analysis was performed. (C) Comparison of AA-containing PEP and PC between ACC DKI and WT platelets. The data are presented as log fold change (squares) relative to ACC WT platelets and with 95% confidence interval (CI) (horizontal lines). Statistical significance was based on false discovery rate *P* values (adjusted *P* values). The different lipid species are arranged from top down in order of increasing adjusted *P* values. Multivariate regression analysis was performed. #*P* ≤ .05 between ACC WT and ACC DKI platelets. (D) Proportions of PE(P-16:0/20:4), PE(P-18:1/20:4), PC(18:2/20:4), and PC(14:0/20:4) relative to all AA-containing PL were evaluated in ACC WT platelets. See also supplemental Figure 6 and 7 and supplemental Table 2 and 3. OR, odds ratio; n.a., not applicable (if no change or if change is OR < 1).

Discussion

Lipids play fundamental roles in platelets, but little is known about the effect of endogenous lipid metabolism on platelet functions and subsequent thrombosis. To assess the importance of endogenous lipid synthesis on platelet function, we characterized a mouse model carrying a genetic mutation that prevents the AMPK-induced phosphorylation of ACC, the first rate-limiting enzyme of lipid synthesis.² We have shown that ACC1 is the predominant isoform in murine and human platelets. These data are consistent with transcriptomic and proteomic analyses that demonstrate ACC1 but not ACC2 transcript and protein expression in platelets.^{34,35} In accordance with the primary

role of ACC1 in lipogenesis, ACC DKI platelets display modified PL content rather than altered lipid oxidation. Indeed, no difference in oxygen consumption, which relies on endogenous lipid oxidation,²⁰ is observed between ACC WT and ACC DKI platelets under both basal conditions and on thrombin or collagen treatment. Consistent with our results, liver-specific inactivation of ACC1 resulted in reduced de novo fatty acid synthesis without alteration of fatty acid oxidation.³⁶

In the first series of experiments, we demonstrated that bleeding time is shorter in ACC DKI mice and thrombosis is increased in vivo and ex vivo under flow on collagen. Second, platelet functions were analyzed and the results indicate that ACC DKI

platelets exhibit enhanced dense granule secretion in response to collagen, attributed to exacerbated TXA₂ generation. Finally, untargeted lipidomics revealed that lack of AMPK-ACC signaling was associated with an increase in some AA-containing PEP, leading to TXA₂ enrichment. Our findings highlight the critical role of platelet endogenous lipid synthesis in thrombosis and hemostasis, a concept that is supported by a recent study showing that a genetic deletion of acid sphingomyelinase, which converts sphingomyelin to ceramide, leads to an alteration of platelet lipidome, which in turn causes dysregulated granule secretion and in vitro thrombus formation.³⁷

Thrombosis induced by FeCl₃ in the carotid artery is a widely used model that can provide valuable information about the effect of genetic modifications on platelet functions. However, it has a number of limitations regarding the underlying mechanisms that induce thrombus formation, notably about the relative roles of tissue factor and thrombin.^{38–41} Recently, several studies reported that plasma proteins and blood cells, including platelets, aggregate because their negatively charged proteins bind to positively charged iron species.^{42,43} Altogether, these results indicate that FeCl₃-induced thrombosis relies on complex multifaceted, incompletely elucidated, mechanisms.

Collectively, the data presented here demonstrate that AMPK-induced ACC1 phosphorylation in platelets does not control lipid oxidation but, rather, regulates cellular lipid content. Intriguingly, we expect that an increase in AA-containing PL, as observed in ACC DKI platelets, would provide more substrates for mitochondrial respiration in response to thrombin.¹ However, we could not find any modifications of platelet mitochondrial oxygen consumption, indicating some degree of compartmentalization and suggesting that increased PEP16:0/20:4 and 18:1/20:4 in ACC DKI platelets do not significantly contribute to overall platelet bioenergetics.

The view that TXA₂ and dense granules are of central importance in triggering thrombus formation but not platelet activation on collagen surfaces has been advanced in previous studies.^{27,28} In addition, the relationship between TXA₂ and dense granule secretion has been clearly established.^{15,44,45} Our work not only confirms this link in ACC DKI platelets but also underlines that excessive thrombus formation is not systematically associated with an alteration of αIIbβ3-dependent platelet aggregation. This disconnection has been recently reported³⁷ and supports the notion that additional cell surface molecules participate in platelet/platelet interactions and support thrombus growth.^{46,47} Therefore, based on the core-shell thrombosis model,^{48,49} we postulate that lack of AMPK-ACC signaling might potentiate platelet recruitment to the shell structure through increased TXA₂ and ADP secretion, augmenting thrombosis through mechanisms independent of αIIbβ3. In addition, increased PF4 secretion might possibly contribute to a rise in thrombus growth in ACC DKI mice.

Interestingly, the effect of AMPK-ACC signaling on TXA₂ generation and platelet dense granule secretion is collagen-specific. This is in agreement with previous data showing that different mechanisms of AA-containing PL breakdown are operating in response to collagen or thrombin. Indeed, although AA release by thrombin is dependent on cPLA₂,^{1,50} collagen-induced AA generation involves cPLA₂, the low-molecular-weight secreted PLA₂ (sPLA₂) and the Ca²⁺-independent cytosolic PLA₂ (iPLA₂).⁵¹ In line with these observations, thrombin and collagen differentially

degrade AA-containing PL, depending on the nature of the PL.^{37,52} For instance, thrombin is more potent at cleaving AA-containing PI and phosphatidylserine than collagen, whereas the latter is 2 times more efficient toward AA-containing PEP.⁵² These data reinforce our observations and help us to understand the specific response or responses of ACC DKI platelets to collagen.

Importantly, ACC phosphorylation could be detected, even in the absence of any agonist stimulation, in platelets from both healthy mice and volunteers.⁷ This has been confirmed by comparing ACC phosphorylation between ACC WT and ACC DKI platelets under basal conditions. A sustained change in basal ACC phosphorylation was sufficient to induce a decrease in PL, suggesting that ACC phosphorylation already affects lipid composition in resting platelets before activation.

In conclusion, our work provides new insights into the contribution of endogenous lipid synthesis to platelet functions. It reveals that sustained modulation of ACC phosphorylation in platelets can modify specific AA-containing PL content and influence platelet reactivity to collagen. This finding might have far-reaching clinical relevance in the pathological context of diseases such as atherosclerosis. Indeed, this is now being tested in a clinical trial demonstrating that basal ACC phosphorylation dramatically increases in platelets of patients with coronary artery disease (ACCTheroma Clinical Trial identifier: NCT03034148). How and whether such a pathway is involved in regulating platelet function in this context remain to be determined.

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Authorship

Contribution: S.L., M.-B.O., L.B., C.O., C.B., and S.H. provided conceptualization; V.M.D.-U. provided methodology; M.G., J.A., and B.G. provided formal analysis; S.L., S.K., M.O., A.G., M.-B.O., A.H., O.W., S.B., F.S., M.G., and J.H. performed the investigation; G.R.S., B.E.K., and C.O. provided resources; S.L., V.M.D.-U., C.B., and S.H., wrote the original draft and performed a review; and C.B. and S.H. performed supervision.

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ORCID profile: M.G., 0000-0003-1684-1894.

Correspondence: Sandrine Horman, Pôle de Recherche Cardiovasculaire, Institut de Recherche Expérimentale et Clinique, Université catholique

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de Louvain, 55, Avenue Hippocrate, 1200 Brussels, Belgium; e-mail: sandrine.horman@uclouvain.be.

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*S.L. and S.K. contributed equally to this study and are joint first authors.

†C.B. and S.H. contributed equally to this study and are joint last authors.

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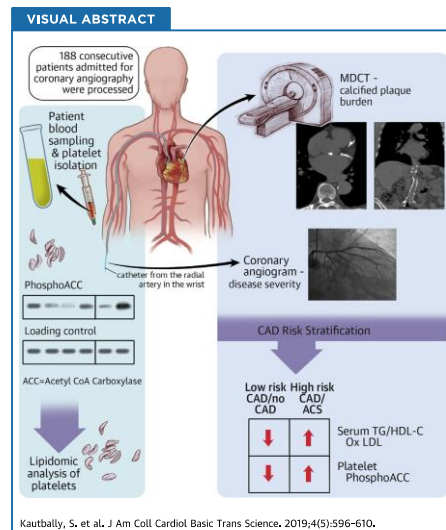
PRECLINICAL RESEARCH

Platelet Acetyl-CoA Carboxylase Phosphorylation



A Risk Stratification Marker That Reveals Platelet-Lipid Interplay in Coronary Artery Disease Patients

Shakeel Kautbally, MD,^{a,*} Sophie Lepropre, PhD,^{a,*} Marie-Blanche Onselaer, PhD,^a Astrid Le Rigoleur, MD,^a Audrey Ginion, MS,^a Christophe De Meester de Ravenstein, PhD,^a Jerome Ambroise, PhD,^b Karim Z. Boudjeltia, PhD,^c Marie Octave, MS,^a Odile Wéra, MS,^d Alexandre Hego, BSc,^d Joël Pincemail, PhD,^e Jean-Paul Cheramy-Bien,^e Thierry Huby, PhD,^f Martin Giera, PhD,^g Bernhard Gerber, MD, PhD,^{a,h} Anne-Catherine Pouleur, MD, PhD,^{a,h} Bruno Guigas, PhD,^{i,j} Jean-Louis Vanoverschelde, MD, PhD,^{a,h} Joelle Kefer, MD, PhD,^{a,h} Luc Bertrand, PhD,^a Cécile Oury, PhD,^k Sandrine Horman, PhD,^{h,†} Christophe Beauloye, MD, PhD^{a,h,‡}



HIGHLIGHTS

- Platelet phosphoACC is a marker for risk stratification in suspected CAD patients. It identifies high-risk CAD patients and correlates with severity of coronary artery calcification.
- The triglycerides/high-density lipoprotein cholesterol ratio is strongly associated with increased phosphoACC in circulating platelets. PhosphoACC is a metabolic signature of the platelet-proatherogenic lipid interplay in CAD patients.
- Phosphorylation and inhibition of acetyl-CoA carboxylase impacts platelet lipid content by down-regulating triglycerides lipid species.

From the ^aPôle de Recherche Cardiovasculaire, Institut de Recherche Expérimentale et Clinique, Université Catholique de Louvain, Brussels, Belgium; ^bCenter for Applied Molecular Technologies, Institut de Recherche Expérimentale et Clinique, Université Catholique de Louvain, Brussels, Belgium; ^cLaboratory of Experimental Medicine (ULB 222 unit), Centre Hospitalier Universitaire de Charleroi, Université Libre de Bruxelles, Charleroi, Belgium; ^dLaboratory of Thrombosis and Hemostasis, GIGA-Cardiovascular Sciences, Department of Cardiology, Université de Liège, Liège, Belgium; ^eDepartment of Cardiovascular Surgery, Surgical Research

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SUMMARY

Adenosine monophosphate-activated protein kinase (AMPK) acetyl-CoA carboxylase (ACC) signaling is activated in platelets by atherogenic lipids, particularly by oxidized low-density lipoproteins, through a CD36-dependent pathway. More interestingly, increased platelet AMPK-induced ACC phosphorylation is associated with the severity of coronary artery calcification as well as acute coronary events in coronary artery disease patients. Therefore, AMPK-induced ACC phosphorylation is a potential marker for risk stratification in suspected coronary artery disease patients. The inhibition of ACC resulting from its phosphorylation impacts platelet lipid content by down-regulating triglycerides, which in turn may affect platelet function.
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Platelets are key players in atherothrombosis. In acute coronary syndrome (ACS), coagulation cascade activation upon plaque rupture leads to thrombin generation (ThG), a crucial platelet agonist enhancing the formation of platelet-rich thrombi (1). The association between increased ThG and ischemic risk in coronary artery disease (CAD) patients renders the coagulation cascade an interesting therapeutic target (2,3).

We previously established the adenosine monophosphate-activated protein kinase (AMPK) to be crucial for platelet activation. In human platelets, thrombin is the major agonist leading to AMPK activation through a calcium-dependent mechanism (4). Once activated, AMPK contributes to platelet secretion, platelet aggregation, and clot retraction by controlling the actin cytoskeleton. AMPK activation likewise leads to phosphorylation of acetyl-CoA carboxylase (ACC) on serine 79 (phosphoACC), its bona-fide substrate, typically used as a marker of AMPK activation in cells and tissues, including

platelets (4). It is tempting to speculate that ThG affects platelet AMPK signaling, resulting in increased phosphoACC in CAD. However, although thrombin is crucial for thrombus formation at the plaque rupture site in ACS, its impact on circulating platelets remains unclear. In CAD patients, the atherogenic environment influences platelet biology and reactivity, mainly through CD36 (5,6). Oxidized low-density lipoprotein (oxLDL) binds to CD36, inducing platelet activation and shape changes via a calcium-dependent mechanism (7). Yet, other factors besides thrombin may affect AMPK-ACC signaling in circulating platelets of CAD patients.

ACC is the first committed enzyme of the fatty acid biosynthesis pathway, while its phosphorylation on serine 79 by AMPK inhibits its activity (8). We demonstrated that AMPK-ACC signaling is a key pathway in controlling platelet lipid content, thereby modulating platelet function and thrombus formation (9). However, ACC contribution to platelet lipid

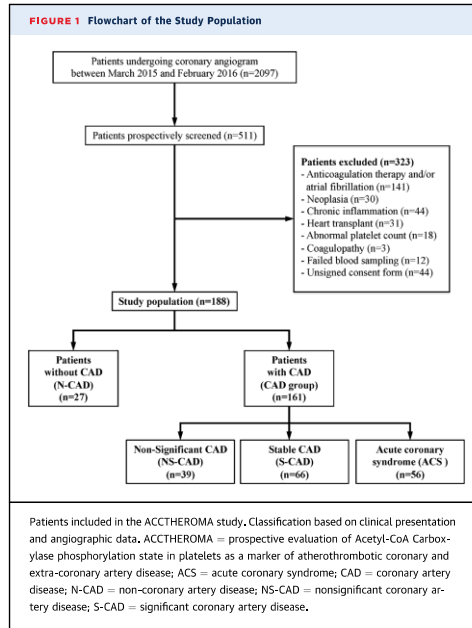
ABBREVIATIONS AND ACRONYMS

ACC = acetyl-CoA carboxylase
AMPK = adenosine monophosphate-activated protein kinase
AoC = extra-coronary calcification score
AU = arbitrary units
CAC = coronary artery calcification
CAD = coronary artery disease
oxLDL = oxidized low-density lipoprotein
phosphoACC = acetyl-CoA carboxylase phosphorylation on serine 79
S-CAD = stable coronary artery disease
TG = triglyceride

Center and Platform Nutrition Antioxydante et Santé, University Hospital of Liège, Liège, Belgium; [†]INSERM UMR_S 1166, Integrative Biology of Atherosclerosis Team, Université Pierre et Marie Curie-Paris 6 and Institute of Cardiometabolism and Nutrition, Pitié-Salpêtrière Hospital, Paris, France; [‡]Center for Proteomics and Metabolomics, Leiden University Medical Center, Leiden, the Netherlands; [§]Division of Cardiology, Cliniques Universitaires Saint-Luc, Brussels, Belgium; ^{||}Department of Parasitology, Leiden University Medical Center, Leiden, the Netherlands; and the [¶]Department of Cell and Chemical Biology, Leiden University Medical Center, Leiden, the Netherlands. *Drs. Kautbally and Lepore contributed equally to this paper and are joint first authors. [†]Drs. Horman and Beauloye contributed equally to this paper and are joint senior authors. This work was supported by grants from the Fonds National de la Recherche Scientifique et Médicale (FNRS) (Belgium) and Louvain Foundation (Louvain-La-Neuve, Belgium), and by unrestricted grants from Bayer and AstraZeneca (Belgium). The Division of Cardiology at Cliniques Universitaires Saint-Luc, Belgium, has received unrestricted research grants from AstraZeneca, Bayer Healthcare, and Daiichi-Sankyo (Belgium). Drs. Kautbally and Lepore were supported by the FNRS (Belgium). Drs. Lepore and Onseleer were supported by grants from the Salus Sanguinis Foundation (UCLouvain, Belgium). Drs. Octave and Wéra have FRIA fellowships from the FNRS (Belgium). Dr. Horman is a research associate, and Drs. Bertrand and Oury are senior research associates at the FNRS (Belgium). Dr. Pouleur is, and Dr. Beauloye was, a clinical master specialist at the FNRS (Belgium). All other authors have reported that they have no relationships relevant to the contents of this paper to disclose.

The authors attest they are in compliance with human studies committees and animal welfare regulations of the authors' institutions and Food and Drug Administration guidelines, including patient consent where appropriate. For more information, visit the JACC: Basic to Translational Science [author instructions page](#).

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metabolism in CAD, in which atherogenic lipids interact with circulating platelets (10), remains unexplored.

Here, we report platelet phosphoACC as a potential risk stratification marker in suspected CAD patients. In consecutive patients admitted for coronary angiography, phosphoACC was significantly increased in circulating platelets of CAD patients and highly associated with acute coronary events. We identified an interplay between platelets and lipids, with oxLDL as a central contributor to increased platelet phosphoACC. Interestingly, the lipidomic data show that sustained phosphoACC regulates triglyceride (TG) lipids in circulating platelets of CAD patients.

METHODS

Methods (including experimental dataset) and reagents are described in the [Supplemental Appendix](#).

CLINICAL COHORT. Study design. From March 2015 to February 2016, 188 consecutive patients admitted for coronary angiography were included in the

ACCTHEROMA (prospective evaluation of Acetyl-CoA Carboxylase phosphorylation state in platelets as a marker of atherothrombotic coronary and extra-coronary artery disease) study (NCT03034148), with at least 2 patients prospectively screened per day, regardless of the indication for angiography. Based on indication and coronary angiography results analyzed by 2 experienced cardiologists, patients were classified into 4 groups. Patients undergoing angiography for chest pain or valvular disease investigation with normal coronary vessels were classed as non-CAD (N-CAD) (reference population). The presence of at least 1 plaque with <50% luminal stenosis was classed as nonsignificant CAD (NS-CAD). Patients presenting with stable disease with significant stenosis (>50%) were classed as stable CAD (S-CAD). ACS comprised unstable angina (n = 30), non-ST-segment elevation myocardial infarction (n = 22), and ST-segment elevation myocardial infarction (n = 4). Further details on patient classifications are provided in the [Supplemental Appendix](#) and study flowchart in [Figure 1](#). The study was approved by the institutional ethics committee (2015/08JAN/010) and complied with the Declaration of Helsinki and good clinical practice guidelines. All participants provided written informed consent.

Blood sampling and phosphoACC analysis. All patients had been fasting for at least 6 h before angiography, except for 4 ST-segment elevation myocardial infarction patients. Blood samples drawn from the arterial sheath were collected in citrated tubes before any drug administration in the catheterization laboratory, including heparin. In a subgroup of patients (n = 8) undergoing right heart catheterization in addition to angiography, a venous blood sample was collected from the femoral access site to compare the level of ACC phosphorylation in arterial and venous blood simultaneously. All samples were immediately processed for platelet isolation. Using flow cytometry, we verified that platelet preparations did not contain any leukocytes ([Supplemental Figure 1](#)). Platelets were lysed in Laemmli buffer before phosphoACC analysis by Western blotting. A standard positive control for phosphoACC was prepared with washed platelets isolated from a healthy volunteer and stimulated with a high dose of thrombin (0.5 U/ml) for 2 min. This standard positive control was used for all the Western blots, placed 4 times on each gel to validate the signal reproducibility. For each patient, band intensities were normalized to corresponding loading controls (gelsolin) on the same gel. The normalized phosphoACC value was compared with the standard positive control. Western blot analyses were confirmed

by electrochemiluminescence immunoassay (Meso Scale Diagnostics, Rockville, Maryland).

Multidetector computed tomography. Thoracoabdominal multidetector computed tomography (MDCT) was performed in a subgroup of patients (n = 68) to assess calcified plaque burden. Arbitrarily, the first and third patient on the list for planned coronary angiogram underwent MDCT for calcium scoring. MDCT was done just before coronary angiogram. Scans were taken with a 256-slice multidetector-row CT scanner (Brilliance iCT 256, Philips Healthcare, Cleveland, Ohio) with 3.0-mm slice collimation, 120-kV tube voltage, and 100-mAs tube current using a prospectively gated “step and shoot” protocol. Coronary artery calcification (CAC) was expressed by means of the Agatston score using calcium scoring software (Philips Healthcare) with a threshold of 130 Hounsfield units. The degree of CAC was classified as mild (Agatston score <100), moderate (between 100 and 400), or severe (>400) (11). An extracoronary calcification score (aorta calcification [AoC]) was measured from the aortic root (excluding the aortic valve) to the common iliac artery in all patients. The AoC score was divided into tertiles for analysis.

Platelet lipidomics. To characterize the phosphoACC impact on regulating platelet lipid homeostasis, we performed a quantitative lipidomic study on 31 samples from patients with the lowest (n = 12) and highest (n = 19) platelet phosphoACC values. Lipids were extracted from a platelet pellet by the methyl-*tert*-butylether method and analyzed using Lipidizer, a direct infusion-tandem mass spectrometry (DI-MS/MS)-based platform (Sciex, Redwood City, California).

STATISTICAL ANALYSIS. Clinical cohort. Analyses were conducted using SPSS version 24 (IBM Corporation, Armonk, New York). Continuous variables were expressed as mean $\pm$ SD or median (interquartile range [IQR]) depending on data distribution, and categorical variables were expressed as number and percentage. CAC and AoC scores, D-dimers, high-sensitivity C-reactive protein, and TG and high-density lipoprotein-cholesterol (HDL-C) ratio were normalized by log-10 transformation. Categorical variables were analyzed using the chi-square test or Fisher’s exact test and continuous variables were analyzed using an unpaired Student’s *t*-test or the Mann-Whitney *U* test, as appropriate. Data were subjected to the Kolmogorov-Smirnov normality test and Bartlett’s test for homogeneity of variance. Group comparisons were made using either 1-way analysis of variance with the *F* test (Bonferroni correction) or Kruskal-Wallis test. Correlations were presented as Pearson (Rp) or Spearman (Rs) coefficients.

Multivariable logistic regression analysis (backward elimination) included variables with *p* value < 0.05 on univariable analysis, with odds ratio (OR) and 95% confidence interval (CI) calculated to determine independent factors associated with ACS. With the receiver-operating characteristic curve, we determined a threshold phosphoACC value for CAD by maximizing sensitivity and specificity. C-statistics were used to describe diagnostic discrimination. The prognostic value of platelet phosphoACC for ischemic outcomes, including cardiovascular death and recurrent myocardial infarction/revascularization procedures, was assessed during patient follow-up. Multivariable Cox regression analysis was used to identify independent predictors for events. Hazard ratios with 95% CI are presented. Event-free survival according to platelet phosphoACC levels was computed using the Kaplan-Meier method.

Lipidomics. Lipid species concentrations obtained from DI-MS/MS (Lipidizer) were analyzed using R software version 3.4.2 (R Foundation for Statistical Computing, Vienna, Austria) according to the following bioinformatics pipeline. Missing DI-MS/MS data were imputed using probabilistic principal component analysis from *pcaMethods* Bioconductor package (12). The data were normalized using total lipid abundance, with a log-2 transformation applied to normalized concentrations. The *limma* Bioconductor package was used to build a multivariable regression model for each lipid species with predictors including platelet phosphoACC, aspirin intake, diabetes, and plasma TG levels. Fold-change estimates and corresponding *p* values were derived from regression models for each lipid species and each predictor. To control for multiple testing, all *p* values were further adjusted for Benjamini-Hochberg false discovery rate, with a false discovery rate <0.05 considered statistically significant (13). Considering that 865 lipid species belong to 11 lipid classes, namely cholesterol ester, diacylglycerol, free fatty acid, lysophosphatidylcholine, lysophosphatidylethanolamine, phosphatidylcholine, phosphatidylethanolamine, plasmalogen phosphatidylethanolamine, plasmalogen phosphatidylethanolamine, sphingomyelin, and TG, lipid class enrichment analysis was performed using Fisher’s exact tests to identify lipid classes with a high proportion of differentially regulated lipid species.

RESULTS

POPULATION BASELINE CHARACTERISTICS AND GLOBAL ATHEROSCLEROTIC CALCIFIED PLAQUE BURDEN. The study population comprised 188 individuals (70% men, 65 $\pm$ 12 years) admitted for

TABLE 1 Baseline Characteristics of the ACCTHEROMA Cohort

	Overall Population (N = 188)	NCAD (n = 27)	CAD			p Value
			NS-CAD (n = 39)	S-CAD (n = 66)	ACS (n = 56)	
Clinical characteristics						
Age, yrs	65 ± 12	59 ± 9	68 ± 11	66 ± 12	66 ± 14	0.029*
Male	131 (69.7)	15 (55.6)	23 (59.0)	48 (72.7)	45 (80.4)	0.046
BMI, kg/m ²	27.6 ± 4.9	27.4 ± 4.0	28.0 ± 5.3	27.4 ± 5.5	27.6 ± 4.2	0.94
Hypertension	119 (63.3)	8 (29.6)	21 (53.8)	52 (78.8)	38 (67.9)	<0.001
Smoking	104 (55.3)	12 (44.4)	17 (43.6)	39 (59.1)	36 (64.3)	0.13
Diabetes	43 (22.9)	1 (3.7)	9 (23.1)	14 (21.2)	19 (33.9)	0.022
Prior history of CAD	62 (33.0)	0 (0)	0 (0)	36 (54.5)	26 (46.4)	<0.001
MI	35 (18.6)	0 (0)	0 (0)	19 (28.8)	16 (28.6)	<0.001
PCI	47 (25.0)	0 (0)	0 (0)	26 (39.4)	21 (37.5)	<0.001
CABG	16 (8.5)	0 (0)	0 (0)	10 (15.2)	6 (10.7)	0.017
Aortic valvular disease	34 (18.1)	4 (14.8)	14 (35.9)	16 (24.2)	0 (0)	<0.001
Mitral valvular disease	12 (6.4)	7 (25.9)	3 (7.7)	2 (3.0)	0 (0)	<0.001
Biological						
Hemoglobin, g/dl	14.1 ± 1.6	13.7 ± 1.3	14.1 ± 1.5	14.2 ± 1.4	14.0 ± 1.9	0.62
Fasting glycemia, mg/dl	100 (92-114)	98 (92-105)	99 (91-123)	101 (93-108)	105 (95-134)	0.61
Creatinine, mg/dl	1.0 (0.8-1.1)	0.9 (0.8-1.1)	1.0 (0.8-1.0)	1.0 (0.9-1.2)	0.9 (0.8-1.1)	0.22
CRI	22 (11.7)	2 (7.4)	3 (7.7)	12 (18.2)	5 (8.9)	0.24
Non-HDL, mg/dl	120 ± 45	124 ± 49	123 ± 39	112 ± 45	127 ± 46	0.32
HDL, mg/dl	50.4 ± 15.3	53.8 ± 15.6	56.5 ± 17.8	50.2 ± 13.6	44.7 ± 13.4	0.002
TG, mg/dl	102 (76-152)	84 (66-132)	95 (78-140)	95 (75-146)	126 (91-161)	0.017†
hsCRP, mg/l	1.4 (0.6-3.4)	0.9 (0.5-2.1)	1.2 (0.6-3.0)	1.3 (0.5-3.6)	1.7 (0.8-5.6)	0.13
Platelet count (×10 ³)/μl	244 ± 61	211 ± 38	268 ± 65	248 ± 66	240 ± 54	0.002§
Multiplate analysis						
ASPI test, AU*min	515 ± 278	549 ± 319	631 ± 258	507 ± 287	427 ± 228	0.004†
ADP test, AU*min	638 ± 217	626 ± 163	692 ± 132	643 ± 243	601 ± 249	0.24
TRAP test, AU*min	1,101 ± 255	1,007 ± 258	1,177 ± 232	1,057 ± 226	1,146 ± 280	0.011*
D-dimers, ng/ml	407 (281-686)	282 (250-435)	398 (288-573)	407 (290-775)	443 (349-706)	0.018‡
Medication						
ACE inhibitor/ARB	86 (45.7)	6 (22.2)	16 (41.0)	43 (65.2)	21 (37.5)	<0.001
Beta-blockers	97 (51.6)	9 (33.3)	15 (38.5)	39 (59.1)	34 (60.7)	0.022
Lipid-lowering treatment	111 (59.0)	13 (48.1)	16 (41.0)	48 (72.7)	34 (60.7)	0.008
Aspirin	141 (75.0)	13 (48.1)	21 (53.8)	55 (83.3)	52 (92.9)	<0.001
Dual antiplatelet therapy	30 (16.0)	0 (0)	0 (0)	12 (18.2)	20 (35.7)	<0.001
Clopidogrel	15 (8.0)	0 (0)	0 (0)	6 (9.1)	9 (16.1)	0.013
Ticagrelor	13 (6.9)	0 (0)	0 (0)	3 (4.5)	10 (17.9)	<0.001
Prasugrel	4 (2.1)	0 (0)	0 (0)	3 (4.5)	1 (1.8)	0.35

Values are mean ± SD, n (%), or median (interquartile range). *Pairwise significant difference (p < 0.05) between NS-CAD and NCAD, †Pairwise significant difference (p < 0.05) between ACS and NS-CAD, ‡Pairwise significant difference (p < 0.05) between ACS and NCAD, §Pairwise significant difference (p < 0.05) between S-CAD and NCAD.

ACCTHEROMA = prospective evaluation of Acetyl-CoA Carboxylase phosphorylation state in platelets as a marker of atherothrombotic coronary and extra-coronary artery disease; ACE = angiotensin-converting enzyme; ACS = acute coronary syndrome; ARB = angiotensin receptor blocker; ASPI = aspirin channel; AU = arbitrary units; BMI = body mass index; CABG = coronary artery bypass grafting; CAD = coronary artery disease; CRI = chronic renal insufficiency; hsCRP = high-sensitivity C-reactive protein; MI = myocardial infarction; NCAD = non-coronary artery disease; NS-CAD = nonsignificant coronary artery disease; PCI = percutaneous coronary intervention; S-CAD = significant coronary artery disease; TG = triglycerides; TRAP = thrombin receptor activating peptide.

coronary angiography. They were divided into 4 groups according to clinical presentation and coronary anatomy (Table 1). The N-CAD group included 27 (14.4%) patients with normal coronary arteries, with about 41% undergoing angiography for valvular disease. In the CAD group (n = 161), 122 (76%) patients had significant coronary stenosis above 50%, 46% of whom had ACS. Most ACS patients (93%) were taking aspirin at enrollment, with only 36% of them on dual antiplatelet therapy, as most unstable angina and

non-ST-segment elevation myocardial infarction patients did not receive P2Y₁₂ inhibitors before angiography. Accordingly, mean platelet reactivity assessed using the Multiplate Analyzer (Roche Diagnostics International Ltd., Rotkreuz, Switzerland) showed decreased platelet aggregation in response to arachidonic acid in ACS patients. Laboratory parameters revealed significantly increased platelet counts in CAD patients, yet within the normal range. As previously reported (2), our data confirmed a significant

increase in ThG, assessed by D-dimer levels, in ACS patients.

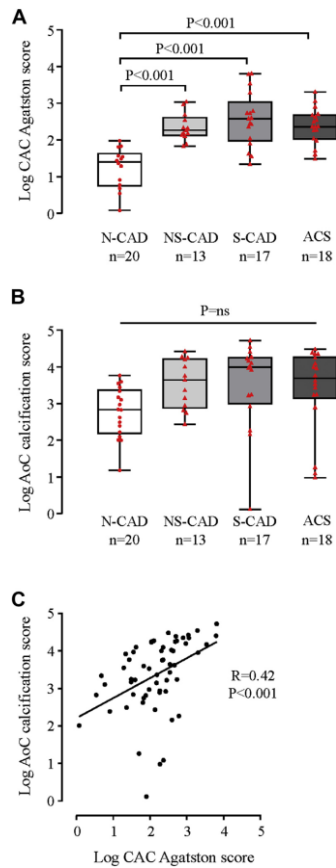
Thoracoabdominal MDCT with prospective electrocardiography gating aimed to evaluate calcified plaque burden, including coronary (CAC Agatston) and extracoronary (AoC) calcification scores. We confirmed that N-CAD patient (reference population) classification based on angiographic data was consistent with low CAC Agatston scores (median 7.0 [interquartile range: 0.0 to 33.9]) (Figure 2A). Although N-CAD patients had low CAC Agatston scores, their AoC scores were fairly high (median 543 [interquartile range: 107 to 2,001]) (Figure 2B). Accordingly, we observed a modest correlation between CAC and AoC calcification scores ($R_p = 0.42$; $p < 0.001$), indicating a heterogeneous atherosclerotic development or arterial calcifications (Figure 2C).

IDENTIFICATION OF HIGH-RISK CAD PATIENTS BY PLATELET phosphoACC. Platelet phosphoACC was studied in consecutive patients by Western blotting (Figure 3A). Quantification of band intensities showed significant increased phosphoACC in circulating platelets of CAD compared with N-CAD patients (median 0.48 [IQR: 0.29 to 0.73] vs. 0.22 [IQR: 0.11 to 0.45]; $p < 0.001$) (Figure 3B). Platelet phosphoACC in N-CAD patients was almost never >0.5 arbitrary units (AU), a threshold value estimated from receiver-operating characteristic analysis (Supplemental Figure 2) with a 96% positive predictive value for CAD. PhosphoACC signal was fairly similar in arterial and central venous blood in a subset of patients, undergoing left and right heart catheterization (Supplemental Figure 3). Of note, phosphorylation of protein kinase C substrates, a readout of platelet activation, was nearly undetectable in the platelets of CAD patients (Supplemental Figure 4), suggesting that phosphoACC occurred independently of platelet activation.

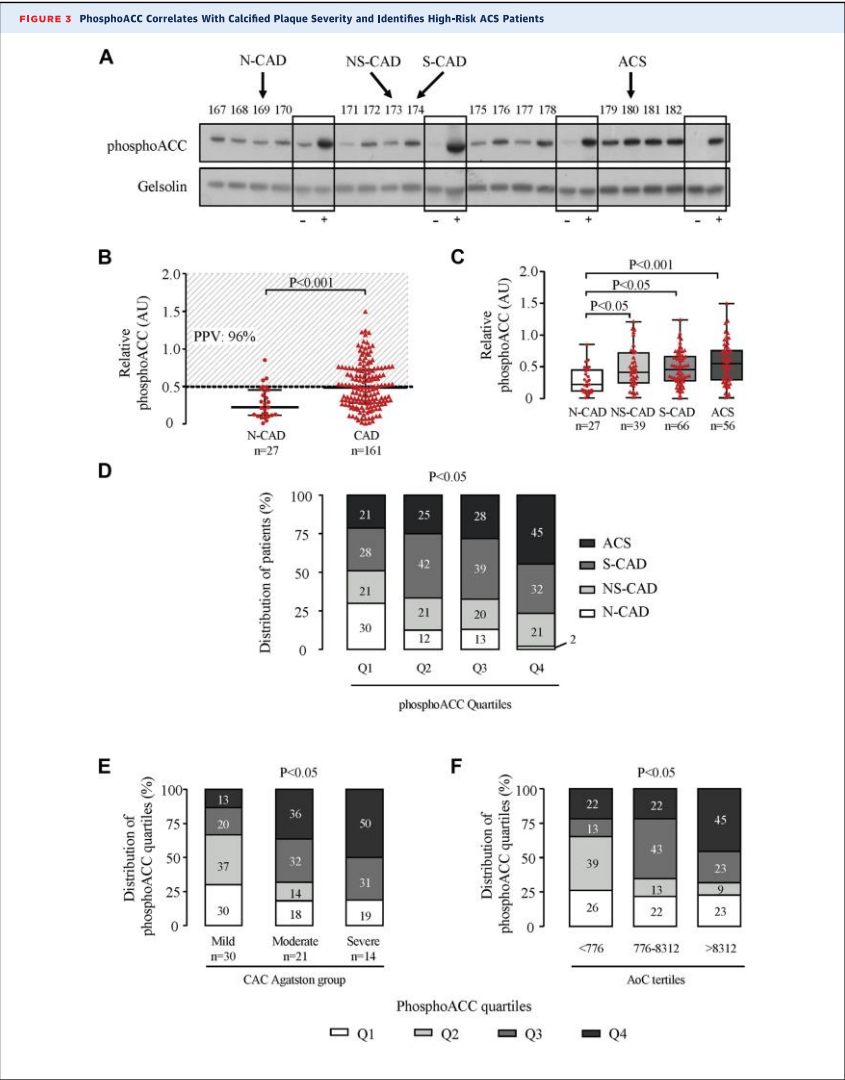
These data were further confirmed by quantitative electrochemiluminescence analysis. Platelet phosphoACC was analyzed twice to test interexperiment reproducibility (intraclass correlation coefficient) and found to be high (0.90), with a bias of -0.03 (95% CI: -0.10 to 0.04) (Supplemental Figure 5A). Furthermore, phosphoACC correlated with Western blot results, confirming that it was significantly increased in circulating platelets of CAD patients (Supplemental Figures 5B and 5C).

Among the CAD subgroups, the highest platelet phosphoACC level was found in ACS patients (median 0.55 [IQR: 0.29 to 0.76]) (Figure 3C). Indeed, quartile analysis of platelet phosphoACC demonstrated that the fourth quartile included 98% of CAD patients, with

FIGURE 2 Assessment of Coronary and Aortic Calcified Plaque by Prospective Electrocardiography-Gated Multidetector Computed Tomography



Box-plot representation of log-transformed (A) coronary artery calcification (CAC) Agatston and (B) aorta calcification (AoC) scores in the N-CAD and CAD subgroups of patients. Red dots (N-CAD, reference population) and triangles (CAD patients) represent individual values. Boxplots represent medians and corresponding whiskers represent extremes of the distribution. (C) Correlation between log-transformed CAC Agatston and AoC scores. Abbreviations as in Figure 1.



a large proportion of high-risk ACS patients (45%) (chi-square test; $p = 0.020$) (Figure 3D). Multivariable logistic regression results coincided with this, showing that, in addition to D-dimer levels (OR: 5.7; 95% CI: 1.8 to 17.8; $p = 0.003$) and the TG/HDL-C ratio (OR: 7.9; 95% CI: 2.1 to 29.9; $p = 0.002$), platelet phosphoACC (OR: 4.8; 95% CI: 1.5 to 15.8; $p = 0.009$) was independently associated to ACS (Table 2).

Prognostic value of platelet phosphoACC levels was assessed for ischemic events (cardiovascular death, recurrent myocardial infarction, and revascularization procedures) during a mean follow-up of 3.7 ± 0.4 years. In our cohort, 28 (14.8%) patients experienced with ischemic events. Multivariable Cox regression analysis identified increased platelet phosphoACC as an independent predictor of events (hazard ratio: 3.7; 95% CI: 1.1 to 12.1; $p = 0.034$), in addition to previous history of MI (hazard ratio: 3.2; 95% CI: 1.4 to 7.3; $p = 0.006$) and atherosclerotic cardiovascular disease score (hazard ratio: 10.1; 95% CI: 1.9 to 54.0; $p = 0.007$) (Supplemental Table 1). Kaplan-Meier event-free survival curves confirmed that patients with high phosphoACC levels (above 0.5 AU) had a higher events rate compared with low phosphoACC levels (log-rank test; $p = 0.036$) (Figure 4).

RELATIONSHIP BETWEEN PLATELET phosphoACC, CALCIFIED PLAQUE BURDEN, AND THG MARKERS. In patients who underwent MDCT, platelet phosphoACC, in addition to age and D-dimer levels, was independently associated to coronary calcification severity (global $R^2 = 0.47$; $p < 0.001$) (Supplemental Table 2). Accordingly, as depicted in Figure 3E, 50% of patients with high phosphoACC (fourth quartile) exhibited severe CAC Agatston scores (chi-square test; $p = 0.023$). Similar results were obtained with the AoC score (Figure 3F). Altogether, these findings highlight the potential of platelet phosphoACC for identifying disease severity and high-risk CAD patients. We next confirmed such observations in an animal model developing spontaneous atherosclerosis, which is

TABLE 2 Univariable and Multivariable Models of Factors Associated With ACS				
	Univariable Analysis		Multivariable Analysis	
	OR (95% CI)	p Value	OR (95% CI)	p Value
ASCVD score	3.61 (0.80-16.24)	0.09		
hsCRP (log transformed)	1.99 (1.11-3.55)	0.020		
D-dimer (log transformed)	3.14 (1.16-8.51)	0.024*	5.69 (1.82-17.76)	0.003*
Non-HDL	1.00 (0.99-1.01)	0.23		
TG/HDL-C ratio (log transformed)	7.57 (2.32-24.72)	0.001*	7.95 (2.11-29.90)	0.002*
Platelet phosphoACC	4.02 (1.39-11.56)	0.010*	4.83 (1.48-15.81)	0.009*

*Statistical significance when p value < 0.05 in univariable and multivariable analysis.
ASCVD = atherosclerotic cardiovascular disease; CI = confidence interval; HDL-C = high-density lipoprotein cholesterol; OR = odds ratio; phosphoACC = acetyl-CoA carboxylase phosphorylation on Ser79; other abbreviations as in Table 1.

enhanced by a Western diet, the SR-B1^{flox/flox}/ApoE^{-/-} hypercholesterolemic mice (Figures 5A and 5B). As in human platelets, phosphoACC levels drastically increased with the severity of atherosclerotic plaque burden (Figure 5C).

ThG markers correlated with the severity of calcified plaque, given that D-dimer levels (chi-square test; $p = 0.014$), thrombin antithrombin complexes (chi-square test; $p = 0.036$), or fragments 1.2 (chi-square test; $p = 0.004$) had a significant positive association with the severity of both CAC Agatston (Supplemental Figure 6A) and AoC score (Supplemental Figure 6B). However, we did not find any correlation between ThG markers (D-dimer levels) and platelet phosphoACC across the entire population (Supplemental Figure 6C), although thrombin is a major agonist leading to increased phosphoACC in platelets in vitro. Therefore, other factors besides thrombin should contribute to platelet phosphoACC in atherosclerosis.

CONTRIBUTION OF INFLAMMATION AND ATHEROGENIC oxLDL TO phosphoACC IN PLATELETS. As inflammatory cytokines may affect platelets (14) and are established circulating actors of atherosclerosis, we explored whether interleukin-1 β , interleukin-6, interleukin-10, interleukin-17A, and tumor necrosis

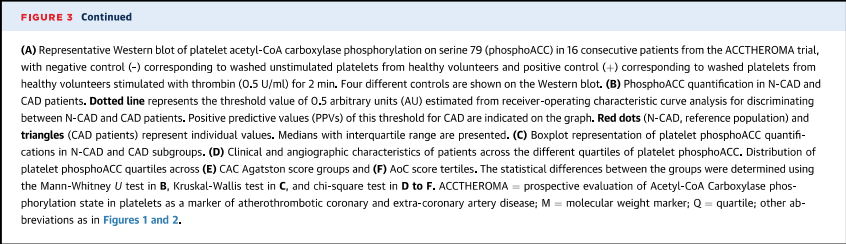


TABLE 3 Factors Associated With Increased Platelet phosphoACC in CAD Patients (>0.5 AU Threshold)

	OR (95% CI)	p Value
Aspirin treatment	1.16 (0.54-2.51)	0.70
DAPT	1.50 (0.68-3.34)	0.32
D-dimer (log transformed)	0.62 (0.20-1.94)	0.41
hsCRP (log transformed)	1.00 (0.58-1.71)	0.99
Non-HDL	1.00 (0.99-1.01)	0.53
TG	1.00 (1.00-1.01)	0.048*
HDL	0.97 (0.96-1.00)	0.043*
TG/HDL-C ratio (log transformed)	3.97 (1.25-12.61)	0.019*

*Statistical significance when p value <0.05.
DAPT = dual antiplatelet therapy; other abbreviations as in Tables 1 and 2.

factor- α impacted phosphoACC level in washed platelets from healthy volunteers. Although their respective receptors are present in human platelets (15), none of these cytokines affected platelet phosphoACC (Figure 6A).

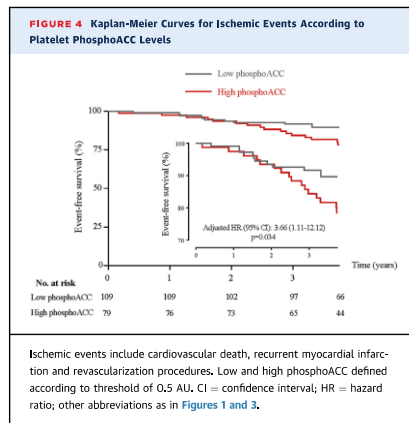
We continued our analysis to identify biological mediators of increased platelet phosphoACC in the CAD group (above 0.5 AU) (Figure 3B). Whereas high-sensitivity C-reactive protein and ThG markers were not associated with increased phosphoACC, we identified a striking positive association between TG/HDL-C and platelet phosphoACC, suggesting atherogenic lipids to affect phosphoACC in circulating platelets (OR: 4.0; 95% CI: 1.3 to 12.6; $p = 0.019$) (Table 3). TG/HDL-C ratio, a well-known atherogenic marker, indicates LDL particle size (16), while TGs were shown to correlate with plasma oxLDL levels

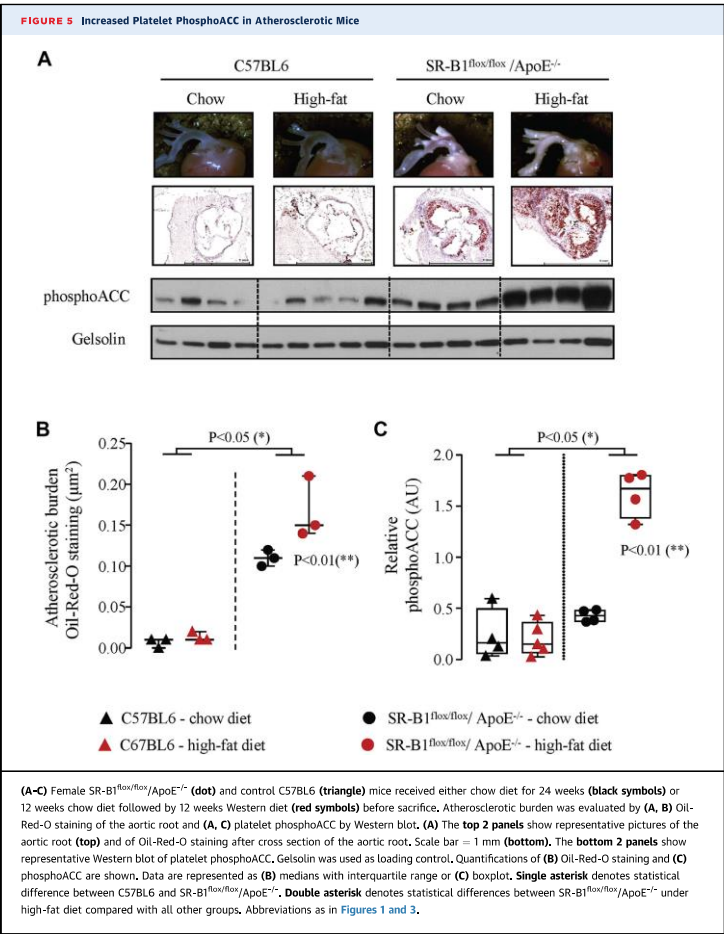
(17). In our cohort of patients, TG/HDL-C ratio correlated with oxLDL levels ($R_p = 0.23$; $p = 0.002$). We thus studied the effect of oxLDL on AMPK-ACC signaling. PhosphoACC was assessed in platelets from healthy volunteers treated with copper-oxidized LDL and myeloperoxidase-oxidized LDL. Both induced a time- and dose-dependent increase in platelet phosphoACC (Figures 6B to 6D, Supplemental Figure 7A). Myeloperoxidase-oxidized LDL-induced phosphoACC was prevented by preincubating platelets with a blocking anti-CD36 antibody (Figure 6E). Accordingly, oxidized choline glycerophospholipids (OxPC^{CD36}), a specific high-affinity ligand for CD36, increased platelet phosphoACC (Figure 6F).

LIPIDOMIC PROFILING OF CIRCULATING PLATELETS AND METABOLIC REGULATION OF INTRAPLATELET TG LEVELS BY phosphoACC IN CAD PATIENTS. We investigated the impact of ACC phosphorylation or inhibition on platelet lipid composition through unbiased lipidomic analysis. To this end, we selected platelet lysates from patients displaying the lowest and highest phosphoACC levels. Baseline characteristics of these patients are provided in Supplemental Table 3. Multivariable regression analysis first showed that diabetes and plasma TG levels were independently associated with up-regulation of intraplatelet TG lipid species. Of the 490 TG lipid species detected within platelets, 253 (52%) were significantly up-regulated (Figure 7A) when plasma TG levels increased. Contrarily, increased phosphoACC was independently associated with significant down-regulation of 66 (14%) intraplatelet TGs (Figure 7B, Supplemental Table 4). This was confirmed by lipid class enrichment analysis revealing a significant proportion of down-regulated lipid species belonging to TG class in platelets of patients with high phosphoACC levels (OR: 27.0; 95% CI: 7.1 to 228.3; $p < 0.001$) (Supplemental Figure 8). Furthermore, we characterized changes in fatty-acid-chain composition of TGs. Increased platelet phosphoACC was associated with down-regulation of TGs containing 14 carbons (C14:0, myristic acid) (Figure 7C). These findings support that circulating platelets interact with the hyperlipidemic environment in atherosclerosis and, more importantly, that ACC phosphorylation or inhibition affects endogenous platelet lipid content, by regulating TG lipid species and their fatty-acid-chain composition.

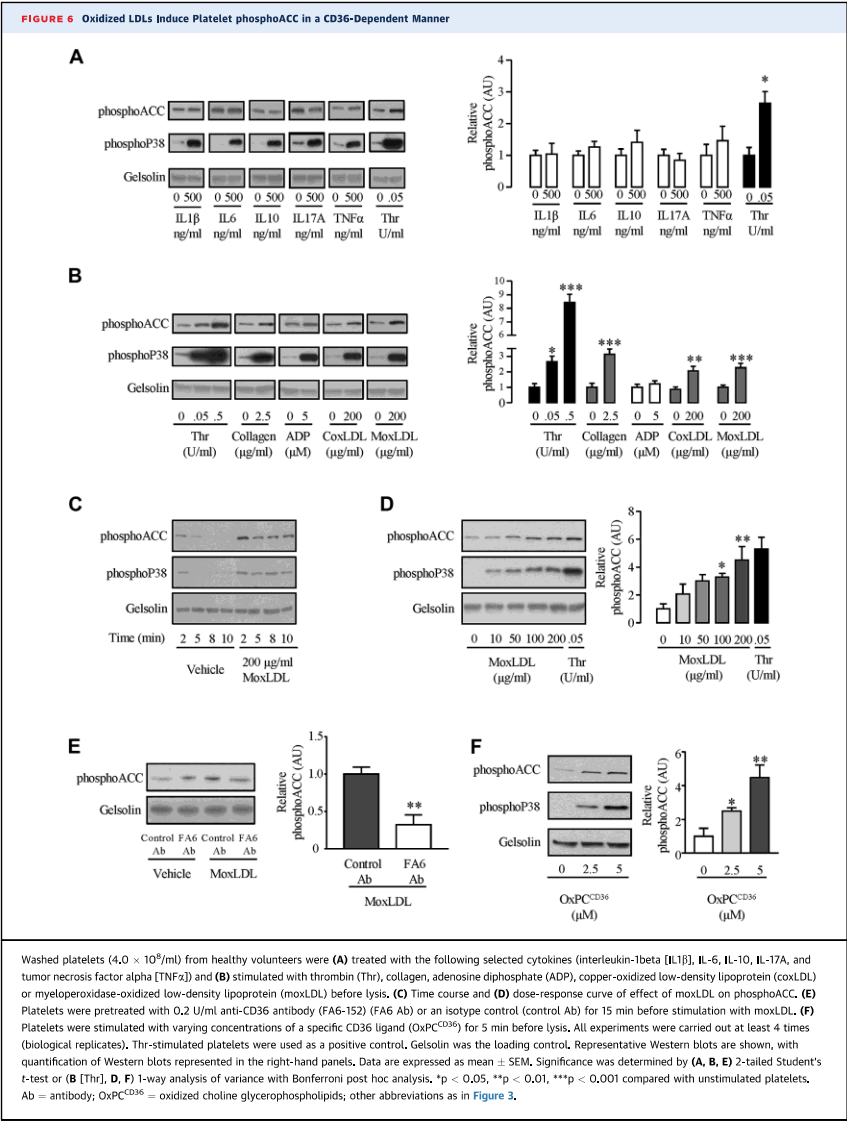
DISCUSSION

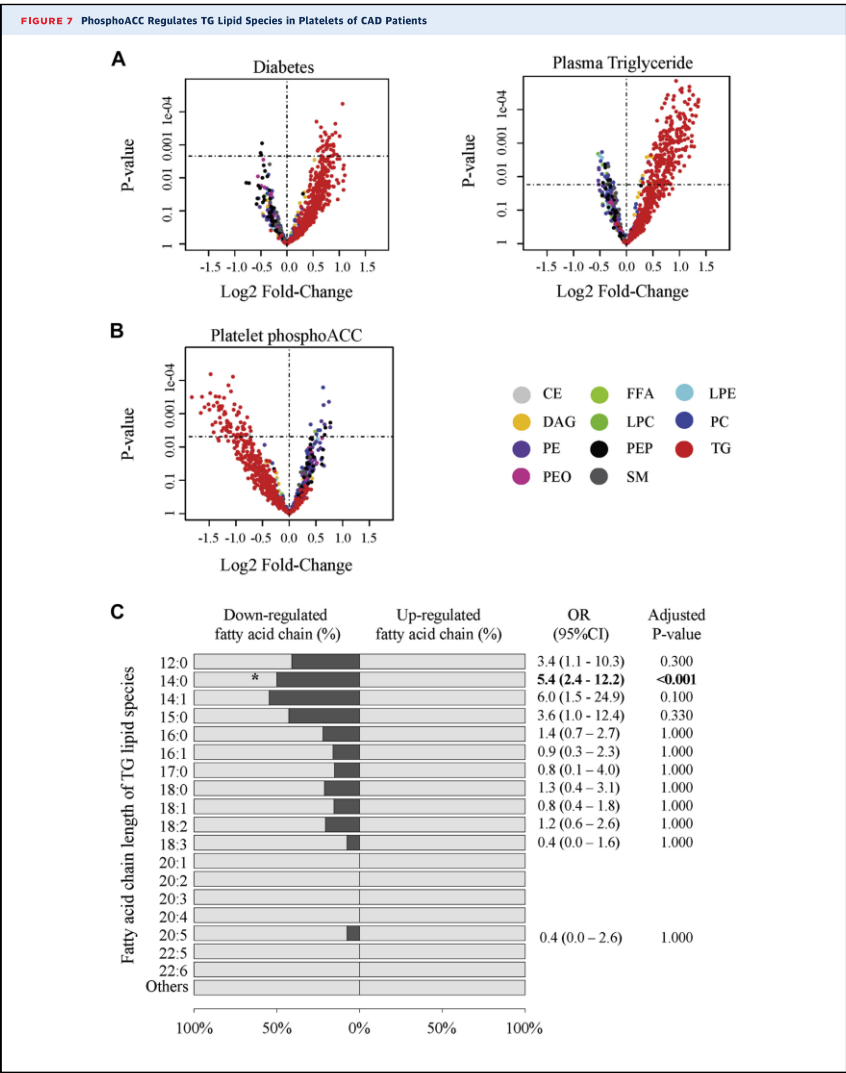
This study highlights that platelet phosphoACC is a risk marker in patients with suspected CAD and





provides additional evidence of an interplay between platelets and lipids in atherosclerosis. We demonstrate a clear relationship between platelet phosphoACC and CAC Agatston score severity, and acute coronary events in CAD. In a molecular point of view, oxLDL participates in increasing phosphoACC in circulating platelets via a CD36-dependent pathway. ACC phosphorylation/inhibition impacts endogenous platelet lipid synthesis by regulating TG lipid species. **PLATELET phosphoACC LEVELS AND RISK ASSESSMENT.** Despite the LDL cholesterol-lowering drugs and platelet inhibitors currently available, substantial





Continued on the next page

residual risk remains (18), and many patients should be given more aggressive treatment (3,19). These patients must be identified by measurable risk markers. Our study brings platelet phosphoACC to the fore as a marker for identifying high-risk patients. More importantly, higher phosphoACC states were detected in platelets of ACS patients, even if treated with aspirin, P2Y₁₂ inhibitors, or statins. The long-term influence of treatments on platelet phosphoACC remains to be determined in this high-risk population.

Numerous studies have reported that high on-treatment platelet reactivity and platelet activation indices reveal high-risk patients in CAD population (20). Particularly, increased circulating activated platelets, evidenced by monocyte- or neutrophil-platelet aggregates or P-selectin expression, can be detected in high-risk patients versus normal subjects. However, this increase is marginal and involves a small proportion of the total platelet pool (21-23). In our study, AMPK-ACC signaling activation was observed in circulating platelets. The absence of protein kinase C substrate phosphorylation, a sign of platelet activation (24), supports the theory that increased phosphoACC and platelet activation are unrelated. Even if thrombin is the principal agonist of platelet AMPK activation *ex vivo* (4), we found no association between ThG markers and phosphoACC. Therefore, platelet phosphoACC is not caused by thrombin-induced platelet activation in patients. Thus, our findings suggest that platelets of high-risk CAD patients exhibit altered metabolic phenotypes characterized by activation of AMPK-ACC signaling, independent of platelet activation state.

LIPID-PLATELET INTERACTION. Changes in LDL phenotypes, such as tendency to aggregate, oxidative status, or size and density, influence cardiovascular risk and can determine residual post-treatment risk (25,26). In line with previous studies (10), our work further supports that this atherogenic environment influences platelet phenotype in CAD. A novel finding of our study is the significant positive correlation between platelet phosphoACC and TG/HDL-C ratio, a

marker of a proatherogenic lipid profile, including small LDL particles known to be more oxidizable (16,17). Based on our data, oxLDL mediates, at least in part, the phosphoACC increase in CAD patients. PhosphoACC in response to oxLDL occurs downstream of CD36. Numerous *in vitro* studies suggested that oxLDL binds to platelet CD36 promoting intracellular signals, including the recruitment of Src family kinases Fyn and Lyn (27), cytoskeleton rearrangement (7), and nicotinamide adenine dinucleotide phosphate oxidase activation (28). Interestingly, an increased binding of oxLDL on platelets has been reported in patients with ACS, in line with our observation (29). Additionally, CD36 is an established receptor for platelet interaction with atherogenic environments, and CD36 expression variability may determine platelet reactivity and thrombotic risk (5,6).

ACC AND PLATELET LIPID CONTENT. Given the central role of ACC in lipid biosynthesis, we performed lipidomic profiling to investigate the impact of its phosphorylation/inhibition on lipid content. Plasma TG levels were associated with an up-regulation of TG lipid species in platelets of CAD patients, highlighting the strong interaction between platelets and plasma lipids. Our results corroborate recent data revealing platelet lipid content to be altered in ACS patients (10). We further demonstrated that increased platelet phosphoACC in high-risk CAD patients is associated with platelet TG down-regulation, reflecting the inhibitory impact of phosphoACC on platelet lipid synthesis. Similar findings were reported in hepatocytes, where liver-specific pharmacological inhibition of ACC lowered lipogenesis and reduced liver TG (30). The down-regulation of fatty acid chains containing 14 carbons (C14:0, myristic acid) provides further evidence that endogenous platelet lipid synthesis is altered upon ACC phosphorylation. In line with our data, ACC inhibition in cancer cells decreases *de novo* fatty acid synthesis, leading to a reduction in fatty acids containing 14 to 18 carbons (31). To our knowledge, this is the first

FIGURE 7 Continued

(A, B) Volcano plot representations of the 865 lipid species detected in platelets by lipidomic profiling. Log fold-changes and p values were calculated from the multivariate regression model. Lipids above the horizontal dotted line were up- or down-regulated with significant adjusted p values. Relationship among (A) diabetic status and plasma triglyceride (TG) levels and (B) platelet phosphoACC and intraplatelet lipid species content. (C) Class enrichment analysis of fatty-acid-chain constituents of TG lipid species in platelets with respect to increased phosphoACC. Bars (dark gray) represent the percentage of down-regulated fatty acid-containing TG. Odds ratio (OR) and adjusted p values derived from Fisher's exact test are shown. CE = cholesterol ester; CI = confidence interval; DAG = diacylglycerol; FFA = free fatty acid; LPC = lysophosphatidylcholine; LPE = lysophosphatidylethanolamine; OR = odds ratio; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PEO = plasmalogen phosphatidylethanolamine; PEP = plasmalogen phosphatidylethanolamine; SM = sphingomyelin; other abbreviations as in Figure 3.

study to report the impact of ACC phosphorylation/inhibition on the regulation of lipid content in circulating platelets of CAD patients.

STUDY LIMITATIONS. First, phosphoACC was determined using immunoblotting in whole platelet lysates. Given the circulating platelets' heterogeneity (32), it is still unclear whether increased phosphoACC occurs in the entire platelet population or in a subgroup only. Moreover, in this setting, platelets must be purified before phosphoACC is measured, and quantitative assessment of phosphoACC remains challenging. However, we employed a more quantitative method, electrochemiluminescence immunoassay, to confirm the phosphoACC increase. Second, the ACCTHEROMA trial was a single-center trial, with a limited patient number. This sample size allowed us to fully address our primary endpoint (i.e., increase in phosphoACC in CAD patients). However, despite this limited number of patients, we identified phosphoACC as an independent predictor for adverse cardiac events and more particularly ischemic events, during follow-up, reinforcing the link between phosphoACC and disease severity. Multicenter studies may be required to validate the prognostic impact of increased phosphoACC in peripheral blood and determine signal variability over time, as with LDL cholesterol (25).

CONCLUSIONS

Our study identifies platelet phosphoACC as a risk stratification marker that reflects the interaction between proatherogenic lipids and circulating platelets. In CAD patients, phosphoACC contributed to regulating endogenous lipid synthesis. We recently provided new insights into AMPK-ACC signaling's key role in regulating platelet lipid composition and function using a genetic mouse model. The absence of phosphoACC (and inhibition) by AMPK in platelets

resulted in increased phospholipid content, with enhanced platelet reactivity and thrombus formation. It is tempting to speculate that increased phosphoACC is a counter-regulatory mechanism limiting lipogenesis and platelet reactivity in CAD patients within a proatherogenic environment.

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ADDRESS FOR CORRESPONDENCE: Dr. Christophe Beauloye, Division of Cardiology, Cliniques Universitaires Saint-Luc, Pôle de Recherche Cardiovasculaire, Institut de Recherche Expérimentale et Clinique (IREC), Université catholique de Louvain, Avenue Hippocrate, 55 (B1.55.05), 1200 Brussels, Belgium. E-mail: christophe.beauloye@uclouvain.be.

PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: The circulating platelets of high-risk CAD patients exhibit altered metabolic phenotypes characterized by phosphorylation and inhibition of ACC, the first committed enzyme of the fatty acid biosynthesis pathway. Platelet phosphoACC is thus a potential marker for risk stratification in suspected CAD patients, indicating an interplay between platelets and atherogenic lipids.

TRANSLATIONAL OUTLOOK 1: Additional studies are needed to further define the role of endogenous lipogenesis in the control of platelet function.

TRANSLATIONAL OUTLOOK 2: Large multicenter studies must investigate whether individual platelet phenotypes, including phosphoACC, influences major adverse outcomes in CAD.

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KEY WORDS AMPK, acetyl-CoA carboxylase, coronary artery disease, lipidomics, platelet

APPENDIX For expanded Methods and References sections as well as supplemental tables and figures, please see the online version of this paper.



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Anti-GPVI (HY101) activates platelets in a multicolor flow cytometry panel

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Anti-GPVI (HY101) activates platelets in a multicolor flow cytometry panel

Marie-Astrid van Dievoet¹, Davide Brusa², Marie Octave³, Sandrine Horman³, & Xavier Stephenne¹

¹Laboratoire d'Hépatologie Pédiatrique et Thérapie Cellulaire, Unité Pedi, Institut de Recherche Expérimentale et Clinique (IREC), Université Catholique de Louvain (Uclouvain), Brussels, Belgium, ²Flow Cytometry Platform, IREC, UCLouvain, Brussels, Belgium, and ³Pôle de Recherche Cardiovasculaire (CARD), IREC, UCLouvain, Brussels, Belgium

Abstract

The platelet transmembrane receptor GPVI can be assessed together with other platelet membrane markers in a whole blood multicolor flow cytometry panel. The advantage of combining multiple antibodies in a single tube is the possibility of distinguishing multiple platelet subgroups. In this short communication, we describe an activation problem encountered with anti-GPVI, clone HY101. Activation of platelets was seen after the addition of anti-GPVI in a flow cytometry panel, highlighted by the expression of the activation markers CD62P, PAC-1, CD63, and CD107a. This was also confirmed by platelet aggregation studies.

Keywords

Anti-GPVI, flow cytometry, HY101, platelet

History

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Introduction

Glycoprotein (GP)-VI is transmembrane protein, a member of the immunoglobulin superfamily and one of the important platelet collagen receptors [1]. The expression of GPVI is restricted to platelets and megakaryocytes. GPVI is associated with the dimeric Fc-receptor γ chain [2], which contains an immunoreceptor tyrosine-based activation motif (ITAM). Upon ligand binding, GPVI dimerization leads to phosphorylation of the tyrosine residues and further signal transduction ensues [3]. Finally, phospholipase C γ 2 becomes activated and the calcium (Ca²⁺) level rises. Next to collagen, several other endogenous GPVI ligands exist, for example, fibrin and adiponectin [4,5]. The synthetic triple-helical collagen-related peptide (CRP), an exogenous ligand, consists of the glycine–proline–hydroxyproline (GPO)n tandem repeats that mediate binding of collagen to GPVI [6]. GPVI contains a cleavage site, which enables shedding of the extracellular domain via the disintegrin and metalloproteinase 10 (ADAM10) upon activation [7].

GPVI on the platelet membrane can be assessed by flow cytometry. The availability of commercial anti-GPVI is still scarce. In the present work, we would like to highlight an activation problem with anti-GPVI, clone HY101, directed against an epitope in the extracellular domain of GPVI. This antibody coupled to a fluorochrome was used in a multicolor platelet flow cytometry protocol assessing both resting and activated states.

Methods

Flow Cytometry

Peripheral blood was collected in a citrate tube (Monovette plastic 3.0 mL, 3.2% citrate, 106 mM (Sarstedt, Numbrecht, Germany); 10:1 blood:citrate ratio) after drawing a discard tube. Preanalytical processing was performed according to Södergren et al. [8]. In brief,

blood samples were kept for 1 hour at room temperature before adding a mix of fluorochrome-coupled Abs and HEPES buffer. Brilliant Stain Buffer (BD) was used in one experiment. For platelet activation experiments, agonists, Ca²⁺, and GPRP were added to the mix. Samples were incubated for 10 minutes at room temperature. Incubation was stopped by adding 1 mL of HEPES-Ca²⁺ buffer and analyzed within 1 hour. The following platelet surface markers were included: CD41a phycoerythrin (PE, clone: HIP8, BD), CD42b BV711 (clone: HIP1, BD), CD42a APC-Vio® 770 (clone: REA209, Miltenyi Biotec, Bergisch Gladbach, Germany), PAC-1 fluorescein isothiocyanate (FITC, binds active conformation of α Ib β 3, BD), CD62P BV786 (anti-P-selectin, α -granule release, clone: AK-4, BD), CD107a PE-CF594 (anti-lysosomal-associated membrane protein 1 (LAMP-1), clone: H4A3, BD), and CD63 BV510 (anti-LAMP-3, clone: H5C6, BD). This mix was tested both with and without anti-GPVI BV421 (clone: HY101, BD). In one analysis, we also used anti-GPVI PE (HY101; BD) instead of anti-GPVI BV421. A BV421 Mouse IgG1, k isotype control was also included in the experiment (BD).

Anti-GPVI BV421 (0.2 mg/mL; 2 μ L or 0.4 μ g in a final volume of 60 μ L; +1.000.000 platelets) was tested at different dilutions: 1/10, 1/20, and 1/30.

An experiment in which paraformaldehyde (1% final concentration) was used to fix platelets before adding anti-GPVI BV421 was also executed.

For platelet activation, α -thrombin (T6884, Sigma-Aldrich, St. Louis, Missouri, US) and synthetic cross-linked collagen-related peptide (CRP-XL; CambCol Laboratories, Littleport, Cambridgeshire, UK) were used at final concentrations of 5 U/mL and 5 μ g/mL, respectively. Fibrin polymerization was blocked with GPRP (G5779, Sigma-Aldrich, 2 mM final concentration). Analysis was performed on the FACS ARIA II (Becton Dickinson (BD), Franklin Lakes, NJ, US). Compensation was executed with BD CompBeads (ref: 552843) and Miltenyi MACS CompBeads anti-REA (130–104-693). Acquisition was stopped after obtaining 10,000 platelets in a temporary gate. Platelets were selected on a forward/side scatter plot, and after removing doublets, the gate was set on the CD41 positive events.

Correspondence: Marie-Astrid van Dievoet, Département des Laboratoires Cliniques, Service d'hématologie Biologique, Université Catholique de Louvain, 54 Avenue Hippocrate, Brussels B-1200, Belgium. Email: marie-astrid.vandievoet@uclouvain.be

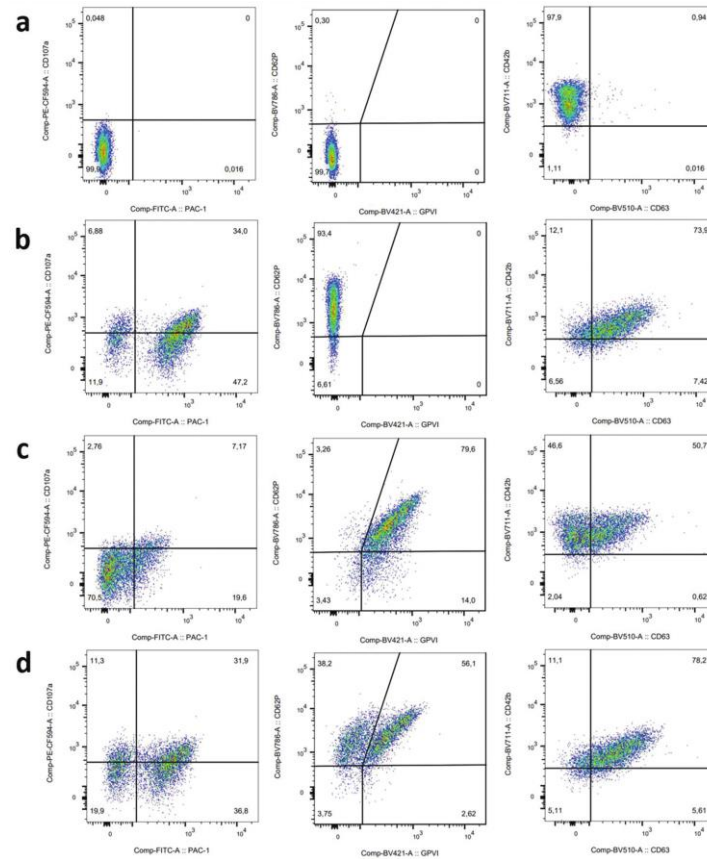


Figure 1. Flow cytometry plots of different platelet surface markers in four different conditions: without GPVI in resting state (a), without GPVI after stimulation by thrombin 5 U/mL and CRP-XL 5 µg/mL (b), with GPVI [6,67 µg/mL] in resting state (c), with GPVI [6,67 µg/mL] after stimulation by thrombin 5 U/mL and CRP-XL 5 µg/mL (d). (n = 4 biological replicates)

Platelet Aggregation

Platelet aggregation experiment was performed using light transmission aggregometry (Chrono-log 700, STAGO). Blood was drawn on a CPDA tube (1/10; citrate, phosphate, dextrose, and adenine) and centrifuged at 330 x g for 20 min. Eptifibatide (4 µg/mL) and apyrase (1 U/mL) were added to platelet-rich plasma (PRP) and centrifuged at 800 x g for 10 min. Platelet pellets were washed once in modified Tyrode's buffer (135 mM NaCl, 12 mM NaHCO₃, 3 mM KCl, 0.3 mM Na₂HPO₄, 1 mM MgCl₂, 5 mM D-glucose, 10 mM Hepes, 0.35% BSA, pH 7.4, 37°C) containing eptifibatide (4 µg/mL) and apyrase (1 U/mL) at 1000 x g for

10 min. Platelets were resuspended at a density of $2.5 \times 10^5/\mu\text{l}$ in modified Tyrode's buffer.

Light transmission of washed human platelets (52.2×10^6), stirred at 1200 rpm and 37°C, was recorded after the addition of 2 mM CaCl₂ and GPVI Ab. A BV421 Mouse IgG1 isotype control was also tested.

Results

Flow Cytometry

Results are depicted in Figure 1.

In the flow cytometry panel without anti-GPVI BV421, no expression of platelet activation markers was seen in the resting state. When anti-GPVI was added in the resting state, activation was demonstrated with every platelet activation marker, reflecting α -granule release, lysosomal release, and α IIb β 3-activated form. Compared to the positive control (addition of thrombin and CRP-XL), platelet activation marker expression was less pronounced in the resting panel with anti-GPVI.

Activation diminished with lower concentrations of anti-GPVI (Supplementary Figure 1) although CD62P was still activated at 55.8% for a 1/30 dilution. Mean fluorescence intensity at different dilutions and in comparison with the isotype control is shown in Supplementary Figure 2.

Replacing anti-GPVI BV421 with anti-GPVI PE also resulted in platelet activation (Supplementary Figure 3). Adding the Brilliant Stain Buffer did not have an impact on results (Supplementary Figure 4).

Fixation with paraformaldehyde 1% before addition of anti-GPVI abolished activation of platelets demonstrated by negative activation markers (Supplementary Figure 5).

Platelet Aggregation

Results are depicted in Figure 2.

Platelet aggregation was performed using a high (6.67 μ g/mL) and a low (0.2 μ g/mL) anti-GPVI Ab concentration. In agreement with the flow cytometry experiment, only the high concentration of anti-GPVI Ab induced platelet aggregation.

Discussion and Conclusions

A whole blood multicolor platelet flow cytometry panel is a valuable tool to map the platelet phenotype in a rather easy and rapid way. The advantage of combining multiple Abs in a single tube is the possibility of distinguishing multiple platelet subgroups. With platelets being easily activated, it is paramount to thoroughly validate the technique used beforehand to avoid any (pre-) analytical problems [9].

In this short communication, we describe an activation problem encountered with anti-GPVI, clone HY101, in a whole blood platelet flow cytometry panel. This activation issue was confirmed with a platelet aggregation study. Neither replacing anti-GPVI BV421 (HY101) with anti-GPVI PE (HY101) nor the addition of Brilliant Stain Buffer, recommended when using >1 brilliant dye, had an impact on flow cytometry results. Fixating the platelets before the addition of

anti-GPVI abolished the activation problem, but complicates the staining protocol, especially for agonist activation assays.

The anti-GPVI monoclonal antibody, recognizing the extracellular domain of GPVI, was generated for the first time by Chen et al. [10]. The authors state that binding of HY101 on platelets does not interfere with collagen or convulxin signaling, suggesting a binding region distinct from the ligand binding site. However, the article did not mention experiments studying the ability of HY101 to aggregate nor activate platelets. Trifiro et al. [11] state that "HY101 is a neutral monoclonal Ab that neither activates GPVI nor inhibits ligand binding to GPVI." It is, however, not clear if this was tested by the authors. In a paper looking at aggregation and shedding properties of different anti-GPVI Abs, HY101 was unfortunately not included [12]. In this article, the majority of Abs identified against the extracellular domain of GPVI ($n = 8$) were able to induce aggregation, except for the 11A7 clone. Fab fragments from the same Abs, except for 11A7, did not induce aggregation but were able to inhibit collagen- and CRP-induced aggregation. Another study, describing a GPVI-deficient patient, used anti-GPVI HY101 to evaluate membrane GPVI expression. Other platelet membrane markers including the activation marker CD62P were analyzed. Again, it is not clear if anti-GPVI was added to the cocktail of Abs. If not, platelet activation in the resting state or inhibition of activation in collagen-stimulated conditions by anti-GPVI HY101 would not have been detected in the control samples. The majority of papers mentioned above did not test anti-GPVI HY101-induced aggregation nor activation, possibly explaining why the activating property of HY101 has not been described yet.

The mechanism of platelet activation by the monoclonal anti-GPVI Ab is not clear. Given that HY101 clone is described as neutral and activates platelets in a dose-dependent manner, it is very likely that platelets are activated by clustering Fc γ RIIa receptor. Why some monoclonal Abs cluster Fc γ RIIa receptor while others do not is not known [13]. This cluster effect diminishes with lower concentrations of Ab. By assessing aggregation in the presence of IV.3 (Fc γ RIIa blocker), the article by Al-Tamimi et al. concluded a direct agonist role independent of the Fc γ RIIa receptor for the GPVI antibodies studied [12].

Recently, Biocytex (Stago, Asnières, France) developed a quantification assay using anti-GPVI 1G5 clone. It is believed that this assay should be used to establish international standards for GPVI expression at the platelet surface [14]. However, the

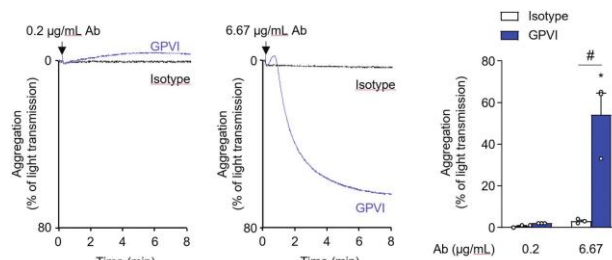


Figure 2. Aggregation was analyzed by turbidimetry (Chrono-Log) in platelets stimulated with various concentrations of GPVI-BV421 (HY101) antibody (Ab) or its isotype (BV421 Mouse IgG1k). Profiles are shown on the left. Data are expressed as means $\pm$ SEM ($n = 3$). * p -value ≤ 0.001 in relation to 0.2 μ g/mL Ab concentration. # p -value ≤ 0.001 in relation to isotype Ab. Data were analyzed using 2-way ANOVA.

same clone (1G5) has been described to induce aggregation and GPVI shedding [12]. In our opinion, this is not a suitable clone for multicolor platelet flow cytometry panels using platelet agonists. A nonactivating GPVI antibody like anti-GPVI clone 11A7 could be a good alternative for utilization in multicolor platelet agonist experiments. To our knowledge, this clone is not commercially available.

Also of interest, Jung et al. [15] described GPVI dimer-specific antibodies, m-Fab-F and 204-11 Fab. They imply that GPVI dimer is the collagen-binding form of GPVI. These Fab fragments, however, inhibit/block collagen binding.

Another alternative for avoiding anti-GPVI-induced activation is the “fix-first method” described by Spurgeon et al. [16] where the antibody cocktail is added after formaldehyde fixation. The disadvantage of this technique is a possible decrease in staining intensity in, for example, anti-CD62P. PAC-1 staining may be abolished after formaldehyde fixation.

Author Contributions

DB, MO, and MAVD performed the experiments for this original paper, which were further refined with SH and XS. MAVD did the writing of the paper with support of all other authors. All the authors approved the final version.

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Supplementary Material

Supplemental data for this article can be accessed on the publisher's website.

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Jeremie Joffe,
University of California, San Francisco,
United States

*Correspondence:

Christophe Beauloye
christophe.beauloye@uclouvain.be

[†] These authors have contributed
equally to this work and share first
authorship

[‡] These authors have contributed
equally to this work and share last
authorship

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Inflammation-Induced Coagulopathy Substantially Differs Between COVID-19 and Septic Shock: A Prospective Observational Study

Mélanie Dechamps^{1,2†}, Julien De Poortere^{1†}, Manon Martin³, Laurent Gatto³, Aurélie Daumerie⁴, Caroline Bouzin⁴, Marie Octave¹, Audrey Ginion¹, Valentine Robaux¹, Laurence Piroton¹, Julie Bodart¹, Ludovic Gerard^{5,6}, Virginie Montiel⁵, Alessandro Campion¹, Damien Gruson⁷, Marie-Astrid Van Dievoet⁷, Jonathan Douxfils^{8,9}, Hélène Haguet^{8,9}, Laure Morimont^{8,9}, Marc Derive¹⁰, Lucie Jolly¹⁰, Luc Bertrand¹, Laure Dumoutier¹¹, Diego Castaneres-Zapatero^{1,5}, Pierre-François Laterre⁵, Sandrine Horman¹² and Christophe Beauloye^{1,12‡}

¹ Pôle de Recherche Cardiovasculaire, Institut de Recherche Expérimentale et Clinique, Université Catholique de Louvain, Brussels, Belgium, ² Department of Cardiovascular Intensive Care, Cliniques Universitaires Saint-Luc, Brussels, Belgium, ³ Computational Biology and Bioinformatics Unit, de Duve Institute, Université Catholique de Louvain, Brussels, Belgium, ⁴ IREC Imaging Platform, Institut de Recherche Expérimentale et Clinique, Université Catholique de Louvain, Brussels, Belgium, ⁵ Department of Intensive Care, Cliniques Universitaires Saint-Luc, Brussels, Belgium, ⁶ Pôle de Pneumologie, Institut de Recherche Expérimentale et Clinique, Université Catholique de Louvain, Brussels, Belgium, ⁷ Department of Clinical Biology, Cliniques Universitaires Saint-Luc, Brussels, Belgium, ⁸ Department of Pharmacy, Namur Research Institute for Life Sciences, Namur, Belgium, ⁹ Qualiblood, s.a., Namur, Belgium, ¹⁰ Inotrem s.a., Vandoeuvre-les-Nancy, France, ¹¹ Experimental Medicine Unit, de Duve Institute, Université Catholique de Louvain, Brussels, Belgium, ¹² Division of Cardiology, Cliniques Universitaires Saint-Luc, Brussels, Belgium

Critical COVID-19, like septic shock, is related to a dysregulated systemic inflammatory reaction and is associated with a high incidence of thrombosis and microthrombosis. Improving the understanding of the underlying pathophysiology of critical COVID-19 could help in finding new therapeutic targets already explored in the treatment of septic shock. The current study prospectively compared 48 patients with septic shock and 22 patients with critical COVID-19 regarding their clinical characteristics and outcomes, as well as key plasmatic soluble biomarkers of inflammation, coagulation, endothelial activation, platelet activation, and NETosis. Forty-eight patients with matched age, gender, and co-morbidities were used as controls. Critical COVID-19 patients exhibited less organ failure but a prolonged ICU length-of-stay due to a prolonged respiratory failure. Inflammatory reaction of critical COVID-19 was distinguished by very high levels of interleukin (IL)-1 β and T lymphocyte activation (including IL-7 and CD40L), whereas septic shock displays higher levels of IL-6, IL-8, and a more significant elevation of myeloid response biomarkers, including Triggering Receptor Expressed on Myeloid cells-1 (TREM-1) and IL-1ra. Subsequent inflammation-induced coagulopathy of COVID-19 also differed from sepsis-induced coagulopathy (SIC) and was characterized by a marked increase in soluble tissue factor (TF) but less platelets, antithrombin, and fibrinogen

consumption, and less fibrinolysis alteration. In conclusion, COVID-19 inflammation-induced coagulopathy substantially differs from SIC. Modulating TF release and activity should be evaluated in critical COVID-19 patients.

Keywords: COVID-19, septic shock, inflammation, coagulopathy, platelet, NETosis, endothelium

INTRODUCTION

The coronavirus disease 2019 (COVID-19) varies from asymptomatic to a severe form with acute respiratory distress syndrome (ARDS) and the pathophysiology of the severe form has yet to be fully elucidated. The variability of host immune responses suggests that dysregulated systemic inflammation, classically called a “cytokine storm,” could contribute to the pathogenesis of severe cases (1, 2). In addition, COVID-19 is associated with a high incidence of arterial and deep venous thrombosis leading to pulmonary embolism (3, 4) and pulmonary microthrombosis (5).

Several mechanisms underlying the pathophysiology of COVID-19 have been proposed. First, ribonucleic acid (RNA) viruses like SARS-CoV-2 usually initiate innate immunity through their detection by pattern recognition receptors (PRRs), triggering subsequent inflammatory and immune responses via the secretion of various cytokines (6). Supporting this hypothesis, critical COVID-19 has been associated with high levels of interferon- γ (IFN- γ), tumor necrosis factor- α (TNF- α), numerous interleukins (ILs), macrophage inflammatory proteins (MIPs), and IFN γ -induced protein 10 (IP-10) (1, 7, 8). Second, internalization of SARS-CoV-2 within endothelial cells after binding to angiotensin-converting enzyme 2 (ACE2) contributes to endotheliitis (9). Both direct injury and activation of the endothelium by dysregulated inflammation may lead to loss of endothelial barrier integrity and to the occurrence of a prothrombotic phenotype (10). Indeed, plasmatic level of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1) (11), plasminogen activator inhibitor-1 (PAI-1) (12), tissue factor (TF) (13), and von Willebrand factor (vWF) (4, 14) antigens and activities are all elevated in severe COVID-19 cases. However, a simultaneous increase in tissue plasminogen activator (tPA) or tissue factor pathway inhibitor (TFPI) could mitigate thrombosis formation (15).

Critical COVID-19-associated coagulopathy is characterized by high D-dimers due to increased thrombin generation and increased fibrinolysis (14, 16). In addition, platelets are hyperactivated (8, 17) and cooperate with the endothelium and immune cells to promote pulmonary microthrombosis through neutrophil extracellular trap (NET) formation. Dysregulated NETosis contributes to hypercoagulability and thrombosis (18). Accordingly, NET-containing microthrombi have been detected in COVID-19 pulmonary autopsies, and increased NET formation correlates with COVID-19-related ARDS and disease severity (19). Moreover, the interaction of platelets with monocytes triggers TF expression in severe COVID-19 patients, further exacerbating the hypercoagulable state (20).

Interestingly, sepsis and septic shock are well-known models of cytokine storms and coagulopathy (21). Like infection by RNA viruses, sepsis is initiated by activation of the innate immune system through recognition of pathogen-associated molecular patterns and damage-associated molecular patterns by PRRs. This inflammatory reaction enhances endothelial dysfunction and contributes to the procoagulant state with microvascular thrombi formation and, eventually, organ damage (22). The procoagulant state of sepsis is further amplified by NETs (23). Sepsis-induced coagulopathy (SIC) occurs as a continuum, progressing to disseminated intravascular coagulopathy (DIC) if the underlying etiology of the sepsis is not resolved. The clinical characteristics of critical COVID-19 and septic shock patients are extensively discussed in the literature although the entities have never been systematically compared in a prospective clinical setting including control patients with matched age, gender, and co-morbidities (24–26).

The current study prospectively compared patients with septic shock and those with critical COVID-19 on admission to the ICU regarding their clinical characteristics and outcomes, as well as key plasmatic soluble biomarkers of inflammation, coagulation, endothelial activation, platelet activation, and NETosis. Here, we show both coagulopathy and inflammatory pattern substantially differ between patients with septic shock and critical COVID-19, highlighting the specific actors involved in their respective pathogenesis.

METHODS

Aim, Design, and Setting of the Study

This study comparing clinical outcomes, inflammatory reaction, and coagulopathy between critical COVID-19 and septic shock was a monocenter, prospective, translational observational study. Adult patients were systematically included between February 1, 2019, and June 1, 2020. The ethics committee approved the study protocol, and all patients signed their informed consent (B403201938590, NCT04107402). Protocol amendment was done to include COVID-19 patients in the ongoing study. All authors had full access to primary clinical data.

Population

Patients with critical COVID-19 were those admitted to the ICU for moderate or severe ARDS due to SARS-Cov-2 infection; they were included within 5 days of admission. Acute respiratory distress syndrome was diagnosed according to the Berlin definition (27), and SARS-Cov-2 infection was demonstrated by real-time reverse transcription PCR on nasopharyngeal swabs. Septic shock was defined according to the Sepsis-3 definition as sepsis with vasopressor therapy needed to elevate the mean arterial pressure ≥ 65 mmHg and lactate levels >2 mmol/L

despite adequate fluid resuscitation of 30 ml/kg of intravenous crystalloid within 6 h (28). A similar protective ventilation strategy (including positive end expiratory pressure above 5 cmH₂O, maximum tidal volume of 6 ml/kg, and maximal plateau pressure of 30 cmH₂O) was applied in both COVID-19 and septic shock patients with ARDS. Prone positioning and inhaled nitric oxide were used for severe ARDS, and venovenous extracorporeal membrane oxygenation (VV-ECMO) for refractory hypoxemia despite optimal treatment (29). Venovenous extracorporeal membrane oxygenation was never used in septic shock patients. Patients with septic shock admitted to the ICU were included within 2 days of admission. Control patients with matched age, gender, and co-morbidities were recruited at a central laboratory consultation. Similar exclusion criteria were applied to all groups: therapeutic anticoagulation (oral or parenteral, including heparins, fondaparinux, vitamin K antagonists, and direct oral anticoagulants), recent (within <1 month) chemotherapy, active inflammatory disease, hemophilia and other coagulopathies, previous history of thrombocytopenia (<100,000 platelets/mm³), cirrhosis (Child–Pugh >A), recent (within <48 h) major surgery (behalf, for septic shock, for infection source control), cardiac arrest during ICU stay, and decision of care limitation. All septic and COVID-19 cases received thromboprophylaxis using low-molecular-weight heparin (LMWH; nadroparin 3,800 IU/days subcutaneously). Sampling was performed at least 6 h after LMWH injection. For the patients with COVID-19, patients on antibiotics for any suspected or confirmed bacterial coinfections were formally excluded.

Clinical Outcomes

Patient baseline characteristics and clinical outcomes were compared. Patient prognosis was assessed using acute physiologic assessment and chronic health evaluation II (APACHE II) (30) and sequential organ failure assessment (SOFA) (31) scores. Moreover, disseminated intravascular coagulation (DIC) and SIC were diagnosed using the International Society of Thrombosis and Hemostasis scoring at inclusion (32, 33). Data were collected from central medical records, including biological datasets that were routinely performed in patients admitted in ICU such as platelet count, CRP (C-reactive protein) level, coagulation assessment, renal function, and liver enzymology. Clinical outcomes were assessed 30 days after ICU admission. Bleeding complications were assessed with Thrombolysis in Myocardial Infarction (TIMI) bleeding criteria, frequently used for cardiovascular trials (34). A major bleeding is defined by the following criteria: any intracranial bleeding, clinically overt signs of hemorrhage associated with a drop in hemoglobin of ≥ 5 g/dl or a $\geq 15\%$ absolute decrease in haematocrit and fatal bleeding.

Sampling

Blood samples were collected through the central venous catheter in all ICU patients and by venous puncture in the control group. Venous blood was collected using vacutainer tubes containing CPDA. After two centrifugation runs enabling platelet isolation, plasma was collected, apportioned into 1 ml aliquots and stored at -80°C until use.

Measurement of Biomarkers

Soluble biomarkers of inflammation, coagulation, endothelial and platelet activation, and NETosis were measured using enzyme-linked immunosorbent assay (ELISA) or suspension array sandwich immunoassays according to regulatory requirements for commercially available research use only ELISA assays. Frozen platelet poor plasma was thawed at room temperature the day of the experiment. The details of each markers analyzed are listed in the **Supplementary Table 1**. Each analytical run was performed in duplicate. Methods respected their respective validated lower limit of quantification and upper limit of quantification. Cytokines and chemokines were measured using Bio-Plex Pro Human Cytokine 27-Plex Panel (27-Plex) and Bio-Plex Human ICAM-VCAM (hICAM-hVCAM) following the manufacturer's protocol.

Lung Biopsies and Autopsies

Lung biopsies and autopsies were obtained from the biobank of Cliniques Universitaires Saint-Luc. A "Human Material Transfer Agreement for research purpose" was elaborated between CUSL and UCLouvain. Use of Residual Human Body Material was approved by the local Ethic committee (B403201938590). Control patients, patients with ARDS from septic shock, and COVID-19 were compared. COVID-19 specimens were lung autopsies obtained from patients who died from respiratory failure at intensive care unit (ICU). Septic shock samples were biopsies taken owing to ARDS due to bacterial pulmonary or extra-pulmonary infection. Control specimens were archived tissues from patients who underwent lung surgery. Details about the methods and analyses are provided in the **Supplementary Material (Supplementary Methods)**.

Statistical Analyses

The analyses were conducted using GraphPad Prism Version 9 (GraphPad Software, San Diego, California). Continuous variables were expressed as mean $\pm$ standard deviation (SD) and categorical variables were expressed as number and percentage. The data were subjected to the Kolmogorov–Smirnov normality test and Bartlett's test for homogeneity of variance. Log transformations were performed when appropriate. The categorical variables were analyzed using the Chi-squared test or Fisher's exact test, and the continuous variables using Tukey's ordinary one-way ANOVA or an unpaired Student's *t*-test, as appropriate. A log-rank test was applied to compare ICU length of stay and ventilation duration. All *p*-values were two-sided, and *p* < 0.05 was considered statistically significant.

A principal component analysis (PCA) was conducted in R (35), based on the patients with COVID-19 or septic shock and study outcomes. As an exploratory multivariate analysis, PCA provides a condensed overview of the main sources of variability in a dataset composed of individuals (here, the patients) with a large number of variables measured (here, the study outcomes). The core idea is to reduce data dimensionality by building new latent variables—called the principal components (PCs)—from the measured ones. The PCs, which are orthogonal between each other, capture the main sources of data variability in a decreasing manner (PC1 captures more variability than PC2,

etc.). Each original variable contributes with a certain weight (called a loading) to the construction of these PCs. Thereafter, the individuals are projected onto these PCs, with these projections called the scores. From this graphical representation, one can inspect the data structure with respect to the main sources of variability. This technique enables to explore the variables that seek to recover the grouped structure of the patients from the measured variables that are segregating the patients and the relationship between the variables of interest. Prior to PCA, the data were standardized, then imputed with the missMDA package (36), so that imputed values would not impact the factorial analysis results.

RESULTS

Baseline Characteristics and Clinical Outcomes of Critical COVID-19 and Septic Shock

Overall, 118 patients were enrolled, including 48 with septic shock, 22 with COVID-19, and 48 controls matched for age, gender, and main co-morbidities (flowchart, **Figure 1**). Type of infection and culture in septic shock patients are detailed in **Supplementary Table 2**. The baseline characteristics of all groups, as well as the clinical outcomes of septic shock and COVID-19 patients are detailed in **Table 1**.

The demographic characteristics and past medical history were similar among the three groups, except that the COVID-19 group included less smokers and oncologic patients. Before inclusion into the study, most COVID-19 patients had been treated with hydroxychloroquine ($n = 18$, 82%) but only one had received corticosteroids (methylprednisolone) as compared with five septic shock patients receiving low-dose hydrocortisone. At the end of the ICU stay, five COVID-19 patients (19%) and 22 septic shock patients (46%) had been treated with corticosteroids. The time delay between symptom onset and ICU admission was longer for COVID-19 compared with septic shock (2.6 ± 2.4 days and 7.3 ± 3.2 days, respectively, $p < 0.01$). The patients with septic shock displayed worse severity scores due to multiple organ failure. By contrast, the COVID-19 patients presented with more severe respiratory failure, as indicated by a lower $\text{PaO}_2/\text{FiO}_2$ (arterial oxygen partial pressure/fractional inspired oxygen) ratio, a higher rate of mechanically ventilated patients, longer ventilation duration and ICU length of stay. The 30-day mortality did not differ between both groups.

Coagulopathies in Critical COVID-19 and Septic Shock Patients

The critical COVID-19 and septic shock cases were compared with the matched controls considered as a reference. Circulating levels of ICAM-1, reflecting endothelial dysfunction, were similarly increased in both COVID-19 and septic shock patients, compared to matched controls (**Figure 2A**). Other endothelial biomarkers differed between COVID-19 and septic shock. Circulating TF was higher in COVID-19 patients than septic shock patients, while TFPI was similarly elevated in both groups (**Figures 2B,C**). By contrast, vWF, PAI-1, and tPA were

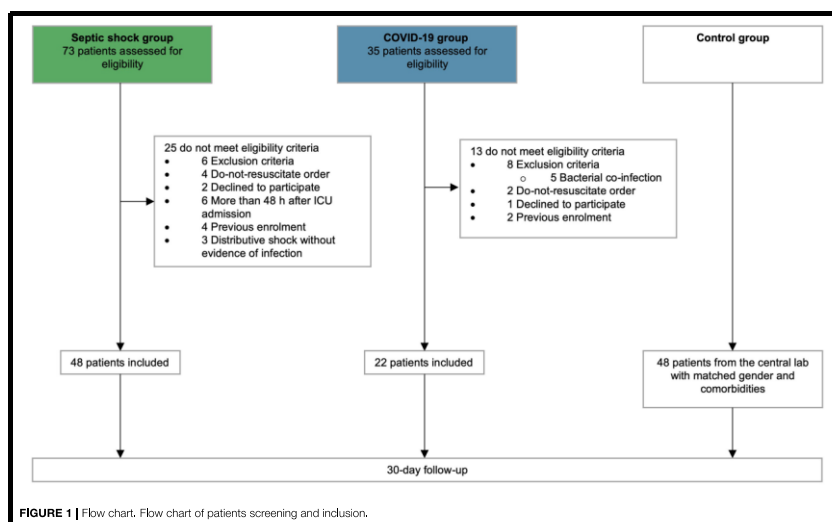
predominant in septic shock (**Figures 2D–F**). The difference in TF levels did not impact thrombin generation, as reflected by the thrombin–antithrombin complex (TAT), which was similarly increased in critical COVID-19 and septic shock, compared to matched controls (**Figure 2G**). Both critical conditions led to increased international normalized ratio (INR) and antithrombin consumption, while these changes were more pronounced in septic shock (**Figures 2H,I**). Interestingly, platelet and fibrinogen consumption mainly occurred in septic shock (**Figures 2J,K**). Accordingly, SIC and DIC were diagnosed in 24 and 16% of septic shock cases, respectively, but in none of the critical COVID-19 cases ($p < 0.05$). D-dimers were significantly elevated in both COVID-19 and septic shock patients compared with matched controls, but further in septic shock compared with COVID-19 patients (**Figure 2L**). Of interest, the levels of TAT and D-dimers that were measured in several control patients with co-morbidities were like those found in some critically ill patients, whether they had COVID-19 or not.

Platelet Activation and NETosis in Critical COVID-19 and Septic Shock Patients

Platelet activation soluble biomarkers P-selectin (sCD62P) and triggering receptor expressed on myeloid cells (TREM)-like transcript-1 (sTLT-1) were significantly increased in septic shock, but not in COVID-19 (**Figures 3A,B**). Levels of circulating NE and Cit-H3, reflecting neutrophil activation and NETosis, respectively, were increased in both diseases compared to matched controls but there were no differences between septic shock and COVID-19 (**Figures 3C,D**). This result was supported by the histopathological analysis of lung sections from control patients and patients with critical COVID-19- and septic shock-induced ARDS (**Figure 3E**). The baseline characteristics of patients included in the histopathological analysis are described in the **Supplementary Table 3**. As already described (37–39), vascular immunothrombosis containing platelets and neutrophils was observed in the lungs of patients with COVID-19 and septic shock, but not in the controls.

Inflammation and Immune Response in Critical COVID-19 and Septic Shock Patients

The inflammation and immune response substantially differed between COVID-19 and septic shock cases. The CRP elevation and peak were similar in both groups (**Figure 4A**; **Table 1**). The septic shock group exhibited a higher neutrophil count, whereas the lymphocyte count at inclusion or nadir during follow-up was similar in both groups (**Table 1**). Ubiquitous proinflammatory cytokines like $\text{TNF-}\alpha$, IL-1 β , IL-6, and IL-8 were discrepantly increased in both groups (**Figures 4B–E**). Septic shock was characterized by a higher level of myeloid-derived cytokines including IL-1 receptor antagonists (IL-1ra), monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein-1 α (MIP-1 α), and the soluble form of TREM-1 (**Figures 4F–I**). In contrast, COVID-19 was distinguished by a lymphocyte T cytokine response (40), as reflected by an increase in IL-2, IL-4, IL-5, IL-7, IL-13, IL-17, and



soluble CD40L (sCD40L) (Figures 4J–P). Of note, IFN- γ , IL-10, and IP-10, although elevated, did not differ between COVID-19 and sepsis (Figures 4Q–S).

Exploratory Multivariate Analysis

A PCA was done on the basis of 53 variables to assess the main relations between study outcomes (correlations), patients (similarities among patients from the same group), and between the outcomes and patients. The first two PCs of the PCA captured 39.65% of the total data variability and inflammatory and immune response biomarkers as well as SOFA, PAI-1, NE, and antithrombin ranked among their main contributors (Supplementary Figures 1A–C). The first two PCs allowed the discrimination of COVID-19 and septic shock patients (Figure 5A), consolidating results of the univariate analysis. COVID-19 patients were characterized by an increased IL1 β , increased lymphoid activation biomarkers, increased TF, and higher fibrinogen, antithrombin, and platelet count. Septic shock patients were characterized by an increased IL-6 and IL-8, increased myeloid activation biomarkers, endothelial activation biomarkers (except TF), a prolonged coagulation, increased D-Dimers, and platelets activation biomarkers (Figures 5B–G).

DISCUSSION

For the first time, the inflammation-related coagulopathy of critical COVID-19 and septic shock was prospectively

and extensively compared. This study overcame two major limitations of the literature, given that bacterial coinfections were formally excluded from the COVID-19 cohort, while pathological changes were assessed using a control group matched for age, gender, and comorbidities instead of healthy volunteers.

This study highlighted essential clinical differences between critical COVID-19 and septic shock cases. The patients with COVID-19 displayed a longer time delay between symptom onset and ICU admission, and a much longer ICU length of stay, reflecting an extended disease course. Critical COVID-19 patients, in the absence of any bacterial coinfection, rarely presented with hypotension or organ failure. These results contrast with previous observational studies that did not exclude coinfection, demonstrating a higher rate of vasopressor use (41, 42).

Inflammation-induced coagulopathy remarkably differed between COVID-19 and septic shock patients. For instance, COVID-19 was characterized by high circulating TF levels, no platelet or fibrinogen consumption, and lower levels of PAI-1, tPA, and D-dimers, suggesting less fibrinolysis activation. Although TF is recognized as one of the main actors in the inflammatory response during sepsis (43), an increase in circulating TF has never been described outside the DIC context (44). However, an increase in extracellular vesicle TF activity has been shown in COVID-19 (13, 45) and moreover, other viral diseases including human dengue infection and HIV

TABLE 1 | Baseline characteristics of prospective cohort of patients.

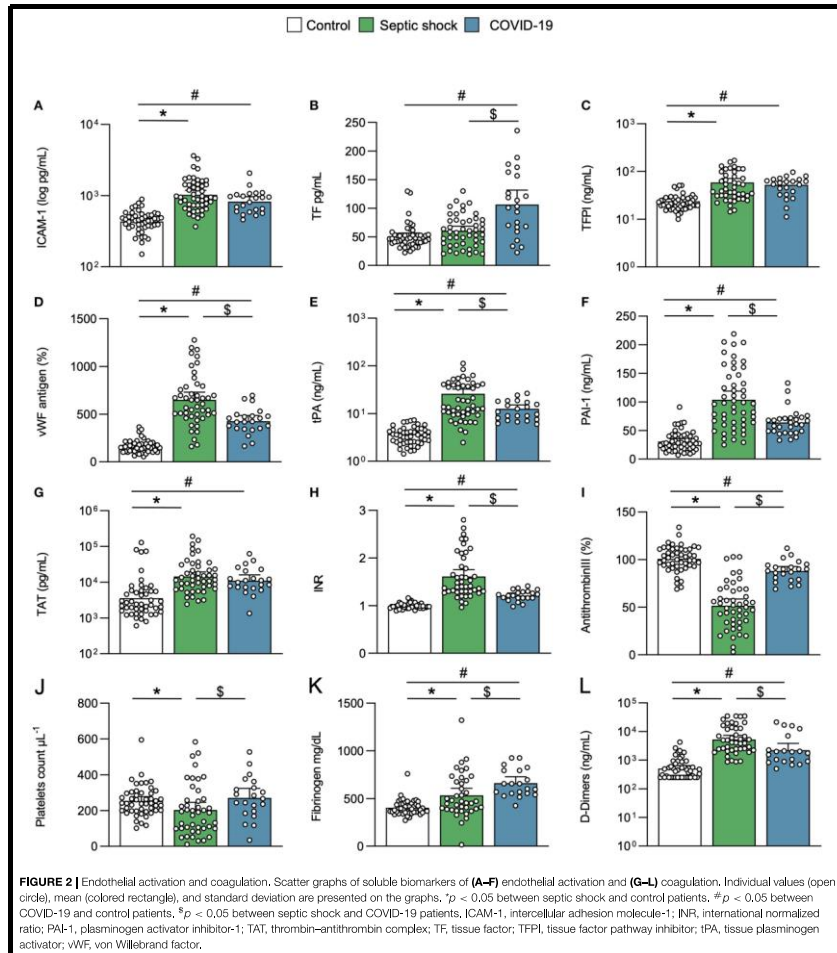
	Control <i>n</i> = 48	COVID-19 <i>n</i> = 22	Septic shock <i>n</i> = 48	<i>p</i> -Value
Demographics				
Men	26 (54)	15 (68)	24 (50)	0.36
Women	22 (46)	7 (32)	24 (50)	
Age (years)	61.9 ± 14.5	59.9 ± 10.3	65.0 ± 14.2	0.53
Medical history				
Hypertension	20 (42)	12 (56)	25 (52)	0.48
BMI >25	26 (58)	14 (74)	26 (54)	0.34
Diabetes	11 (23)	8 (36)	5 (10)	0.71
History of smoking	10 (21)	1 (5)	15 (31)	0.04
COPD	4 (8)	3 (14)	5 (10)	0.75
CKD	9 (19)	0 (0)	10 (21)	0.07
Cancer	15 (31)	0 (0)	9 (19)	0.01
Delay symptoms-ICU admission (days)				
		7.3 ± 3.2	2.6 ± 2.4	<0.01
Routine laboratory testing				
Highest CRP (mg/dl)		323 ± 119	313 ± 122	0.75
Creatinine (mg/dl)		0.91 ± 0.59	2.19 ± 1.91	<0.01
Hemoglobin (g/dl)		11.62 ± 1.90	10.34 ± 2.05	0.02
Neutrophils (10 ³ /μl)		8.0 ± 3.0	15.5 ± 10.2	<0.01
Lymphocytes (/μl)		993 ± 319	779 ± 521	0.33
Lowest lymphocytes (10 ³ /μl)		484 ± 335	469 ± 310	0.86
Platelet count (10 ³ /μl)		271 ± 117	203 ± 143	0.04
Organ failure, severity scores, and complications				
PaO ₂ /FIO ₂		103 ± 37	225 ± 119	<0.01
Mechanical ventilation		17 (77)	25 (52)	0.06
VV ECMO		5 (23)	0 (0)	<0.01
Norepinephrine (μg/kg/min)		0.049 ± 0.105	0.330 ± 0.350	<0.01
Norepinephrine duration (days)		1.2 ± 3.4	4.8 ± 6.1	<0.01
Renal replacement therapy		1 (5)	13 (27)	0.04
Apache II score		15 ± 4	20 ± 7	<0.01
SOFA score		4 ± 1	9 ± 3	<0.01
SIC		0 (0)	11 (24)	0.01
DIC		0 (0)	7 (16)	0.09
Thromboembolic events		6 (27)	4 (8)	0.06
TIMI major bleeding events		5 (23)	1 (2)	0.01
Outcome				
30-day mortality		6 (27)	22 (46)	0.45
Ventilation duration (days)		27 ± 24	4 ± 7	<0.01
ICU length of stay (days)		29 ± 30	8 ± 9	<0.01

Values are numbers (percentages) or mean ± standard deviation. Major bleeding complications have been defined according to the TIMI definition. All bleeding complications in COVID-19 group occurred in ECMO-treated patients. APACHE, acute physiology and chronic health evaluation; BMI, body mass index; COPD, chronic obstructive pulmonary disease; CKD, chronic kidney disease; CRP, C-reactive protein; DIC, disseminated intravascular coagulopathy; ICU, intensive care unit; PaO₂/FIO₂, arterial oxygen partial pressure/fractional inspired oxygen; SIC, sepsis-induced coagulopathy; SOFA, sepsis-related organ failure assessment; VV ECMO, venovenous extracorporeal membrane oxygenation.

are characterized by an increased soluble TF release (46, 47). Altogether, these results indicate that coagulation abnormalities of COVID-19-associated coagulopathy promote a procoagulant state. In contrast, SIC and DIC are septic shock features, in which the coagulation imbalance can be either pro- or anti-coagulant (48). Therefore, the current study formally supports that COVID-19-associated coagulopathy should not be assimilated to SIC or DIC (49). Given the prolonged disease course, a

procoagulant state may explain the high rate of thrombotic complications observed in COVID-19 patients (3, 4).

The histopathological data on COVID-19- and septic-shock-induced ARDS confirmed the presence of lung microthrombi in both diseases. These microthrombi contain numerous Cit-H3⁺-NE⁺ neutrophils at early stages of NET formation, as reported by others (37–39). Neutrophil extracellular traps represent a mechanism by which neutrophils



participate to thrombus formation in COVID-19 (19), possibly leading to organ dysfunction (50) and lung injury (51). The similar level of NETosis formation in ARDS due to COVID-19 vs. septic shock suggests that this parameter could be equally involved in the pathophysiology of both

diseases. This hypothesis was reinforced by the plasmatic measurements in this clinical cohort revealing identical levels of circulating Cit-H3.

The secretion of soluble markers of platelet activation, namely CD62P and TLT1, was increased in septic shock but unaltered in

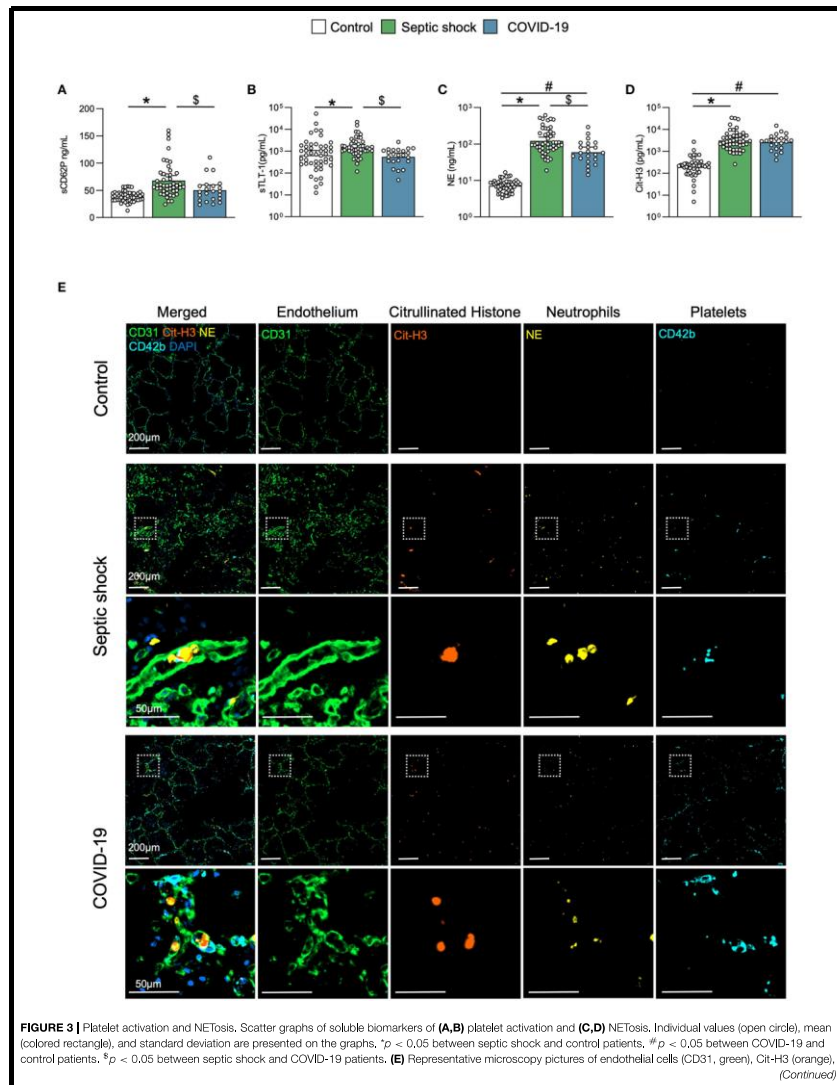


FIGURE 3 | neutrophils (NE, yellow), platelets (CD42b, light blue), nucleus (DAPI, dark blue), and merged staining, from control, septic shock, and COVID-19 lungs. sCD62P, soluble p-selectin; Cit-H3, citrullinated histone 3; NE, neutrophil elastase; NET, neutrophil extracellular traps; MPO, myeloperoxidase; sTLT-1, soluble triggering receptor expressed on myeloid cells (TREM) like transcript-1.

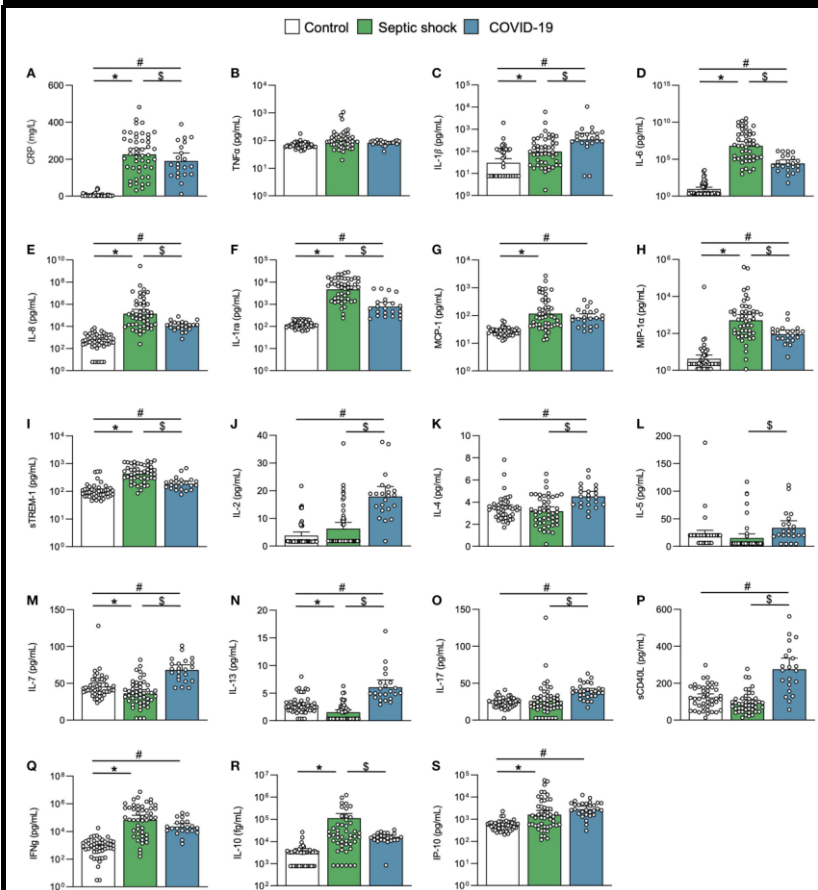


FIGURE 4 | Inflammation and immune response, Scatter graphs of (A–E) ubiquitous proinflammatory cytokines, (F–I) myeloid inflammatory cytokines, and (J–S) lymphoid inflammatory cytokines. Individual values (open circle), mean (colored rectangle), and standard deviation are presented on the graphs. * $p < 0.05$ between septic shock and control patients. # $p < 0.05$ between COVID-19 and control patients. S $p < 0.05$ between septic shock and COVID-19 patients. CD40L, CD40 ligand; CRP, C-reactive protein; IFNγ, interferon gamma; IL, interleukin; IL-1ra, IL-1 receptor antagonist; IP-10, interferon gamma-induced protein 10; MCP-1, monocyte chemoattractant protein 1; MIP-1α, Macrophage inflammatory protein-1α; sTREM-1, soluble triggering receptor expressed on myeloid cells 1.

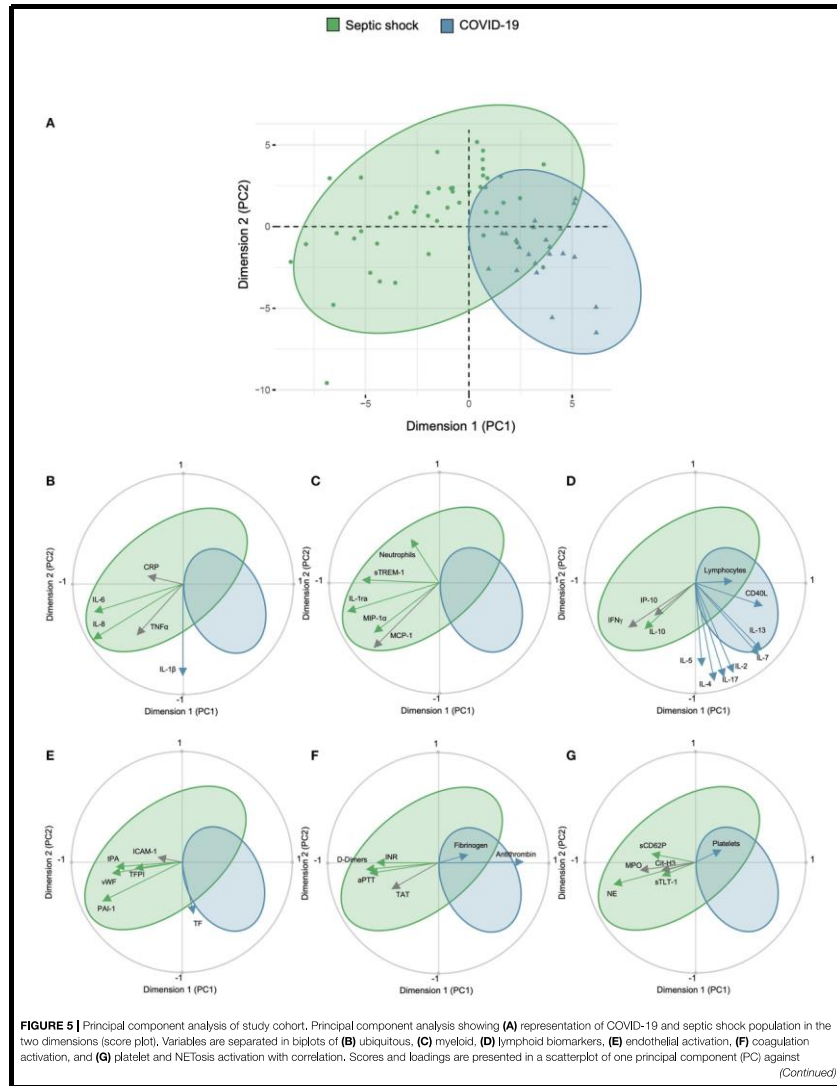


FIGURE 5 | another. The loadings are represented in a circle of correlations; the closer the arrow of a loading is to the circle, the more the variable is well-represented in the space of the two plotted PCs and contributed to the building of these PCs. This graph also indicates the positive or negative links between the variables and groupings of variables. Arrow colors correspond to the between-group comparison as mentioned in the scatter graphs, CD40L, CD40 ligand; 1Ct-H3, citrullinated histone H3; CRP, C-reactive protein; ICAM-1, intercellular adhesion molecule-1; IFN γ , interferon gamma; IL, interleukin; IL-1ra, IL-1 receptor antagonist; INR, international normalized ratio; IP-10, interferon gamma-induced protein 10; MCP-1, monocyte chemoattractant protein 1; MIP-1 α , macrophage inflammatory protein-1 α ; PCA, principal component analysis; MPO, myeloperoxidase; NE, neutrophil elastase; PAI-1, plasminogen activator inhibitor-1; TAT, thrombin-antithrombin complex; TF, tissue factor; TFPI, tissue factor pathway inhibitor; sTLT-1, soluble TREM like transcript-1; tPA, tissue plasminogen activator; sTREM-1, soluble triggering receptor expressed on myeloid cells 1; TT, thrombin time; VCAM-1, vascular cell adhesion protein 1; VWF, von Willebrand factor; WBC, white blood cells.

COVID-19 cases, thereby corroborating previous findings (17). However, this observation remains controversial in the literature (52, 53). The discrepancies could be explained by a small sample size or the possible presence of bacterial coinfections in the critical COVID-19 cohort. Nevertheless, platelets from COVID-19 patients have been reported to display a hyperactive phenotype upon agonist stimulation (8, 17) and it must be noted that soluble CD62P does not inform on the presence of the protein on platelet surface neither on platelet reactivity.

The immune response was characterized by lymphocyte T activation in critical COVID-19 cases, whereas those with septic shock exhibited a greater activation of myeloid cells. Variables related to the immune and inflammatory responses were the most discriminating in distinguishing COVID-19 from septic shock at ICU admission. It has already been shown for patients infected with SARS-CoV-1 that elevation of T helper Type 2 (Th2) cytokines (IL-4, IL-5, IL-10) is correlated with disease severity (54). Interestingly, here, sCD40L also differentiated critical COVID-19 from septic shock patients. sCD40L is expressed by cells of the immune system, especially by activated CD4⁺ T lymphocytes and activated platelets (55). In COVID-19, the elevation of sCD40L was dissociated from the raise of the other platelet activation biomarkers sCD62P and sTLT1, suggesting that its source could be the T lymphocytes. A similar result was recently found in another study (56) and corroborates the elevation of other lymphocyte T-derived ILs. Finally, sCD40L–CD40 interaction promoted endothelial cell and monocyte TF expression (57, 58), linking these two COVID-19 specific observations.

The study had limitations. First, the COVID-19 group was relatively small, so the results must probably be validated in a larger confirmation cohort. However, prospective and systematic enrollment of patients with either COVID-19 or septic shock within a closed time period has limited inclusion bias. Second, the patients with COVID-19 were included during the first wave of the pandemic, when the systematic use of corticosteroids was not yet recommended. It is therefore possible that the study findings would differ in patients that were systematically treated with dexamethasone. Finally, the observations were based on a single time point, namely early after ICU admission. A longitudinal assessment of these specific biomarkers could better define the dynamic changes in inflammatory and coagulation processes over time, which remain to be clarified, particularly in the COVID-19 context.

In conclusion, coagulopathy and inflammation patterns of critical COVID-19 substantially differ from septic shock at ICU

admission. Critical COVID-19 is distinguished by very high levels of IL-1 β and T lymphocyte activation (including IL-7), whereas septic shock displays higher levels of IL-6, IL-8 and a more significant myeloid response (including TREM-1 and IL-1ra). Moreover, COVID-19-associated coagulopathy is formally different from SIC and DIC, with a marked increase in soluble TF, yet less platelet, antithrombin, and fibrinogen consumption, and less fibrinolysis alteration. Hence, coagulation imbalance of severe COVID-19 almost exclusively tends toward a procoagulant state, and in the context of a prolonged disease course, this could explain the very high rate of thrombo-embolic complications of the disease. A better understanding of critical COVID-19 pathophysiology suggests potential therapeutic strategies, in particular recombinant IL-1ra and recombinant TFPI could modulate these two over-expressed pathways.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comité d'Ethique Hospitalo-Facultaire Saint-Luc-ULouvain. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SH and CB: had full access to all of the study data and takes responsibility for the data integrity and the accuracy of data analysis. MDec, DC-Z, P-FL, SH, and CB: concept and design. CBo, AD, SH, LGe, MDec, JDP, MO, LP, VR, AG, JB, DG, M-AVD, JD, HH, LM, MDer, and LJ: acquisition, analysis, or interpretation of data. MDec, JDP, P-FL, SH, and CB: drafting of the manuscript. VM, LD, and LB: critical revision of the manuscript for important intellectual content. MDec, JDP, MM, and LGa: statistical analysis. MDec, P-FL, LB, SH, and CB: obtained funding. P-FL and CB: administrative, technical, or material support. P-FL, SH, and CB: supervision. MDec, JDP, P-FL, AC, and VM: other—monitoring of the study progress, supporting patient recruitment, data clarifications, and data

entry. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmed.2021.780750/full#supplementary-material>

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Conflict of Interest: JD is the CEO and founder of QUALIblood s.a., a Belgian Contract Research Organization. LM and HH were employed by the company QUALIblood s.a. and authors MDer and LJ were employed by the company Inotrem s.a.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Bioreactivity of Stent Material: *In Vitro* Impact of New Twinning-Induced Plasticity Steel on Platelet Activation

Carole Verhaegen¹, Sophie Lepropre¹, Marie Octave¹, Davide Brusa², Luc Bertrand¹, Christophe Beauloye^{1,3}, Pascal J. Jacques⁴, Joelle Kefer^{1,3}, Sandrine Horman^{1*}

¹Pôle de Recherche Cardiovasculaire, Université catholique de Louvain (UCLouvain), Brussels, Belgium

²Plateforme de Cytométrie de Flux, Université catholique de Louvain (UCLouvain), Brussels, Belgium

³Division of Cardiology, Cliniques Universitaires Saint-Luc, Université catholique de Louvain (UCLouvain), Brussels, Belgium

⁴Department of Materials and Process Engineering, Institute of Mechanics, Materials and Civil Engineering, Université catholique de Louvain (UCLouvain), Brussels, Belgium

Email: *sandrine.horman@uclouvain.be

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Abstract

A current challenge concerns developing new bioresorbable stents that combine optimal mechanical properties and biodegradation rates with limited thrombogenicity. In this context, twinning-induced plasticity (TWIP) steels are good material candidates. In this work, the hemocompatibility of a new TWIP steel was studied *in vitro* via hemolysis and platelet activation assessments. Cobalt chromium (CoCr) L605 alloy, pure iron (Fe), and magnesium (Mg) WE43 alloy were similarly studied for comparison. No hemolysis was induced by TWIP steel, pure Fe, or L605 alloy. Moreover, L605 alloy did not affect CD62P exposure, αIIbβ3 activation at the platelet surface, or phosphorylation of protein kinase C (PKC) substrates upon thrombin stimulation. In contrast, TWIP steel and pure Fe significantly decreased platelet response to the agonist. Given that similar inhibitory effects were obtained when using a conditioned medium previously incubated with TWIP steel, we postulated TWIP steel corrosion to be likely to release components counteracting platelet activation. We showed that the main ion form present in the conditioned medium is Fe²⁺. In conclusion, TWIP steel resorbable scaffold displays anti-thrombogenic properties *in vitro*, which suggests that it could be a promising platform for next-generation stent technologies.

Keywords

Stent, TWIP Steel, Platelet, Biomaterial, Hemocompatibility

1. Introduction

Endothelial dysfunction due to dyslipidemia, hypertension or pro-inflammatory molecules leads to atherosclerotic plaques formation inside the vessel's intima layer which may cause coronary artery disease (CAD) [1] [2]. CAD, primarily responsible for myocardial infarction, is currently the first mortality cause worldwide [3] [4]. Coronary angioplasty with stent implantation is one of the most frequently performed therapeutic interventions to treat symptomatic CAD [5]. Stents are tubular scaffolds that are placed and expanded inside the coronary artery, designed to restore normal blood flow by local relief of obstructive lesions. However, harmful events, such as exacerbated smooth muscle cell proliferation in the neointima, chronic inflammatory local reactions, and thrombogenicity, contribute to lumen re-narrowing [6]. The mechanical characteristics, as well as the hemocompatibility of blood-contacting materials, must thus be investigated before considering them appropriate for *in vivo* applicability [7].

Blood flow through artificial stent-provided surfaces causes hemodynamic stress that may lead to erythrocytes rupture, called hemolysis [8], with associated consequences, including a reduction in oxygen transport to tissues, as well as free hemoglobin toxicity, notably altering kidney function [9]. In addition, the biomaterial exposure to the blood promotes the rapid adsorption of plasma proteins, including fibrinogen or von Willebrand factor, which both interact with the $\alpha\text{IIb}\beta\text{3}$ platelet integrin receptor, inducing platelet activation [7]. Stable integrin-dependent platelet adhesion leads to further platelet activation, with subsequent release from their granules (α -, dense, and lysosomal) of a broad range of biomolecules that act in both autocrine and paracrine ways to amplify the activation process [10]. Moreover, degranulation alters platelet plasma membrane composition and results in surface exposure of P-selectin (CD62P), a protein involved in the interaction of platelets with endothelial cells and leukocytes [11]. Finally, the artificial stent-provided surfaces can activate the intrinsic coagulation pathway, thereby leading to thrombin generation [7]. Thrombin is not only a potent platelet agonist but also cleaves fibrinogen into fibrin, which accumulates on the biomaterial surface, being recognized as a late stent thrombosis feature [12] [13]. Importantly, platelets and the entire coagulation system have been considered as processes that all interact in multifaceted ways [14]. Altogether, this indicates that platelet activation is undoubtedly an essential part of hemocompatibility testing [7].

Until recently, stent technology was based on using permanent bare metal stents (BMS). Cobalt-chromium (CoCr) has so far been considered to be the backbone of several stent generations [6]. However, the permanent delivery of a metallic implant has been demonstrated to be associated with several drawbacks, including vessel caging, vasomotion impairment, late stent thrombosis, and nonpermissive characteristics for later surgical revascularization [15] [16]. Given that a stent's scaffolding effect must only persist for 6 - 12 months, *i.e.* the time required to achieve arterial remodeling, the development of bioresorbable stents

is a promising approach enabling these limitations to be overcome [17]. Poly(lactic acid) bioresorbable scaffolds failed to demonstrate a better outcome than conventional metallic permanent stents, combining a poor radial force and a higher delayed thrombogenicity. In contrast, iron (Fe) and magnesium (Mg) alloys were reported to be suitable metals for bioresorbable stents in terms of undesirable effects [18] [19] [20] [21] [22]. Pure Fe stent implantation in animals was shown to be associated with good biocompatibility [23] [24] [25]. Mg-based stent has shown a good safety profile in patients [26]. While stents based on some Fe alloys present mechanical properties comparable to those of CoCr alloy, Mg alloy stents suffer from lower mechanical properties [27]. Moreover, Fe and Mg alloys were reported to be associated with an uncontrollable degradation rate [19] [28]. This highlights the need to further pursue investigations in this field, with the aim to modify or better control the degradation rate, particularly by optimizing the chemical compositions of the alloys.

Amongst the Fe-based alloys for bioresorbable stenting solutions, Fe-Mn alloys were so far considered based on their improved mechanical properties [18]. Some of them constitute twinning-induced plasticity (TWIP) steels [29] [30] [31]. The present *in vitro* study sought to characterize the impact of new TWIP steel composed of Fe, Mn, C with minor additions of Si, and Al on hemolysis and platelet activation. CoCr L605 alloy and Mg WE43 alloy, and pure Fe were tested for comparison.

2. Materials and Methods

2.1. Scaffolds Preparation

In this work, TWIP steel (73.5 wt.% Fe, 24 wt.% Mn, 0.4 wt.% C, 1 wt.% Al, 1 wt.% Si) was compared with CoCr L605 alloy (65 wt.% Co, 27.5 wt.% Cr, 6.2 wt.% Mo and minor additions (<0.5 wt.%) of Fe, Mn, Ni and Si), 99.99% pure Fe, and Mg WE43 alloy (93 wt.% Mg, 4 wt.% Y, 0.5 wt.% Gd, 2 wt.% Nd, 0.5 wt.% Dy, 0.5 wt.% Zr). Ingot of TWIP steel was first cast after induction melting of the different pure elements (provided by Goodfellow) added in the right proportion. This ingot was then reheated to 1250°C for 30 min before being hot rolled. The same procedure was applied for pure Fe ingot. CoCr L605 alloy was provided by Biotronik. Mg WE43 alloy was provided by Magnesium Elektron. All metallic samples were cut into rectangular specimens of 1 mm × 4 mm × 10 mm. For surface preparation, the TWIP steel and pure Fe specimens were chemically etched in a solution of HF2.9M and H₂O₂4.3 M for 3 minutes (min) and rinsed in water. They were then electropolished at 20 V in a solution of HClO₄ 575 mM and CH₃COOH 16.6 M for 1 min. The L605 alloy was electropolished at 4 V in a solution of H₃PO₄ 11.6 M for 6 min. The WE43 alloy was electropolished at 15 V in a solution of CH₃OH 20.6M, CH₃(CH₂)₃OCH₂CH₂OH 1272 mM, Cl₂MgO₈ 83 mM, and LiCl208mM for 2 min. All metallic samples were rinsed in denatured C₂H₅OH 99% (v/v), then dried and kept in a desiccator until use.

2.2. Determination of Blood Cell Number

Whole blood was collected from healthy volunteers using a 21-gauge butterfly needle in a tube containing 1/10 volume of citrated solution (citrate, phosphate, dextrose, and adenine). Metallic samples were immersed in whole blood at 37°C for 1 hour. The tubes were gently mixed every 10 min to avoid blood cell decantation. The numbers of platelets, white blood cells, and red blood cells were measured using Cell-DYN Emerald (Abbott Diagnostics), before and after incubation with the metallic samples.

2.3. Hemolysis Assay

Blood from healthy donors was collected using a 21-gauge butterfly needle in a tube containing 1/10 volume of citrated solution, then diluted with normal saline at a volume ratio of 4:5. Five specimens of L605 alloy, TWIP steel, pure Fe, and WE43 alloy were dipped in 10 mL of normal saline for 30 min at 37°C. Next, 200 µL of diluted blood were added to the tubes containing the samples and incubated for 60 min at 37°C. After the incubation time, the metallic samples were removed, and the blood was centrifuged at 800 g for 5 min. The supernatant was transferred to a 96-well plate for spectroscopic analysis at 540 nm with Victor X4 (Perkin Elmer). An increase in optical density (OD) of the supernatant reflects hemoglobin release from red blood cells via hemolysis. Deionized water was employed as the positive control because its hypotonicity which induces complete hemolysis. Similarly, normal saline was set as the blank control because its isotonicity [9] [32]. The percentage of hemolysis was then calculated as the ratio of OD obtained with the test material with blank subtraction to OD obtained with the positive control with blank subtraction [33], as in Equation (1).

$$\text{Hemolysis rate} = \frac{\text{OD of test} - \text{OD of blank control}}{\text{OD of positive control} - \text{OD of blank control}} \times 100 \quad (1)$$

2.4. Platelet Isolation

Venous blood from healthy volunteers was collected into tubes containing 1/10 volume of citrated solution using a 21-gauge butterfly needle. Platelet-rich plasma (PRP) was obtained by centrifugation at 330 g for 20 min at 22°C. For platelet activation measurement, platelets were isolated from PRP by centrifugation at 800 g for 10 min, in the presence of eptifibatide (4 µg/mL) and apyrase (1 U/mL). The pellet was washed in modified Tyrode's buffer (135 mM NaCl, 12 mM NaHCO₃, 2.9 mM KCl, 0.3 Na₂HPO₄, 1 mM MgCl₂, 5 mM D-glucose, 10 mM Hepes, and 1.5% BSA, pH 7.4, and 37°C) containing eptifibatide (4 µg/mL) and apyrase (1 U/mL). The platelets were then isolated by centrifugation at 1000 g for 10 min at 22°C and re-suspended in modified Tyrode's buffer. The platelet concentration was measured by means of Cell-DYN Emerald (Abbott Diagnostics) and adjusted to 2.5×10^5 platelets/µL.

2.5. Flow Cytometry Analysis

For the CD62P and αIIbβ₃ analyses, washed platelets were treated for 60 min at

37°C with L605 alloy, TWIP steel, pure Fe, or WE43 alloy at a ratio of 1 metallic sample per 500 μL . After incubation, the metallic samples were removed, with the platelets counted using Cell-DYN Emerald (Abbott Diagnostics). Next, treated platelets (6.25×10^6) were incubated with PE mouse anti-human CD62P (BD Biosciences, #555524) and FITC mouse PAC-1 antibody (BD Biosciences, #340507) at saturating concentrations (1:12.5) for 15 min at room temperature. After adding 2 mM CaCl_2 , the platelets were stimulated with thrombin (0.03, 0.1, or 0.3 U/mL) for 2 min at 37°C. To stop the reaction, they were fixed in 4% formaldehyde. The samples were analyzed using the BD Canto II flow cytometer, with 20,000 events recorded. For preparing the TWIP steel-conditioned medium, TWIP steel specimens were dipped in modified Tyrode's buffer for 1 hour at 37°C (1 specimen/1 mL). The platelets were then re-suspended in the conditioned medium at a concentration of 2.5×10^5 platelets/ μL . CD62P and PAC-1 quantification was done using flow cytometry after stimulation with thrombin (0.03, 0.1, and 0.3 U/mL), as described before. The results were compared with those obtained when the metallic samples were in direct contact with the platelets.

2.6. Inductively Coupled Plasma and Colorimetric Tests

Metallic specimens of TWIP steel were dipped into modified Tyrode's buffer for 1 hour at 37°C (1 specimen/1 mL), as previously described. The conditioned medium thus produced was collected immediately after incubation. The total Fe was measured in the conditioned medium by inductively coupled plasma (ICP). Moreover, colorimetric detection of Fe^{2+} and Fe^{3+} ions was performed in the conditioned medium using a spectrophotometer. The organic matter from the modified Tyrode's buffer reacted to create a trouble solution with Fe^{2+} . Therefore, only Fe^{2+} was quantified and Fe^{3+} was deduced by subtracting the Fe^{2+} concentration from the total Fe concentration. Modified Tyrode's buffer with no metallic incubation was employed to set the blank.

2.7. Western Blotting

For the lysate preparation, platelets were centrifuged, and the pellet was lysed in SDS-Laemmli sample buffer $1 \times$ (25 mM Tris-HCl, pH 6.8, 12.5% (w/v) glycerol, 3% (w/v) SDS, 0.01% (w/v), bromophenol blue, and 6.25% (v/v) β -mercaptoethanol). Whole platelet lysates were subjected to Western blotting. Antibody dilutions in 5% BSA were 1:200,000 for the rabbit anti-phospho-PKC substrates antibody (Cell Signaling Technology, #2261S) and 1:30,000 for the HRP-conjugated anti-rabbit antibody (Sigma-Aldrich, #A0545). For the loading control, membranes were treated using azide 0.05%, with the primary antibody being the rabbit anti-gelsolin antibody (Cell Signaling Technology, #12953S) diluted 1:80,000 and the secondary antibody being the HRP-conjugated anti-rabbit antibody diluted 1:30,000. Band intensities were quantified using Image J, and those obtained with the anti-phospho-PKC substrate antibody were normalized to those of the

gelsolin loading control on the same gel.

2.8. Statistical Analysis

All experiments were performed in at least three independent replicates. Results were expressed as mean $\pm$ SEM. The statistical analyses were conducted using a one-way or two-way analysis of variance (ANOVA), followed by Tukey's test for multiple comparison. $P < 0.05$ values were considered statistically significant. All statistical analyses were carried out using GraphPad Prism (GraphPad Software).

3. Results and Discussion

At first, we assessed the number of platelets, leukocytes, and erythrocytes after the blood had been in contact with the metals, in comparison with the blood not previously exposed to any metal. This was performed using the hematology analyzer Cell-DYN (Abbott Diagnostics). Platelet number (mean platelet count $\times 10^5/\mu\text{L} \pm \text{SEM}$) was 2.14 ± 0.11 before treatment, 2.13 ± 0.10 after 1 hour without metal, 2.12 ± 0.11 with L605 alloy, 2.10 ± 0.10 with TWIP steel, 2.07 ± 0.11 with pure Fe and 2.09 ± 0.10 with WE43 alloy. Leukocyte number (mean leukocyte count $\times 10^3/\mu\text{L} \pm \text{SEM}$) was 4.5 ± 0.5 before treatment, 4.4 ± 0.5 after 1 hour without metal, 4.4 ± 0.6 with L605 alloy, 4.4 ± 0.6 with TWIP steel, 4.3 ± 0.6 with pure Fe and 4.4 ± 0.6 with WE43 alloy. Erythrocyte number (mean erythrocyte count $\times 10^6/\mu\text{L} \pm \text{SEM}$) was 3.7 ± 0.1 before treatment, 3.5 ± 0.1 after 1 hour without metal, 3.4 ± 0.3 with L605 alloy, 3.6 ± 0.1 with TWIP steel, 3.5 ± 0.3 with pure Fe and 3.5 ± 0.1 with WE43 alloy. There was no change in blood cell number, indicating that the tested biomaterials were not thrombogenic and did not induce hemolysis (Figures 1(a)-(c)). The stability of the platelet count was confirmed using the washed platelets (Figure 2). In addition, we measured absorbance at 540 nm in diluted whole blood supernatant in order to assess the release of hemoglobin from damaged erythrocytes. Figure 1(d) shows that, even if the hemolysis rates of TWIP steel and pure Fe were slightly higher compared to that of L605 alloy, the three metals did not induce significant hemolysis according to the ASTM F756-08 standard (hemolysis rate below 5%) [33]. This proved to be in agreement with previous studies showing the low hemolysis rates induced by pure Fe as well as by Fe-3.3 wt.% Mn and Fe-35 wt.% Mn alloys [18] [19] [34] [35]. It is worth noting that the mean hemolysis induced by the WE43 Mg alloy was 8.9%. This observation confirmed the results obtained in several studies demonstrating that Mg alloys displayed hemolysis rates close to [36] or higher [20] [37] [38] [39] [40] than 5%. Importantly, pure Mg was shown to induce a very high hemolysis rate, estimated at 37% [34]. The high hemolysis rate of pure Mg and several of its alloys may be accounted for by the fast corrosion rate of Mg associated with a release of Mg^{2+} ions, as well as an essential pH variation [20] [40]. In another study, the corrosion of pure Fe did not induce significant pH value changes, which possibly explains why it does not affect hemolysis [41].

Platelet activation resulting from interaction with artificial surfaces is a second

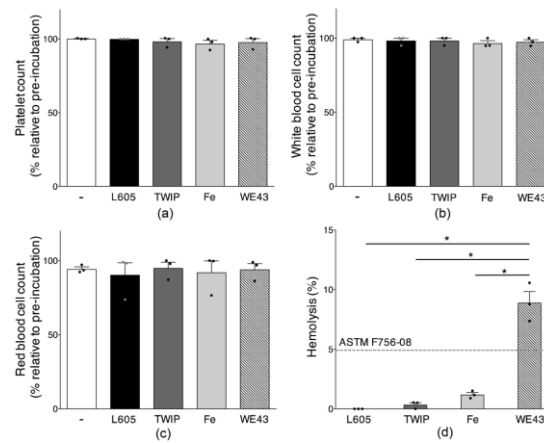


Figure 1. Blood cell count in total blood and hemolysis rate. (a), (b), and (c) L605 alloy, TWIP steel, pure Fe, and WE43 alloy were dipped into total blood for 1 hour at 37°C. Blood with no metallic treatment was used as the negative control (-). Platelets, white blood cells, and red blood cells were counted before and after metallic immersion, and the percentage of cells recovered after incubation was calculated. (d) Metallic specimens were dipped in diluted human blood for 1 hour. Following centrifugation, the optical density of the supernatant was measured at 540 nm, and the hemolysis rate was calculated. A hemolysis rate under 5% (ASTM F756-08) represents a criterion for excellent blood compatibility. The results were expressed as mean% $\pm$ SEM. The data underwent a one-way analysis of variance (ANOVA) and Tukey's multiple comparison test. * indicates values statistically different with $P < 0.05$, $n = 3$.

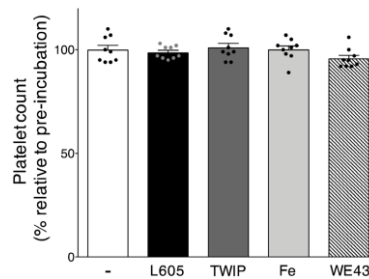


Figure 2. Isolated platelet count. Platelets were isolated and set at a final concentration of 2.5×10^5 platelets/ μ l. After immersion of the metallic specimens for 1 hour at 37°C, platelets were counted, and the percentage of platelets recovered after incubation was calculated. Platelets with no metallic treatment were used as the negative control (-). Results were expressed as mean% $\pm$ SEM, $n = 9$.

essential indication of blood incompatibility, as it may lead to thrombotic complications under *in vivo* conditions [7]. According to ISO-10993-4, platelet activation can be assessed by measuring activated $\alpha\text{IIb}\beta_3$, along with the presence of CD62P at the platelet surface, by means of flow cytometry [42]. Platelets were incubated for 1 hour with the metals prior to treatment using different thrombin concentrations. As expected, thrombin dose-dependently increased PAC-1 binding and CD62P surface exposure in platelets not previously incubated with metal. While L605 alloy did not affect platelet response upon thrombin stimulation, PAC-1 binding and CD62P exposure were drastically reduced in the presence of TWIP steel, pure Fe, and WE43 alloy (Figure 3(a) and Figure 3(b)). This observation suggests that the three resorbable scaffolds were able to display anti-thrombogenic properties *in vitro*. Pure Fe exhibited the same effect as TWIP steel, which implies that Fe itself is likely to be responsible for the TWIP

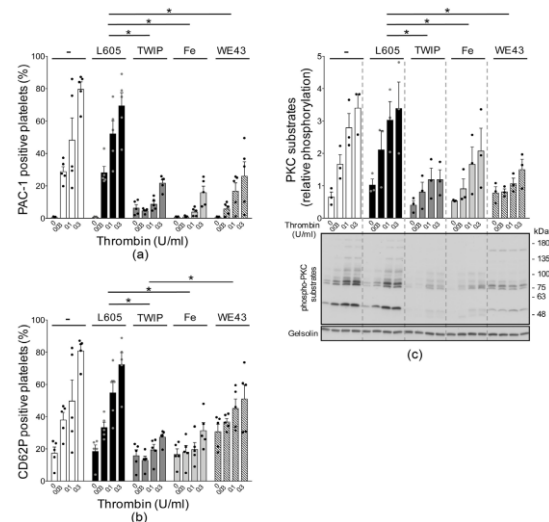


Figure 3. Platelet activation. Metallic specimens were immersed in washed platelets for 1 hour at 37°C. Platelets with no metallic treatment were used as the negative control (-). ((a) and (b)) Platelets were stimulated with thrombin at the indicated concentrations for 2 mins at 37°C. Binding of PAC-1 and surface exposure of CD62P were quantified by flow cytometry ($n = 5$). (c) Platelets were stimulated with thrombin at the indicated concentrations for 2 mins at 37°C, and proteins were extracted. The relative phosphorylation of the PKC substrates was analyzed with Western blotting ($n = 3$). Gelsolin was used as the loading control. The results are expressed as mean $\pm$ SEM. The data underwent a two-way ANOVA and Tukey's multiple comparison test. * indicates values statistically different with $P < 0.05$.

steel-induced antithrombogenic effect. Since the effects of thrombin on platelets are mediated, at least to some extent, through the activation of protein kinase C (PKC) [43], we performed a Western blot analysis of PKC phosphorylated substrates using protein extracts from platelets treated as described above. In line with our previous results, Figure 3(c) shows that the increased phosphorylation state of the PKC substrates in response to increasing thrombin doses was not affected by L605 alloy, whereas it was significantly reduced in the presence of TWIP steel, pure Fe, and WE43 alloy. Moreover, the unchanged platelet count measured following the metallic samples' immersion in washed platelets for 1 hour indicated that the diminution of CD62P exposure, PAC-1 binding, and phosphorylation state of PKC substrates were not due to a decreased platelet number but rather to a real change in platelet activation. This study is the first to describe CD62P, active α IIb/ β 3, and the phosphorylation state of PKC substrates following incubation of a Fe-based metallic sample in washed platelets. Platelet adhesion, shape change, and spreading of pseudopodia-like structures also reflected platelet activation [18] [44]. As previously shown, platelets can adhere to pure Fe or Fe-3.3 wt.% Mn alloy but conserve a normal round shape with only a few pseudopodia-like structures, indicating no induction of thrombogenicity [19] [34] [35]. A lower platelet reactivity was described following immersion of stents composed of Fe-35 wt.% Mn alloy in a model of circulating porcine blood, as compared to stainless steel used as reference material. In the latter study, the Fe-35 wt.% Mn alloy stent decreased platelet adhesion, secretion of β -thromboglobulin (a marker of platelet activation), and thrombin generation. It has been suggested that the decreased platelet adhesion resulted from the formation of large amounts of corrosion products (oxide layer) on the surface of the Fe-35 wt.% Mn alloy, rendering the surface nonadherent [18]. We, therefore, investigated whether platelet activation inhibition was due to a direct contact between TWIP steel and platelets or if it resulted from the production of corrosion products released into the incubation medium. To test this, platelets were incubated for 1 hour with a TWIP steel-conditioned medium. We found that the effect of conditioned medium on platelet activation reproduced the effect of direct contact with the metal in regard to both PAC-1 (Figure 4(a)) and CD62P (Figure 4(b)), indicating that TWIP steel corrosion might release components counteracting platelet activation. Moreover, this observation supported the fact that the decrease in platelet activation did not result from a modified metal surface. Fe corrosion produces Fe^{2+} and OH^- , which react to form $\text{Fe}(\text{OH})_2$. This is easily oxidized to $\text{Fe}(\text{OH})_3$ [45]. We found Fe^{2+} (1.3 ± 0.2 mg/L) and Fe^{3+} (13.2 ± 1.4 mg/L) in the TWIP steel-conditioned medium, suggesting that Fe^{3+} might contribute to the inhibition of platelet activation. This was surprising, given that some studies have highlighted the thrombogenic effect of Fe^{3+} -containing molecules *in vivo* through a mechanism involving induction of oxidative stress in platelets [46] [47]. Moreover, FeCl_3 is currently being used in animal models to induce thrombosis in the arteries. Of note is, however, that FeCl_3 -induced

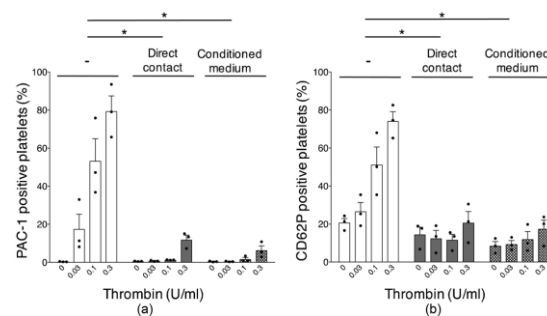


Figure 4. Effect of TWIP steel-conditioned medium on platelet activation. The conditioned medium was prepared by immersion of TWIP steel into a modified Tyrode's buffer for 1 hour at 37°C. Washed platelets were suspended in the conditioned medium at a concentration of 2.5×10^5 platelets/ μ L. Platelets were stimulated with thrombin at the indicated concentrations for 2 min at 37°C. Binding of PAC-1 (a) and surface exposure of CD62P (b) were quantified using flow cytometry. The results were compared with those obtained with platelets in direct contact with the metal or without metallic incubation (-). The results were expressed as mean% $\pm$ SEM. The data underwent a two-way ANOVA and Tukey's multiple comparison test. * indicates values statistically different with $P < 0.05$, $n = 3$.

thrombosis relies on complex multifaceted and incompletely elucidated mechanisms. It was historically accepted that spherical bodies filled with Fe^{3+} would bud from endothelial cells and support platelet adhesion, triggering their aggregation [48]. Recently, however, several studies have reported that plasma proteins and red blood cells contribute to platelet aggregation, owing to their negatively-charged proteins bind to positively-charged iron species [49] [50] [51]. Therefore, working on isolated platelets might explain the lack of Fe^{3+} -induced platelet activation in our study. In contrast, an antithrombotic effect of Fe^{3+} was previously described by Miron *et al.*, in accordance with our findings. In this latter study, the addition of nonphysiological concentrations of ferric nitrate ($\text{Fe}(\text{NO}_3)_3$) to human platelets was shown to activate the ectonucleoside triphosphate phosphohydrolase responsible for the hydrolysis of ADP, as well as the 5'-nucleotidase, responsible for the hydrolysis of AMP into adenosine. These two enzymes led to ADP level depletion and, therefore, decreased platelet activation [52].

4. Conclusion

Our study showed the relative *in vitro* hemocompatibility of new bioresorbable TWIP steel compared to traditional stent materials. Unlike WE43 alloy, TWIP steel does not induce significant hemolysis. Moreover, it inhibits platelet activation compared to L605 alloy, suggesting that TWIP steel presents antithrombotic

properties. Fe^{3+} released as a corrosion product could be responsible for this effect. Our results indicated that TWIP steel is a good potential candidate for cardiovascular stent applications. However, as *in vitro* tests are unable to reproduce physiological environments, *in vivo* compatibility evaluation is necessary to confirm our encouraging findings.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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