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Investigating the platelet metabolic, AMPK-ACC signaling pathway in Coronary Artery Disease

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Summary

Despite modern antiplatelet strategies and powerful lipid lowering treatments, a residual risk of recurrent ischemic events persists in coronary artery disease (CAD) patients presenting acute coronary syndrome (ACS). High-on treatment platelet reactivity, persistent thrombin generation, activation of the inflammatory pathways (IL-1 β , IL-6) and cardiometabolic abnormalities (hyperlipemia with increased triglycerides (TG) levels despite achieved low-density lipoprotein (LDL) cholesterol goals) are the major factors determining this residual risk. The identification of such patients needing more aggressive treatment strategies remains a difficult task.

Our group previously established that AMP-activated protein kinase (AMPK) α 1 is activated in platelets upon thrombin or collagen stimulation and, as consequence, phosphorylates and inhibits its *bona-fide* substrate, the acetyl-CoA carboxylase (ACC). ACC is a key enzyme involved in the *de novo* fatty acid synthesis in cells, including platelets. Given the impact of the hyperlipidemic environment on platelet biology and function, and the crucial role of ACC in regulating lipid metabolism in platelets, we explored the activation of the AMPK-ACC signaling pathway in the setting of CAD. Circulating platelets of CAD patients displayed increased ACC phosphorylation (phosphoACC) compared to non-CAD. Importantly, phosphoACC was associated with acute coronary events on admission and correlated with calcified plaque burden on thoraco-abdominal MDCT (coronary and extra-coronary (aortic) calcification score). Our data revealed a key interaction between atherogenic lipids and circulating platelets, as shown by the association between the triglyceride/high-density lipoprotein cholesterol (TG/HDL-C) ratio and increased phosphoACC. Activation of the AMPK-ACC signaling by atherogenic lipids (oxidized LDL) occurs downstream the CD36

pathway. Lipidomic profiling of circulating platelets furthermore support the interplay between lipids and platelets (upregulation of platelet TG lipid species when plasma TG is elevated). However, increased phosphoACC was associated with a down-regulation of TG containing C14:0 (myristic acid) lipid species, thereby showing that platelet endogenous lipid synthesis is inhibited when ACC is phosphorylated.

In platelets, lipids fulfill three primary roles: structure, signaling and energy storage. Since ACC is crucial for fatty acids synthesis, we hypothesized that this enzyme plays a central regulatory role in platelet function. In this second part of the project, we studied the role of ACC in a double knock-in (DKI) mouse model where the AMPK phosphorylation sites Ser79 on ACC1 and Ser212 on ACC2 were mutated to prevent AMPK-signaling to ACC. Impaired AMPK-ACC signaling promoted injury-induced arterial thrombosis in vivo and enhanced thrombus growth ex vivo on collagen-coated surfaces under flow. DKI platelets exhibit a modified phospholipid content, marked by an increase in arachidonic acid (AA)-containing phosphatidylethanolamine plasmalogen lipid species. Increase in AA-containing phospholipids promotes thromboxane generation and dense granule secretion, thereby contributing to platelet prothrombotic phenotype.

In conclusion, we report phosphoACC as an interesting risk assessment marker in suspected CAD patients. PhosphoACC is a metabolic signature of the platelet-proatherogenic lipid interplay and contributes to the regulation of TG lipid species in CAD. Since the AMPK-ACC signaling pathway is a key mechanism regulating platelet prothrombotic phenotype, phosphoACC can be a counter-regulatory mechanism limiting platelet reactivity within a pro-atherogenic environment.

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LIST OF ABBREVIATIONS

ABBREVIATIONS	FULL NAME
AA	arachidonic acid
AC	adenylate cyclase
ACC	acetyl-coA carboxylase
ACS	acute coronary syndrome
ADP	adenosine diphosphate
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
ATP	adenosine triphosphate
BC	biotin carboxylase
BCCP	biotin-carboxyl carrier protein
Ca ²⁺	calcium
CAC	coronary artery calcification
CAD	coronary artery disease
CaMKK β	Ca ²⁺ /calmodulin-dependent protein kinase kinase beta
cAMP	cyclic AMP
CD40L	CD40 ligand
CD62P	P-selectin
CE	cholesterol esters
cGMP	cyclic guanosine monophosphate
COX-1	cyclooxygenase 1
CoxLDL	copper-oxidized low density lipoprotein
cPLA ₂	cytosolic phospholipase A ₂

CPT-1	carnitine palmitoyl transferase 1
Cu ²⁺	copper
DAG	1,2-diacylglycerol
DI-MS/MS	direct infusion-tandem mass spectrometry
DKI	double knock-in
DMS	demarcation membrane system
DTS	dense tubular system
ECLIA	electrochemiluminescence immunoassay
ECM	extracellular matrix
ERK5	extracellular signal-regulated kinase 5
F1.2	fragments 1+2
FAS	fatty acid synthase
FDR	false discovery rate
Fe ³⁺	iron
FFA	free fatty acid
GLUT1	glucose transporter 1
GLUT3	glucose transporter 3
GP	glycoprotein
GPCR	G-protein coupled receptor
HDLc	high density lipoprotein cholesterol
HR	hazard ratio
ICC	inter-experiment reproducibility
IL	interleukine
IP-3	inositol 1,4,5-triphosphate

IQR	interquartile Range
KAT	lysine acetyltransferase
KDAC	lysine deacetylase
KI	knock-in
LDLc	low density lipoprotein cholesterol
LKB1	liver kinase B1
LPC	lysophosphatidylcholine
LPE	lysophosphatidylethanolamine
MCD	malonyl-coA decarboxylase
MDCT	multi-detector computed tomography
MKs	megakaryocytes
MLC	myosin light chain
MLCK	myosin light chain kinase
MLCP	myosin light chain phosphatase
MoxLDL	myeloperoxidase oxidized LDL
NADPH	nicotinamide adenine dinucleotide phosphate hydrogen
NAFLD	nonalcoholic fatty liver disease
NETs	neutrophils extracellular traps
NOX	NADPH oxidase
N-CAD	non-coronary artery disease
NS-CAD	non-significant coronary artery disease
NSTEMI	non ST-elevation myocardial infarction
OCR	oxygen consumption rate
OCS	open canalicular system

OR	odds ratio
oxLDL	oxidized LDL
OxPL	oxidized phospholipids
PAD	peripheral artery disease
PAR	protease-activated receptor
PAT	palmitoyl acyl transferase
PC	phosphatidylcholine
PDGF	platelet derived growth factor
PE	phosphatidylethanolamine
PEO	plasmeynl phosphatidylethanolamine
PEP	plasmalogen phosphatidylethanolamine
PF4	platelet factor 4
PG	prostaglandin
PhosphoACC	ACC phosphorylation on serine 79 (AMPK site)
PI	phosphatidylinositol
PI3K	phosphoinositide-3 kinase
PIP2	phosphatidylinositol 4,5-biphosphate
PKA	cAMP-dependent protein kinase
PKC	protein kinase C
PLC	phospholipase C
PS	phosphatidylserine
PSGL1	P-selectin glycoprotein ligand 1
RANTES	regulated on activation normal T-cell expressed and secreted

ROC	receiver operating characteristic
ROCK	Rho-associated protein kinase
ROS	reactive oxygen species
RP _s	reticulated platelets
sCD40L	soluble CD40 ligand
SD	standard Deviation
SERCA	sarco-endoplasmic Ca ²⁺ ATPase
SFK	src family of kinase
SM	sphingomyelin
STEMI	ST elevation myocardial infarction
S-CAD	stable coronary artery disease
TATc	thrombin-antithrombin complex
TF	tissue factor
TG	triglyceride
TG/HDLc	triglyceride/high density lipoprotein cholesterol ratio
ThG	thrombin generation
TNF α	tumor necrosis factor alpha
TP	thromboxane/prostaglandin
TPO	thrombopoietin
TSP-1	thrombospondin 1
TxA ₂	thromboxane A ₂
VEGF	vascular endothelial growth factor
VSMCs	vascular smooth muscle cells
vWF	von willebrand factor

CHAPTER I - INTRODUCTION

1.1 Platelet overview

Platelets are central players of hemostasis and thrombosis. Following vascular injury, collagen and proteins of the sub endothelial extracellular matrix (ECM) are exposed to the blood stream, leading to platelet activation and formation of a hemostatic plug, thereby preventing blood loss. In the setting of coronary artery disease (CAD), ruptured atherosclerotic plaque leads to platelet activation, aggregation and thrombus formation. Myocardial infarction (MI) is the consequence of the (sub-)occluded coronary vessel. Antiplatelet therapies are therefore a cornerstone in the management of acute coronary syndrome (ACS). However, despite modern antithrombotic strategies, patients presenting with ACS remain at risk of relapsing cardiovascular ischemic events. Factors influencing this residual risk include high-on treatment platelet reactivity, persistent activation of the coagulation cascade (thrombin generation), activated inflammatory pathways and hyperlipemia despite intensified statin therapy. The study of platelet phenotypes in this clinical setting can improve our knowledge on residual risk, identify risk stratification markers and tailor more aggressive treatments in selected patients.

1.1.1 Thrombopoiesis

Platelets are anucleated cell fragments produced by the cytoplasmic division of megakaryocytes (MKs). MKs are large, polyploid cells that originate from the hematopoietic stem cells. MKs production from hematopoietic stem cells is regulated by thrombopoietin (2) and various transcription factors (3). MKs undergo various processes of maturation before fragmentation, releasing functional platelets in the blood stream (4,5). These processes include: the formation of polyploidy MKs via endomitosis, consisting of several chromosomal duplication without cell division (4), increase in platelet specific organelles and the generation of a demarcation membrane system which constitutes a reservoir for the future released platelets (6). Once mature, MKs release functional platelets via a mechanism called thrombopoiesis. This process involves migration of MKs from the bone marrow niche to the bone marrow sinusoids or to the lung capillaries (7), into which the demarcation membrane system extends to form long pseudopodial extensions called proplatelets that fragment into platelets (8). Upon division, platelets inherit classical cellular organelles (endoplasmic reticulum, lysosomes, Golgi apparatus and mitochondria) and specific structures, namely a highly organized cytoskeleton structure and two distinct types of granules (alpha and dense) from MKs. Each MK releases about 5000-10 000 platelets. Normal platelet count ranges from 150 000-400 000 per microliter in human adult whereas their concentration in mice is approximately 1 000 000 per microliter (9). Platelets half-life is about 8-12 days in humans and 3-4 days in mice.

1.1.2 Platelet structure

Platelets are the smallest circulating elements in blood, with a diameter of 2-4 μm in humans and 0.5 μm in mice. Besides classical cellular organelles, they contain specific structures necessary for platelet physiological role: a plasma membrane and the Open Canalicular System (OCS), the Dense Tubular System (DTS), a highly organized cytoskeleton and two specific secretory granules (alpha and dense) (Figure 1).

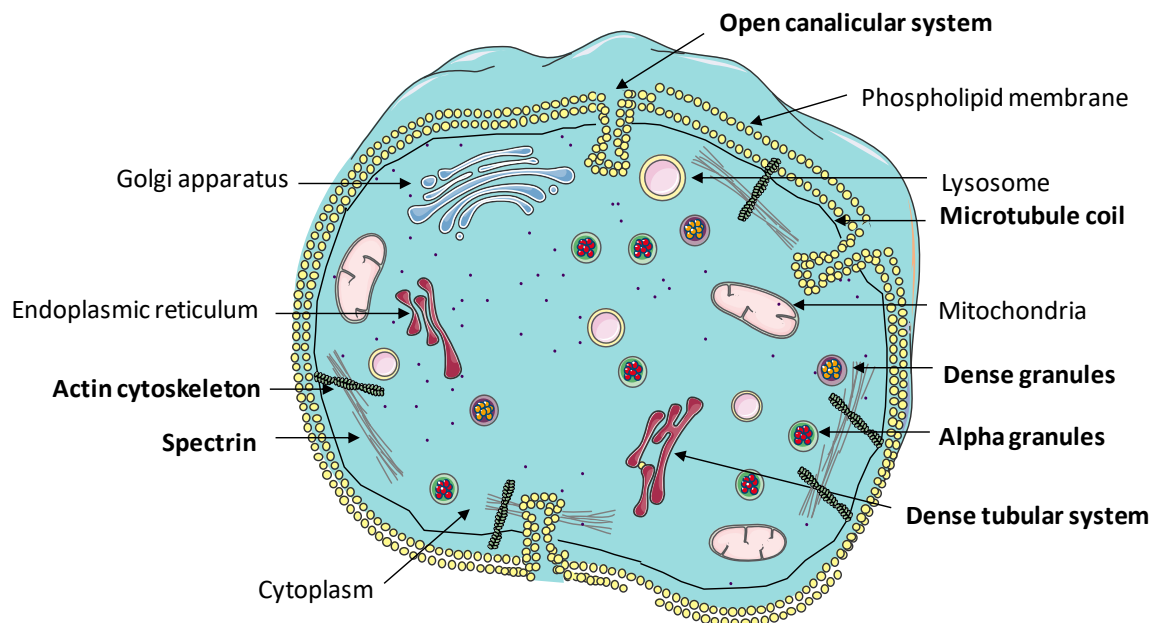


Figure 1. Platelet structure. Platelets are anucleated cell fragments that contain specific structures namely: open canalicular system, dense tubular system, highly organized cytoskeleton, alpha and dense granules (shown in bold letters).

1.1.2.1 Plasma membrane and open canalicular system

Platelets are delimited by a typical phospholipid bilayer plasma membrane. There are two principal classes of phospholipids: glycerophospholipids and sphingomyelin (SM). The phosphate moiety of glycerophospholipids can attach additional groups

such as ethanolamine, choline, serine or inositol, generating subclasses phosphatidylethanolamines (PE), phosphatidylcholines (PC), phosphatidylserines (PS) or phosphatidylinositol (PI). In platelets, PC and PE represent the major pool of phospholipids, about 40% and 28% respectively, followed by SM (18%), PS (10%) and PI which represents 3-5% (10). Importantly, beyond their structural role, glycerophospholipids constitute an important reservoir of lipids necessary for signaling and energy production during platelet activation. The availability of these lipids depends on the activity of phospholipases, namely phospholipase A₂ and phospholipase C. The role of platelet phospholipids will be detailed in section 1.5.

The OCS constitutes an extensive plasma membrane reservoir required when platelets spread on a surface and form plasma membranes protrusions, called filipodia and lamellipodia, that tremendously increase platelet surface area (11). In addition, the OCS supports granules secretion and facilitates the uptake of plasma components into the platelet (12).

1.1.2.2 Dense tubular system

The DTS is a reticular membrane network originating from the endoplasmic reticulum of the MKs and constitutes a major reservoir for intraplatelet releasable calcium. The sarcoendoplasmic reticulum calcium ATPase (SERCA) uses ATP to pump cytosolic calcium in the DTS, maintaining a calcium gradient between the cytosol (100nM) and the intracellular stores (1mM) (13). Regulation of cytosolic calcium level is a crucial phenomenon in order to prevent inadvertent platelet activation.

1.1.2.3 Cytoskeleton

In the resting state, platelets have a smooth discoid shape, maintained by a well-organized and robust cytoskeleton to withstand high shear forces in the blood stream. Upon activation, drastic structural changes occur leading to shape change and platelet spreading. Platelet cytoskeleton consists of 3 major elements:

(1) A peripheral microtubule coil composed of polymerized α - β tubulin dimers that form a rigid tubular structure allowing platelets to maintain their discoid shape (14). Beyond this structural role, the microtubule is also essential for proplatelets formation from MKs and for transporting platelet vesicles (15,16).

(2) A spectrin-based membrane skeleton that associates with actin filaments to provide structural support to the plasma membrane.

(3) A cytoplasmic actin cytoskeleton formed by the action of filamin and α -actinin, 2 proteins that promote actin filaments branching, organizing them into a densely packed network.

1.1.2.4 Secretory granules

Platelets contain two major types of secretory granules: alpha and dense granules. These granules constitute a major reservoir of active molecules that are released upon platelet activation, due to the fusion of granules with the plasma membrane.

1.1.2.4.1 Alpha granules

Alpha granules are the most abundant granules (40-80/ platelet), having a diameter of 200-500 nm. The content of these granules is a mix of either endogenous proteins (from the parent MKs) or exogenous plasmatic proteins that are captured by

endocytosis or pinocytosis via a mechanism involving the $\alpha\text{IIb}\beta 3$ integrins (17).

Alpha granule content and their respective functional role are summarized in table 1.

Alpha granules content	Functional role
Adhesive protein: fibrinogen, fibronectin, von Willebrand factor (vWF), vitronectin, thrombospondin (TSP-1)	Platelet adhesion on the injured endothelium
Chemokines and cytokines: RANTES-CCL5, β -thromboglobulin (CXCL7), platelet factor 4 (PF4), interleukine 8 (IL-8), tumor necrosis factor (TNFa)	Recruitment of neutrophils, interaction between platelets and inflammatory pathways
Pro- (vascular endothelial growth factor – VEGF) and anti-angiogenic factors (angiostatin, TSP-1, PF4)	Regulation of microvascular integrity
Growth factors (platelet derived growth factor (PDGF), transforming growth factor- β (TGF- β))	Infarct healing and tumor metastasis and stability
Hemostatic factors (factor V, vWF, factor XI, factor XIII,...)	Regulation of hemostasis
Anticoagulant factors (antithrombin, tissue factor pathway inhibitor (TFPI), protein S	Regulation of thrombosis

Table 1: Alpha granules content and their respective functional role during platelet activation.

Importantly, alpha granules fusion with plasma membrane following platelet activation, expose specific markers on platelet surface. Among these proteins, P-selectin (CD62P) is the most highly expressed and its detection on platelet surface is an interesting marker of platelet activation and granule secretion. Increased detection of platelet CD62P has been described in CAD patients and more

importantly in patients with unstable angina (18,19). Furthermore, P-selectin exposure on platelet surface is crucial for platelet-neutrophils interaction, since neutrophils express the P-selectin glycoprotein ligand1 (PSGL1) receptor. Furthermore, circulating platelet-monocyte aggregates is also an interesting marker of platelet activation in CAD (20,21). Biomarkers of platelet activation are detailed in section 1.3.3.

1.1.2.4.2 Dense granules

Dense granules number is about 10 times less than alpha granules ranging from 3 to 8 per platelet (22). They belong to a family of lysosome-related organelles and contain small molecules that play an important role in amplifying platelets response in a positive feedback mechanism. Dense granules contain calcium, the nucleotides adenosine diphosphate (ADP) and adenosine triphosphate (ATP), pyrophosphates, polyphosphates and serotonin. ADP and serotonin are key secondary mediators of platelet activation. By binding to their respective receptors, P2Y₁₂ and 5HTR2A, they amplify platelet activation and participate in platelet recruitment to the growing thrombus (23-25).

1.1.2.5 Organelles

Platelets contain classical cellular organelles such as the endoplasmic reticulum, lysosomes, the Golgi apparatus and mitochondria. Among these, the mitochondria plays a crucial role in providing energy for platelet production from MKs (26) and during platelet activation. The transition of platelets from a quiescent to an activated state requires drastic changes in ATP availability (27-29). Platelet bioenergetics under resting and activated state will be detailed under section 1.5.2.

1.2 Physiological role of platelets - Hemostatic plug formation

The major physiological role of platelets is to ensure a hemostatic plug at the site of vascular injury to prevent blood loss. Exposure of the sub endothelial ECM proteins after vascular lesion is the trigger for platelet adhesion, activation and aggregation. Release of secondary mediators from dense granules recruit additional platelets for thrombus growth. There is a simultaneous activation of the coagulation cascade leading to thrombin generation and fibrin formation. Activated platelets become procoagulant through PS externalization, which serve as a platform to anchor coagulation factors promoting thrombin formation. Thrombin, being a major platelet agonist, contributes to amplify platelet activation and thrombus formation. The different steps of platelet and coagulation activation following vascular injury are represented below (Figure 2 and 3).

1.2.1 Platelet rolling and adhesion

Platelet adhesion to the injured endothelium is mediated by the interaction of platelet glycoprotein (GP) Ib-IX-V and the high molecular weight plasma protein von Willebrand factor (vWF) bound to the exposed collagen. This pathway is mostly involved in platelet adhesion under elevated shear conditions ($>500 \text{ sec}^{-1}$) as found in small arteries, which induce a conformational change of vWF, allowing its tight binding to GP Ib-IX-V. However, in conditions of low shear rate ($<500 \text{ sec}^{-1}$) as in large veins/arteries, the contribution of vWF/ GP Ib-IX-V is not essential. Instead, $\alpha_2\beta_1$ and GPIIb/IIIa (binding to collagen), $\alpha_5\beta_1$ (binding to fibronectin), $\alpha\text{IIb}\beta_3$ (binding to vWF, fibrinogen and fibronectin), $\alpha_6\beta_1$ (binding to laminin), all constitute platelet membrane receptors that bind to their respective ECM-associated protein (30,31).

The binding of vWF to its receptor GPIb-IX-V and collagen to GPVI initiate platelet activation. The collagen/GPVI interaction is crucial for strong and sustained platelet activation.

1.2.2 Platelet spreading

Following platelet activation, major reorganization of platelet cytoskeleton occurs, leading to drastic morphological changes, including the formation of filopodia and lamellipodia. Filopodia are long finger-like structures that extend from the platelet cytoplasm and lamellipodia are actin-rich sheets that maximize platelet surface area to induce strong platelet adherence and stabilize the aggregate (32). Interestingly, our group previously established the key role of the AMP-activated protein kinase (AMPK) in the regulation of platelet cytoskeleton remodeling during platelet activation (detailed in section 1.6.3) (33).

1.2.3 Platelet activation and Granules secretion

Platelet activation is triggered by various agonists, including sub endothelial collagen, thromboxane A₂ (TxA₂) and ADP released from activated platelets, and thrombin generated by the coagulation cascade. Although these agonists act on different platelet receptors and trigger different signaling pathways, all lead to an increase in the intracellular calcium concentration (34,35). Release of calcium from DTS involves a phospholipase (PL) C-mediated production of inositol-1,4,5-trisphosphate (IP₃), which stimulates the release of calcium through IP₃ receptor channels (36,37). Calcium is a central and common second messenger downstream of most signaling pathways in platelets.

Alpha and dense granules release are necessary to recruit additional platelets, amplify platelet activation and further potentiate thrombus growth. The mechanisms

regulating granules release involve the actin cytoskeleton (38), different proteins of the exocytosis machinery (39,40) and protein kinase C (PKC) isoforms (41,42). It is important to note that mechanisms behind alpha and dense granules release are independent of each other.

1.2.4 Aggregation – Integrin $\alpha\text{Ib}\beta 3$ activation

Increase in intracellular calcium concentration and diacylglycerol (DAG) formation following platelet activation, are essential for the integrin $\alpha\text{Ib}\beta 3$ activation. The conformational change of the $\alpha\text{Ib}\beta 3$ receptor allows a high binding affinity state for fibrinogen. This pathway is characterized as the “inside-out” signaling (43). Fibrinogen bound to activated $\alpha\text{Ib}\beta 3$ induces integrin clustering and phosphorylation of downstream proteins (c-Src), activating Syk, thereby mediating cytoskeleton remodeling, clot retraction and platelet spreading (44). This process is called the “outside-in” signaling. Binding of fibrinogen to $\alpha\text{Ib}\beta 3$ results in platelet aggregation, by crosslinking adjacent activated platelets. The integrin $\alpha\text{Ib}\beta 3$ is a potential therapeutic target, characterized by a strong inhibition of platelet aggregation (detailed in section 1.3.2).

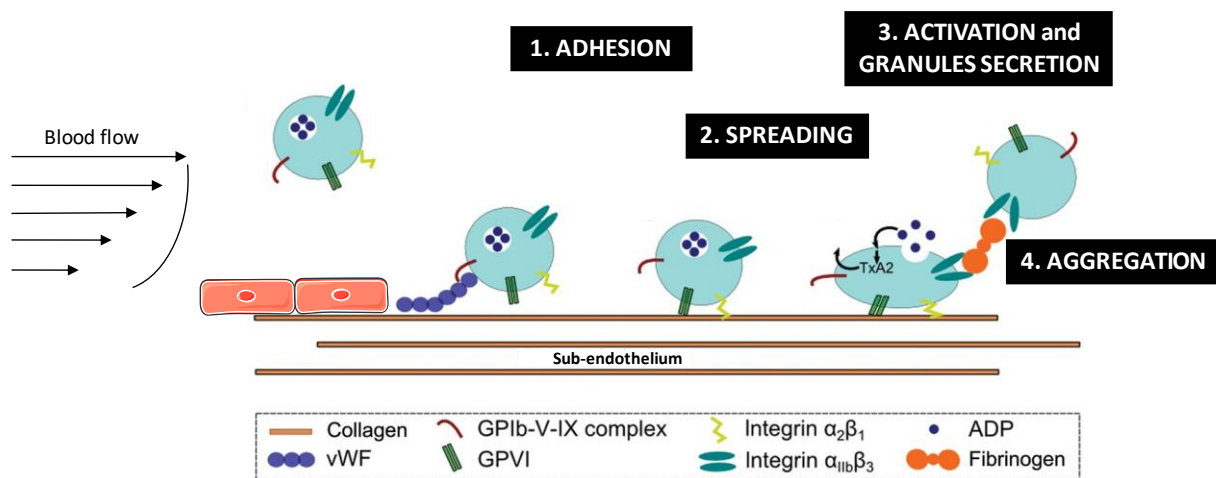


Figure 2: Major steps leading to platelet activation and aggregation. Upon vessel injury, the sub-endothelium containing proteins such as collagen fibers and the vWF binding protein, are exposed to circulating

platelet agonist. Upon binding to its receptor (protease activated receptor, mainly PAR-1 in human platelets), thrombin activates phospholipase C generating IP3 and DAG that induces cytoskeletal remodeling and platelet activation (45,46). Inhibiting thrombin generation by anticoagulant drugs affect platelet prothrombotic functions and is a potential target for intervention in the management of CAD (see section 1.3.2).

Platelet populations within the thrombus are heterogeneous, with highly activated and packed platelets found in the “core” and loosely adherent platelets found in the “shell”. This hierarchical organization of platelets in the hemostatic plug is driven by the level of platelet activation and the nature of the agonist and its distribution. Platelets in the “core” region are highly activated since thrombin is generated at the site of vascular injury. It is surrounded by a shell composed of loosely attached platelets mainly recruited by the action of ADP and thromboxane (TXA₂), secondary agonists, secreted by the activated platelets in the core. ADP signaling plays a role in initial platelet deposition and aggregation, while TXA₂ is only important for the latter (23). Accordingly, treatment of platelets, *in vitro*, with acetylsalicylic acid (ASA, aspirin), a cyclooxygenase-1 (COX-1) inhibitor preventing TXA₂ generation, and ADP-receptor (P2Y₁₂) antagonist reduce platelet deposition and aggregate formation (23,47-49). *In vivo*, this combination (aspirin and P2Y₁₂ inhibitors) is of high clinical relevance and is actually recommended in CAD patients presenting with acute coronary syndrome (50).

AGONIST DISTRIBUTION

HETEROGENEOUS PLATELET ACTIVATION

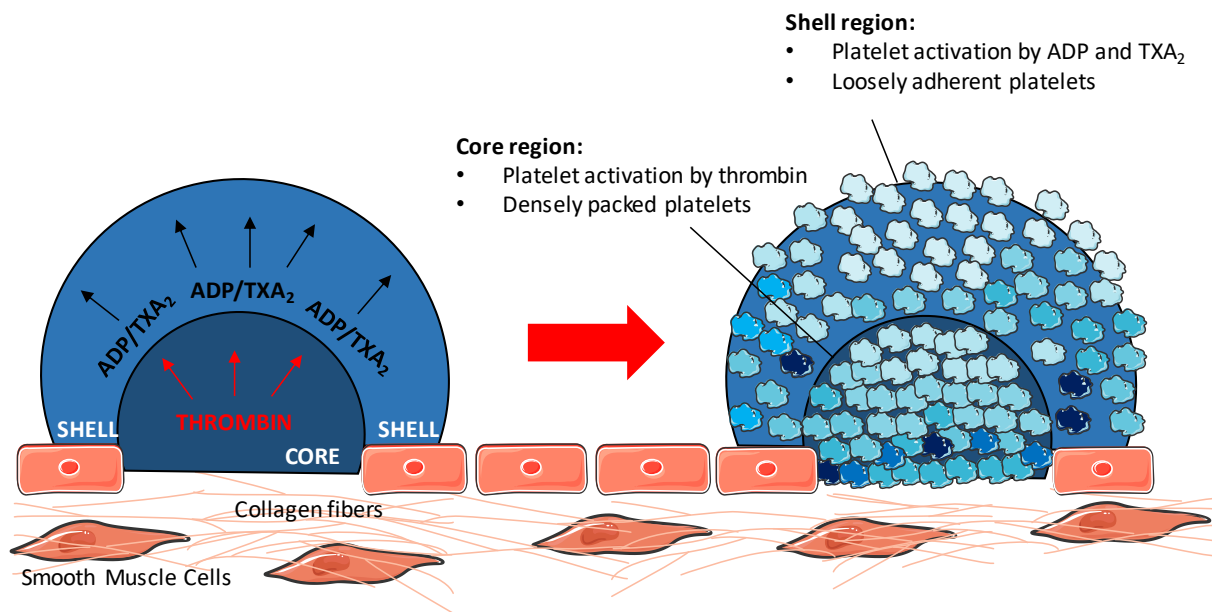


Figure 4. Hierarchical organization of platelets in the thrombus. Thrombus is composed of a core region in contact with the ruptured endothelium, where platelets are densely packed and highly activated by thrombin. Secretion of secondary agonists, ADP and TXA₂, by activated platelets from the core region recruits and activate additional platelets forming a shell. There is a gradient in the distribution of secondary agonists and platelet activation levels in the shell region, which contains loosely adherent platelets.

1.2.6 Fibrinolysis

Fibrinolytic process is the dissolution of the fibrin network, constituted by the cross-linking of fibrin monomers. Plasminogen is transformed to plasmin by the tissue plasminogen activator (tPA) produced by endothelial cells, or the urokinase plasminogen activator (uPA) from kidney cells. Active plasmin cleaves fibrin and forms fragments composed of 2 D-domains, called D-dimers (measurable marker of thrombin generation, see section 1.3.3).

1.3 Platelets and Coronary Artery Disease

Platelets play a key role in the development, progression of atherosclerosis and more importantly in the pathophysiology of atherothrombosis. The development of atherosclerosis, detailed in this section below, consists of lipids accumulation in the artery walls, together with an inflammatory reaction, platelet and coagulation activation, causing the narrowing of blood vessel. Ischemic heart disease can be classified as stable coronary artery disease (S-CAD) or acute coronary syndrome (ACS). In S-CAD, progressive narrowing of coronary arteries without sudden occlusion, results in stable angina pectoris, decreased heart function (left ventricular dysfunction), heart failure and cardiac death (1-2% cardiac death per annum (51,52)). In ACS, coronary plaque rupture leads to a sudden (sub) occlusion of the artery (atherothrombosis) and acute necrosis of the heart muscle (acute myocardial infarction (MI)). Acute MI is a life-threatening condition, which requires urgent treatment strategies such as reperfusion therapy. Despite major advances in the management of MI (rapid reperfusion therapy - primary percutaneous coronary intervention (PCI), modern antithrombotic therapy), mortality remains substantial (10% at 1 year) (53,54). Furthermore, CAD patients presenting with ACS remain at high risk of relapsing cardiovascular events; about 20% death rates at 5 years (55,56).

In the pathophysiology of atherosclerosis and atherothrombosis, there is an important crosstalk between activated platelets, coagulation cascade and inflammation (57). Guidelines recommend antiplatelet strategies in the management of CAD (50,58,59). In patients presenting with ACS, the combination of P2Y₁₂ inhibitors and aspirin, and more importantly Ticagrelor and Prasugrel compared to Clopidogrel, drastically reduce death rate, recurrent MI and stroke (22% relative

reduction rate) (58,59). More recent evidence suggests that anticoagulant drugs (60,61) and therapies targeting the inflammatory pathway (62) can further reduce cardiovascular ischemic burden. These data highlight the ultimate need to identify CAD patients with high residual risk of ischemic events, in order to tailor more aggressive therapies. Several biomarkers for risk stratification have been studied in CAD patients. However, the routine clinical use of these markers remains limited.

In this section, we will provide an overview of the development and assessment of atherosclerosis, the role of platelets in atherothrombosis and their interaction with coagulation and inflammation, management of antithrombotic drugs in CAD and finally described some risk stratification markers.

1.3.1 Atherosclerotic development

1.3.1.1 Endothelial dysfunction

Vascular integrity is maintained by endothelial cells, producing among many different molecules, antithrombotic (prostacyclin, antithrombin, thrombomodulin, ect.) and vasodilator mediators (nitric oxide-NO, prostacyclin). The overall contribution of these molecules is to limit platelet activation and vasoconstriction and to regulate inflammation and proliferation of smooth muscle cells. Reduced bioavailability of endothelium derived NO is a hallmark of endothelial dysfunction and a recognized trigger for plaque development. Impaired NO availability is the consequence of several cardiovascular risk factors including diabetes, hypertension, smoking, hypercholesterolemia, activating the vascular NADPH oxidase, thereby leading to reactive oxygen species (ROS) formation (63-65). The altered vascular endothelium, under the pro-inflammatory environment, express surface adhesion molecules for leucocytes and monocytes and display disrupted intercellular junctions (66). These modifications lead to the attachment and infiltration of inflammatory cells in the sub endothelial space, but also promote the accumulation of atherogenic lipids (low-density lipoprotein- LDL and oxidized-LDL) in the intima (67).

1.3.1.2 Lipids in circulation

Cholesterol and triglycerides (TG) are insoluble in water and therefore these lipids must be transported in association with proteins in the circulation, called lipoproteins. Plasma lipoproteins can be divided into five classes based on size, lipid composition, and apolipoproteins (Table 2) (68).

	CM	VLDL	IDL	LDL	HDL ₂	HDL ₃
Density (g/mL)	0.93	0.95-1.010	1.008-1.019	1.019-1.060	1.063-1.125	1.125-1.210
Size (nm)	75-1200	30-80	25-35	18-25	9-10	7-9
Proteins (%)	2-3%	6-9	20	20-25	40	55
Triglycerides (%)	85-90	55-60	20-30	5-8	5-6	3-5
Free Cholesterol (%)	2	6-8	8-12	7-10	5-6	4-5
Cholesterol esters (%)	3-5	10-12	25-30	40-45	15-17	12-15
Phospholipids (%)	5-7	15-18	15-20	20-25	25-30	25
Apolipoproteins	B-48, A-I, A-II, A-IV, C-I, C-II, C-III, E	B-100, C-I, C-II, C-III, E	B-100, E	B-100	A-I, C-II, C-III, E	A-I, C-II, C-III, E

Table 2. Classes and characteristics of different lipoproteins. CM: chylomicrons, VLDL: very low-density lipoprotein, IDL: intermediate density lipoprotein, LDL: low-density lipoprotein, HDL: high-density lipoprotein.

Lipid interventions in CAD patients after ACS have focused primarily on the lowering of low density lipoprotein cholesterol (LDLc) or raising high density lipoprotein cholesterol (HDLc). Triglyceride-rich lipoproteins comprise a third category of particles that may influence cardiovascular risk. In this chapter, we will focus particularly on these three types of lipids to define their role in atherogenesis, as potential therapeutic targets and as prognostic markers in CAD.

1.3.1.2.1 LDL-C and oxidized LDL

LDL particles are derived from VLDL and IDL, where they are further enriched in cholesterol esters. LDL carries the majority of cholesterol in our circulation. The predominant apolipoprotein is apoB-100 and each LDL particle contain one apoB-100 molecule. LDL particles are of different sizes: small dense LDL particles are potentially more atherogenic (69,70) and are associated with hypertriglyceridemia,

low HDL-C levels, increased TG/HDL-C ratio (71), obesity and type 2 diabetes. Small dense LDL particles are more atherogenic for a number of reasons: (1) they have a decreased affinity for the LDL receptor resulting in a prolonged retention time in the circulation, (2) they enter more easily in the arterial wall and bind more avidly to intra-arterial proteoglycans, which traps them in the arterial wall, (3) small dense LDL particles are more susceptible to oxidation, which could result in an enhanced uptake by macrophages.

The atherogenic effect of LDL particles is mainly due to its modification and particularly oxidation into oxLDL, since native LDL does not accumulate in macrophages while modified lipoprotein (oxLDL) does (72,73). However, the exact process of LDL oxidation *in vivo* is controversial. Metal ion-dependent oxidation of LDL, namely iron (Fe^{3+}) and copper (Cu^{2+}), has been extensively used for *in vitro* experiments, but Cu^{2+} -mediated oxLDL seems to target mostly the lipid moiety of LDL and not apoB-100 (74). Metal ion-dependent oxidation mechanisms *in vivo* assume a high concentration of cations at the site of oxidation, a subject which is controversial (75). On the other hand, some studies have shown an increase in both copper and iron levels in the intima of lesions compared with healthy controls (76) and a correlation between Fe^{3+} and cholesterol levels (77). In summary, these cations are present in the intima but their implication in atherosclerosis remains a point of contention.

Several lines of evidence suggest that the myeloperoxidase (MPO)-dependent mechanism of LDL oxidation might be more physiologically relevant than the copper-dependent oxidation of LDL (78,79). The first evidence of MPO-mediated oxidized LDL (MoxLDL) was described in 1994, where MPO was detected in atherosclerotic lesions (80). Several studies report that MPO levels are associated

with cardiovascular risk in CAD patients (81-83), providing evidence that MPO is a key element for oxidative damages in atherosclerosis. Mechanistically, MPO produces hypochloric acid by the enzymatic system $\text{MPO}/\text{H}_2\text{O}_2/\text{Cl}^-$. Modification of LDL by HOCl has been studied by different groups and it clearly appeared that the protein moiety apoB-100 is the major target of hypochloric acid (84). The main reason for this is the fact that MPO rapidly adsorbs at the surface of LDL and seems to have a strong interaction with the protein moiety of LDL (79,85). Mechanisms of LDL oxidation are illustrated in Figure 5.

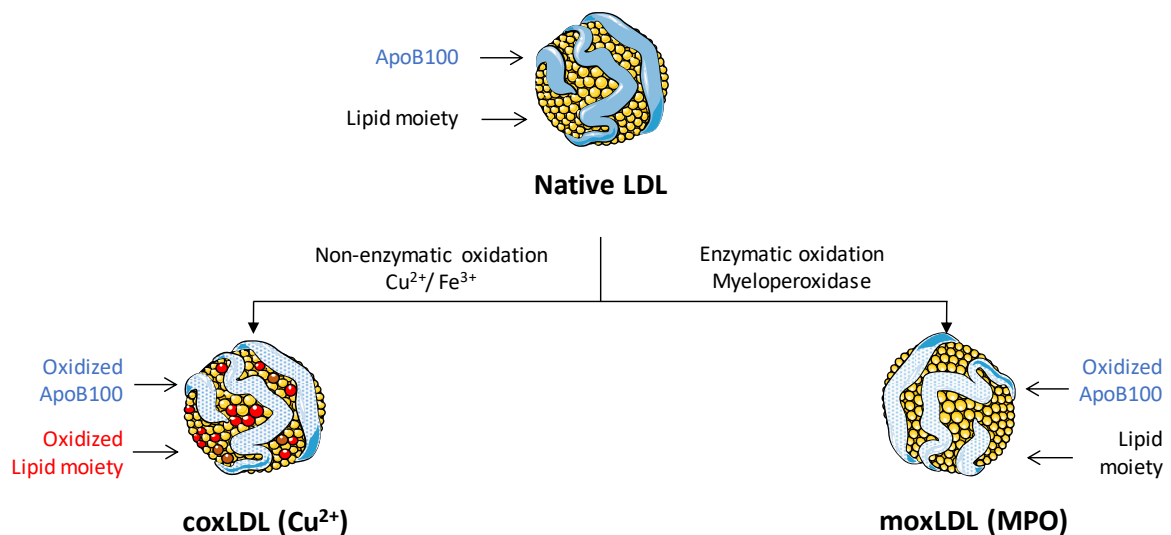


Figure 5. Mechanisms of LDL oxidation. Native LDL is composed of a specific apolipoprotein, ApoB100 and a lipid moiety. Copper mediated LDL oxidation, mainly used for in vitro experiments, involves the ApoB100 and lipid moiety oxidation. Myeloperoxidase oxidation of LDL modifies only the ApoB100.

LDL: low-density lipoprotein, coxLDL: copper oxidized low-density lipoprotein, MPO: myeloperoxidase, moxLDL: myeloperoxidase oxidized low-density lipoprotein

The modified apoB-100 (moxLDL) is no longer recognized by the LDL receptor, but by scavenger receptors like CD36, SR-A1, SR-B1 or LOX-1. Scavenger receptors are present on endothelial cells, monocytes/macrophages, smooth muscle cells and interestingly on platelet surface. CD36 is the fourth most abundant receptor on platelet surface (10 000-25 000 copies on platelet surface and alpha granules) (86,87) and is a key receptor for the platelet-lipid interplay in hyperlipidemic

conditions (88). The role of platelet CD36, its contribution to platelet mediated thrombosis and platelet-lipid interaction in CAD patients is fully detailed in section 1.4.

In agreement with its critical role in atherogenesis, LDL is a major therapeutic target in the management of CAD. Statin therapy (inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase) reduces both LDL-C levels and cardiovascular events (89-91). There is a clear relation between intensified statin therapy and reduction rates of non-fatal cardiovascular events (92-94). Because of residual risk of ischemic events despite intensive statin therapy and achievement of target LDL-C levels, additional lipid modifying therapy has been studied (95-98). The addition of ezetimibe, an inhibitor of the Niemann-Pick C1 like 1 protein for cholesterol absorption from intestine (IMPROVE-IT trial) (97), or an inhibitor of the proprotein convertase subtilisin–kexin type 9 (PCSK9), on top of statin to further reduce LDL-C levels (FOURIER trial) (98), have been shown to reduce cardiovascular events. The ODYSSEY trial randomized 18 924 patients after hospitalization for acute MI or unstable angina, treated with statins, and with LDL-C (≥ 70 mg/dL), non-HDL cholesterol (≥ 100 mg/dL), or ApoB ≥ 80 mg/dL, to receive injections of alirocumab (PCSK9 inhibitor) or matching placebo. In this study, there was a 15% relative reduction in the primary outcome (composite of CHD death, non-fatal MI, ischaemic stroke, or unstable angina requiring hospitalization) (HR 0.85, 95% CI 0.78–0.93) after a median follow-up of 2.8 years (99). The recently revised guidelines for the management of dyslipidaemias for secondary prevention recommend a 50% reduction of LDL-C from baseline and an LDL-C goal of < 55 mg/dl in very high-risk patients (100). Even if guidelines recommend more aggressive strategies in very high-risk population, the identification of such patients remains challenging and

there are still some gaps in the evidence. Whether very low LDL-C levels achieved with the combination of statin, ezetimibe, and PCSK9 inhibitor reduce the need for further PCI remains to be addressed in outcome studies.

Risk assessment by classical lipid markers include total cholesterol, LDL-C, HDL-C, non-HDLc, TG, ApoB100 and Lp(a). LDL-C is the primary lipid analysis method for screening, diagnosis, and management. In addition, assessment of oxLDL has been described as a potential risk marker for ischemic events (101,102) and correlates with severity of CAD (101,103,104). Several methods for measuring oxLDL levels, using antibodies binding specific epitopes of oxLDL, have been reported (104-106), but their results are not easily comparable limiting the interpretation of these data across different studies. Furthermore, controversial data showed that circulating oxLDL, measured with antibody 4E6, is not an independent predictor of CAD after adjustment for lipid markers and is less predictive in development of CAD than apoB and total cholesterol/HDL-C ratio (106). Routine measurement of oxLDL levels is therefore not recommended for risk assessment and in the management of dyslipidemia in CAD patients (107). MPO activity has also been described to be associated with severity of CAD (81,82), however its assessment and interpretation remain challenging. While MPO expression give information about the abundance of the molecule, MPO activity varies considerably between individuals even if MPO levels is similar (108).

Taken together, even if intensified therapy for lowering LDL-C levels is recommended in CAD patients, a considerable residual risk of events remains, suggesting an impact of other lipids species on cardiovascular risk.

1.3.1.2.2 HDL-C

HDL are the smallest (5–17 nm) and densest (1.063–1.210 kg/l) lipoproteins in the plasma. Apolipoprotein (Apo) A1, the major protein in HDL, is synthesized in the liver and the small intestine. The principal role of HDL is to transport cholesterol from the periphery (e.g. from macrophages in blood vessel walls) to the liver, a process called the reverse cholesterol transport (RCT) (109,110). In the setting of CAD, epidemiologic studies have shown that, in addition to elevated LDL-C levels, low HDL-C levels are an independent predictor of ischemic heart disease (111,112). Several previous studies highlighted the potential cardiovascular benefit of increasing HDL-C levels, even in patients with normal LDL-C levels (113,114); however more recent studies showed controversial results. The AIM-HIGH study showed no incremental benefit for the increase in HDL-C levels by niacin, in patients with intensively controlled LDL-C levels, on cardiovascular events (96). Cholesteryl ester transfer protein (CEPT) inhibitor is an attractive pharmacological approach to lower LDL-C levels and increase HDL-C. However, even if the CEPT inhibitor, Torcetrapib, achieved a decrease of 20% of LDL-C and increase in 70% of HDL-C levels, the ILLUMINATE trial was terminated earlier because the drug increased the rate of cardiovascular ischemic events (115). A second CEPT inhibitor, Dalcatrapib, that mainly increase HDL-C levels without affecting LDL-C, was also stopped earlier because of lack of efficacy (95). Until now, there is no robust evidence that a modification in HDL-C metabolism by treatment strategies can affect cardiovascular risk. The current policy concerning HDL-C is: an increase in HDL-C through lifestyle change (smoking cessation, physical exercise) is recommended, but HDL-C is currently not a valid target for drug therapy.

1.3.1.2.3 Triglycerides

In plasma, most of the triglycerides (TG) are vehicled by chylomicrons, VLDL and/or remnants. TG and remnant cholesterol (cholesterol contained in TG rich lipoproteins) accumulates in arterial wall foam cells leading to development of atherosclerosis. There is a clear relationship between increased TG levels and low HDL-C measured in plasma of patients. Increased TG/HDL-C ratio is a well-known pro-atherogenic condition (71,116,117). Indeed, studies have reported association between increased TG levels and risk of ischemic heart disease, stroke and all-cause mortality (118,119). Recent data on more than 15 000 patients clearly establish the link between levels of fasting TGs and recurrent ischemic events in ACS patients on statin treatment. The relation between TG levels and cardiovascular risk was independent of LDL-C levels (120). Collectively, there is strong evidence that even at LDL-C goal, patients with cardiometabolic abnormalities remain at high risk of cardiovascular events due to increased TG levels. However, similar to studies targeting HDL-C, TG reduction (by niacin, fibrates or omega-3 fatty acid) has not been shown to lower risk for such events. The recently published REDUCE-IT trial (more than 8000 patients) with a new molecule for TG reduction (high-dose icosapent ethyl) has shown impressive efficacy on cardiovascular risk reduction of events (25% risk reduction in cardiovascular death, non-fatal MI, revascularization procedures, stroke) (121). Other randomized trials with moderate doses of icosapent ethyl are still ongoing. This molecule is a potential additional therapy, on top of statins, to reduce ischemic events.

1.3.1.3 Plaque formation and atherosclerotic assessment

Atherogenic lipids accumulate in the sub endothelial compartment where they are oxidized and taken up by macrophages forming foam cells. With time, the foam cells die, contributing their lipid-filled contents to the necrotic core of the lesion. Inside the plaque, activated leucocytes and macrophages release several mediators (cytokines, chemokines, growth factors, ect.) promoting the proliferation and migration of vascular smooth muscle cells (SMCs). Under the influence of inflammatory mediators, SMCs contributes to the formation of the fibrous cap by producing the extracellular matrix. Initially, the lesions grow towards the adventitia (positive remodeling) until a critical point is reached, after which they begin to expand outwards and encroach on the lumen (negative remodeling).

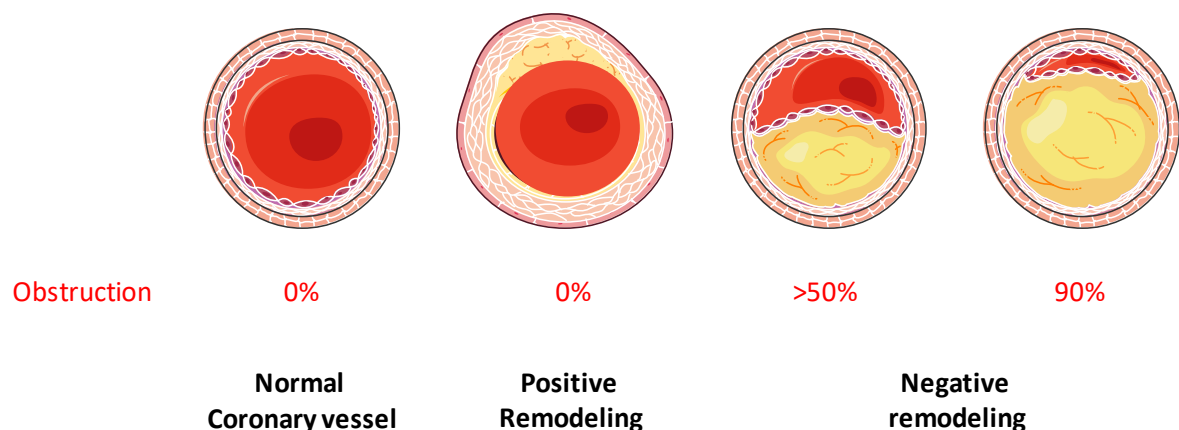


Figure 6. Arterial remodeling during atherosclerotic development. During the initial phase of atherosclerotic progression, the lesion grow in the wall of the artery without affecting the lumen size (positive remodeling of the artery). The growing atherosclerotic plaque reach a critical point where it starts to reduce the artery lumen. Significant coronary artery obstruction is defined as a stenosis above 50% and critical stenosis above 90%.

The necrotic core of the growing atherosclerotic plaque, under the influence of several factors (inflammation, medications: warfarin, metabolic disorders: renal insufficiency, hypercalcemia, or hormonal disorders), undergo calcification process. These factors induce the expression of bone morphogenetic protein, leading to

osteogenic differentiation of pluripotential, pericyte like cells located in the arterial intima, which then produce bonelike matrix and hydroxyapatite mineral (122). Vascular calcification and more importantly coronary artery calcification (CAC) is a highly specific feature of coronary atherosclerosis and is one of the most thoroughly studied and widely available tests in cardiovascular medicine (123). While vascular calcification has been associated for a long time with the passive aging process, the development of CAC is now considered as an active process and its evaluation is an interesting tool for risk assessment. Currently, CAC is assessed by a multi-detector computed tomography (MDCT) with a cardiac gating to obtain the quantitative CAC Agatston score. CAC quantification is a validated anatomic measure of the atherosclerotic plaque burden, since numerous studies have shown correlation between CAC and plaque volume, histopathologic studies of coronary arteries from autopsy heart and IVUS measurement of plaque area (124-126). CAC Agatston score is classified as negative (CAC=0), low (CAC<100), moderate (CAC: 100-400), high (CAC>400) and very high (CAC>1000). CAC Agatston score (127,128) and its progression over time (129,130), have been associated with increased risk of MI and all-cause mortality.

In preventive cardiology, clinical practice guidelines for risk assessment recommend the use of equations taking into account plasma lipid levels, blood pressure, age, smoking history, and diabetic status (Atherosclerotic Cardiovascular disease 10 year-risk estimate: ASCVD score). However, it is now established that CAC improves statistical risk reclassification that is, modifying risk estimates in subjects with low CAC score into lower risk categories and those with high CAC score into higher risk categories of coronary events (net reclassification index) (131,132). There is also evidence that CAC might directly guide the selection of appropriate

preventive therapies (133), that is avoiding statin therapy in patients with CAC score = 0 and low 10-year event rates (low ASCVD score) and recommending statins in patients with intermediate ASCVD score but CAC score >0 (134,135). In line with these studies, inclusion of CAC results in the MESA (Multi-Ethnic Study of Atherosclerosis) risk score offered significant improvements in risk prediction (136).

Atherosclerotic Cardiovascular disease risk estimate (ASCVD score)- 10 year risk	<5%	5-7.5%	7.5-20%	>20%
ASCVD risk score only	Statins not recommended	Consider Statins	Recommend statins	Recommend statins
ASCVD risk score + CAC Agatston score If CAC=0	Statins not recommended	Statins not recommended	Statins not recommended	Recommend statins
If CAC > 0	Statins not recommended	Consider Statins	Recommend statins	Recommend statins

Table 3: CAC Agatston score on top of ASCVD score to guide statin therapy for primary prevention. Assessment of CAC Agatston score improves risk stratification and management of preventive therapies. When CAC score is 0 in patients with intermediate ASCVD risk score, there is no need for statins.

CAC: coronary artery calcification, ASCVD: atherosclerotic Cardiovascular disease risk estimate.

Adapted from Greenland P et al. J Am Coll Cardiol. 2018 Jul 24;72(4):434-447.

However, the need for a new CAC score is a matter of current debate since the “traditional” CAC Agatston score has limitations. Calcifications observed on other vascular beds are not taken into account in the CAC Agatston score. This may explain why events are observed in patients with CAC Agatston score of 0, while many older patients with high CAC scores do not experience cardiovascular events (137). One of the major strategies to improve risk assessment by calcium scoring, is to consider extra coronary calcifications (number of lesion site, calcium density, lesion size, diffusivity) (137). Recently, extra coronary calcifications (mitral valve, aortic valve, aortic root, thoracic aorta and abdominal aortic calcifications) has been shown to improve risk assessment in addition to traditional risk factors (138,139).

Taken together, these data suggest that even if CAC Agatston score is implemented in the guidelines to guide preventive therapies, there is still room for improvement in terms of risk prediction.

1.3.2 Atherothrombosis – Acute Coronary Syndrome

Plaque rupture is the consequence of the fibrous cap degradation, a process which is largely influenced by the inflammatory pathway. Vulnerable plaques have thin fibrous cap and increased number of inflammatory cells. Production of interferon(IFN)- γ by T-cells inhibit production of extracellular matrix by SMCs and macrophages produce different proteases that degrade extracellular matrix. Thrombus formation is driven by activated platelets and thrombin generation pathways. Platelets adhere to exposed sub endothelial collagen and von Willebrand factor (vWF), become activated, and then release ADP and thromboxane A₂ (TXA₂), which activate other platelets. Platelet activation induces conformational changes of the integrin α IIb β 3, which increases the affinity for its ligands fibrinogen and vWF, and thereby mediates platelet aggregation and thrombus formation (see section 1.2).

The sudden total occlusion of the coronary vessel leads to the clinical presentation of ST-elevation myocardial infarction (STEMI), which requires urgent revascularization strategy (primary percutaneous coronary intervention preferred when available). Actually, guidelines recommend a 90 minutes delay between the first medical contact (ambulance) and reperfusion, when STEMI diagnosis is made in the out-of-hospital setting (140). Reperfusion is obtained after wiring of the culprit coronary artery, after which a stent is placed to maintain the vessel open. Besides reperfusion, antiplatelet therapies are essential to prevent stent thrombosis and recurrent ischemic events. Platelet signaling pathways and respective inhibition by antiplatelet drugs are described below.

1.3.2.1 Overview of platelet surface receptors, downstream signaling pathways and antiplatelet drugs

1.3.2.1.1 Platelet Adhesion receptors

1.3.2.1.1.1 vWF receptor - GP Ib-IX-V

The GP Ib-IX-V complex is highly expressed on platelet surface (25 000 copies/platelet) (141). Following binding of vWF, Src and Lyn associate with the GP Ib-IX and phosphoinositide 3 kinase (PI3K) leading to stimulation of phospholipase C (PLC γ 2), TXA₂ generation, granules secretion and α IIb β 3 activation (142). Phospholipase stimulation is a mechanism common to many intracellular signaling pathways and has therefore a central impact on platelet activation. Once activated, PLC cleaves membrane phosphatidylinositol 4,5-bisphosphate (PIP₂) into inositol 1,4,5-trisphosphate (IP₃) and 1,2-diacylglycerol (DAG). IP₃ activates its receptor in the endoplasmic reticulum membrane, increasing cytosolic Ca²⁺, a master regulator of platelet activation. In addition to binding vWF, GPIb-IX-V also interacts with E- and P-selectin, present on activated endothelial cells and platelets respectively (143), with the coagulation factors FXII and FXI and with thrombin (144). In the setting of atherosclerosis, genetic polymorphism of the GP Ib-IX-V receptor has been described to be associated with increased risk for prevalence and severity of CAD (145) and increased risk of stroke (146).

1.3.2.1.1.2 Collagen receptors – GPVI and $\alpha_2\beta_1$

Two major collagen receptors are expressed by platelets: GPVI and the integrin $\alpha_2\beta_1$. GPVI mainly participates in platelet activation while $\alpha_2\beta_1$ is involved in platelet adhesion. Binding of collagen fibrils on GPVI induces receptor clustering and cross-

linking, leading to phosphorylation of the immunoreceptor tyrosine-based activation motif (ITAM) of the FcR γ -chain by the two Src kinases, Fyn and Lyn, which are permanently associated to GPVI cytoplasmic tail. ITAM phosphorylation triggers the binding and activation of Syk, which induces the formation of the “LAT signalosome”, a complex protein network that activates PI3K and PLC γ 2, leading to complete platelet activation. The $\alpha_2\beta_1$ is almost exclusively considered as an adhesion receptor even though it also increases collagen binding to GPVI. $\alpha_2\beta_1$ and GPVI are therefore regarded as complementary for maximal platelet adhesion and activation in response to collagen.

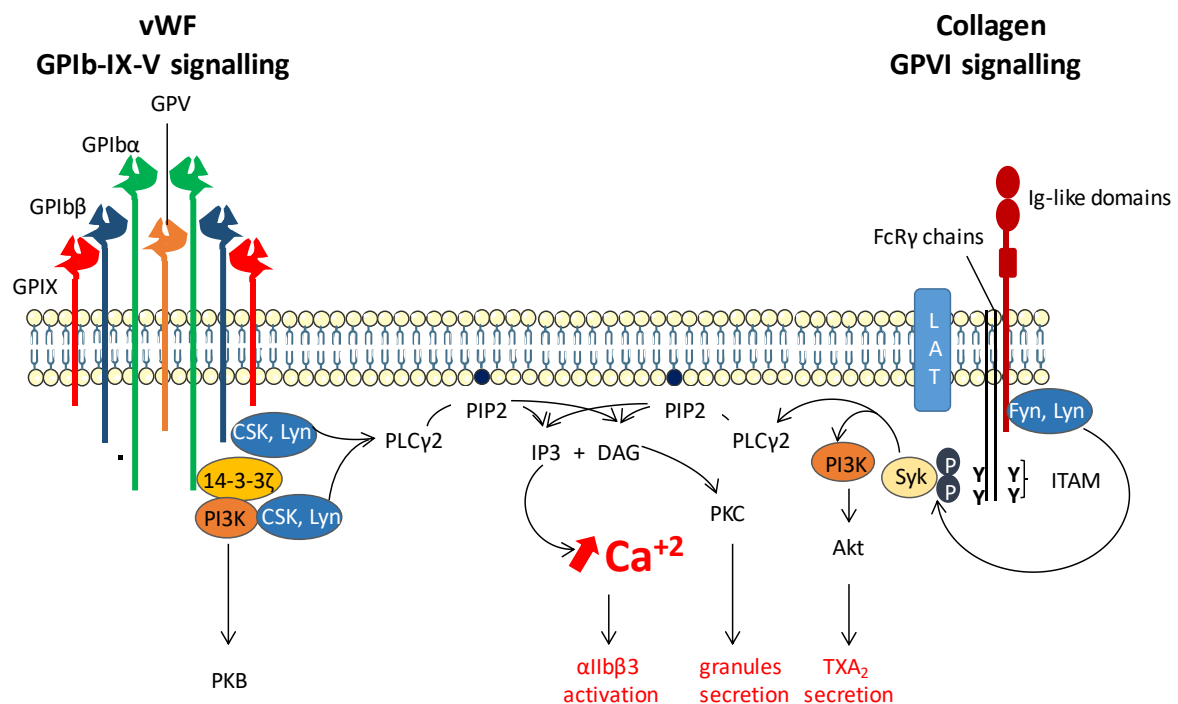


Figure 7: Adhesion receptors and their signaling pathway. GPIb-IX-V and GPVI are the two major platelet adhesion receptors to the extracellular matrix. The GPIb-IX-V complex binds the vWF and activates PLC γ 2 via CSK, leading to increase in intracellular calcium. GPIb-IX-V also activates the PI3K/PKB pathway. The binding of collagen to its receptor GPVI leads to a drastic increase in intracellular calcium levels, via tyrosin kinases (Fyn, Lyn, Syk) and adaptative proteins (LAT).

CSK: c-Src kinase, PI3K: phosphoinositide 3 Kinase, PKB: protein kinase B, PKC: protein kinase C, PLC γ 2: phospholipase C gamma 2, PIP2: phosphatidylinositol-4,5-diphosphate, IP3: Inositol-1,4,5-triphosphate, DAG: diacylglycerol

More recent data report that the GPVI receptor can interact with other macromolecules. The GPVI receptor can bind fibrin, thereby promoting amplification of thrombin generation (enhanced exposure of phosphatidylserine- procoagulant platelets) and recruitment of circulating platelets to the clot (147,148). GPVI can also interact with D-dimer which induces platelet spreading through activation of Src and Syk tyrosine kinases (149). In contrast, when platelets are activated in suspension, soluble D-dimer inhibits platelet aggregation induced by fibrin and collagen. Given the complexity of the GPVI receptor, further studies are needed to fully characterize the specific binding of these molecules to GPVI and their impact on platelet prothrombotic functions.

Interestingly, the platelet GPVI receptor has been studied in the setting of CAD. Enhanced GPVI expression levels on hospital admission are associated with the development of ACS and myocardial infarction. In this study, GPVI surface expression is elevated in CAD patients and more importantly in ACS. Its expression was also correlated with platelet activation marker CD62P. Given that GPVI levels may be elevated while markers of myocardial necrosis is still negative in patients presenting with ACS, GPVI expression can be an interesting marker for risk assessment in CAD patients (150). Furthermore, high GPVI levels correlates with increased residual platelet aggregation despite antiplatelet therapy and can therefore identify the subgroup of patients with a high risk for coronary stent thrombosis and thrombo-ischemic events (151).

Antiplatelet agents targeting the GPVI receptor has been studied in CAD patients, and some studies are still ongoing. Several strategies may be employed to inhibit GPVI-triggered platelet activation: collagen-binding molecules (GPVI mimics),

GPVI-binding compounds (aptamers, small molecules, and antibodies) or by GPVI depletion (152). Revacept (Dimeric Glycoprotein VI-Fc), inhibiting specifically collagen-induced platelet aggregation without affecting general hemostasis, has been studied in a phase 1 trial and proved to be safe and well-tolerated (153). Other GPVI blockers (exemple: Trowaglerix Venom Polypeptides) are still under study (154).

1.3.2.1.2 Soluble agonist receptors

The majority of these agonists are secondary auto- or paracrine mediators, generated by platelets themselves (thrombin, ADP, ATP and TXA₂) that transduce signal via binding to their respective receptors, which are mostly G-protein coupled receptors (GPCR), except the ATP-receptor, which is a channel. The signaling pathways downstream each of these platelet agonists and corresponding antiplatelet drugs are illustrated in Figure 8.

1.3.2.1.2.1 Thrombin receptors – Protease Activated Receptors (PARs)

Thrombin is a soluble plasma serine protease, generated via the coagulation cascade on the surface of activated platelets (see section 1.2.5). It is a major platelet agonist that leads to strong stimulation (46) via its binding to the protease-activated receptor (PAR). Four PARs members have been characterized: PAR-1, PAR-2, PAR-3, and PAR-4. Among these, human platelets express PAR-1, the major thrombin receptor (155), and PAR-4. PAR-1 is activated by lower concentrations of thrombin than PAR-4 and mediates a more rapid platelet-activation response (156). In mice platelets, PAR-4 is the major thrombin receptor. Mice platelets also express PAR-3 which does not trigger signaling cascade but rather acts as a co-factor that binds thrombin and localizes it in proximity to PAR-4 (46). Thrombin mediates PAR

activation via a proteolytic cleavage of its N terminal extracellular (157). Both PAR-1 and PAR-4 are coupled to the G proteins G α q and G α 12/13, which lead to PLC β activation, triggering IP3 and DAG generation and platelet activation (46,158).

In the setting of CAD, Vorapaxar, a PAR-1 antagonist has been tested in ACS patients on top of conventional treatment (TRACER trial) to prevent thrombin-mediated platelet activation. The addition of a PAR-1 antagonist (on top of aspirin and P2Y₁₂ inhibitors) did not significantly reduce the primary composite end point occurrence of cardiovascular death, MI, stroke, hospitalization for ischemia, or urgent revascularization, but significantly increased moderate and severe bleeding and intracranial hemorrhage (159). However, in the subgroup of patients with peripheral artery disease (PAD) (7% of the population from the TRACER trial), there was a clear trend reduction in ischemic endpoints, peripheral revascularization and amputation. These results suggest that more aggressive antithrombotic strategies can improve ischemic endpoints in high-risk patients despite conventional therapy. However, these strategies warrant further investigation and a careful patient selection to balance efficacy and safety (160). Actually, Vorapaxar is not recommended on top of conventional treatment for ACS because of the increase bleeding risk.

1.3.2.1.2.2 ADP receptors: P2Y₁ and P2Y₁₂

ADP is a secondary platelet agonist, release from dense granules upon platelet activation, and contributes to thrombus build-up (see section 1.2.3 and 1.2.5). The two ADP receptors expressed on platelets are P2Y₁ and P2Y₁₂, which are associated with G α q and G α i, respectively. In order to regulate platelet reactivity towards ADP, both receptors are submitted to endocytic trafficking, which allows

platelets to become desensitized (receptor internalized) and resensitized (receptor is recycled back to the membrane) after agonist disappearance (161). P2Y₁₂ is approximately four times more expressed in platelets as compared to P2Y₁ (162). Both receptors are crucial for a complete and coordinated platelet response to ADP, with P2Y₁ being involved in the early stages of platelet aggregation and shape change, while P2Y₁₂ allows potentiation of platelet function and strong aggregation (162).

Mechanistically, ADP binding to P2Y₁₂, activates protein G α i which inhibits the adenylate cyclase (AC), leading to decreased cyclic AMP (cAMP) generation and therefore to attenuation of the protein kinase A (PKA) activity. PKA is a negative regulator of platelet activation by inhibiting Ca²⁺ mobilization, aggregation, granules secretion and cytoskeletal remodeling (163). In addition to relieving PKA inhibition on platelet function via G α i, P2Y₁₂ coupling to G $\beta\gamma$ i leads to PI3 kinase, PKB and Rap1b activation, increasing platelet aggregation.

The P2Y₁₂ receptor is a very important target for intervention in CAD patients. P2Y₁₂ inhibitors include thienopyridines from three different generations: ticlopidine (actually not recommended in the guidelines because of its toxicity), clopidogrel, Prasugrel and nonthienopyridines (Ticagrelor, cangrelor). Ticagrelor and Prasugrel are the third generation of P2Y₁₂ inhibitors. In the setting of ACS, Ticagrelor and Prasugrel in combination with aspirin, have been shown to reduce cardiovascular ischemic endpoints (MI, stent thrombosis) as well as cardiovascular death and stroke compared to Clopidogrel (50,58,59). However, in patients with S-CAD needing percutaneous coronary intervention (PCI) as well as patients with previous history of stroke or intracranial hemorrhage, ongoing bleeds, clopidogrel is still recommended in combination with aspirin. In general, the type, dose and duration

of treatment by P2Y₁₂ inhibitors are well established according to the various setting of CAD (50).

However, even the accuracy of the current dual antiplatelet therapy, a persistent residual risk remains. Numerous studies have demonstrated variable levels of platelet P2Y₁₂ receptor inhibition in patients treated with clopidogrel and an increased risk of thrombotic events in those with high on-treatment platelet reactivity (164). There are two methods for testing efficacy of platelet inhibition by P2Y₁₂ drugs:

(1) Platelet stimulation and aggregation induced by agonist (Verify Now (Accumetrics, San Diego, CA, USA) and Multiplate assays (Roche Diagnostics, Switzerland). VerifyNow assays study light transmission following platelet stimulation and aggregation induced by agonist (efficient platelet function inhibition shows low light transmission; reduced aggregation) while Multiplate assays analyze the increase in resistance following platelet aggregation induced by agonists, on the electrode. However, there is currently no evidence to support the use of routine platelet testing to individualize antiplatelet therapy. The ANTARTIC trial using the Verify Now assay, showed no benefit for platelet function monitoring with treatment adjustment in elderly patients treated with coronary stenting for an acute coronary syndrome (165).

(2) Study of the vasodilator-stimulated phosphoprotein, VASP, signaling pathway. Phosphorylation assessment of VASP is specific to P2Y₁₂ signaling in platelets. VASP is an intracellular regulatory protein that is not phosphorylated under basal state and its phosphorylation is influenced by the level of cAMP and PGE₁ in platelets. P2Y₁₂ inhibition leads to increased intracellular cAMP and VASP phosphorylation, which can be detected by flow cytometric analysis (166,167). The

PhosphoVASP assay has been used to study the level of P2Y₁₂ inhibition by clopidogrel in patients with ischemic heart disease. This assay demonstrated a wide interindividual variability of the inhibitory response of platelets to clopidogrel and showed that one-third of the patients treated appeared to be 'unprotected' by this therapy (166).

1.3.2.1.2.3 ATP receptor – P2X₁

ATP released by activated platelets binds, in an autocrine way, to P2X₁, a ligand-gated Ca²⁺ channel that belongs to a large family of P2X channels. Upon ATP binding, there is a rapid Ca²⁺ entry. This increase in intracellular Ca²⁺ mediates Ca²⁺-calmodulin-dependent Erk2 activation, which amplifies myosin light chain phosphorylation in response to collagen, leading to cytoskeletal remodeling, shape change, granule secretion and pseudopods formation (168,169).

1.3.2.1.2.4 Thromboxane A₂ receptor – TP

During platelet activation, TXA₂ is generated after arachidonic acid (AA) - containing phospholipids are cleaved by phospholipase A₂ (PLA₂). Similar to ADP, TXA₂ stimulates and recruits additional platelets to the thrombus. The synthesis of TXA₂ from AA includes the sequential metabolism of AA into prostaglandin (PG) H₂, via the COX-1, and of PGH₂ into TXA₂ via the TXA synthase. TXA₂ crosses the plasma membrane and activates surrounding platelets in an auto- or paracrine way. TXA₂ is extremely unstable, has therefore a very short half-life and is rapidly converted to TXB₂. The latter binds to the TP (Thromboxane/Prostaglandin) receptor, which has two isoforms, TP_α and TP_β, which are associated with G_{αq} and G_{α12/13}, and are both expressed on platelets (170).

Aspirin blocks COX-1 activity via an irreversible acetylation of serine 530, thereby affecting TXA₂ generation. Low dose aspirin (<100mg/daily) is recommended in addition to P2Y₁₂ inhibitors in CAD patients presenting with ACS or after PCI. Even if the role of aspirin in primary prevention is actually downgraded (171,172), it is essential in secondary prevention (50) and recent data suggest that maintaining aspirin in the long term still has beneficial effects on ischemic outcomes (173).

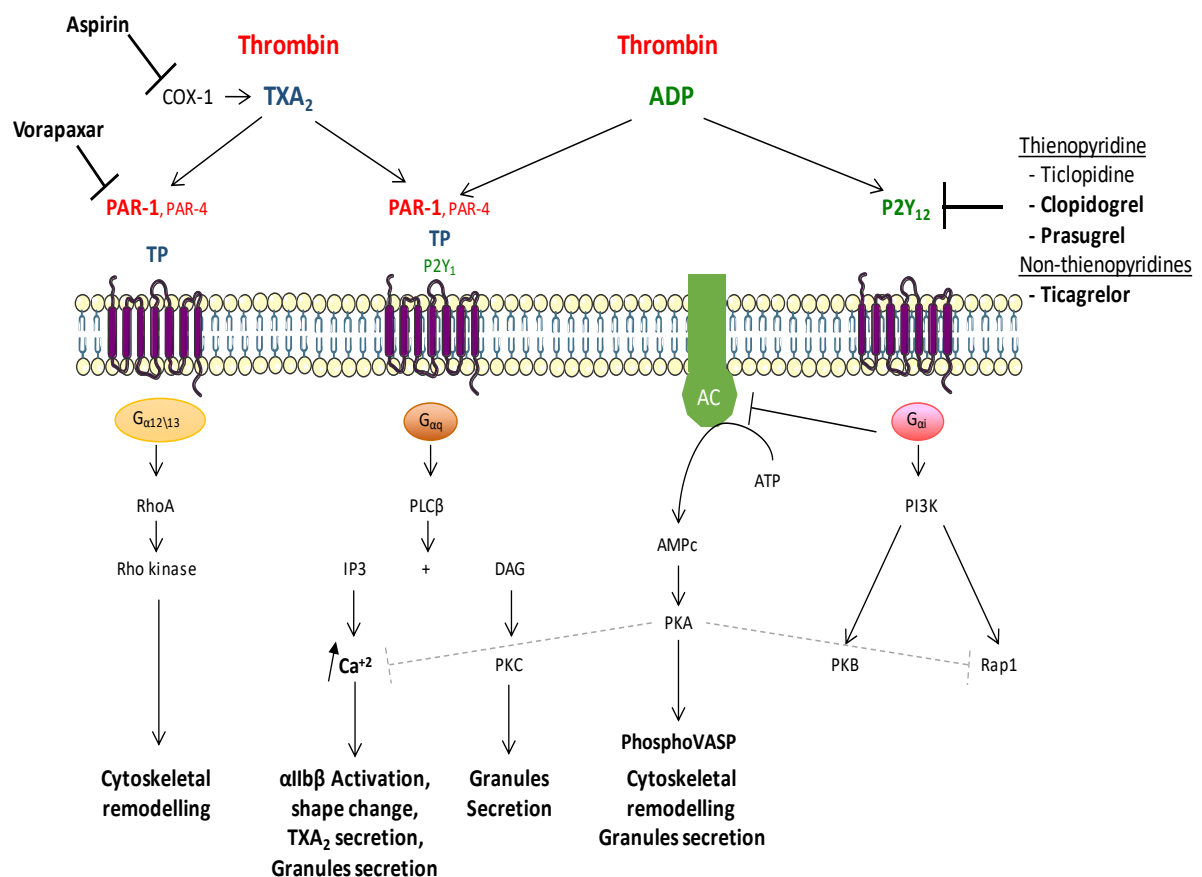


Figure 8: Soluble platelet agonists, signaling pathways and corresponding antiplatelet drugs. Thrombin and the secondary mediators, TXA₂ and ADP, are the principal soluble platelet agonists. They all bind to G-protein coupled receptors (GPCR). Thrombin, the most powerful platelet agonist, binds to its protease-activated receptor (PAR)-1 and PAR-4, which are coupled to G_{αq} and G_{α12/13}. G_{αq} activates a phospholipase Cβ (PLCβ), leading to increase in calcium levels. ADP, in addition to stimulating G_{αq} via its P2Y₁ receptor, also binds to P2Y₁₂ which is coupled to G_{ai}, inhibiting the adenylate cyclase (AC), thereby decreasing protein kinase A (PKA) activity, a negative regulator of platelet activation. PKA phosphorylates VASP (PhosphoVASP), which can be measured to assess P2Y₁₂ inhibition. TXA₂ binds to G_{αq} and G_{α12/13} coupled receptors, similar to PARs.

Platelet agonists and corresponding receptors are represented as: red (Thrombin – PARs), blue (Thromboxane – TP receptor), green (ADP- P2Y₁₂ and P2Y₁ receptors).

1.3.2.1.3 Fibrinogen receptor - integrin $\alpha\text{IIb}\beta 3$

Integrin $\alpha\text{IIb}\beta 3$ activation is the consequence of increase in intraplatelet Ca^{2+} levels following platelet activation. It changes conformation from low to high affinity for binding fibrinogen (“inside-out” signaling) allowing platelet aggregation. Activation of the integrin $\alpha\text{IIb}\beta 3$ can be studied *in vitro* by flow cytometric assays.

Agents that block the function of integrin $\alpha\text{IIb}\beta 3$ are powerful antiplatelet drugs (Eptifibatide, Tirofiban, Abciximab). Since these agents inhibit the final pathway leading to platelet aggregation, they are more potent than the other antiplatelet agents but are associated with increased bleeding events. Integrin $\alpha\text{IIb}\beta 3$ inhibitors provide a rapid and nearly complete platelet aggregation inhibition, overcoming the delayed and poor platelet inhibition induced by a clopidogrel loading dose (174). $\alpha\text{IIb}\beta 3$ inhibitor therapy has been associated with a reduction in adverse cardiovascular events, including MI, at the expense of increased bleeding and thrombocytopenia (175,176). However, recent trials in patients pre-treated with modern P2Y_{12} inhibitors (third generation), have shown contradicting evidence in the benefit of routine use of $\alpha\text{IIb}\beta 3$ inhibitors. With the introduction of modern P2Y_{12} inhibitors and rapid access to primary PCI, the $\alpha\text{IIb}\beta 3$ inhibitors are less frequently prescribed. Currently, the administration of $\alpha\text{IIb}\beta 3$ inhibitors is an accepted treatment option for patients undergoing primary PCI with visible thrombus burden.

1.3.2.2 Anticoagulant drugs in CAD

Plaque rupture stimulates the coagulation cascade (see section 1.2.5), resulting in the formation of thrombin and finally fibrin to stabilize the clot (177). Thrombin being a key platelet agonist, anticoagulant drugs targeting its generation have a major impact on platelet function in addition to the coagulation cascade activity. In the

management of the acute phase of MI, anticoagulant drugs are recommended in addition to antiplatelet agents (140). Fondaparinux, a pentasaccharide blocking the FXa, low molecular weight heparin (LMWH- enoxaparin) or unfractionated heparin (UFH) are the principal anticoagulant drugs recommended in the acute phase of ACS. During primary PCI, UFH is considered as a first choice.

ACS patients remain at high risk of recurrent ischemic events. The Belgian-UK GRACE registry report a 20% risk of death and relapsing ischemic events at 5 years (178). Several lines of evidence demonstrate that patients with recurrent cardiovascular ischemic events exhibit a persistent thrombin generation (179-183). In line with these studies, drugs targeting the coagulation cascade, inhibiting thrombin formation, have been shown to have an impact on ischemic outcomes. Vitamin K antagonists (VKAs, e.g Warfarin) in combination with aspirin or given alone, was superior to aspirin alone in reducing the incidence of death, nonfatal reinfarction, stroke but was associated with a higher risk of bleeding (184-186). More recent data on direct oral anticoagulants (DOACs) suggest a clear benefit on cardiovascular ischemic outcomes compared to placebo. The ATLAS-ACS2-TIMI51 studied the impact of low dose Rivaroxaban, a direct FXa inhibitor, on cardiovascular outcomes after a recent ACS. Low-dose rivaroxaban, on top of conventional treatment, reduced the risk of cardiovascular death, myocardial infarction, or stroke after ACS, but increased the risk of major bleeding and intracranial hemorrhage (60). The COMPASS study showed that the addition of low-dose Rivaroxaban on top of Aspirin lowers the rate of reMI, cardiovascular death and stroke in patients with stable CAD. The study was prematurely stopped for achievement of the primary endpoint, which was the reduction of ischemic events and cardiovascular death. However, there was an increase in major bleeds but no

significant increase in intracranial bleeding or other critical organ bleeding (61). In agreement with the increased risk of events in patients with PAD, low-dose Rivaroxaban also reduces limb events (major amputation) in addition to cardiovascular events (187). These data highlight the potential impact of a persistent thrombin generation on residual risk of ischemic events in CAD patients, and put anticoagulation strategies to the forefront, on top of conventional antiplatelet therapies, to reduce ischemic burden.

1.3.2.3 The inflammatory pathway, a possible target for intervention?

Activation of the inflammatory pathway is a hallmark of atherosclerotic development. Moreover, various inflammatory cell types (e.g. macrophages, neutrophils, lymphocytes) play a crucial role for plaque destabilization and erosion, leading to atherothrombosis. During this process, there is an important crosstalk between inflammatory mediators, the coagulation system and platelets (57). Activated platelets binds leucocytes, through P-selectin interaction with PSGL-1, forming platelet-leucocytes aggregates, which furthermore activates leucocytes. Neutrophils activation releases Neutrophil Extracellular Traps (NETs) which participate in thrombus formation (188). NETs consist of strands of deoxyribonucleic acid (DNA), to which primitive defense proteins may attach, derived from the neutrophil itself (e.g. myeloperoxidase), or from the circulation (e.g. tissue factor procoagulant). NETs can also entrap circulating platelets, fibrin strands and von Willebrand factor, forming a scaffold for clot formation. Interestingly, leucocytes taken from the culprit lesion during ACS are highly activated and readily undergo NETosis (189).

Inflammatory cytokines, increased during atherothrombosis, have been shown to affect platelet reactivity. IL-1 β enhance platelet prothrombotic functions via

activation of the p38 mitogen activated protein kinase (MAPK) signaling pathway (190). IL-6 is involved in the up-regulation of tissue factor that brings about initiation of coagulation. IL-8 promotes procoagulant activity, by triggering platelet activation (191). Two major inflammatory cytokines, IL-1 β and IL-6, increased during ACS have been studied as potential target for intervention to reduce ischemic events. The Canakinumab Anti-inflammatory Thrombosis Outcomes (CANTOS) Study has provided convincing evidence that an anti-inflammatory intervention with the IL-1 β monoclonal antibody, Canakinumab, reduces cardiovascular events (nonfatal myocardial infarction, nonfatal stroke, or cardiovascular death) after a previous MI. These effects were more importantly observed in patients with higher reduction in inflammation (hs-CRP levels) and was independent on LDLc levels (192). However, even if there was a higher incidence of fatal infection and sepsis with Canakinumab than with placebo, cancer mortality was significantly lower among patients assigned to receive Canakinumab than among those in the placebo group, a finding that is consistent with experimental data relating interleukin-1 to the progression and invasiveness of certain tumors, particularly lung cancer. Another alternative approach to inflammation inhibition, by low-dose methotrexate, has been tested in the CIRT (Cardiovascular Inflammation Reduction) trial. However, this trial was prematurely stopped because of adverse events (elevations in liver-enzyme levels, reductions in leukocyte counts and hematocrit levels, and a higher incidence of non-basal-cell skin cancers) without significant reduction in cardiovascular events (193). Colchicine, a classic anti-inflammatory drug used to treat gout, is also being tested for cardiovascular protection. In a preliminary open-label trial of 532 patients, low-dose colchicine showed promise for secondary prevention and, interestingly, the active intervention significantly reduced the primary endpoint of recurrent ACS,

cardiac arrest or non-embolic stroke. The recently published Colchicine Cardiovascular Outcomes Trial (COLCOT) randomized 4,745 patients to receive low dose colchicine (0.5mg daily) versus placebo, within 30 days after an ACS. Low-dose colchicine was effective at preventing major adverse cardiovascular events, particularly by reducing the incidence of stroke and urgent hospitalization for unstable angina leading to revascularization (194).

Another emerging molecule that modulates lipoprotein and inflammatory factors mediating atherosclerosis is the BET (bromodomain and extra-terminal) inhibitor, Apabetalone. BET inhibition is an epigenetic mechanism that can regulate disease-causing genes. Pooled analysis of short-term studies (ASSERT, ASSURE, SUSTAIN trials) demonstrated fewer cardiovascular events among patients treated with apabetalone, than among those treated with placebo (195). Cardiovascular benefit was mainly observed in diabetic patients and subjects with elevated hs-CRP levels. However, the BETonMACE trial (Phase 3 trial) failed to show significant reduction in cardiovascular events.

Taken together, these studies provide strong evidence of the role of inflammation in atherosclerosis/atherothrombosis but treatment strategies with these anti-inflammatory drugs remain to be determined.

1.3.3 Prognostic markers in CAD

Considering the residual risk of ischemic events and the need to identify patients needing more aggressive treatments, several studies have assessed markers for risk stratification.

1.3.3.1 Platelet markers

High-on treatment platelet reactivity is a major factor contributing to relapsing ischemic events in CAD patients. Platelet function testing in patients treated with aspirin and P2Y₁₂ inhibitors include: the PFA-100 analyzer, aggregation measurement by light transmittance aggregometry (LTA), VerifyNow, Multiplate Analyzer, platelet signaling pathways (VASP phosphorylation) and platelet released molecules (serum thromboxane B₂, urinary 11-dehydro thromboxane B₂) (see Table 4 below). Even if the Verify Now and Multiplate assays are the two functional tests most commonly used in clinical practice, identification of high risk patients and guiding antiplatelet therapies remain difficult because of their lack of precision.

Besides platelet function testing, platelet activation markers have also been studied to determine risk of ischemic events in patients. These tests include: surface receptors detection (P-selectin expression (CD62P), activated α IIb β 3, leucocyte-platelet aggregates, GPVI expression) and detection of released molecules upon platelet activation (platelet derived microvesicles and microRNA (miRNAs), soluble CD40 ligand). Among platelet activation indices, CD62P and platelet-leucocytes aggregates can be detected in high-risk patients compared to normal subjects (20,196,197). Circulating platelet-leucocyte aggregates seems to be a more sensitive marker of in vivo platelet activation than CD62P in CAD patients (20). Platelet derived microvesicles are known to increase with cardiovascular risk factors

hypercholesterolemia (198), hypertension (199), diabetes (200) and progression of atherosclerosis (201). Interestingly, these microparticles are increased in CAD and in ACS (202-204) including STEMI patients (205). Platelets are also a major source of circulating miRNAs and miRNA expression profiles correlate with platelet reactivity (206,207), raising the exciting possibility of novel therapeutic targets and disease biomarkers. Among miRNA, miR-223 is highly expressed in platelets and is associated with P2Y₁₂ surface expression, and thereby may regulate platelet function (206). Detection of plasma miR-223 can predict platelet response to P2Y₁₂ inhibitors. Decreased miR-223 has been observed in clopidogrel low responders (208,209). Furthermore, serum miR-223 levels were inversely associated with risk of future myocardial infarction and showed a stronger association with fatal than nonfatal events (210).

However, all these markers are of limited routine clinical use because their evaluation are time-consuming, requires flow-cytometer (platelet surface markers detection), real-time polymerase chain reaction (miRNA detection), an experienced staff and are expensive.

Tests and methods	Ability to monitor antiplatelet therapies		Predicts CV outcomes	Routine Clinical Use
	Aspirin	P2Y ₁₂ inhibitors		
PFA-100- depends on shear stress (vWF evaluation)	Yes	No	Yes	No
<u>Platelet Aggregation</u> A. Light transmittance Aggregometry (LTA) – study of platelet aggregation in PRP after stimulation with agonists B. VerifyNow- Study of platelet aggregation in whole blood after stimulation with agonists C. Multiplate Analyzer – study of platelet aggregation in whole blood (by impedance) after stimulation with agonists	Yes Yes Yes	Yes Yes Yes	Yes Yes Yes	No Yes* Yes*
<u>Platelet signaling pathways</u> Vasodilator-stimulated phosphoprotein (VASP) phosphorylation - flow cytometric detection in whole blood	No	Yes	Yes	No
<u>Platelet released molecules</u> A. Serum TXB ₂ , B. Urinary 11-dehydro TXB ₂	Yes Yes	No No	No Yes	No No

Table 4. Platelet function tests and monitoring in Coronary Artery Disease. PFA: platelet function analyzer, PRP: plasma-rich platelets, TXB₂: thromboxane B₂. * identifies the most commonly used in clinical practice.

1.3.3.2 Coagulation markers

Persistent thrombin generation is associated with increased risk of ischemic events. Thrombin assessment markers studied in CAD are D-dimers, thrombin anti-thrombin complex (TATc) and prothrombin fragment 1.2 (F1.2). *Merlini et al.* showed that during the acute phase of unstable angina and MI, prothrombin F1.2 was increased and remained high over the next 6 months (179). TATc and F1.2 have been described in many other studies to be associated with ACS and recurrent coronary events (180-183). An association between in vivo thrombin generation and severity of coronary vessel disease on the angiogram (211) or coronary calcification on the computed tomography scan (212) has been reported. However, even if, in general, persistent thrombin generation is detected in CAD patients with high risk of

ischemic events, its assessment on an individual basis scale to guide antithrombotic therapies remain limited. Other factors (cancer, infectious disease, chronic inflammation) influence thrombin generation and renders the interpretation of its markers difficult.

1.3.3.3 Inflammation markers

Increase in IL-1 β , IL-6, IL-18 (203,213,214), myeloperoxidase (215,216), metalloproteinase-9 (217), soluble CD40 ligand (218,219) are some of inflammatory biomarkers associated with acute coronary events. However, clinical application of these findings largely focuses on high-sensitivity C-reactive protein (hsCRP), a biomarker of innate immunity, produced by the liver, easily assessed in outpatient clinical settings. In primary prevention, numerous studies have evaluated the role of hsCRP as a determinant of vascular risk, and all reported positive findings. In addition to global assessment algorithms such as Framingham Risk score, HsCRP improved risk prediction (220-222). When evaluated as a group and despite highly diverse patient populations, each study showed the importance of inflammation beyond traditional approaches to risk prediction. The cut-off of HsCRP used in these studies were: <1mg/L (low), 1-3 mg/L (medium), >3mg/L (high). Even if there is an association between insulin resistance and HsCRP in type 2 diabetes mellitus, multiple studies show that hsCRP levels predict vascular events beyond the components of the metabolic syndrome or the presence of diabetes (223,224). In addition to primary prevention, multiple studies have found that hsCRP levels in stable patients after MI can predict recurrent infarction and cardiovascular death (225,226), a finding consistent with data from patients undergoing elective PCI or surgical revascularization. However, in the guidelines, routine evaluation of HsCRP as a risk biomarker is a class IIb recommendation because of its lack of

precision (narrow diagnostic window), lack of specificity, lack of specific therapeutic strategies, higher costs (227,228).

1.4 The concept of platelet-lipid interplay in coronary artery disease

Several lines of evidence suggest that the hyperlipidemic environment increases the risk of thrombosis by modulating platelet biogenesis and reactivity. Hyperlipidemia is associated with increased platelet activation and agonist induced platelet reactivity (229,230). Multiple mechanisms contribute to hyperlipidemia-mediated platelet activation but the platelet CD36 receptor-signaling pathway seems to play a major role. OxLDL or oxidized phospholipids, which are increased in hyperlipidemia, serve as ligands for platelet CD36 and activate platelets (231-236). Recent data clearly demonstrate that in the clinical setting of ACS, levels of platelet-bound oxLDL are increased, with a significantly altered intraplatelet lipidome (increased oxidized lipid metabolites, ceramides, sphingomyelins, cholesteryl esters, phospholipase metabolites, acylcarnitines, triglycerides), which influence platelet prothrombotic functions (88).

1.4.1 Platelet CD36 receptor and its ligands

In mononuclear phagocytes, endothelial cells, SMCs and platelets, CD36 is a multifunctional membrane receptor that serves as a scavenger receptor for oxidized phospholipids, apoptotic cells and certain microbial pathogens. In macrophages, the role of CD36 scavenger receptor and its interaction with oxLDL has been largely studied during atherosclerotic development. However, in platelets, in spite of its abundant presence, the function of CD36 has remained unclear for a long time.

The protein CD36, or glycoprotein (GP) IV, is a major platelet protein with 10 000–25 000 copies expressed on the platelet surface and additional copies present in the α -granules (86,87). CD36 has two short N- and C-terminal cytoplasmic domains and a large glycosylated extracellular domain. Previous studies reported that CD36

receptor was a primary receptor for platelet-collagen adhesion (237) but it was shown later on that patients with CD36-deficient platelets display normal platelet adhesion to collagen (238,239). Ligands for platelet CD36 are thrombospondin-1 (TSP1) present in alpha granules, oxidized low-density lipoproteins (oxLDL), oxidized forms of phospholipids or oxPC_{CD36} and long-chain fatty acids. The specificity of these ligands to CD36 has been demonstrated by blocking the CD36 receptor with a monoclonal antibody and by using platelets from CD36 null mice or CD36-deficient human subjects (231). When platelets are incubated with oxLDL at concentrations likely to be present in patients with atherosclerosis, they become more sensitive to activation by low doses of classic agonists, such as ADP. This can be detected as an increase in platelet aggregation responses and by increased expression of platelet activation markers, such as P-selectin and integrin $\alpha\text{IIb}\beta 3$ activation. These effects were not seen in CD36 null platelets (231).

1.4.2 CD36 signaling mediates platelet activation and thrombosis

The active contribution of platelet CD36 signaling to platelet-mediated thrombus formation was studied in ApoE and CD36 null strains. The ApoE null mice fed with high-fat “western” diet, a commonly used model to study hyperlipidemia and atherosclerosis, displays enhanced coagulability and prothrombotic state (shorter tail bleeding time and more rapid occlusive thrombi after vascular injury by ferric chloride) (240). However, the genetic deletion of CD36 in ApoE null strain “rescued” the prothrombotic phenotype (tail bleeding time and vascular injury challenge by ferric chloride were comparable with wild-type strains). Moreover, when platelets from wild type mice, but not CD36 null mice, were washed and re-suspended in plasma from the high fat diet-fed apoE null mice, they became more sensitive to activation by low doses of ADP (231). While these studies clearly support the link

between hyperlipidemia and prothrombotic phenotype *in vivo*, the signaling pathways activated downstream CD36 for platelet activation, remained largely unexplored.

Recent studies showed that the signaling pathway in platelets activated by CD36 is similar to that seen in macrophages (241). OxLDL induces recruitment of specific members of the src family kinases, Fyn and Lyn to CD36 with preferential accumulation of the activated, phosphorylated form of Fyn. Similar to other cell types, oxLDL interaction with CD36 induced activation of different members of MAPK (ErK2, ErK5, p38, JNK). OxLDL-induced JNK2 phosphorylation is mediated by src family kinases (241). Importantly, activation of the MAPK p38 by oxLDL has been described as a key mechanism promoting platelet activation, thereby triggering ischemic events in the setting of CAD. These recent data provide a link between proprotein convertase subtilisin/kexin 9 (PCSK9) levels, increased oxLDL levels, platelet activation and ischemic events (242,243). PCSK9 activity has an important influence on LDL metabolism, and its regulation is complex. PCSK9 is expressed mainly in the liver, where it regulates LDL receptor and LDL cholesterol levels. Gain-of-function mutations of the gene encoding PCSK9 have been clearly associated with hypercholesterolemia and an increased risk of coronary artery disease (244,245). Increase in PCSK9 levels is associated with increased LDL levels and consequent oxLDL levels. The binding of oxLDL to CD36 on platelets activates cytosolic phospholipase A2 (cPLA2) through the p38 MAPK pathway. cPLA2 releases arachidonic acid (AA) from membrane phospholipids that is subsequently converted to TxA₂ by cyclooxygenase (COX)-1/thromboxane synthase activity. TxA₂ further stimulates platelet activation and recruits additional platelets to the thrombus. The correlation observed in this study between PCSK9 levels and platelet

activation marker, urinary 11-dehydro thromboxane B₂ (resulting from the conversion of unstable TxA₂ to TxB₂), provide evidence of a platelet-lipid interplay in the setting of CAD (242). Besides this interaction, oxLDL binds to endothelial cells and leucocytes, promoting the synthesis of adhesion and inflammatory molecules (IL-2, IL-6, IL-8, TNFα). Inflammation is also supported by P-selectin expression on activated platelet surface (PSGL-1 mediated platelet interaction with leucocytes). Altogether, enhanced inflammation induced by oxLDL leads to increased coagulation cascade activity, thrombin generation and fibrin formation. Activated platelets and coagulation by oxLDL contribute to increase risk of ischemic events (243).

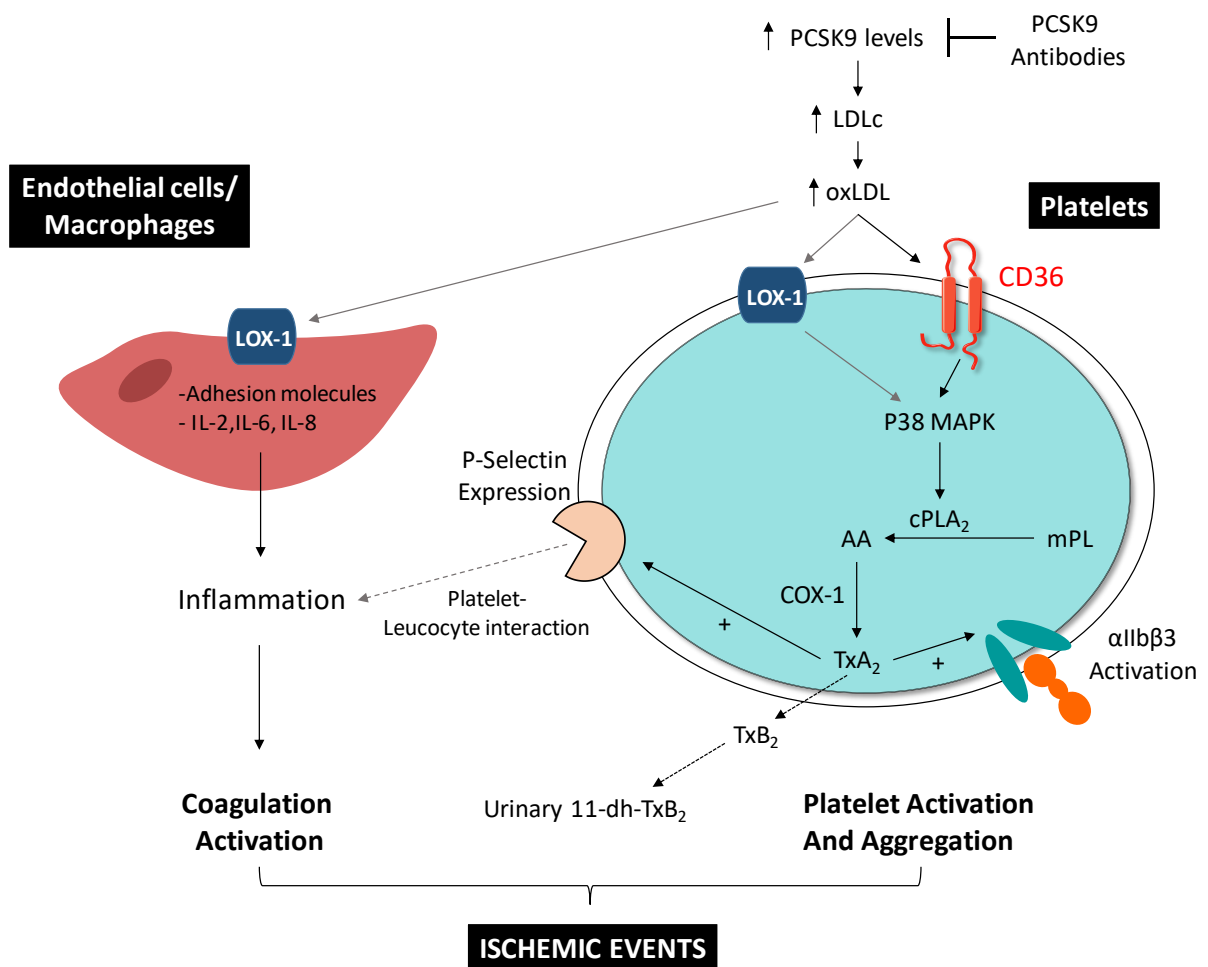


Figure 9: Interaction between oxidized LDL, platelets, endothelial cells and macrophages. Increased PCSK9 levels contributes to elevated LDL and oxLDL. OxLDL binding to platelets activates the cytosolic

phospholipase A₂ (cPLA₂) via the P38 mitogen-activated protein kinase (MAPK) pathway. cPLA₂ releases arachidonic acid (AA) from membrane phospholipids (mPL), which is transformed to thromboxane A₂ by the cyclo-oxygenase 1 (COX-1). TxA₂ promotes platelet activation and aggregation. The unstable TxA₂ is rapidly converted to TxB₂, which is then transformed to urinary 11-dh-TxB₂ (measurable biomarker). OxLDL also activates endothelial cells and macrophages, induces adhesion molecules and inflammatory cytokines that stimulates the coagulation pathway.

AA: arachidonic acid, COX-1: cyclo-oxygenase 1, cPLA₂: cytosolic phospholipase A₂, IL: interleukine, LOX-1: Lectin-like oxidized low-density lipoprotein receptor-1, mPL: membrane phospholipids, PCSK9: proprotein convertase subtilisin/kexin 9, TxA₂: thromboxane A₂, TxB₂: thromboxane B₂

Two other important signaling pathways downstream CD36 have also been described in platelets, contributing to platelet activation. First, Wraith et al. demonstrate that oxLDL influence platelet cytoskeleton remodeling during platelet activation by the phosphorylation of myosin light chain (MLC). The binding of oxLDL on platelet CD36 activates SyK and phospholipase Cy2, inducing an increase in calcium levels and subsequent activation of the myosin light chain kinase (MLCK) by a calcium-calmodulin dependent mechanism. In addition, simultaneous SFK activation stimulates the Rho kinase (ROCK) thereby inhibiting the MLC phosphatase (MLCP). Activation of these pathways amplify MLC phosphorylation, promoting platelet shape change, granules secretion and aggregation (233). Second, the increase in calcium levels leads to the activation of PKC and the NADPH oxidase 2 (NOX2), and ROS production. While ROS has been shown to activate the MAPK Erk5 pathway promoting pro-coagulant platelets (increase PS exposure) (236), they also inhibit the NO/GMPc/PKG pathway, which plays a role in limiting platelet activation (232). These different pathways contributing to platelet activation following platelet-lipid interaction are represented in Figure 10.

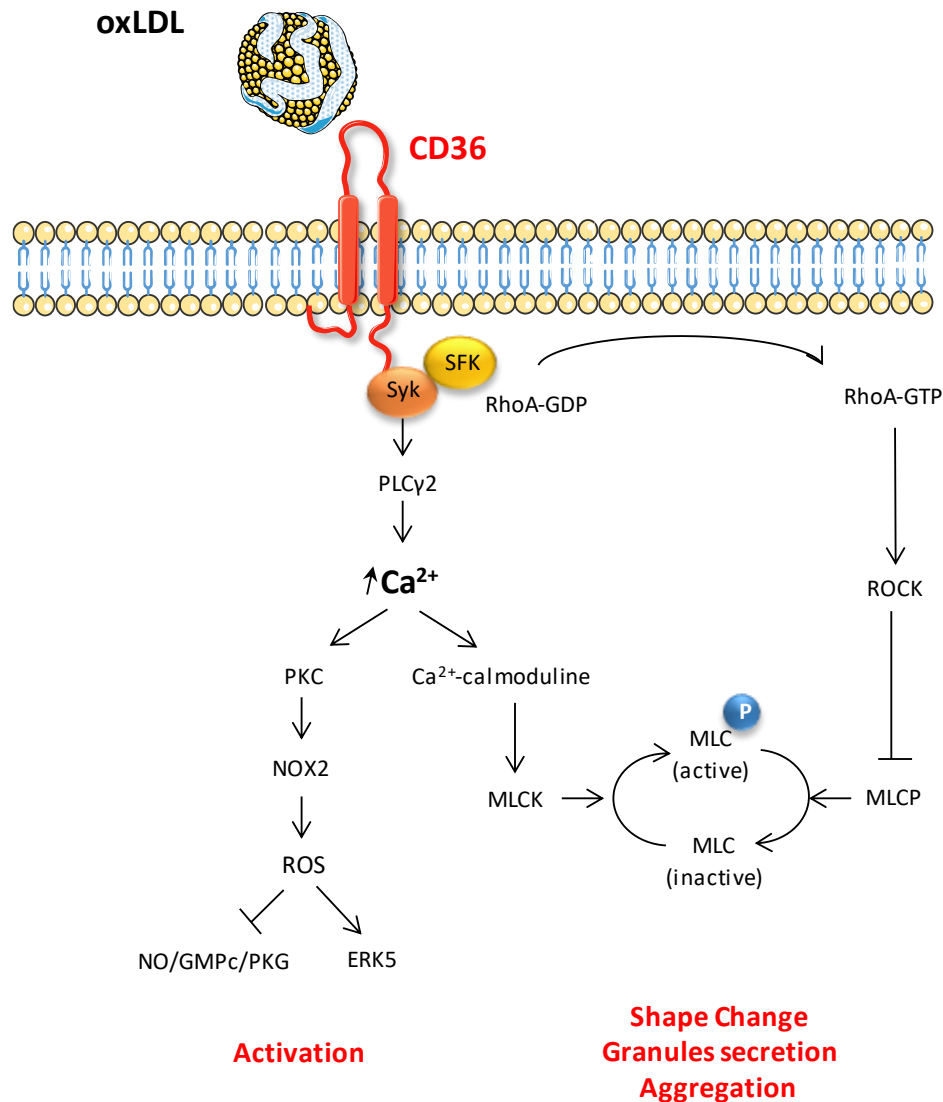


Figure 10. Downstream signaling pathways triggered by oxLDL binding to CD36. Syk and SFK are activated upon binding of oxLDL to CD36, which leads to the inhibition of MLCP, therefore increasing MLC phosphorylation (necessary for platelet shape change and activation). Simultaneously, Syk stimulates the MLCK, promoting MLC phosphorylation, and activates PKC and NOX2, ROS production via a calcium-dependent mechanism. ROS production inhibit the NO/GMPc/PKG pathway and activate the ERK5 MAPK pathway, responsible for platelet hyperreactivity. SKF: src family of kinase; PLCγ2: phospholipase Cγ2; PKC: protein kinase C; NOX2: NADPH oxidase 2; ROS: reactive oxygen species; ERK5: extracellular signal-regulated kinase 5; NO: nitrogen monoxide; cGMP: cyclic GMP; PKG: protein kinase G; MLCK: myosin light chain kinase; MLC: myosin light chain; ROCK: Rho-associated protein kinase; MLCP: myosin light chain phosphatase.

However, controversial data showed that oxLDL fail to promote platelet aggregation in platelet rich plasma (PRP) (246) and that the influence of oxLDL on platelet reactivity depends on the levels of oxidation of LDL (247). This variability of platelet responses to oxLDL can also be explained by the wide differences in CD36 expression levels between individuals. Analysis of more than 500 subjects revealed

that CD36 expression levels were consistent in individual donors over time but varied widely among donors (200-14 000 molecules per platelet). There is a high correlation between platelet activation responses to oxLDL and CD36 expression levels. Therefore, the inheritance of specific genetic polymorphism of platelet CD36 influence platelet responses to oxLDL and may affect thrombotic risk (248).

1.4.3 Platelet lipidomics in coronary artery disease

Since the mid 2000's, the advent of 'omics', driven by the development of sensitive mass liquid chromatography- mass spectrometry, have largely contributed to improve our knowledge on platelet lipid content. The new advances in technology allow a nearly complete characterization and quantitation of all lipid species. However, the remaining challenge is to establish the functional significance of all these lipids in platelets and how their regulation may influence platelet functions.

Recently, lipid profiling in platelets, assessed by liquid chromatography hyphenated to high-resolution mass spectrometry, has revealed an altered platelet lipidome in CAD patients compared to healthy subjects. There was enhanced intraplatelet-oxidized phospholipids, cholesteryl esters (CE), sphingomyelin (SM), ceramides (Cer), di (DAG) - and triacylglycerols (TG), acylcarnitines in CAD patients compared with age-matched controls. Increased platelet lipid species were even higher in patients presenting with STEMI compared to match controls. Elevated plasma Cer levels, which has been shown to have prognostic significance on cardiovascular mortality (249), were significantly up-regulated in CAD platelets. Elevated platelet SM in CAD patients suggest that catabolism of SM is altered, probably reflecting platelet activation status. Two specific acylcarnitines showed significant increase in STEMI patients compared to S-CAD patients, which can be an additional peripheral

biomarker for risk stratification (88). Collectively, these recent data highlight the potential of lipidomics in the improvement of our knowledge on platelet lipid metabolism and identify new risk stratification markers.

1.5 Overview of the platelet lipid metabolism

Fatty acids are generated either via *de novo* synthesis or by phospholipids cleavage.

1.5.1 De novo lipogenesis

Like other cell types, platelets contain all the essential machinery for synthesizing fatty acids (250,251). *De novo* lipogenesis can be sub-divided into three steps; (1) palmitate synthesis from acetyl-CoA, (2) palmitate elongation and (3) palmitate desaturation yielding oleic acid, considered as the end product.

(1) Palmitate synthesis occurs in the cytosol and consists of a cycle of elongation steps that starts with acetyl-CoA on which two-carbon units from malonyl-CoA are added at each cycle, which ends when the fatty acid has reached 16 carbons (C 16, palmitate).

The carboxylation of acetyl-CoA into malonyl-CoA is the first rate-limiting step for the *de novo* lipogenesis, which is catalyzed by Acetyl-CoA Carboxylase (ACC) (structure, isoforms and regulation of ACC activity are detailed in section 1.6). Accordingly, several studies have established that ACC is a key regulator of the *de novo* lipogenesis in cells. Specific pharmacological inhibition of liver ACC, by the MK-4047, leads to reduced fatty acid synthesis, particularly liver triglycerides (TGs). These results were confirmed in the liver specific ACC1 and ACC2 knock out mice. However, if liver TGs content was decreased, there was an increased plasma TGs level, which was explained by the activation of sterol regulatory element binding protein (SREBP) and VLDL secretion. In cancer cells, chronic ACC inhibition decreases *de novo* fatty acid synthesis, leading to a reduction in fatty acids containing 14 to 18 carbons (252) and mostly TGs containing lipid pools (253). ACC inhibitors have also been studied in the management of metabolic syndrome and

type 2 diabetes. Even if in general fatty acid synthesis is reduced, the benefit of ACC inhibition on metabolic endpoints and the future of ACC inhibitors in clinical treatment of metabolic disorders are uncertain.

(2) Palmitate elongation

Palmitate can be elongated to form long- or very-long-chain fatty acids. The elongation reaction involves elongases, which are localized on the cytosolic side of the endoplasmic reticulum

(3) Palmitate desaturation

Fatty acids of varying carbon lengths can be converted to unsaturated products by fatty acyl-CoA desaturase.

1.5.2 Lipids: major actors for platelet function

Lipids fulfill three primary roles in platelets: signaling, structural roles and energy storage. The platelet lipidome and respective functional roles are described in Figure 11 below.

1.5.2.1 Lipids for signaling role

The majority of lipids involved in platelet signaling are cleaved from membrane phospholipids. In this context, phospholipases (PLA₂ and PLC) are essential for generating phospholipid-derived signaling molecules. However, there is an overlap between the use of certain lipid classes for energy production and signaling roles (e.g. eicosanoids). Among lipid-derived signaling molecules, DAG, TXA₂ and oxidized phospholipids are the major lipids playing an essential role in platelet activation.

1.5.2.1.1 Thromboxane A₂

TXA₂ is abundantly produced and released following platelet activation. TXA₂ derives from AA-containing phospholipids, which are cleaved by PLA₂ upon platelet activation by various agonists, described in section 1.3.2.1. TXA₂ potentiates platelet activation by binding to its platelet receptor TP, which activates a signaling cascade that ultimately leads to dense granules secretion (254). As a consequence, a defect in TXA₂ generation results in impaired dense granule secretion (255).

1.5.2.1.2 1,2-diacylglycerol and Phosphatidylinositol-3,4,5-trisphosphate

DAG, generated from PIP₂ by PLC cleavage in response to platelet stimulation, is a master regulator of platelet activation by activating several PKC isoforms. The study of PKC substrates phosphorylation state (activation) is a read-out of platelet activation.

Phosphatidylinositol-3,4,5-trisphosphate (PIP₃) is a phosphatidylinositide that represents an essential bioactive lipid species generated from PIP₂ phosphorylation by PI3K, following GPCRs activation. It binds to a region of ≈ 120 amino acids, known as a pleckstrin homology (PH) domain, which is present on a multitude of proteins involved in cytoskeleton remodeling, signaling, aggregation, adhesion, integrin activation and Ca²⁺ mobilization (10). PIP₃ recruits these proteins to the plasma membrane and contributes to their activation.

1.5.2.1.3 Oxidized phospholipids

Human platelets generate significant quantities of oxidized phospholipids (oxPL) via enzymatic mechanisms involving cPLA₂ -dependent AA generation, AA oxidation by 12-lipoxygenase or COX-1, and its re-esterification by an acyl-CoA ligase, forming

hydroxy-eicosatetraenoic (HETE)-PC or HETE-PE (256). These oxPL have been shown to promote coagulation factors binding, via alteration of membrane charge, and therefore to enhance thrombin generation (256).

1.5.2.2 Lipids for structural role

Membrane phospholipids play a central structural role in almost all steps of platelet activation. They do not only serve as a scaffold for shape change and spreading, but are also required for platelet secretion, a process requiring the fusion of two membranes, and finally for appropriate signaling of receptors, which experience lateral movements and clustering in the plasma membrane, a process mediated by lipid rafts.

1.5.2.2.1 Role in adhesion and spreading

When platelets adhere on the injured endothelium, they undergo irreversible morphological changes such as spreading, with the formation of lamellipodia and filopodia, which do not only require cytoskeletal remodeling but also an important lipid membrane reorganization to allow increase in platelet surface area.

1.5.2.2.2 Role in platelet secretion

Phospholipids have been described to modulate platelet secretion in three ways. First of all, they serve as a matrix for the fusion of two opposing membranes, secondly their nature influences membrane fluidity and curvature and thirdly they provide binding sites for exocytic machinery proteins.

Granule secretion occurs via the formation of a fusion pore between two membranes. Clearly the lipid composition of the two opposing membranes influences the secretion process. It has been shown that exocytosis mainly occurs

in cholesterol and sphingolipid-rich microdomains, called lipid rafts (257). Indeed, they do not only stabilize the fusion pore but they also allow the concentration of specific proteins involved in platelet secretion (t-SNAREs, SNAP-23 and syntaxin-11).

Given its degree of saturation, the length of its fatty acid chains and the nature of its polar headgroup, each phospholipid adopts a specific geometry and a preferential curvature that influence fusion pore formation and stability. Conical lipids (larger tail), such as PE, tend to form negative membrane curvature while inverted conical lipids (larger head), such as PS, preferentially support positive curvature. The fusion pore is an extremely curved structure in which lipids associated with positive curvature assemble on the outer leaflet whereas negatively curved lipids are present in the inner leaflet. Platelets incubation with PS increases fusion pore stability, probably by providing additional lipids supporting positive curvature (258).

1.5.2.2.3 Role of lipid rafts for receptor signaling

Lipid rafts are very small membrane domains enriched in sphingolipids, cholesterol and proteins, which are involved in signaling due to their association with GPCRs and their ability to cluster signaling proteins. Therefore, platelet rafts are critical for mediating platelet activation in response to agonists. They have been shown to be essential for GPIb-IX-V, GPVI, P2Y₁₂ and TP signaling as well as for clot retraction as they constitute a platform for fibrin concentration. Thrombin or collagen platelet stimulation induces cholesterol rearrangement, the lateral movements of lipid rafts and their fusion (259). Since these domains are enriched in many signaling molecules, this lateral clustering has a great impact on receptor activation. Among them, GPVI signaling has been shown by several authors to involve lipid rafts. Lipid

rafts have been shown to constitute a platform connecting extracellular fibrin and intracellular actomyosin cytoskeleton via $\alpha\text{IIb}\beta_3$, which promotes “outside-in” signaling and clot retraction (260).

The Platelet Lipidome

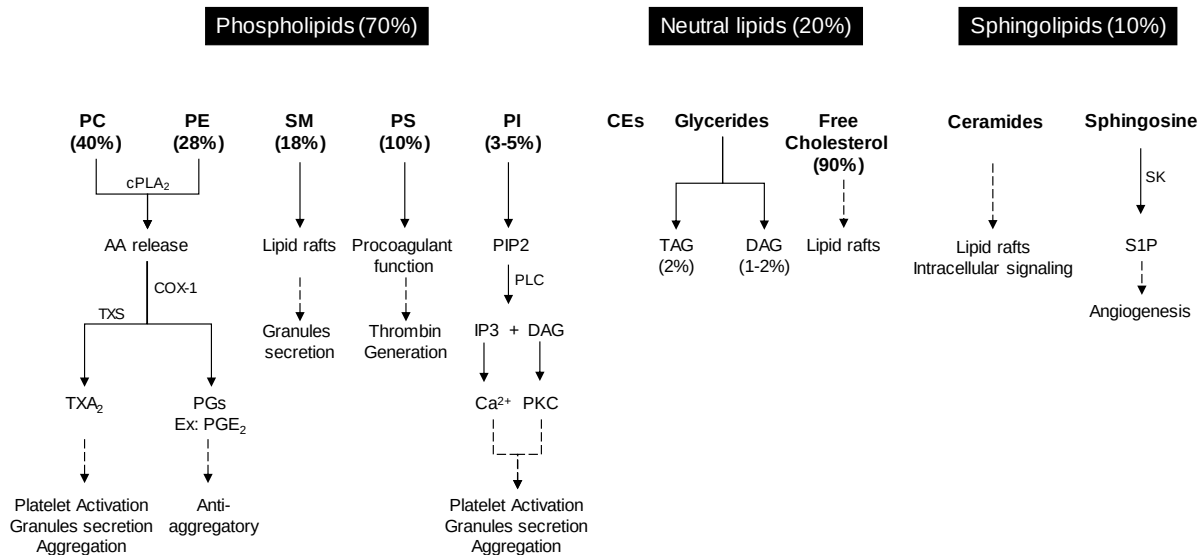


Figure 11. Lipid characterization in platelets and their respective functional role. Phospholipids (PL) represents more than 70% of lipid species in platelets. Among PL, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the most abundant. They provide arachidonic acid (AA) upon cPLA₂ (cytosolic phospholipase A₂) activation, which generate a key secondary mediator, thromboxane A₂ (TXA₂) necessary for platelet activation and thrombus growth. Phosphatidylserine (PS) exposure on platelet surface upon activation, anchor coagulation factors necessary for thrombin formation. Phosphatidylinositides (PI), via phosphoinositol (4,5)P₂ (PIP₂), generate Inositol (1,4,5)P₃ that triggers calcium release and di-acylglycerol (DAG) that activates protein kinase C (PKC). Among neutral lipids, free cholesterol represents more than 90%, and is involved in lipid rafts during platelet activation.

AA: arachidonic acid, CEs: cholesterol esters, COX-1: cyclo-oxygenase 1, cPLA₂: cytosolic phospholipase A₂, DAG: di-acylglycerol, IP₃: inositol (1,4,5) trisphosphate, PC: phosphatidylcholine, PE: phosphatidylethanolamine, PG: prostaglandins, PI: phosphoinositide, PIP₂: phosphoinositide (4,5)bisphosphate, PKC: protein kinase C, PLC: phospholipase C, PS: phosphatidylserine, S1P: sphingosine 1 phosphate, SK: sphingokinase, SM: sphingomyelin, TAG: tri-acylglycerol, TXA₂: thromboxane A₂, TXS: thromboxane synthase.

1.5.2.3 Lipids as energetic substrate for platelets

The transition of platelets from a quiescent to an activated state requires drastic changes in ATP availability (27-29). The major metabolic pathways in platelets for ATP production are glycolysis, glycogenolysis and mitochondrial oxidative phosphorylation. Among oxidized substrates, lipids, glutamine and glucose

contribute for 37%, 7% and 4% respectively, to total ATP production under basal conditions (261).

Modern technique, measuring oxygen consumption (OCR) and extracellular acidification (ECAR) rate after sequential addition of mitochondrial inhibitors with the extracellular flux (XF) analyzer (Seahorse metabolic flux assay), has brought a better comprehension of platelet bioenergetics regulation. The XF analyzer measures OCR, which reflects mitochondrial respiration as well as the change in pH (ECAR), which can be related to glycolysis (262-264). Under basal state, both oxidative phosphorylation and glycolysis play a role in energy production in platelets (265-267). Upon activation and aggregation, mitochondrial fatty acid oxidation and glycolysis are increased but their distinct contribution in energy production has been largely debated. Some studies demonstrated that mitochondrial function is essential to provide the ATP necessary for platelet aggregation, by inhibiting components of the mitochondrial respiratory chain (268-270). However, other reports have stated that glycolysis is the major source of ATP in driving platelet aggregation, and that mitochondria play only a minor role (29,267,271). More recent study with the XF analyzer have highlighted the concept of metabolic plasticity, both in resting and activated platelets, where inhibition of mitochondrial oxidative phosphorylation can be compensated by enhanced glycolysis (272,273).

1.5.2.3.1 Platelet metabolism under resting condition

Under basal state, ATP is mainly needed to control calcium homeostasis in platelets. The sarcoendoplasmic reticulum calcium ATPase (SERCA) uses ATP to pump cytosolic calcium in the dense tubular system, maintaining a calcium gradient between the cytosol (100nM) and the intracellular stores (1mM) (13). Calcium

homeostasis is also maintained by the calcium-ATPases on the plasma membrane, which transport calcium out of the platelet in the presence of ATP (274). Regulation of cytosolic calcium level is a crucial phenomenon in order to prevent inadvertent platelet activation.

In resting platelets, more than 50% of ATP synthesis comes from mitochondrial oxidative phosphorylation (266). Among oxidized substrates, lipids are a major metabolic fuel for platelets (261). In contrast to other blood cells (neutrophils, monocytes and lymphocytes), platelets have the highest basal OCR, suggesting that they are mostly oxidative. Upon inhibition of mitochondrial ATP synthase by oligomycin, platelets display the highest shift to glycolytic metabolism as illustrated by the clear increase in ECAR (266). The small size to high surface area ratio renders platelets one of the most metabolically active circulating “cells”. Platelets have therefore significant metabolic fuels and flexibility, which are necessary when they are activated.

1.5.2.3.2 Platelet bioenergetics and the concept of metabolic plasticity during activation

Glycolysis and oxidative phosphorylation are the two active metabolic pathways in platelets. In thrombin-activated platelets, there is a rapid stimulation of both metabolic pathways. However, inhibition of fatty acid oxidation or glutamine depletion, which are the essential fuels for oxidative phosphorylation, lead to a drastic compensatory increase in glycolytic metabolism. Interestingly, the combined effect of fatty acid inhibition and glutamine depletion was not sufficient to prevent platelet aggregation, while inhibitors of glycolysis decrease aggregation by 50% (272). In line with this observation, previous studies demonstrated that inhibition of glucose metabolism (inhibition of glycolysis, glycogenolysis and glucose oxidation)

prevent platelet activation (267,270). These results highlight the concept of metabolic plasticity and provide evidence of the crucial role of glycolysis for platelet activation and aggregation.

1.5.2.3.3 Role of glucose metabolism on platelet functions

While the role of glucose metabolism during platelet activation has been largely studied on washed platelets *in vitro*, recent *in vivo* data reinforce this evidence. Glucose enters platelets via the facilitative glucose transporter 1 (GLUT 1) and glucose transporter 3 (GLUT 3) (275,276). Glucose uptake allows platelet to constitute an important glycogen store, which is rapidly degraded by glycogen phosphorylase to provide energy for platelet activation (277,278). While GLUT 3 seems to be the major glucose transporter under thrombin stimulation (277), deletion of both GLUT transporters (DKO) abolished glucose uptake and increased OCR under basal and thrombin-stimulated condition. Decreased ECAR, lactic acid release and glycogen content observed in DKO platelets demonstrate that glucose uptake and metabolism are largely abolished. In the absence of glucose, DKO platelets showed increase AMP to ATP ratio and thereby activation of the AMPK-ACC signaling, suggesting metabolic stress (26).

Importantly, altered glucose metabolism has an impact on platelet thrombotic phenotype. DKO platelets display decreased alpha granule number and impaired secretion that account for reduced platelet activation. Calcium homeostasis is also affected when glucose metabolism is altered in platelets. This has an impact on platelet prothrombotic functions. Tail-bleeding time was longer in DKO mice. Time to arterial occlusion (arterial thrombosis induced by ferric chloride) was significantly increased in DKO mice. Impaired platelet glucose metabolism reduces platelet

activation and thrombus formation *in vivo*, thereby supporting the link between metabolism and platelet function.

1.5.2.3.4 Role of lipid metabolism on platelet functions

Platelets have all the necessary machinery for the *de novo* lipid synthesis (250,251). Lipids found in platelets come from either, the *de novo* fatty acid synthesis (endogenous) (250,251) or from the circulation (exogenous) (88). Interestingly, exogenous lipids do not contribute to platelet basal and thrombin-stimulated oxygen consumption; oxidation of endogenous lipids is the main ATP provider (272). Lipids enter mitochondria via the carnitine palmitoyl transferase 1 (CPT1), where they are oxidized to generate acetyl-CoA, NADH and FADH₂ which act as substrates for the mitochondrial respiratory chain to produce ATP. Treatment of platelets with etomoxir, a CPT1 inhibitor, decreases platelet basal and thrombin-stimulated mitochondrial respiration (272). However, it does not influence aggregation (272) while slightly increasing PS exposure and procoagulant activity (279).

Taken together, these data illustrate the crucial role of lipid and glucose metabolism for platelet activation. While stimulation of glycolysis is critical for platelet activation and aggregation, lipids constitute a major metabolic fuel for platelets under basal state. The production of signaling molecules and platelet shape change during activation are highly dependent on lipid metabolism. The link between metabolism and platelet function under basal and activated state is represented in Figure 11 below. The role of ACC in regulating lipid metabolism and its impact on platelet function have been established by our group and illustrated in the results section (part 2).

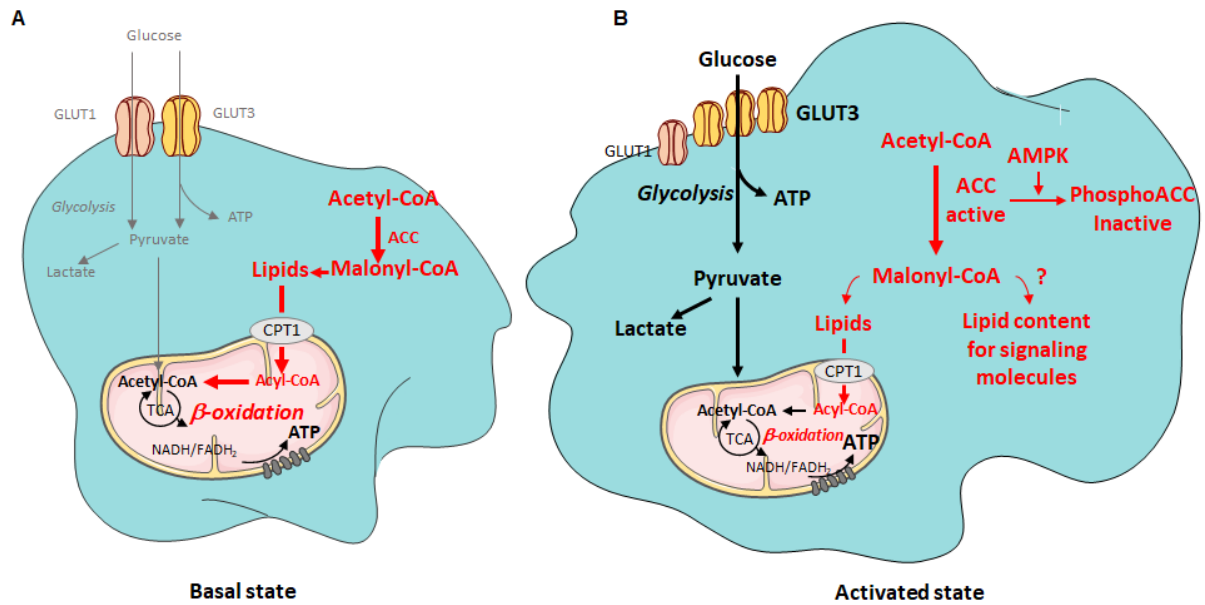


Figure 12. Role of glucose and lipid metabolism on platelet bioenergetics and function.

(A) Under basal state, platelets are more oxidative (lipid metabolism for energy production represented in red). (B) During platelet activation, glycolysis is massively stimulated for ATP production. While beta-oxidation is also increased during platelet activation, the role of lipids for energy production can be compensated by enhanced glycolysis if lipid metabolism is inhibited (concept of metabolic plasticity). Lipids represent an important source of signaling molecules during activation, which can be affected by the AMPK-ACC signaling.

ACC: Acetyl-CoA Carboxylase, AMPK: adenosine monophosphate-activated protein kinase, ATP: adenosine triphosphate, CPT-1: carnitine palmitoyl transferase 1, GLUT: glucose transporter.

1.6 Acetyl-CoA Carboxylase (ACC): a key regulator of platelet lipogenesis

ACC catalyzes the carboxylation of acetyl-CoA to malonyl-CoA, the first rate-limiting step of the *de novo* lipogenesis. Malonyl-CoA is a powerful CPTI inhibitor, which controls fatty acids entry in the mitochondria for their oxidation, providing ACC the dual role of being both involved in lipid synthesis and oxidation. Given the key role of lipids in platelets, it is tempting to speculate that regulation of the enzymatic activity of ACC has an impact on platelet functions. However, even if ACC has been described in platelets (250), its exact role remains unexplored.

1.6.1 Acetyl-CoA Carboxylase structure, isoforms and role

ACC belongs to the family of biotin-dependent carboxylases which are characterized by a common structure consisting of three subunits: the biotin carboxylase (BC), the carboxyltransferase (CT), and the biotin-carboxyl carrier protein (BCCP), giving it a high molecular weight of ~250 kDa. The BC and CT domains are responsible for carrying out the enzymatic activity of ACC while BCCP has a crucial structural role since it carries a lysine residue that forms an amide bond with biotin, an essential co-factor for ACC catalysis. A particular characteristic of ACC is that it is one of the most highly biotinylated protein.

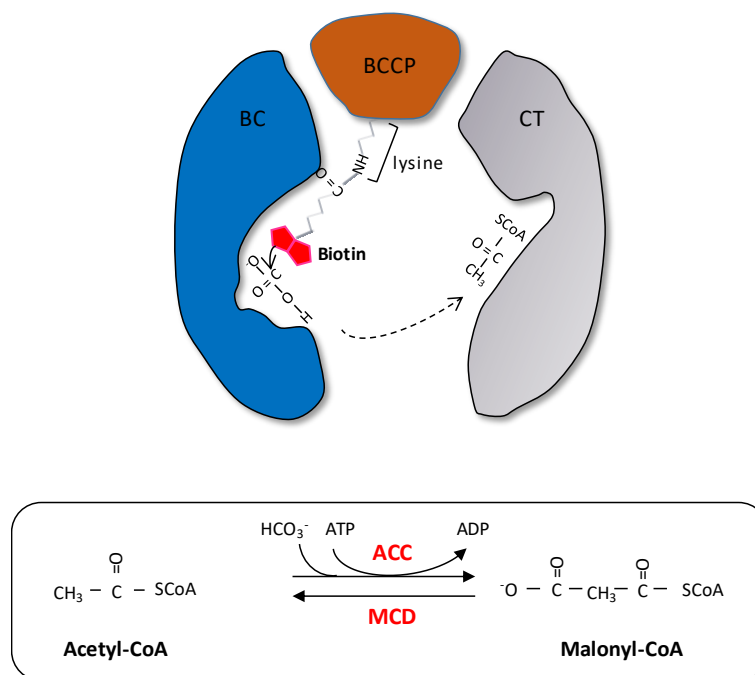


Figure 13. Acetyl-CoA Carboxylase structure. ACC catalyzes the transformation of Acetyl-CoA to Malonyl-CoA. It is composed of three subunits: the BC and CT domains (for enzymatic reactions) and the BCCP domain (structural role). BCCP binds biotin via a lysine residue, essential for the carboxylation of Acetyl-CoA to Malonyl-CoA. The decarboxylation of malonyl-CoA into acetyl-CoA is catalyzed by MCD.

ACC: Acetyl-CoA Carboxylase, BC: biotin carboxylase, CT: carboxyltransferase, BCCP: biotin-carboxyl carrier protein, MCD: malonyl-CoA decarboxylase

Mammals express two ACC isoforms, known as ACC1 and ACC2, which have different tissue distributions, cellular localizations and roles, even though they catalyze the same reaction. ACC1 is cytoplasmic and mainly expressed in lipogenic tissues such as the liver and adipose tissue. On the other hand, ACC2 is more common in oxidative tissues such as the heart and skeletal muscle and is associated with the outer mitochondrial membrane. Indeed, as compared to ACC1, ACC2 has an extra N-terminal of 140 residues, of which the 20 first amino acids are highly hydrophobic, allowing its attachment to the mitochondrial membrane (280). In view of these different cellular localizations, it has been suggested that two different pools of malonyl-CoA co-exist and it was therefore shown that the two isoforms have distinct roles (281,282). While malonyl-CoA produced from ACC1 is mostly involved

in *de novo* lipogenesis (281), malonyl-CoA generated from ACC2 inhibits CPTI (282), the essential enzyme allowing fatty acids entry in the mitochondria for their oxidation.

1.6.2 Regulation of Acetyl-CoA carboxylase activity

ACC activity, like many metabolic enzymes, is regulated both by allosteric and covalent (phosphorylation) modifications. While citrate is the most important allosteric regulator activating the enzyme by promoting its polymerization (283), fatty acid is an allosteric inhibitor of ACC. Covalent modifications by phosphorylation inhibit the enzyme. Both ACC isoforms contain up to eight phosphorylated residues. Among these, Ser79, Ser1200, Ser1215, all located in the N-terminal extension of the BC domain, have been shown to be phosphorylated by the AMPK and Ser77 and Ser1200 by the PKA (284). Of these phosphorylated residues, only Ser77 and Ser79 phosphorylation lead to ACC inhibition (285). Ser79 residue is found on ACC1 isoform while its corresponding residue is Ser212 on ACC2 (286). While PKA-mediated Ser77 phosphorylation only induces modest changes of ACC activity (285), AMPK-induced ACC inhibition is dependent on Ser79 phosphorylation. Accordingly, a single mutation of Ser79 to alanine totally prevents ACC inactivation by AMPK (287). Therefore, AMPK-dependent ACC inactivation is mediated both by Ser79 and Ser212 phosphorylation on ACC1 and ACC2, respectively.

Protein phosphatase-2A (PP-2A) orchestrates ACC dephosphorylation, thereby promoting ACC activation. In the liver, glutamate and bivalent cations, Mn^{2+} and Mg^{2+} , are the major actors stimulating the ACC phosphatase activity (288). Incubation of hepatocytes with amino acids activates ACC by a phosphatase-dependent mechanism but did not modify AMPK activation, which in turn can still

phosphorylates and inhibits ACC. Of note, the Serine 79 phosphorylation site of ACC (AMPK phosphorylation site) is specifically dephosphorylated by the glutamate-dependent type-2A protein phosphatase (289). Interestingly, platelet PP-2A has been described to regulate phosphorylation state of several proteins (ex: myosin light chain), that affects platelet functions (290,291).

1.6.3 Role of AMPK-ACC signaling in regulating lipid metabolism

1.6.3.1 Liver

ACC being a key enzyme for the *de novo* lipogenesis, its essential role in regulating lipid metabolism has been largely studied in the liver. ACC double knock-in (DKI) mice carrying alanine mutations in both ACC1 (at Ser79) and ACC2 (at Ser212), to prevent AMPK induced ACC inhibition, have been characterized (292). Along with increased liver ACC1 and ACC2 activity, ACC DKI mice display increased hepatic *de novo* lipogenesis and lower fatty acid oxidation. As a result, ACC DKI liver DAG and triglycerides (TG) contents are increased, leading to clinical signs of nonalcoholic fatty liver disease (NAFLD), such as hepatic fibrosis.

1.6.3.2 Heart and skeletal muscles

In contrast, the heart and skeletal muscles are two highly oxidative tissues, which oxidize fatty acids for ATP production. Regulation of fatty acid oxidation by the AMPK-ACC signaling pathway has therefore been investigated in these tissues. In the heart, impaired ACC inhibition by AMPK in DKI mice, showed no effect on cardiac function, morphology and fatty acid oxidation, both under basal state and stress conditions (such as myocardial ischemia) (293). These results indicate that other mechanisms, besides AMPK-ACC, exist for maintaining cardiac fatty acid

oxidation rates. Resting skeletal muscles of ACC2 knock-in (KI) mice, carrying a single alanine mutation in Ser212 of ACC2, display decreased lipid oxidation, associated with an increase in TG and ceramides levels, leading to skeletal muscle insulin resistance (294). In contrast to resting state, ACC2 KI mice had normal rates of fatty acid oxidation during muscle contractions or treadmill exercise, indicating that ACC2 phosphorylation is not essential for controlling lipid oxidation during exercise (295).

1.6.3.3 Platelets

Transcriptomic and proteomic data suggest that ACC1 is the predominant isoform in platelets (296,297). Our group and others demonstrate that ACC is phosphorylated on its Ser79 by AMPK in response to platelet agonists (33,298,299). Thrombin and less importantly collagen are the major platelet agonists leading to strong and sustained AMPK activation and ACC phosphorylation in human and murine platelets both in a time- and dose-dependent manner (33,299).

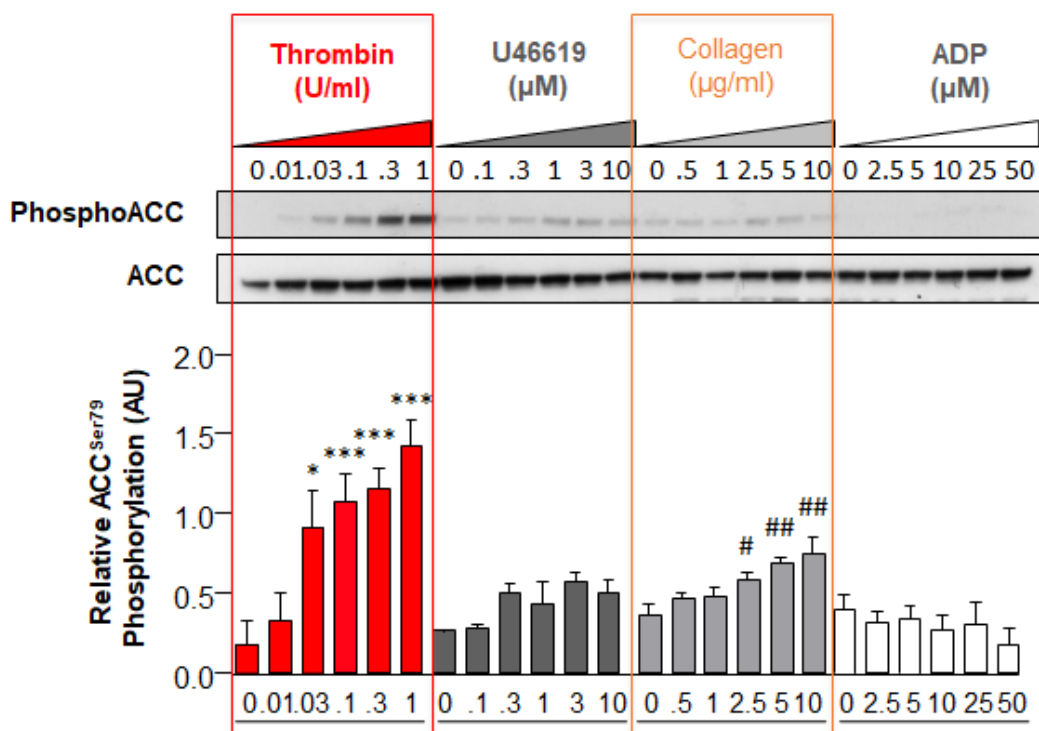


Figure 14. ACC phosphorylation by platelet agonists. Purified human platelets were incubated with increasing concentration of thrombin, U46619 (a stable TxA_2 receptor agonist), collagen and ADP for 2 mins, prior to cell lysis. Ser79 ACC phosphorylation was studied in protein extracts by western blot.

ACC: Acetyl-CoA Carboxylase, ADP: adenosine diphosphate, AU: arbitrary units.

The molecular mechanisms leading to ACC Ser79 phosphorylation in agonist-stimulated platelets have been elucidated by our group. We reported that thrombin, by stimulating the Ca^{2+} /calmodulin-dependent protein kinase kinase β (CaMKK β), an AMPK-activating kinase, induces AMPK activation and subsequent Ser79 ACC phosphorylation (33). We showed that human platelets express exclusively the AMPK $\alpha 1$ subunit, while both subunits $\alpha 1$ and $\alpha 2$ are found in mice platelets. In addition, we showed that AMPK $\alpha 1$ is the main isoform controlling platelet functions by modulating phosphorylation of its downstream cytoskeletal targets, thereby regulating cytoskeleton remodelling.

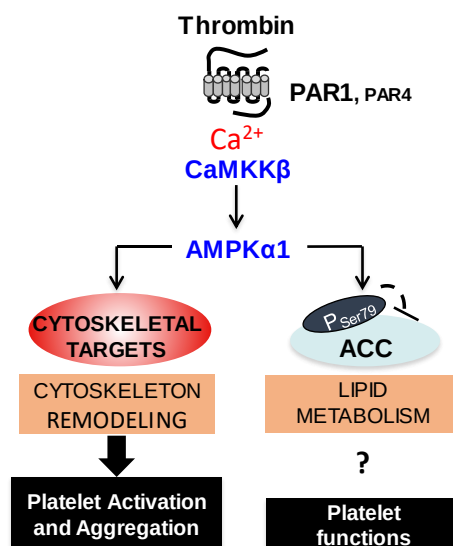


Figure 15. AMPK $\alpha 1$ regulates platelet functions. Thrombin stimulates the CaMKK β pathway leading to AMPK $\alpha 1$ activation, via a Ca^{2+} dependent mechanism. AMPK activation phosphorylates and inhibits its bona fide substrate, ACC. AMPK activation also phosphorylates cytoskeletal targets, thereby controlling cytoskeleton remodelling and platelet functions.

The role of AMPK in platelets has also been studied by other groups. The authors show that the AMPK α 2 subunit is the main regulator of platelet functions as compared to AMPK α 1 (299). While we cannot exclude the possibility that both AMPK α isoforms control murine platelet functions, only AMPK α 1 was present in highly purified human platelets extracts. In addition, our data are supported by a transcriptomic and proteomic analysis demonstrating the exclusive presence of AMPK α 1 transcript or proteins in human platelets (296,297). Accordingly, in a second study led by the same authors, they finally demonstrated the presence of AMPK α 1 in human platelets (300).

While thrombin is the major platelet agonist for AMPK-ACC signaling activation *in vitro*, our group demonstrate that platelet ACC phosphorylation is also detectable *in vivo*. ACC phosphorylation was studied in platelets from patients undergoing a major cardiac surgery (valve replacement/repair, coronary artery bypass graft), where the coagulation cascade is fully activated. During cardiac surgery, patients were subjected to high dose heparin to block thrombin generation, in order to prevent clots in the cardiopulmonary bypass (CPB) machine. Platelet ACC phosphorylation was therefore studied in the same patient at three different time points: basal state (before CPB), during CPB (under high dose heparin) and 4 hours after surgery (after complete normalization of the coagulation activity). ACC phosphorylation increased significantly post-surgery (normal coagulation) compared to CPB (33). Interestingly, preliminary data in this study identified a relationship between the level of basal ACC phosphorylation and the presence of CAD. Patients undergoing coronary artery bypass graft surgery have higher basal phosphoACC than patients with valvular disease needing surgery.

CHAPTER II - AIMS and HYPOTHESIS

2.1 Part 1: Prospective evaluation of Acetyl-CoA Carboxylase phosphorylation state in platelets as marker of atherothrombotic coronary artery disease. ACCTHEROMA study ([NCT03034148](#))

Given the role of the AMPK-ACC signaling on platelet lipid metabolism and function, this pathway could be affected in CAD patients, where the atherogenic environment has a major impact on platelet biology. Our objective was to determine whether the platelet AMPK-ACC signaling pathway is activated in CAD patients and can identify high risk patients with atherothrombotic CAD. The **ACCTHEROMA** (Prospective evaluation of **Acetyl-CoA Carboxylase** phosphorylation state in platelets as marker of atherothrombotic coronary artery disease) study ([NCT03034148](#)) was designed with the following endpoints:

2.1.1 Primary endpoint

The primary endpoint was to demonstrate an increase in ACC phosphorylation state (phosphoACC) in platelets of patients with demonstrated coronary artery disease.

2.1.2 Secondary endpoints

- To correlate platelet phosphoACC and thrombin generation markers in CAD patients.
- To determine factors predicting an increased phosphoACC in the studied population.
- To establish the importance of coronary and extra-coronary atherosclerosis in mediating an increase in platelet phosphoACC.

- To evaluate the prognostic value of platelet phosphoACC on ischemic events (cardiovascular death, recurrent myocardial infarction and revascularization procedures) within 3 years.
- To study the impact of ACC phosphorylation and inhibition on platelet lipid metabolism in CAD patients.

Results of the ACCTHEROMA study are described in the manuscript published in JACC Basic to translational science (DOI: 10.1016/j.jacbts.2019.04.005) “Platelet Acetyl-CoA Carboxylase Phosphorylation: A risk stratification marker that reveals platelet-lipid interplay in coronary artery disease patients.”

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*Contributed equally to the work.

2.2 Part 2: Exploring the role of AMPK-ACC signaling on platelet lipid metabolism, functions and thrombus formation.

Our previous study established that AMPK α 1 is activated in platelets upon thrombin or collagen stimulation and, as consequence, phosphorylates and inhibits its *bona-fide* substrate, ACC. Since ACC is crucial for the synthesis of fatty acids, essential for platelet structure, energy storage and signaling, 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.

The goals of this study were:

- a) To determine the impact of ACC phosphorylation and inhibition on platelet functions, structure, lipid metabolism and bioenergetics.
- b) To assess the role of platelet AMPK-ACC signaling *in vivo*, on primary haemostasis and arterial thrombosis.

Results are described in a manuscript published in Blood journal (DOI: 10.1182/blood-2018-02-831503) “AMPK-ACC signalling modulates platelet phospholipids content and potentiates platelet function and thrombus formation.”

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CHAPTER III - RESULTS (PART 1)

3.1 Platelet Acetyl-CoA Carboxylase Phosphorylation

A risk stratification marker that reveals platelet-lipid interplay in coronary artery disease patients.

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Platelet ACC phosphorylation in CAD patients

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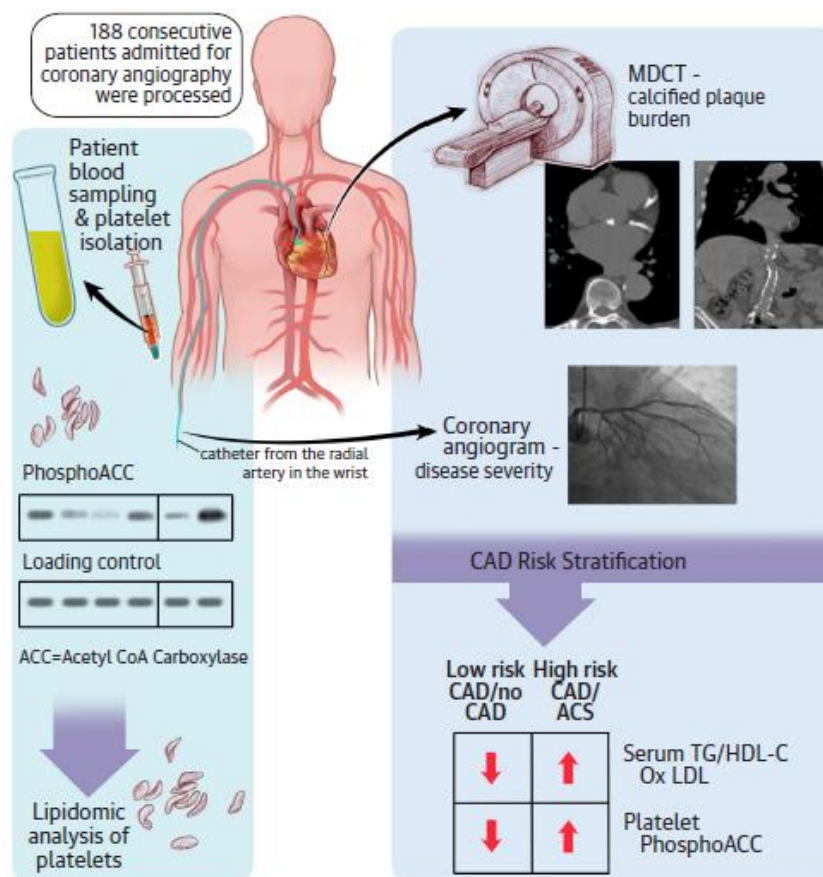
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3.1.1 Condensed abstract:

AMP-activated protein kinase-acetyl-CoA carboxylase (AMPK-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 (phosphoACC) is associated with the severity of coronary artery calcification as well as acute coronary events in coronary artery disease (CAD) patients. PhosphoACC is therefore a potential marker for risk stratification in suspected CAD patients. The inhibition of ACC resulting from its phosphorylation impacts platelet lipid content by down-regulating triglycerides, which in turn may affect platelet function.



HIGHLIGHTS

- Platelet acetyl-CoA carboxylase phosphorylation on Ser79 (phosphoACC) is a marker for risk stratification in suspected coronary artery disease (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.

3.1.2 Structured abstract:

Our recent data revealed the central role of AMP-activated protein kinase (AMPK)-acetyl-CoA carboxylase (ACC) signaling in platelet lipid metabolism and thrombus formation. The **objectives** were to determine whether AMPK-ACC is affected in platelets from coronary artery disease (CAD) patients and to explore its impact on platelet lipid metabolic profile.

Background. Activation of AMPK leads to phosphorylation of ACC on serine 79 (phosphoACC) and its subsequent inhibition, which in turn, limits endogenous fatty acids biosynthesis, including platelets. Given the impact of atherogenic environment on platelet biology, we hypothesized that AMPK-ACC is activated by atherogenic lipids and could be considered as a metabolic signature in high-risk CAD patients.

Methods. Blood samples from 188 consecutive patients admitted for coronary angiography were processed. Platelet lipid extracts were subjected to lipidomic analysis.

Results. PhosphoACC was significantly increased in circulating platelets of CAD patients and was linked to the severity of coronary artery calcification as well as acute coronary syndrome (OR: 6.7, 95% CI: 2.1-21.9; P=0.002). The triglycerides/high-density lipoprotein cholesterol ratio, a well-known atherogenic marker, was strongly associated with increased phosphoACC in our CAD cohort. Mechanistically, oxidized low-density lipoprotein activated AMPK through a CD36-dependent pathway. Lipidomic analysis revealed that increased phosphoACC led

to a down-regulation of intraplatelet triglycerides, particularly of those containing C14:0 fatty acid chains.

Conclusion. Platelet phosphoACC is a marker for risk stratification in suspected CAD patients and reveals an interaction between platelets and lipids. Inhibitory phosphoACC impacts platelet lipid content by down-regulating triglycerides, which in turn may affect platelet function (ACCTHEROMA, NCT03034148).

Keywords:

Coronary artery disease, platelet, AMPK, acetyl-CoA carboxylase, oxidized low-density lipoprotein, lipidomics

3.1.3 Introduction

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 (301). The association between increased ThG and ischemic risk in coronary artery disease (CAD) patients renders the coagulation cascade an interesting therapeutic target (61,179).

We previously established the adenosine monophosphate (AMP)-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 (33). 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, its *bona fide* substrate, typically used as a marker of AMPK activation in cells and tissues, including platelets (33). It is tempting to speculate that ThG affects platelet AMPK signaling resulting in increased ACC phosphorylation (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 (231,248). Oxidized low-density lipoprotein (oxLDL) binds to CD36, inducing platelet activation and shape changes via a calcium-dependent mechanism (233). 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 (302). We demonstrated that AMPK-ACC signaling is a key pathway in controlling platelet lipid content, thereby modulating platelet function and thrombus formation (303). However, ACC contribution to platelet lipid metabolism in CAD, where atherogenic lipids interact with circulating platelets (88), 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.

3.1.4 Materials and Methods

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

3.1.4.1 Clinical Cohort

3.1.4.1.1 Study design

From March 2015 to February 2016, 188 consecutive patients admitted for coronary angiography were included in the ACCTHEROMA study (NCT03034148), with at least two patients prospectively screened per day, regardless of the indication for angiography. Based on indication and coronary angiography results analyzed by two experienced cardiologists, patients were classified into four 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 one plaque with less than 50% luminal stenosis was classed as non-significant 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 (NSTEMI) (n=22), and ST segment elevation myocardial infarction (STEMI) (n=4). Further details on patient classifications are provided in the supplemental appendix, and study flow chart 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.

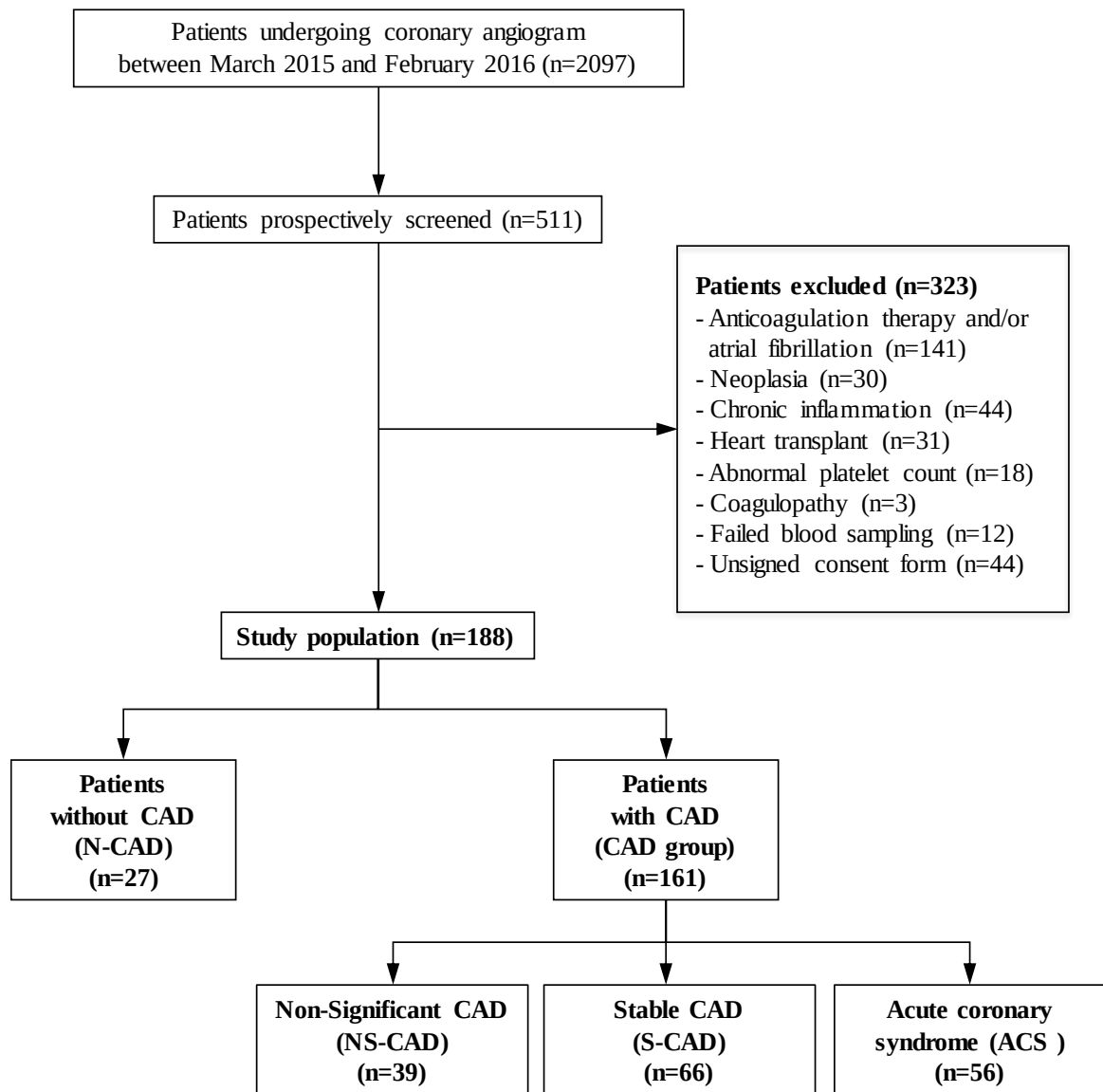


Figure 1. Flow chart of the study population. ACS: acute coronary syndrome, CAD: coronary artery disease, N-CAD: absence of coronary artery disease, NS-CAD: coronary artery disease without significant stenosis, S-CAD: coronary artery disease with at least one lesion >50%.

3.1.4.1.2 Blood sampling and phosphoACC analysis

All patients had been fasting for at least 6 hours prior to angiography, except for 4 STEMI 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 in order 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 prior to 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 minutes. 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 against the standard positive control. Western blot analyses were confirmed by electrochemiluminescence immunoassay (ECLIA, Meso Scale Discovery).

3.1.4.1.3 Multidetector computed tomography

Thoraco-abdominal 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 were done just before coronary angiogram. Scans were taken with a 256-slice multidetector-row CT scanner (Brilliance iCT 256; Philips Healthcare, Cleveland, Ohio, USA) with 3.0mm slice collimation, 120kV tube voltage and 100mAs 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 HU. The degree of CAC was classified as mild (Agatston score < 100), moderate (between 100 and 400), or severe (> 400) (304). An extra-coronary calcification score (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.

3.1.4.1.4 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, USA).

3.1.4.2 Statistical Analysis

3.1.4.2.1 Clinical cohort

Analyses were conducted using SPSS 24 (IBM Corp. 2011, IBM SPSS Statistics for Windows, Armonk, NY, USA). Continuous variables were expressed as mean $\pm$ one standard deviation (SD) or median with interquartile (25th-75th percentiles) range depending on data distribution, and categorical variables as number and percentage. CAC and Aoc scores, D-dimers, Hs-CRP and TG/HDL-C ratio were normalized by log10 transformation. Categorical variables were analyzed using the chi-squared test or Fisher's exact test, and continuous variables using an unpaired 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 one-way ANOVA 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 ratios (OR) and 95% confidence intervals (95% CI) calculated to determine independent factors associated with ACS. With the receiver operating characteristic (ROC) curve, we determined a threshold phosphoACC value for CAD by maximizing sensitivity and specificity. C-statistics were used to describe diagnostic discrimination. 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.

3.1.4.2.2 Lipidomics

Lipid species concentrations obtained from DI-MS/MS (Lipidyzer™) were analyzed using R.3.4.2 according to the following bioinformatics pipeline. Missing DI-MS/MS data were imputed using probabilistic principal component analysis from `pcaMethods` Bioconductor package (305). The data were normalized using total lipid abundance, with a log2 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 (FDR), with a FDR <0.05 considered statistically significant (306). Considering that 865 lipid species belong to 11 lipid classes, namely cholesterol ester (CE), diacylglycerol (DAG), free fatty acid (FFA),

lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), phosphatidylcholine (PC), phosphatidylethanolamine (PE), plasmalogen phosphatidylethanolamine (PEO), plasmalogen phosphatidylethanolamine (PEP), sphingomyelin (SM), 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.

3.1.5 Results

3.1.5.1 Population baseline characteristics and global atherosclerotic calcified plaque burden

The study population comprised 188 individuals (70% men, 65±12 years) admitted for coronary angiography. They were divided into four 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 patients (76%) had significant coronary stenosis above 50 %, 46% of whom had ACS. Most ACS patients (93%) were taking aspirin at enrolment, with only 36% of them on dual antiplatelet therapy, as most unstable angina and NSTEMI patients did not receive P2Y₁₂ inhibitors before angiography. Accordingly, mean platelet reactivity assessed using the Multiplate® Analyzer 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 (179), our data confirmed a significant increase in ThG, assessed by D-dimer levels, in ACS patients.

Table 1. Baseline characteristics of the ACCTHEROMA cohort

	Overall Population n = 188	NCAD n = 27	CAD			<i>p value</i>
			NS-CAD n = 39	S-CAD n = 66	ACS n = 56	
<u>Clinical Characteristics</u>						
Age, yrs	65 ± 12	59 ± 9	68 ± 11	66 ± 12	66 ± 14	0.029 [*]
Male Gender, n (%)	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, n (%)	119 (63.3)	8 (29.6)	21 (53.8)	52 (78.8)	38 (67.9)	<0.001
Smoking, n (%)	104 (55.3)	12 (44.4)	17 (43.6)	39 (59.1)	36 (64.3)	0.13
Diabetes, n (%)	43 (22.9)	1 (3.7)	9 (23.1)	14 (21.2)	19 (33.9)	0.022
Prior history of CAD, n (%)	62 (33.0)	0 (0)	0 (0)	36 (54.5)	26 (46.4)	<0.001
- MI, n (%)	35 (18.6)	0 (0)	0 (0)	19 (28.8)	16 (28.6)	<0.001
- PCI, n (%)	47 (25.0)	0 (0)	0 (0)	26 (39.4)	21 (37.5)	<0.001
- CABG, n (%)	16 (8.5)	0 (0)	0 (0)	10 (15.2)	6 (10.7)	0.017
Aortic valvular disease, n (%)	34 (18.1)	4 (14.8)	14 (35.9)	16 (24.2)	0 (0)	<0.001
Mitral valvular disease, n (%)	12 (6.4)	7 (25.9)	3 (7.7)	2 (3.0)	0 (0)	<0.001
<u>Biological</u>						
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, n (%)	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 (x10 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)	1101 ± 255	1007 ± 258	1177 ± 232	1057 ± 226	1146 ± 280	0.011 [*]
D-dimers, (ng/ml)	407 (281-686)	282 (250-435)	398 (288-573)	407 (290-775)	443 (349-706)	0.018 [§]
<u>Medication</u>						
ACEi/ARB, n (%)	86 (45.7)	6 (22.2)	16 (41.0)	43 (65.2)	21 (37.5)	<0.001
Beta blockers, n (%)	97 (51.6)	9 (33.3)	15 (38.5)	39 (59.1)	34 (60.7)	0.022
Lipid lowering treatment, n (%)	111 (59.0)	13 (48.1)	16 (41.0)	48 (72.7)	34 (60.7)	0.008
Aspirin, n (%)	141 (75.0)	13 (48.1)	21 (53.8)	55 (83.3)	52 (92.9)	<0.001
Dual antiplatelet therapy, n (%)	30 (16.0)	0 (0)	0 (0)	12 (18.2)	20 (35.7)	<0.001
- Clopidogrel, n (%)	15 (8.0)	0 (0)	0 (0)	6 (9.1)	9 (16.1)	0.013
- Ticagrelor, n (%)	13 (6.9)	0 (0)	0 (0)	3 (4.5)	10 (17.9)	<0.001
- Prasugrel, n (%)	4 (2.1)	0 (0)	0 (0)	3 (4.5)	1 (1.8)	0.35

Values are mean±SD, n (%) or median (25th-75th percentile). ACEi/ARB: angiotensin converting enzyme inhibitor/angiotensin receptor blocker, BMI: body mass index, CABG: coronary artery bypass graft, CRI: chronic renal insufficiency, hsCRP: high sensitive C reactive protein, MI: myocardial infarction, PCI: percutaneous coronary intervention, TG: triglycerides. P-values represent statistical difference across groups with pairwise significant differences (p<0.05) denoted as * between NS-CAD and NCAD, † between S-CAD and NCAD, ‡ between ACS and NS-CAD, § between ACS and N-CAD.

Thoraco-abdominal MDCT with prospective ECG-gating aimed to evaluate calcified plaque burden, including coronary (CAC Agatston) and extra-coronary (AoC) calcification scores. We confirmed that N-CAD patient (reference population) classification based on angiographic data was consistent with low CAC Agatston scores (median [25th -75th percentile]: 7.0 [0.0-33.9]) (Figure 2A). Although N-CAD patients had low CAC Agatston scores, their AoC scores were fairly high (median [25th -75th percentile]: 543 [107-2001]) (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).

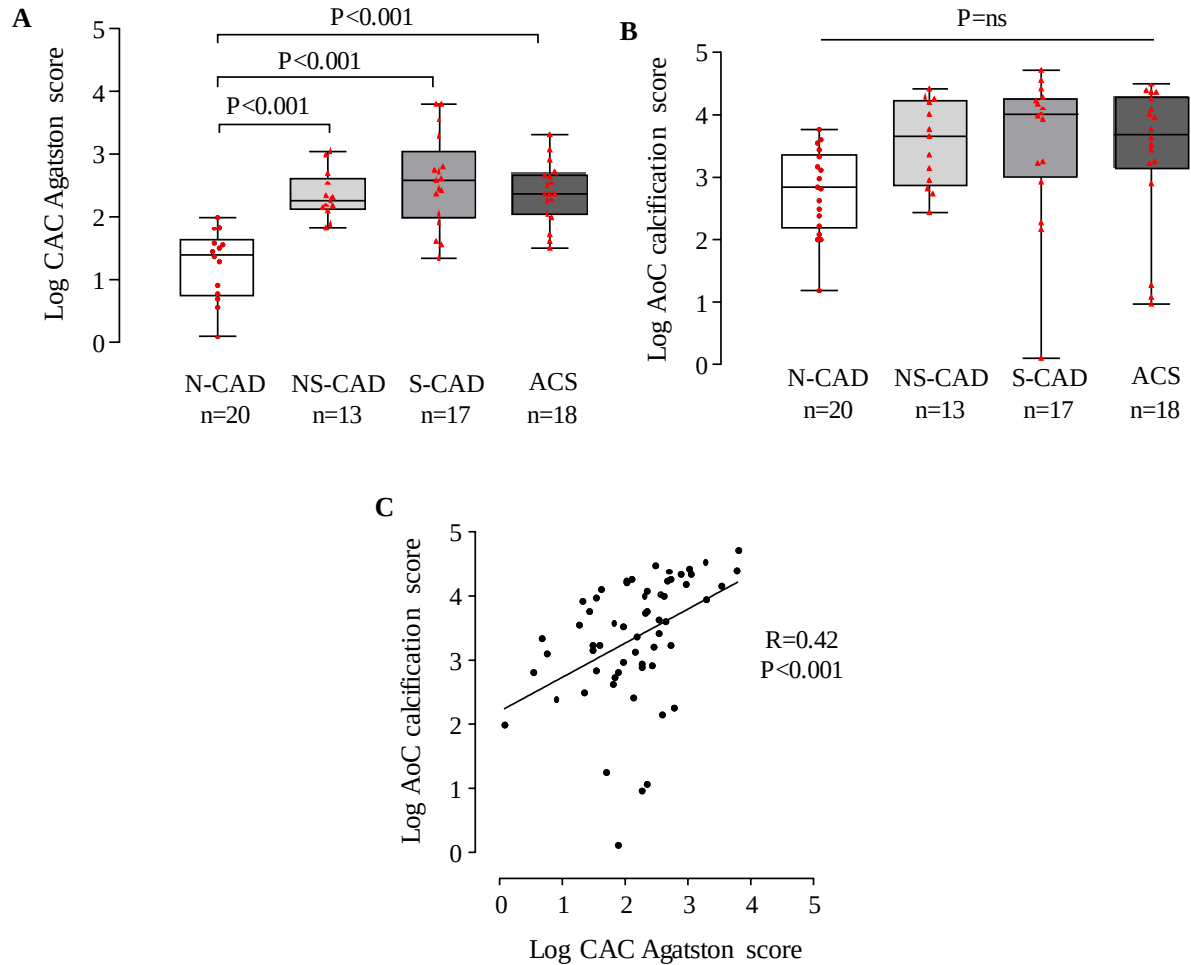


Figure 2. Assessment of coronary and aortic calcified plaque by prospective ECG-gated MDCT.

Box-plot representation of log-transformed (A) CAC Agatston and (B) AoC scores in the N-CAD and CAD subgroups of patients. Red dots (N-CAD, reference population) and triangles (CAD patients) represent individual values. Box-plots represent medians and corresponding (25th – 75th percentile) and whiskers, extremes of the distribution. (C) Correlation between log-transformed CAC Agatston and AoC scores. ACS: acute coronary syndrome, AoC: aortic calcification, CAC: coronary artery calcification, N-CAD: absence of coronary artery disease, NS-CAD: coronary artery disease without significant stenosis, S-CAD: coronary artery disease with at least one lesion >50%.

3.1.5.2 Identification of high-risk coronary artery disease 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 to N-CAD patients (median [25th -75th percentile]: 0.48 [0.29-0.73] vs. 0.22 [0.11-0.45], $p<0.001$) (Figure 3B). Platelet phosphoACC in N-CAD patients was almost never >0.5 AU, a threshold

value estimated from ROC 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 (PKC) substrates, a read-out 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 (ECLIA). Platelet phosphoACC was analyzed twice to test inter-experiment reproducibility (Intraclass Correlation Coefficient, ICC), found to be high (0.90) with a bias of -0.03 (95%CI: -0.10-0.04) (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).

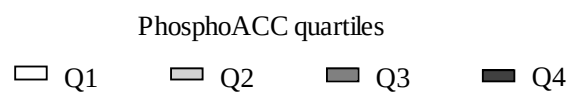
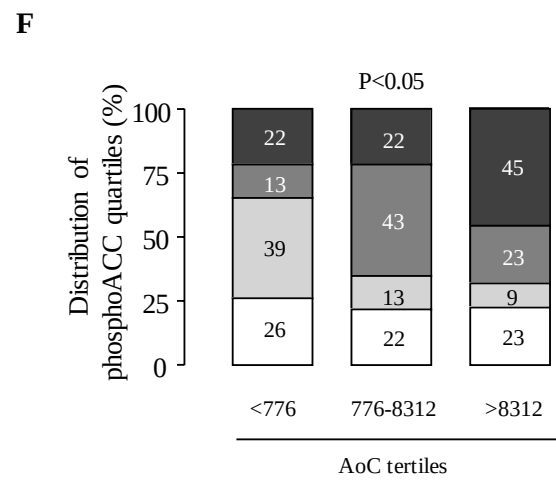
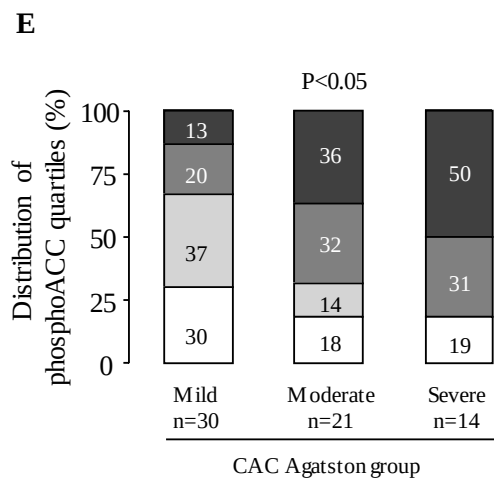
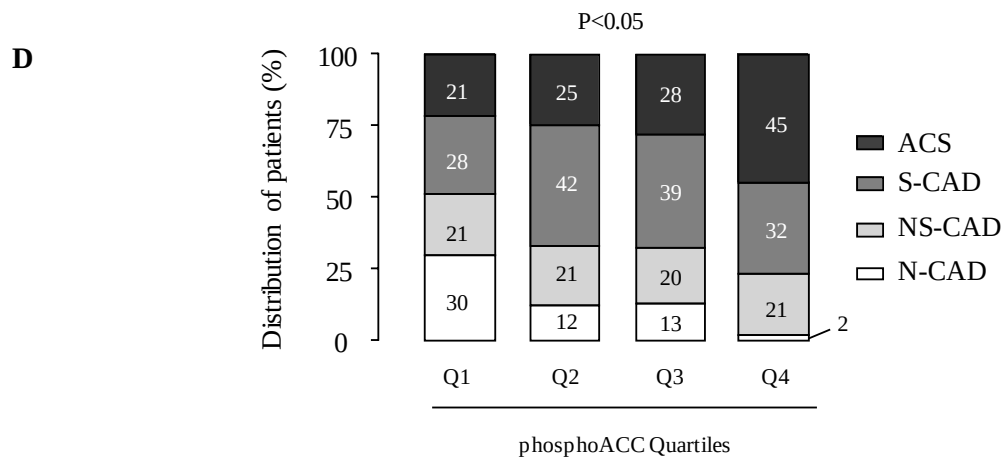
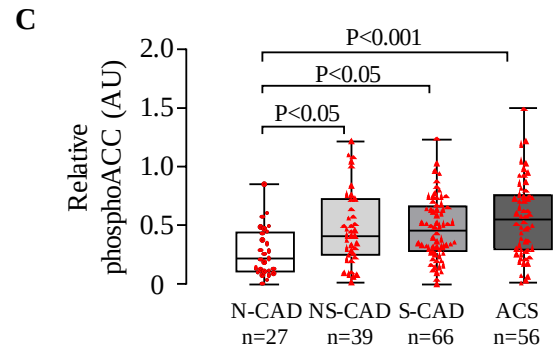
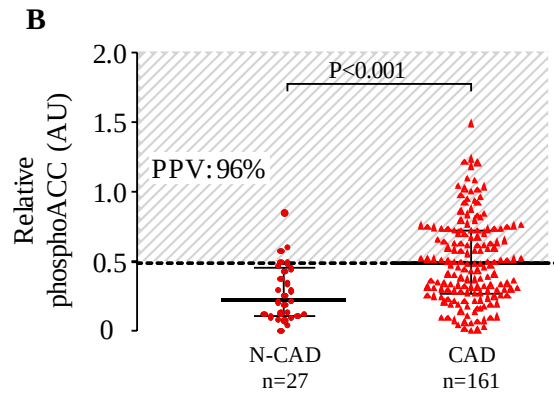
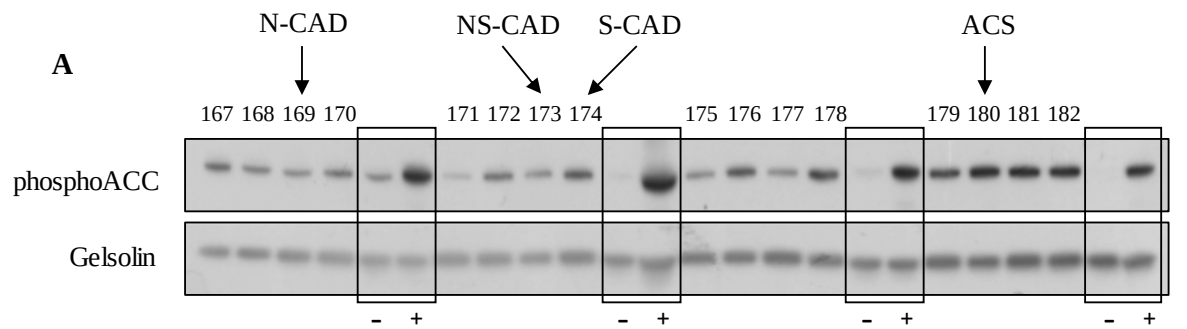


Figure 3. PhosphoACC correlates with calcified plaque severity and identifies high-risk ACS patients.

(A) Representative western blot of platelet phosphoACC in 16 consecutive patients from the ACCTHEROMA trial. (-) negative control corresponding to washed unstimulated platelets from healthy volunteers; (+) positive control corresponding to washed platelets from healthy volunteers stimulated with thrombin (0.5 U/mL) for 2 minutes. Four different controls are shown on the western blot. (B) PhosphoACC quantification in N-CAD and CAD patients. Dotted line represents the threshold value of 0.5 AU estimated from ROC curve analysis for discriminating between N-CAD and CAD patients. Positive predictive values of this threshold for CAD are indicated on the graph. Red dots (N-CAD, reference population) and triangles (CAD patients) represent individual values. Medians with interquartile (25th – 75th percentile) range are presented. (C) Box-plot representation of platelet phosphoACC quantifications in N-CAD and CAD subgroups. (D) Clinical and angiographic characteristics of patients across the different quartiles of platelet phosphoACC. Distribution of platelet phosphoACC quartiles across CAC Agatston score groups (E) and AoC score tertiles (F). The statistical differences between the groups were determined using the Mann-Whitney *U*-test in (B), Kruskal-Wallis test in (C) and chi-squared test in (D, E-F).

ACS: acute coronary syndrome, AU: arbitrary units, M: molecular weight marker, N-CAD: absence of coronary artery disease, NS-CAD: coronary artery disease without significant stenosis, PPV: positive predictive value, Q: quartile, S-CAD: coronary artery disease with at least one lesion >50%.

Among the CAD subgroups, the highest platelet phosphoACC level was found in ACS patients (median [25th -75th percentile]: 0.55 [0.29-0.76]) (Figure 3C). Indeed, quartile analysis of platelet phosphoACC demonstrated that the 4th quartile included 98% of CAD patients, with 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: 1.8-17.8, $p=0.003$) and the TG/high-density lipoprotein-cholesterol ratio (TG/HDL-C) (OR: 7.9, 95% CI: 2.1-29.9, $p=0.002$), platelet phosphoACC (OR: 4.8, 95% CI: 1.5-15.8, $p=0.009$) was independently associated to ACS (Table 2).

Table 2. Univariable and multivariable models of factors associated with ACS

Variable	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 (mg/dl)	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 $p < 0.05$. OR: odds ratio, CI: confidence interval, ASCVD score: 10 year risk of atherosclerotic cardiovascular disease (2013 ACC/AHA Guideline on the Assessment of Cardiovascular Risk), BMI: body mass index, hsCRP: high sensitivity C-reactive protein, TG/HDL-C ratio: triglyceride/high-density lipoprotein cholesterol ratio.

Prognostic value of platelet phosphoACC levels was assessed for ischemic events (cardiovascular death, recurrent MI and revascularization procedures) during a mean follow-up of 3.7 ± 0.4 years (mean $\pm$ SD). In our cohort, 28 patients (14.8%) experienced with ischemic events. Multivariable Cox regression analysis identified increased platelet phosphoACC as an independent predictor of events (HR: 3.7, 95% CI: 1.1-12.1, $p = 0.034$), in addition to previous history of MI (HR: 3.2, 95% CI: 1.4-7.3, $p = 0.006$) and ASCVD score (HR: 10.1, 95% CI: 1.9-54.0, $p = 0.007$) (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 to low phosphoACC levels (Log-rank test, $p = 0.036$) (Figure 4).

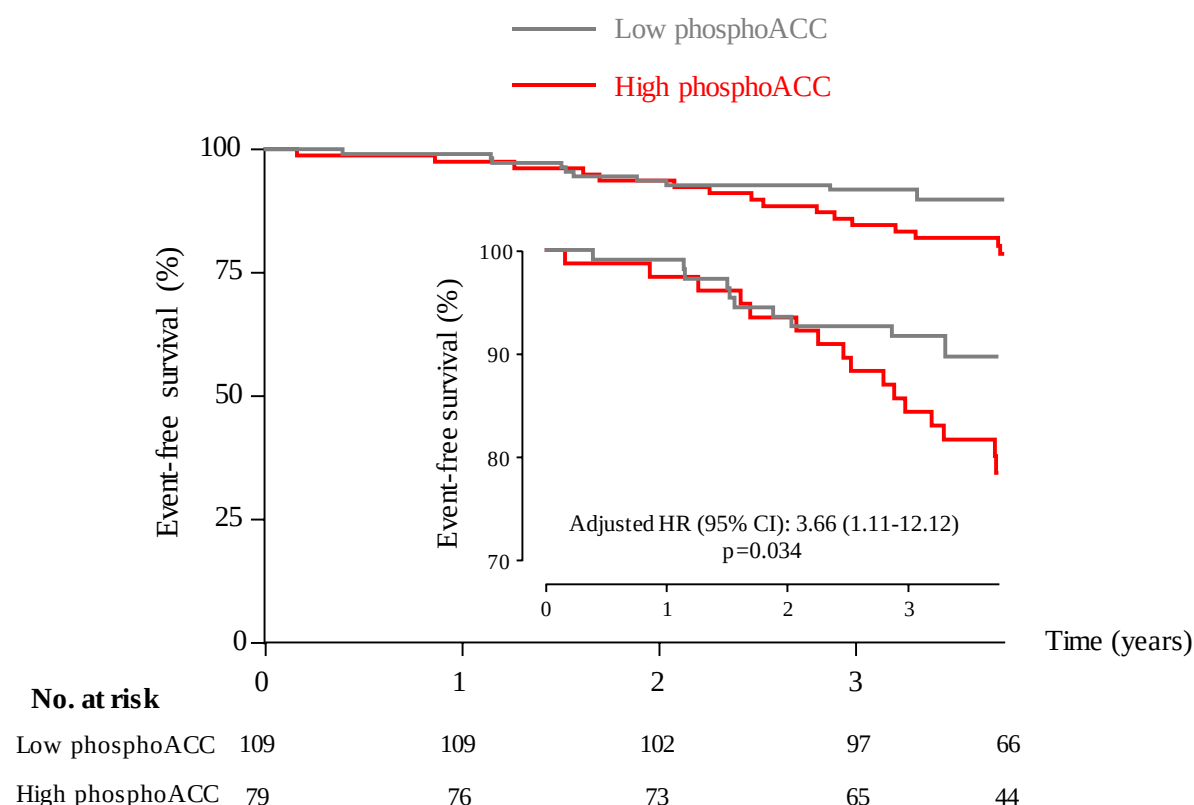


Figure 4. Kaplan-Meier curves for ischemic events according to platelet phosphoACC levels.

Ischemic events include cardiovascular death, recurrent myocardial infarction and revascularization procedures. Low and high phosphoACC defined according to threshold of 0.5 AU.

3.1.5.3 Relationship between platelet phosphoACC, calcified plaque burden, and thrombin generation 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 (4th 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 enhanced by a Western diet, the SR-B1^{fllox/fllox}/ApoE^{-/-} hypercholesterolemic mice (Figures 5A and 5B). As in human platelets, phosphoACC levels drastically increased with the severity of atherosclerotic plaque burden (Figure 5C).

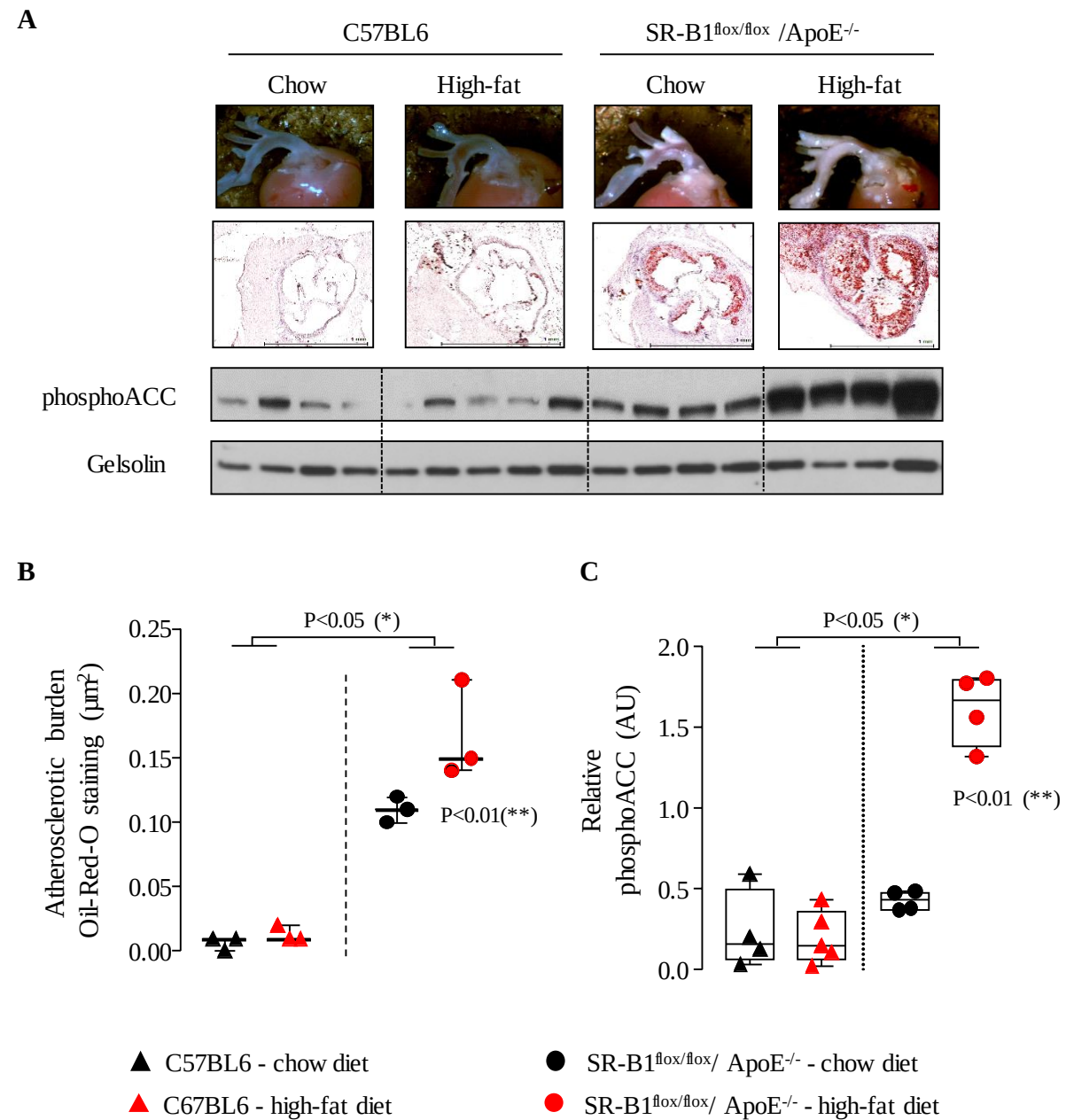


Figure 5: Increased platelet phosphoACC in atherosclerotic mice.

(A-C) Female SR-B1^{flox/flox}/ApoE^{-/-} (dot) and control C57BL6 (triangle) mice received either chow diet for 24 weeks (black symbols) or 12 weeks chow diet followed by 12 weeks western diet (red symbols) before sacrifice. Atherosclerotic burden was evaluated by Oil-Red-O staining of the aortic root (A, B) and platelet phosphoACC by western blot (A, C). (A) Top two panels: representative pictures of the aortic root (top) and of Oil-Red-O staining after cross section of the aortic root, scale bars indicate 1 mm (bottom). Bottom two panels: representative western blot of platelet phosphoACC. Gelsolin was used as loading control. Quantifications of Oil-Red-O staining (B) and phosphoACC (C) are shown. Data are represented as medians with interquartile (25th – 75th percentile) range (B) or Box plot (C). (*) denotes statistical difference between C57BL6 and SR-B1^{flox/flox}/ApoE^{-/-} (***) denotes statistical differences between SR-B1^{flox/flox}/ApoE^{-/-} under high fat diet compared to all other groups
AU: arbitrary unit.

ThG markers correlated with the severity of calcified plaque, given that D-dimer levels (chi-square test, $p=0.014$), thrombin anti-thrombin complexes (TAT) (chi-square test, $p=0.036$), or fragments 1.2 (F1.2) (chi-square test, $p=0.004$) had a significant positive association with the severity of both CAC Agatston (Supplemental Figure 6A) and AoC calcification 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.

3.1.5.4 Contribution of inflammation and atherogenic oxLDL to phosphoACC in platelets

Since inflammatory cytokines may affect platelets (190) and are established circulating actors of atherosclerosis, we explored whether interleukin-1beta, interleukin-6, interleukin-10, interleukin-17A, and tumor necrosis factor-alpha impacted phosphoACC level in washed platelets from healthy volunteers. Although their respective receptors are present in human platelets (296), 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 hsCRP 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: 1.3-12.6, $p=0.019$) (Table 3). TG/HDL-C, a well-known atherogenic marker, indicates LDL particle size (71), while TGs were shown to correlate with plasma oxLDL levels (307). In our cohort of patients, TG/HDL-C correlated with oxLDL levels ($R_p=0.23$, $p=0.002$).

Table 3: Factors associated with increased platelet phosphoACC in CAD patients (>0.5 AU threshold).

Variable	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 (mg/dL)	1.00 (0.99-1.01)	0.53
TG (mg/dL)	1.00 (1.00-1.01)	0.048
HDL (mg/dL)	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 $p<0.05$. DAPT: dual antiplatelet therapy, HDL: high-density lipoprotein, hsCRP: high sensitivity C-reactive protein, TG: triglycerides, TG/HDL-C ratio: triglyceride/high-density lipoprotein cholesterol ratio.

We thus studied the effect of oxLDL on AMPK-ACC signaling. PhosphoACC was assessed in platelets from healthy volunteers treated with copper-oxidized LDL (CoxLDL) and myeloperoxidase-oxidized LDL (MoxLDL). Both induced a time- and

dose-dependent increase in platelet phosphoACC (Figures 6B-D and Supplemental Figure 7A). MoxLDL-induced phosphoACC was prevented by pre-incubating 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).

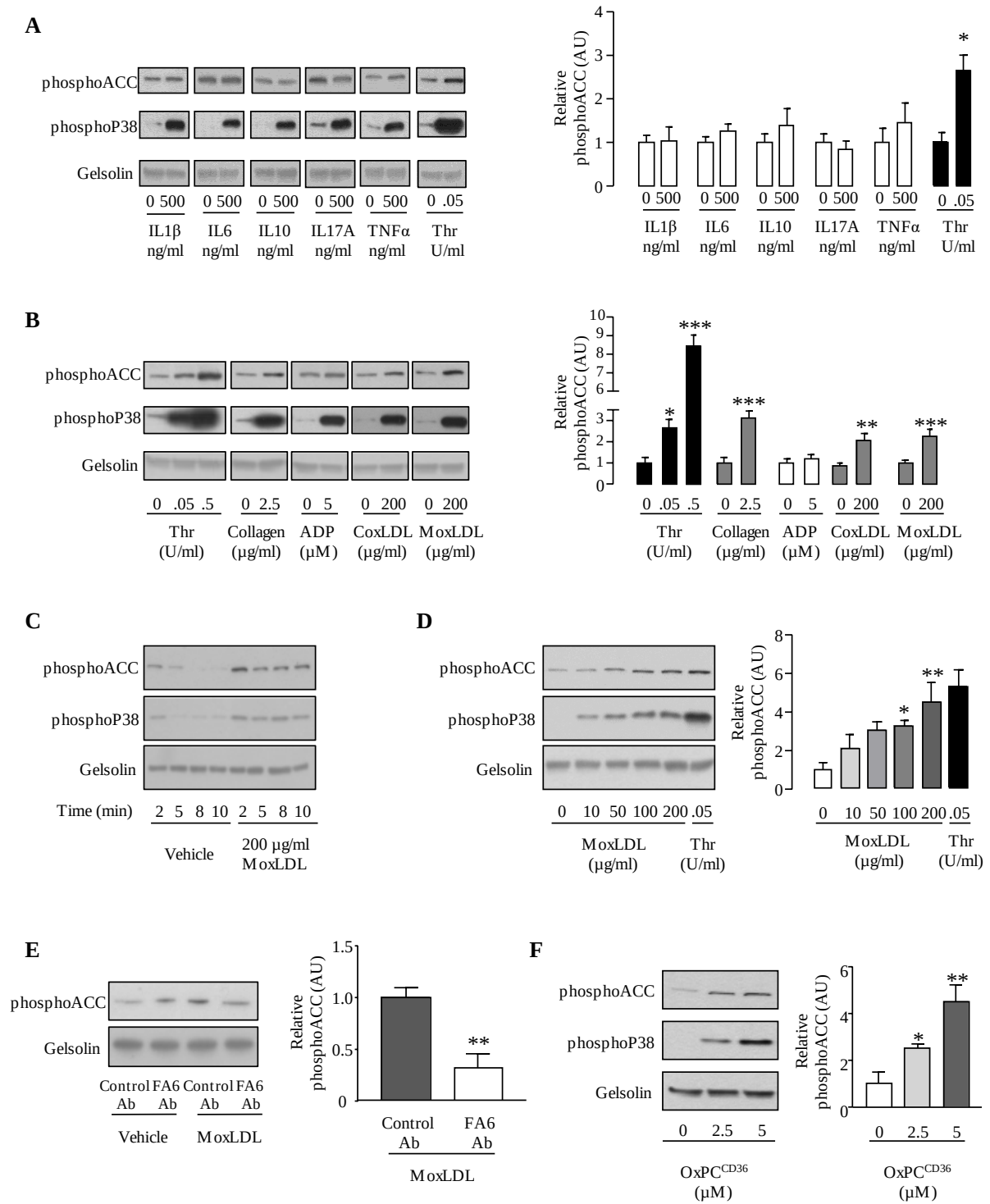


Figure 6. OxLDLs induce platelet phosphoACC in a CD36-dependent manner. Washed platelets (4.0×10^8 /mL) from healthy volunteers were (A) treated with the following selected cytokines (IL1 β , IL6, IL10, IL17A, and TNF α) and (B) stimulated with thrombin, collagen, ADP, coxLDL or moxLDL prior to lysis. Time course (C) and dose response (D) curve of effect of moxLDL on phosphoACC. (E) Platelets were pre-treated with 0.2 U/mL anti-CD36 antibody (FA6-152) (FA6 Ab) or an isotype control (control Ab) for 15 minutes before stimulation with moxLDL. (F) Platelets were stimulated with varying concentrations of a specific CD36 ligand (OxPC^{CD36}) for 5 minutes prior to lysis. All experiments were carried out at least four times (biological replicates). Thrombin-stimulated platelets were used as a positive control. Gelsolin was the loading control. Representative western blots are shown, with quantification of western blots represented in the right-hand panels. Data expressed as mean $\pm$ SEM. Significance was determined by a 2-tailed Student's *t*-test (A, B, and E) or 1-way ANOVA with

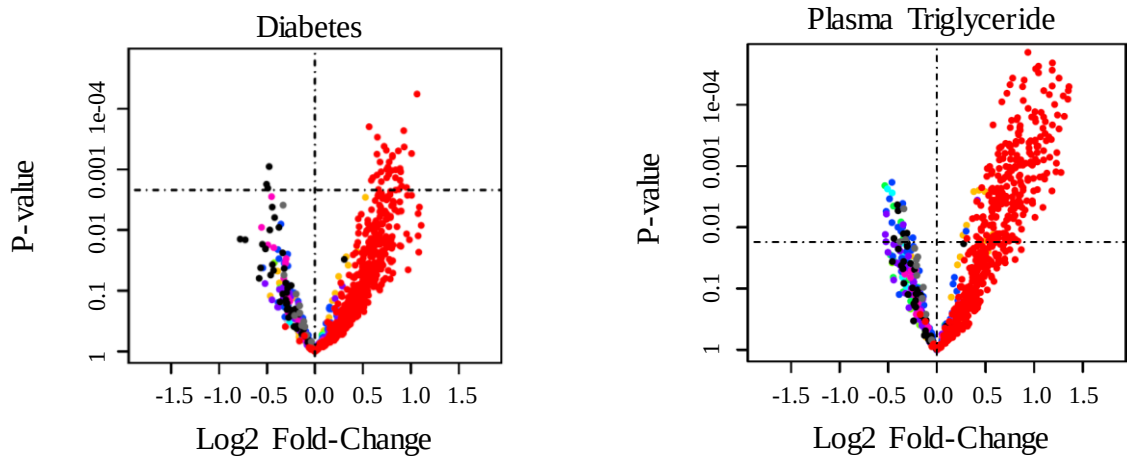
Bonferroni post-hoc analysis (B-thrombin, D, and F). *P<0.05, **P<0.01, ***P<0.001 compared with unstimulated platelets.

Ab: antibody, coxLDL: copper-oxidized LDL, IL1 β : interleukin-1beta, IL6: interleukin-6, IL10: interleukin-10, IL17A: interleukin-17A, moxLDL: myeloperoxidase-oxidized LDL, OxPC^{CD36}: oxidized choline glycerophospholipids, Thr: thrombin, TNF α : tumor necrosis factor-alpha.

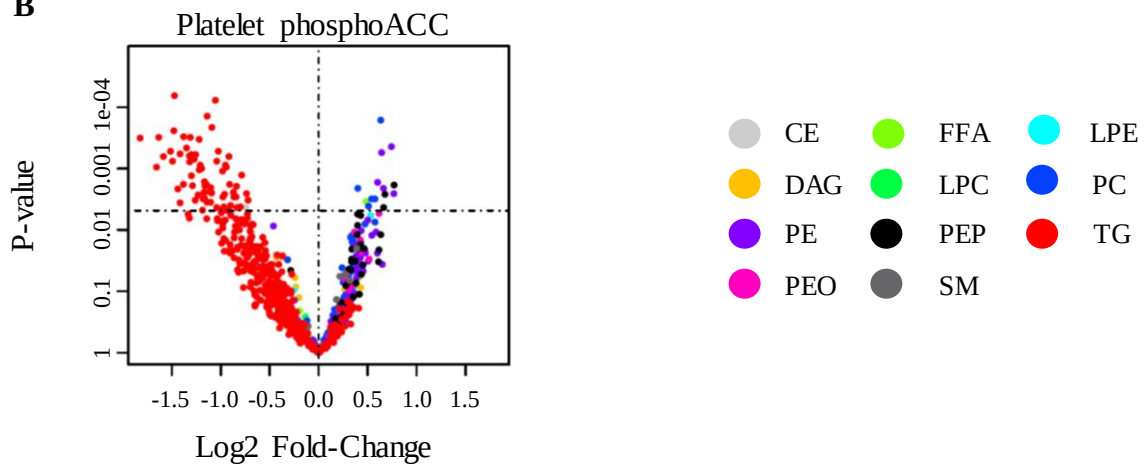
3.1.5.5 Lipidomic profiling of circulating platelets and metabolic regulation of intraplatelet TG levels by phosphoACC in CAD patients

We investigated the impact of ACC phosphorylation/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 (Figure 8A). 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 and 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: 7.1-228.3, 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/inhibition affects endogenous platelet lipid content, by regulating TG lipid species and their fatty-acid-chain composition.

A



B



C

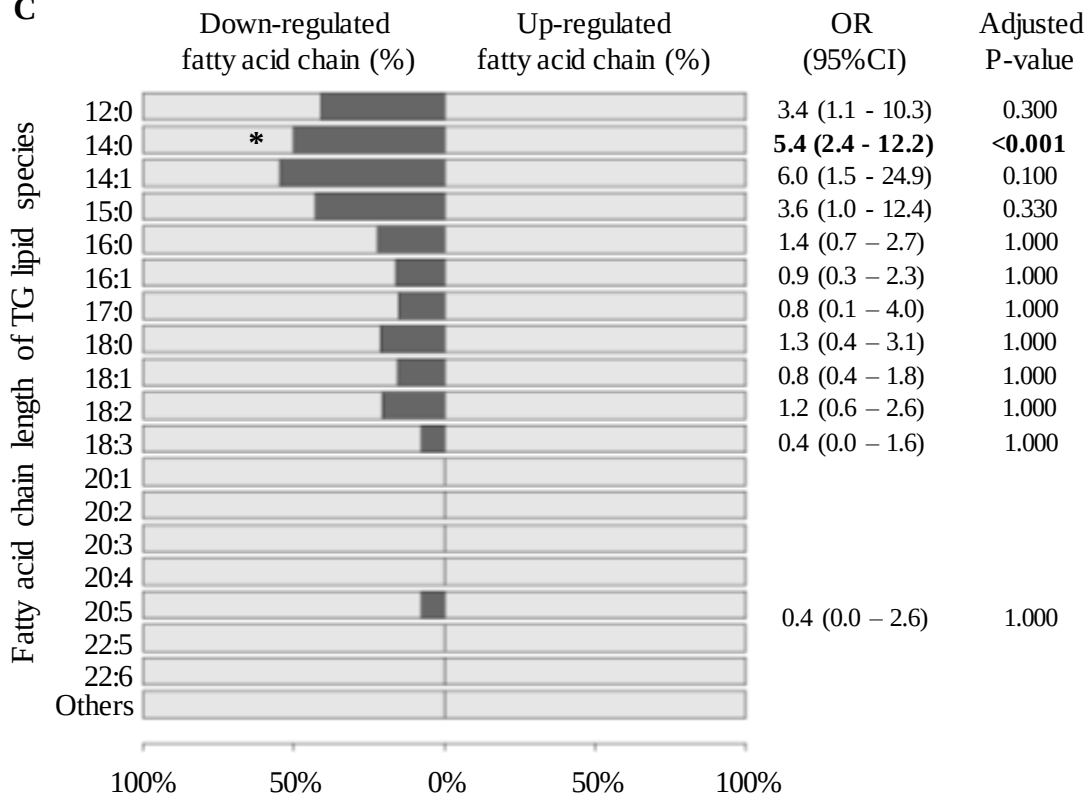


Figure 7. PhosphoACC regulates TG lipid species in platelets of CAD patients. (A-C) Volcano plot representations of the 865 lipid species detected in platelets by lipidomic profiling. Log fold-changes and P values were calculated from the multivariate regression model. Lipids above the horizontal dotted line were up- or down-regulated with significant adjusted P values. Relationship between diabetic status (A), plasma TG levels (B), platelet phosphoACC (C) and intraplatelet lipid species content. (D) 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, PE: phosphatidylethanolamine, LPE: lysophosphatidylethanolamine, OR: odds ratio, PC: phosphatidylcholine, PEO: plasmalogen phosphatidylethanolamine, PEP: plasmalogen phosphatidylethanolamine, SM: sphingomyelin, TG: triglycerides.

3.1.6 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 (central illustration).

3.1.6.1 Platelet phosphoACC levels and risk assessment

Despite the LDL-C-lowering drugs and platelet inhibitors currently available, substantial residual risk remains (308), and many patients should be given more aggressive treatment (61,98). 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 (309). 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 (20,196,197). In our study, AMPK-ACC signaling activation was observed in circulating platelets. The absence of PKC substrate phosphorylation, a sign of platelet activation (41), supports the theory that increased phosphoACC and platelet activation are unrelated. Even if thrombin is the principal agonist of platelet AMPK activation *ex vivo* (33), we found no association between ThG markers and phosphoACC. Therefore, platelet phosphoACC is not caused by thrombin-induced platelet activation in patients. Our findings thus suggest that platelets of high-risk CAD patients exhibit altered metabolic phenotypes characterized by activation of AMPK-ACC signaling, independent of platelet activation state.

3.1.6.2 Lipid-platelet interaction

Changes in LDL phenotypes, like tendency to aggregate, oxidative status, or size and density, influence cardiovascular risk and can determine residual post-treatment risk (69,70). In line with previous studies (88), 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 pro-atherogenic lipid profile, including small LDL particles known to be more oxidizable (71,307). 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 (241), cytoskeleton rearrangement (233), and NADPH oxidase 2 activation (232). Interestingly, an increased binding of oxLDL on platelets has been reported in patients with ACS, in line with our observation (310). Additionally, CD36 is an established receptor for platelet interaction with atherogenic environments, and CD36 expression variability may determine platelet reactivity and thrombotic risk (231,248).

3.1.6.3 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 (88). 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 (311). 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 (252). To our knowledge, this is the first study to report the impact of ACC phosphorylation/inhibition on the regulation of lipid content in circulating platelets of CAD patients.

3.1.6.4 Study limitations

First, phosphoACC was determined using immunoblotting in whole platelet lysates. Given the circulating platelets' heterogeneity (312), 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, ECLIA, 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 (70).

3.1.7 Conclusions

Our study identifies platelet phosphoACC as a risk stratification marker that reflects the interaction between pro-atherogenic 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 pro-atherogenic environment.

3.1.8 Clinical perspectives

Competency in medical knowledge:

The circulating platelets of high-risk CAD patients exhibit altered metabolic phenotypes characterized by phosphorylation and inhibition of acetyl-CoA carboxylase (ACC), the first committed enzyme of the fatty-acid biosynthesis pathway. Platelet ACC phosphorylation 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 ACC phosphorylation, influences major adverse outcomes in coronary artery disease.

3.1.9 Supplemental Material and Methods

3.1.9.1 Reagents and Materials

Apyrase (#A6132), bovine thrombin (#T6634), ADP (#A2754), horse radish peroxidase (HRP)-conjugated anti-rabbit antibodies (#A0545), hematoxylin (#MHS32) and oil-red-O (#O0625) were purchased from Sigma. Eptifibatide (Integrilin) was obtained from GlaxoSmithKline. Collagen (#ABP-COL-1, equine tendon type 1) was obtained from American Biochemical and Pharmaceutical Ltd. Anti-phosphoACC (S79) (#3661), anti-phosphoP38 MAPK (T180/Y182) (#9211), anti-gelsolin (#12953) and anti-phosphoPKC substrate (#2261) antibodies were obtained from Cell Signaling. Bovine serum albumin (BSA) (#8076) was purchased from Roth. GOLD 96-well streptavidin SECTOR plates (#L15SA-5) and goat anti-rabbit sulfo-tag secondary antibody (#R32AB5) were purchased from MSD. A cocktail of protease and phosphatase inhibitors was purchased from Thermo Fisher Scientific. IL1 β (#78034), IL6 (#78050), IL10 (#78024), IL17A (#78032), and TNF α (#78068) cytokines were purchased from STEMCELL Technologies. Oxidized choline glycerophospholipids (OxPC^{CD36}- Kodia-PC, CAS 439904) were purchased from Santa Cruz Biotechnology. Anti-CD36 (FA6-152, ab17044) and the corresponding isotype control (ab18449) antibodies were purchased from Abcam. APC mouse anti-human CD45 (#555485) and FITC mouse anti-human CD41a antibodies were purchased from BD biosciences for flow cytometry experiments.

LDL preparation and oxidation

Native low-density lipoproteins (LDLs) were isolated from the plasma of healthy donors by means of sequential density gradient ultracentrifugation. LDL concentration was adjusted to 1mg/mL in phosphate-buffered saline (PBS). Copper-oxidized LDLs (coxLDLs) were obtained by treating native LDLs (1mg/mL) with

10 μ M CuSO₄ for 24 hours at 37°C. The reaction was stopped on ice with 25 μ M butylated hydroxytoluene (Sigma-Aldrich, St. Louis, MO, USA) (313). Myeloperoxidase-oxidized LDLs (moxLDLs) were produced by mixing 1.6mg of native LDL with PBS containing 4mM HCl, 250nM myeloperoxidase, 1mM H₂O₂ and 3.4mM EDTA for 4 hours at 37°C (313,314). OxLDL and moxLDL were desalted in RPMI-1640 medium without glutamine (Lonza, Belgium) using PD-10 desalting columns (GE Healthcare, Little Chalfont, Buckinghamshire, UK). OxLDL and moxLDL were sterilized using sterile filters (0.2 μ m), resuspended in Tyrode's buffer and stored in the dark at 4°C. Concentrations of both types of oxidized LDL were determined using the Lowry method (315).

3.1.9.2 Clinical Cohort

Patients were classed as follows:

- Non-significant coronary artery disease (NS-CAD) if stenosis less than 50% or displayed irregularities
- Significant CAD (S-CAD) or acute coronary syndrome (ACS) classifications depended on the clinical presentation of the patient and the presence of stenosis of more than 50%. S-CAD included patients with chronic stable chest pain for more than 1 month or patients with valvular disease or heart failure. ACS included patients with unstable angina (*de novo* chest pain for < 1 month, resting angina or worsening symptoms without high sensitivity [hs]-troponin T elevation); non-ST segment elevation myocardial infarction (NSTEMI) (requiring changes in cardiac markers, including hs-troponin T elevation above the 99th percentile in addition to recent chest pain and/or ECG changes); or ST segment elevation myocardial infarction (STEMI) treated by primary angioplasty.

3.1.9.2.1 Eligibility criteria

Inclusion criteria

- Patients > 18 years old
- Signed informed consent
- Angiography scheduled within the following 3 days, whatever the indication

Exclusion criteria

- Patients not able to sign the informed consent form
- Patients on anticoagulation therapy (oral or parenteral) for any reason, including heparins, fondaparinux, vitamin K antagonists, or novel oral anticoagulants
- Hemophilia or other coagulopathy
- Abnormal platelet count ($< 1.5 \times 10^5/\mu\text{L}$ or $> 4 \times 10^5/\mu\text{L}$)
- Active neoplasia or chronic inflammatory disease
- Patients with a life expectancy of less than 3 years
- Heart transplant patients
- Active hepatitis B or C, or HIV patients
- Any contraindication to coronary angiography

3.1.9.2.2 Estimated sample size before inclusion

Based on our preliminary data, we determined that an enrolment of 102 patients would provide a power of 80% at a significance level of 5% for detecting a difference of 0.15 AU in platelet phosphoACC in CAD patients.

3.1.9.3 Blood sampling and analysis

3.1.9.3.1 Human platelet isolation

Blood samples were drawn into citrated tubes (citrate phosphate dextrose adenine, S-Monovette, Sarstedt) after the sheath had been inserted at the catheterization laboratory but before any drugs had been administered. Platelet-rich plasma (PRP) was obtained by 20 minutes' centrifugation at $330 \times g$. Platelets were then counted with a Cell-Dyn Emerald (Abbott) and pelleted by centrifugation at $400 \times g$ for 10 minutes in apyrase (0.5 U/mL) and eptifibatide (4 μ g/mL). The presence of contaminating leucocytes and platelets was tracked by flow cytometry using anti-CD45 and anti-CD41a antibodies respectively, all along the platelet isolation process from blood of 3 healthy volunteers. The platelet pellets were either immediately lysed (400,000 platelets/ μ L) or stored frozen (-80°C) along with plasma samples.

3.1.9.3.2 Western blot analysis

The platelet pellets were lysed in Laemmli buffer (50mM Tris-HCl, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 0.01% (w/v) bromophenol blue, and 6.25% (v/v) β -mercaptoethanol). Protein extracts from whole platelet lysates for each patient were separated on polyacrylamide gels (Criterion TGX, Bio-Rad). The membranes were probed with phosphoACC (1:1000), gelsolin (1:40 000), phosphoP38, or phosphoPKC substrate (1:10 000) antibodies. HRP-conjugated anti-rabbit (1:20 000) antibodies were diluted in 5% bovine serum albumin. Band intensities were quantified using Image J (National Institutes of Health, Bethesda, MD, USA).

3.1.9.3.3 Electrochemiluminescence analysis (ECLIA)

To facilitate the quantitative assessment of platelet phosphoACC in patients, we optimized the ECLIA technique from MSD. Since ACC contains biotin, phosphoACC

quantification was based on the interaction between streptavidin and its biotin group. GOLD 96-well streptavidin SECTOR plate wells (MSD) were blocked for 1 hour, washed and incubated with protein extracts (1µg/µL) from the patients' platelets, which had been previously lysed in 50mM Tris-HCl, pH 7.5, 1mM EGTA, 1mM EDTA, 1% Triton X 100, 0.27M sucrose, 0.1% β-mercaptoethanol, 0.1% SDS and a cocktail of protease and phosphatase inhibitors (1:100). The plate was incubated at room temperature with shaking for 1 hour. After washing, primary rabbit anti-phosphoACC antibody was added (1:250) and incubated overnight at 4°C with shaking. After washing, goat anti-rabbit sulfo-tag secondary antibody (1:250) was added and incubated at room temperature with shaking for 1 hour. The plates were read on the SECTOR Imager 2400 (Meso Scale Discovery) immediately after the read buffer had been added. A positive control of washed platelets from healthy volunteers stimulated with thrombin (0.5 U/mL) for 2 minutes was used as a benchmark in all experiments.

3.1.9.3.4 ThG, oxLDL and platelet reactivity measurement

ThG was assessed in citrated plasma by measuring D-dimer levels using the automated latex-enhanced immunoassay (HemosIL D-dimer HS, Werfen, Milan, Italy), and thrombin anti-thrombin complex (TATc) and fragment 1.2 (F1.2) using the ELISA method (Siemens Healthcare Diagnostics), according to the manufacturers' protocols. OxLDL levels were measured using the ELISA method with the 4E6 monoclonal antibody (Mercodia, Uppsala, Sweden). Platelet reactivity was also evaluated in all patients by the multiple electrode platelet aggregometry method (Multiplate® Analyzer, Roche Diagnostics).

3.1.9.3.5 Platelet lipid extraction and analysis

Lipids were extracted using the methyl-tert-butyl ether (MTBE) method (316,317). Platelets were transferred to a 2mL Eppendorf vial to which 100µL of water and 100µL of an Internal Standard Mix (Sciex, Nieuwekerk aan den IJssel, Netherlands) were added. Next, 160µL of methanol and 500µL of MTBE were added. The extracts were then shaken at room temperature for 30 minutes. Then, 200µL of water was added, after which the samples were briefly vortexed and then centrifuged at $16\ 100 \times g$ for 3 minutes. The upper organic layer (400µL) was then transferred to a fresh glass vial. Another 500µL of MTBE, 100µL of methanol and 100µL of water were added to the original Eppendorf vials and the extraction was repeated. The combined organic extracts were concentrated under a gentle stream of nitrogen and reconstituted in 250µL of running buffer (10mM ammonium acetate in 50:50 dichloromethane:methanol).

Lipidomic analysis was carried out using the commercial Lipidyzer platform according to the manufacturer's instructions (Sciex) (318). Lipid analysis was performed in flow-injection mode. The lipid classes were separated using differential mobility spectroscopy (318), followed by tandem mass spectrometric lipid species analysis on a QTrap 5500 (Sciex) in multiple reaction monitoring mode. The lipid species were identified and quantified based on characteristic mass spectrometric transitions. The Lipidyzer software automatically calculated lipid species concentrations. All samples were analyzed in a randomized fashion. Control and fortified plasma samples were analyzed daily as quality controls. The relative standard deviations (RSD) of the quality control samples were below 15% for all lipid classes, except for sphingomyelin, where a RSD of 25% was observed.

3.1.9.4 Experimental dataset

3.1.9.4.1 Atherosclerotic mouse model

We studied platelet phosphoACC in 20 to 24-week-old female SR-B1^{flox/flox}/ApoE^{-/-} hypercholesterolemic mice. The animals were randomized to receive either 24 weeks of standard chow diet or 12 weeks of a chow diet followed by 12 weeks of a Western diet (1.25% cholesterol, 16% cocoa butter, U8220 version 151, Safe, Augy, France), while 24-week-old female C57BL6 mice were similarly randomized as above and were used as controls. On the day of sacrifice, the mice were bled under ketamine and xylazine anesthesia from the retro-orbital plexus. Blood was processed for platelet isolation as described below. All animals were kept in a 12:12 hour light/dark cycle with free access to food and water. 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 US National Institutes of Health (publication no. 85-23, revised 1996).

3.1.9.4.2 Murine platelet preparation

Blood was collected in EDTA-K tubes (Sarstedt) containing citrate-dextrose solution (1:8), apyrase (1 U/mL) and eptifibatide (4µg/mL). PRP was obtained by centrifugation at 100 × g for 5 minutes. Platelets were counted with a Cell-Dyn Emerald (Abbott). After centrifugation at 400 × g for 5 minutes, platelet pellets were lysed in Laemmli buffer for Western blotting (400,000 platelets/µL).

3.1.9.4.3 Murine atherosclerosis analysis

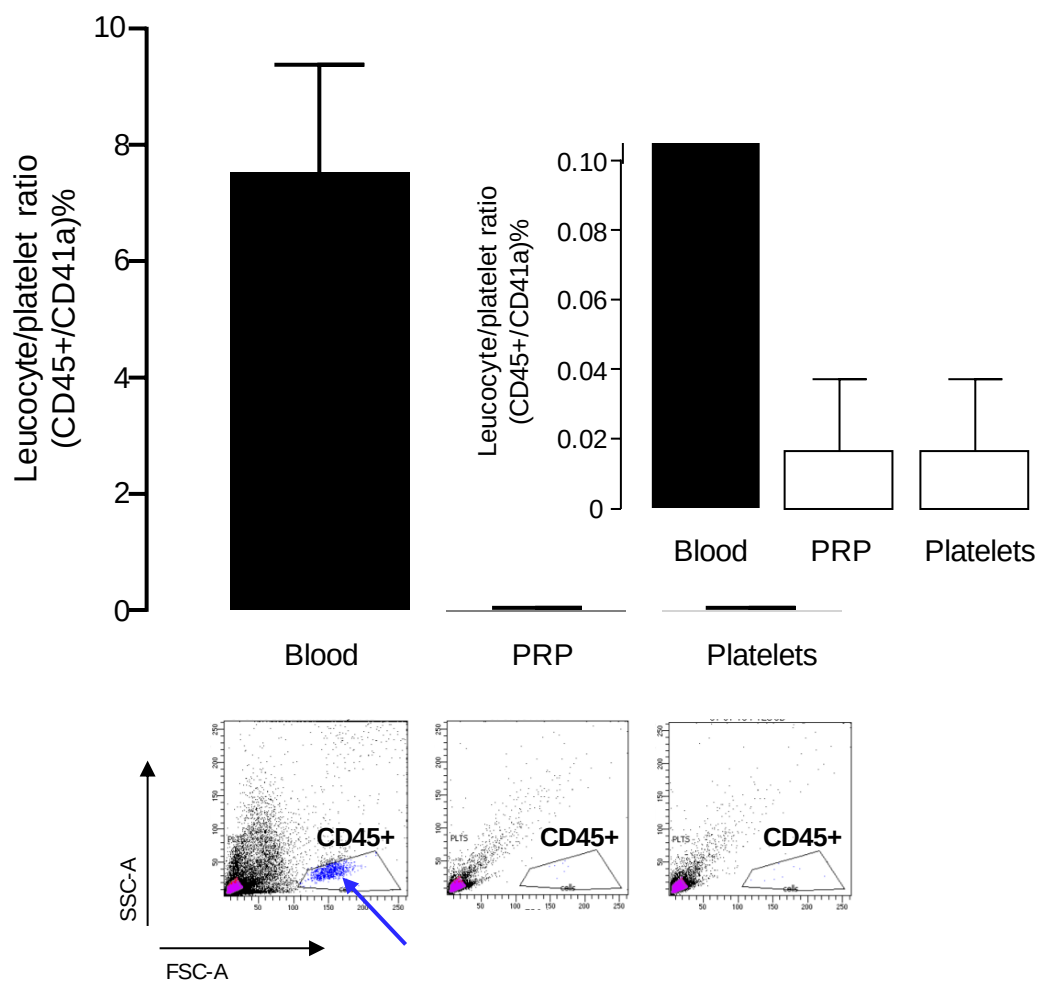
Murine hearts and aortic roots were isolated, embedded in optimum cutting temperature (OCT) compound (Tissue-Tek, Sakura, Torrance, CA, USA) and frozen to quantify atherosclerosis at the aortic sinus. Cryostat sections (10µm) were then cut, fixed with 4% formaldehyde, stained with 0.3% oil-red-O and counterstained with hematoxylin. Lesion sizes were quantified using a Leica SCN400 image analysis program and calculated by dividing the surface area of the lesion by the total surface area of the vessel.

3.1.9.4.4 Ex vivo human platelet signaling

Human platelet preparation

Blood from healthy volunteers was processed as previously described (303). Platelet pellets were washed in modified Tyrode's buffer (135mM NaCl, 12mM NaHCO₃, 2.9mM KCl, 0.3mM Na₂HPO₄, 1mM MgCl₂, 10mM Hepes, 5mM D-glucose, 0.35% bovine serum albumin, pH 7.4, 37°C) before being suspended to a density of 4.0 x 10⁵ platelets/µL. Platelets were treated with selected cytokines for 5 minutes at 37°C and stimulated (in 2mM Ca²⁺) with thrombin, collagen or ADP for 2 minutes at 37°C, or coxLDL or moxLDL for 5 minutes at 37°C (as stated in the figure legends), prior to lysis with Laemmli buffer for western blot analysis. Gelsolin was used as the loading control.

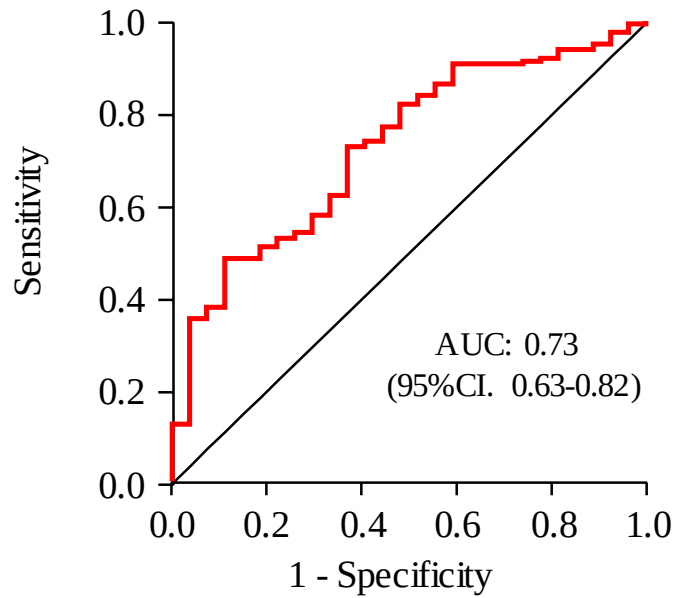
3.1.10 Supplemental Figures and Tables



Supplemental Figure 1. Leukocytes in platelet preparation.

Blood samples from healthy volunteers (n=3) were processed for platelet isolation. Leucocytes (CD45+) and platelets (CD41a+) were detected by flow cytometry in whole blood samples, platelet rich-plasma et platelet samples (used for phosphoACC analysis). Box-plots represents mean $\pm$ SD. Blue arrow indicates leukocyte population

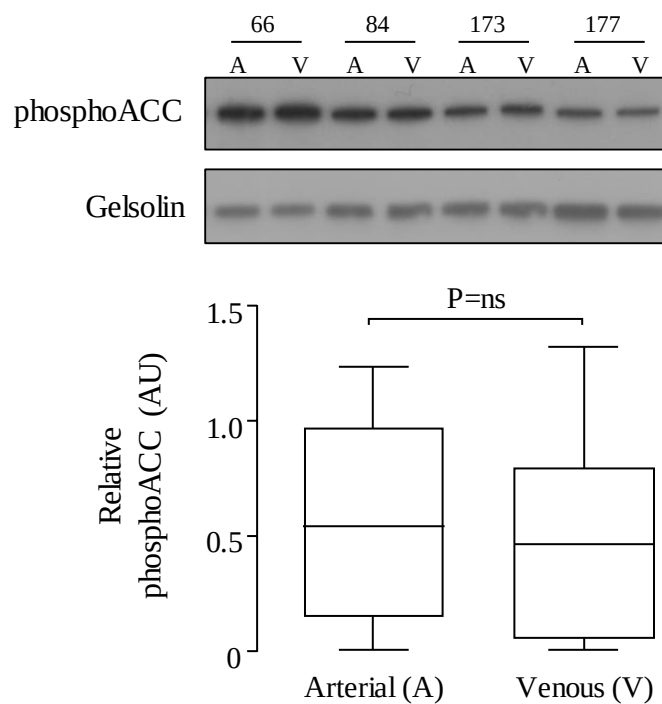
FSC-A: forward-scattered light-area, PRP: platelet rich-plasma, SSC-A: side-scattered light-area.



Supplemental Figure 2. ROC curve analysis of platelet phosphoACC (CAD vs N-CAD).

ROC curve analysis of platelet phosphoACC (CAD vs. N-CAD) to estimate the optimal threshold (0.5 AU) for a maximal sensitivity (48 %) and specificity (90 %).

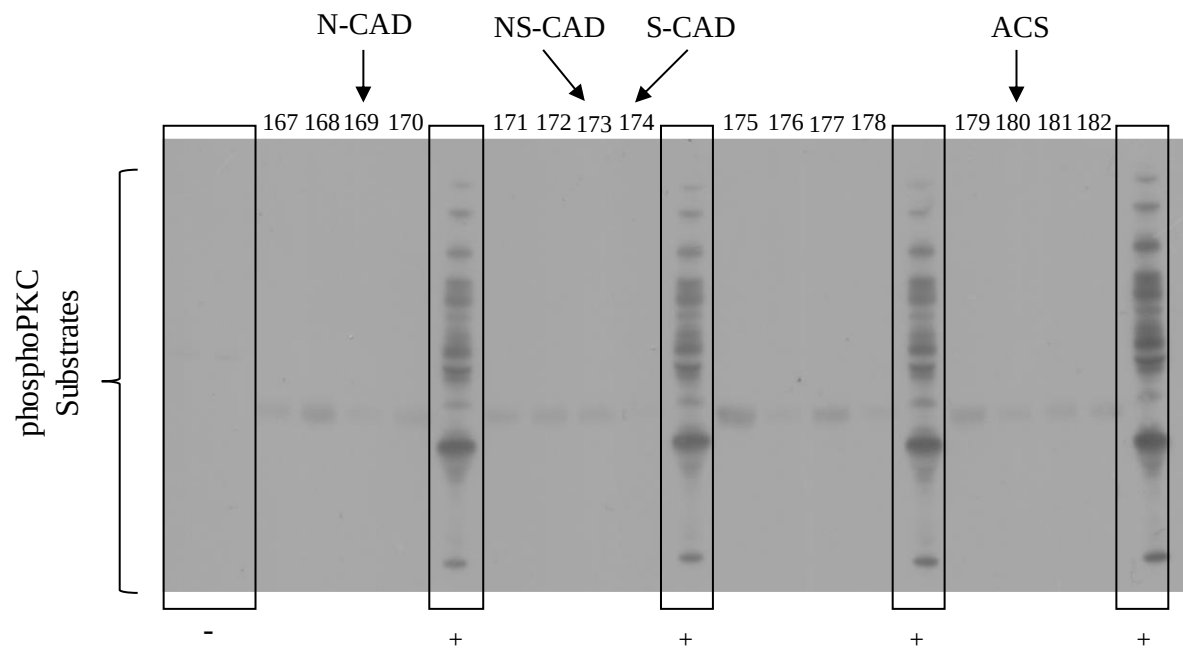
AUC: area under the curve, CAD: coronary artery disease, CI: confidence interval, N-CAD: absence of coronary artery disease, ROC: receiver operating characteristic.



Supplemental Figure 3. Arterio-venous difference in platelet ACC phosphorylation.

Platelet phosphoACC was detected in platelets from venous (V) and arterial (A) blood samples drawn simultaneously from central venous and arterial access site respectively, during left and right catheterization (in 8 patients from ACCTHEROMA cohort). Representative western blot analysis is shown on the top panel and quantification, on bottom panel. Gelsolin was used as loading control. Box-and-whisker plots represents median (interquartile range: P25-P75) and extremes of the distribution.

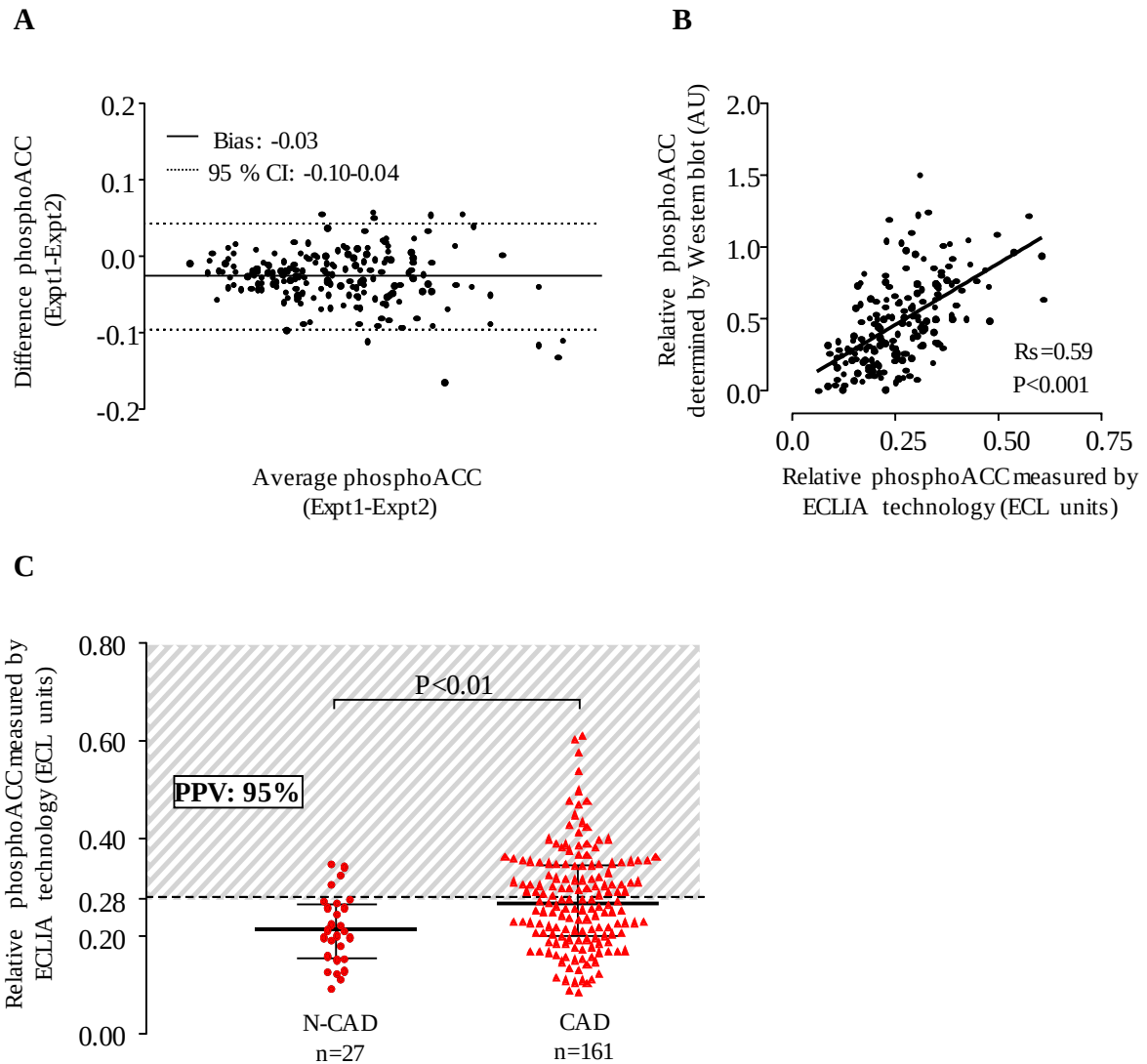
A: arterial, A.U.: arbitrary unit, V: venous.



Supplemental Figure 4. PKC substrates phosphorylation in ACCTHEROMA cohort.

Representative western blot of platelet phosphoPKC substrates of 16 consecutive patients (their corresponding phosphoACC is shown in Figure 3A). (-) negative control corresponding to washed unstimulated platelets from healthy volunteers. (+) positive controls corresponding to washed platelets from healthy volunteer, stimulated ex-vivo with thrombin 0.5 U/ml for 2 min.

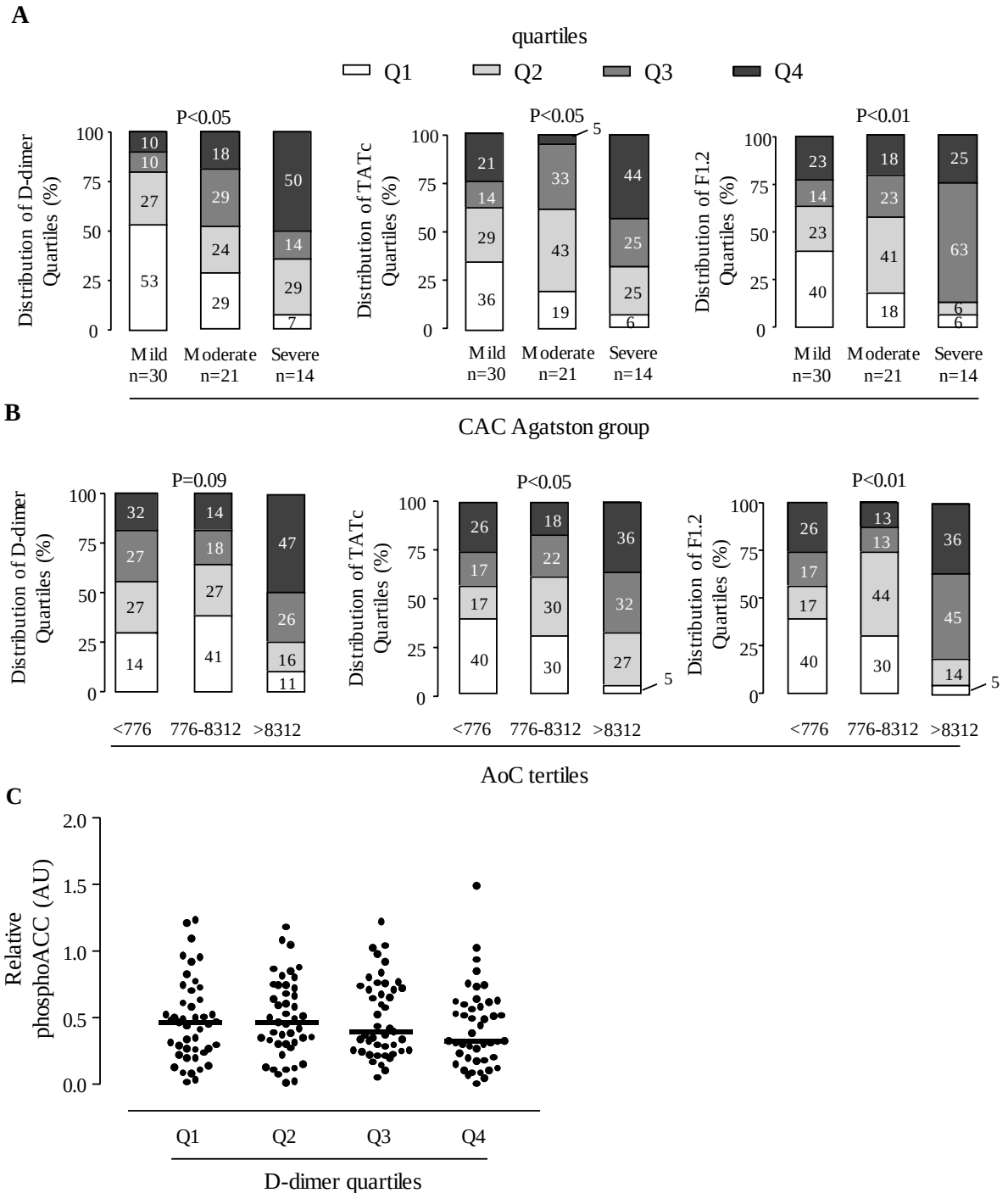
ACS: acute coronary syndrome, CAD: coronary artery disease, M: molecular weight marker, N-CAD: absence of coronary artery disease, NS-CAD: coronary artery disease without significant stenosis, phosphoPKC substrates: phosphorylation of protein kinase C substrates, S-CAD: coronary artery disease with at least one lesion > 50 %.



Supplemental Figure 5. Detection of platelet phosphoACC by electrochemiluminescence.

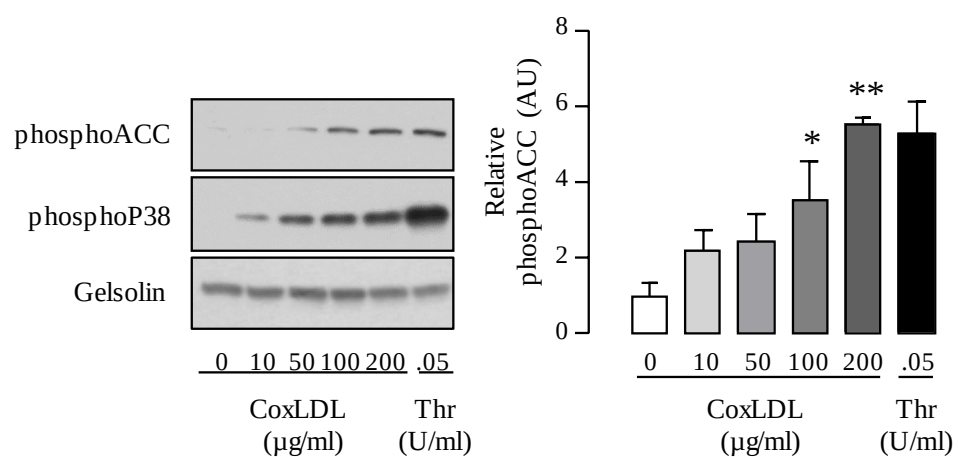
(A) Bland-Altman analysis of two separate quantifications (Expt1-Expt2) of phosphoACC by electrochemiluminescence. (B) Correlation between western blot quantifications and electrochemiluminescence results for platelet phosphoACC (R_s =Spearman coefficient). (C) Electrochemiluminescence quantification of platelet phosphoACC in N-CAD and CAD patients. PPV of optimal threshold (0.28 ECL units for a sensitivity of 47 % and specificity of 85 %) for CAD is indicated on the graph. Red dots (N-CAD, reference population) or triangles (CAD patients) represent individual values. Medians with interquartile range are presented.

AU: arbitrary unit, CAD: coronary artery disease, CI: confidence interval, ECL: electrochemiluminescence, ECLIA: electrochemiluminescence immunoassay, Expt: experiment, N-CAD: absence of coronary artery disease, PPV: positive predictive value.



Supplemental Figure 6. Association between thrombin generation markers and the severity of atherosclerotic burden.

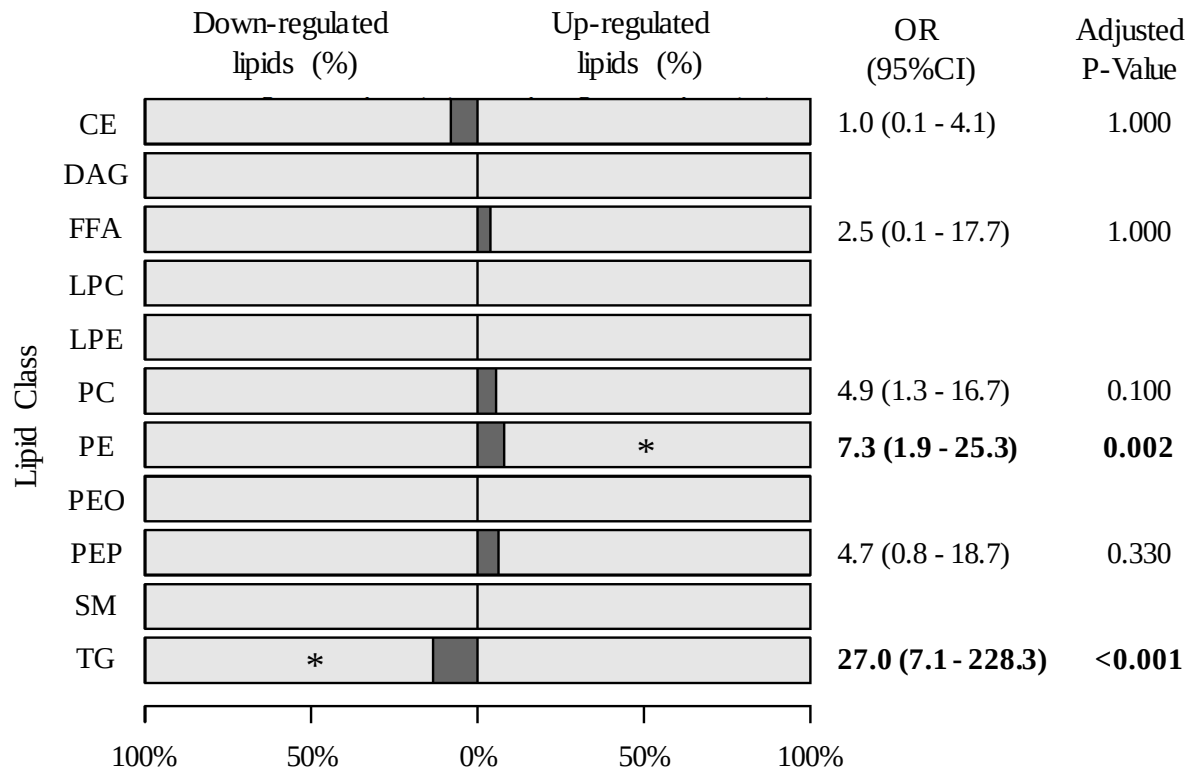
(A, B) Distribution of D-dimers, TATc and F1.2 quartiles among CAC Agatston score groups (A) or AoC score tertiles (B). CAC was classified as mild, moderate or severe if the CAC Agatston score was below 100, between 100-400 or above 400, respectively. (C) Correlation between D-dimer quartiles and platelet phosphoACC in the entire cohort ($P = \text{ns}$). Black dots represent individual values. Medians are presented. AoC: extra-coronary calcification score, AU: arbitrary unit, CAC: coronary artery calcification, F1.2: fragment 1.2, Q: quartile, TATc: thrombin anti-thrombin complex.



Supplemental Figure 7. Platelet phosphoACC-induced by CoxLDL.

Platelet phosphoACC and phosphoP38 were detected in washed platelets from healthy volunteers, after treatment with varying concentrations of CoxLDL (10-200 µg/ml) for 5 min or with thrombin (0.05 U/ml) for 2 min, which was used as reference. Gelsolin was used as loading control. Representative western blots and quantifications are shown. Data are expressed as mean±SEM. Significance was determined by 1-way ANOVA with bonferroni post hoc analysis. *P<0.05, **P<0.01, relative to unstimulated platelets.

AU: arbitrary unit, CoxLDL: copper-oxidized low-density lipoprotein, Thr: thrombin



Supplemental Figure 8. Lipid class enrichment analysis from the lipidomic study.

Bars (dark grey) represent lipid classes with differentially regulated lipid species with respect to increased platelet phosphoACC. * denotes classes with significantly up- or down-regulated lipid species. OR and adjusted P-value derived from Fisher's exact test are represented.

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, TG: triglycerides

Supplemental Table 1: Prediction of ischemic events by platelet phosphoACC in multivariate Cox regression analysis

Variable	Univariable Analysis		Multivariable Analysis	
	HR (95% CI)	P value	HR (95% CI)	P value
Previous history of MI	1.55 [1.15-2.08]	0.004	3.17 [1.38-7.29]	0.006
ASCVD score	1.37 [1.00-1.88]	0.048	10.07 [1.88-54.02]	0.007
ACS at inclusion time	1.42 [1.01-1.99]	0.045		
Platelet count (x10 ³) /μl	1.45 [1.03-2.04]	0.034		
Creatinine (mg/dl)	0.90 [0.46-1.76]	0.76		
HsCRP (log-transformed)	1.41 [0.96-2.09]	0.083		
Non-HDL (mg/dL)	0.82 [0.54-1.25]	0.35		
TG/HDL-C ratio (log-transformed)	0.90 [0.6-1.36]	0.63		
D-dimer (log-transformed)	1.36 [1.01-1.83]	0.043		
Platelet phosphoACC	1.44 [1.02-2.03]	0.036	3.66 [1.11-12.12]	0.034

Ischemic events include cardiovascular death and recurrent myocardial infarction/revascularization procedures. Statistical significance when p<0.05, CI: confidence interval, HR: hazard ratio. ACS: Acute Coronary Syndrome, ASCVD score: 10-year risk of atherosclerotic cardiovascular disease (2013 ACC/AHA Guideline on the Assessment of Cardiovascular Risk), hsCRP: high-sensitive C-reactive protein, HDL-C: high-density lipoprotein cholesterol, MI: myocardial infarction, TG: triglycerides.

Supplemental Table 2: Multivariable linear regression model of factors associated with CAC

Variable	Univariable			Multivariable (global $R^2 = 0.47$, $p < 0.001$)	
	R^2	β Coefficient (95% CI)	P value	β Coefficient (95% CI)	P value
Age	0.219	0.03 (0.01-0.04)	<0.001	0.21 (0.01-0.04)	0.011
Sex (male)	0.025	0.24 (-0.14-0.61)	0.22		
Diabetes	0.001	0.05 (-0.40-0.50)	0.82		
Hypertension	0.137	0.58 (0.21-0.96)	0.003		
Non-HDLc (mg/dl)	0.004	-0.001(-0.005-0.003)	0.62		
D-dimer (log-transformed)	0.127	0.95 (0.17-1.72)	0.018	0.87 (0.17-1.56)	0.017
Platelet phosphoACC	0.088	2.27 (0.38-4.16)	0.019	3.61 (1.57-5.65)	0.001

Statistical significance when $P < 0.05$. CAC: coronary artery calcification, CI: confidence interval, HDLc: high-density lipoprotein cholesterol.

Supplemental Table 3. Baseline characteristics of the lipidomics cohort

	All N = 31	Low phosphoACC N = 12	High phosphoACC N = 19	P
<u>Clinical Characteristics</u>				
Age, years	61.4 ± 10.6	56.2 ± 10.4	64.7 ± 9.5	0.025*
Male sex, n (%)	19 (61.3)	6 (50)	13 (68.4)	0.52†
BMI, kg/m ²	28.4 ± 4.2	27.3 ± 4.7	29.1 ± 3.9	0.25
Hypertension, n (%)	15 (48.4)	5 (41.7)	9 (52.6)	0.82†
Smoking, n (%)	18 (58.1)	8 (66.7)	10 (52.6)	0.70†
Diabetes, n (%)	6 (19.4)	1 (8.3)	5 (26.3)	0.45†
Prior history of CAD, n (%)	10 (32.3)	0 (0)	10 (52.6)	0.004*†
- MI, n (%)	7 (22.6)	0 (0)	7 (36.8)	0.038*†
- PCI, n (%)	10 (32.3)	0 (0)	10 (32.3)	0.004*†
- CABG, n (%)	1 (3.2)	0 (0)	1 (5.3)	> 0.99†
Aortic valve disease, n (%)	3 (9.7)	2 (16.7)	1 (5.3)	0.66†
Mitral valve disease, n (%)	2 (6.5)	2 (16.7)	0 (0)	0.28†
ACS, n (%)	12 (38.7)	0 (0)	12 (63.2)	<0.001*†
<u>Lab results</u>				
Creatinine, (mg/dL)	1.0 (0.8-1.2)	0.9 (0.8-1.3)	1.0 (0.9-1.2)	0.68
CRI, n (%)	2 (6.5)	2 (16.7)	0 (0)	0.30†
Total cholesterol, (mg/dL)	167 ± 47	175 ± 66	163 ± 34	0.51
Non-HDL, (mg/dL)	119 ± 42	122 ± 54	117 ± 35	0.78
Triglycerides, (mg/dL)	125 ± 54	98 ± 44	141 ± 53	0.034*
<u>Medication</u>				
ACEi/ARB, n (%)	13 (41.9)	4 (33.3)	9 (47.4)	0.70†
Beta-blockers, n (%)	14 (45.2)	5 (41.7)	9 (47.4)	> 0.99†
Lipid-lowering treatment, n (%)	18 (58.1)	4 (33.3)	14 (73.7)	0.065†
Aspirin, n (%)	23 (74.2)	6 (50)	17 (89.5)	0.044*†
Dual antiplatelet therapy, n (%)	6 (19.4)	0 (0)	6 (31.6)	0.074†
- Clopidogrel, n (%)	2 (6.5)	0 (0)	2 (10.5)	0.74†
- Ticagrelor, n (%)	3 (9.7)	0 (0)	3 (15.8)	0.43†
- Prasugrel, n (%)	1 (3.2)	0 (0)	1 (5.3)	>0.99†
Platelet phosphoACC	0.8 (0.1-1.0)	0.10 (0.08-0.13)	0.96 (0.85-1.03)	<0.001

Values are mean ± SD, n (%) or median (25th – 75th percentile). ACEi/ARB: angiotensin converting enzyme inhibitor/angiotensin receptor blocker, ACS: acute coronary syndrome, BMI: body mass index, CABG: coronary artery bypass graft, CAD: coronary artery disease, CRI: chronic renal insufficiency, hsCRP: high sensitivity C reactive protein, LDL: low-density lipoprotein, MI: myocardial infarction, PCI: percutaneous coronary intervention, P: P value. Statistical differences between groups are denoted as * between low- and high-phosphoACC. † denotes calculated mid-P value (Fischer's test).

Supplemental Table 4. List of the significantly down-regulated TG lipid species in platelets with increased phosphoACC.				
Lipid Species	N data	Log FC	P	Adj P
TAG40.0.FA16.0	23	-1.383	0.002	0.038
TAG42.0.FA14.0	30	-1.657	0.001	0.027
TAG42.0.FA16.0	31	-1.824	0.000	0.025
TAG42.1.FA14.0	20	-1.351	0.000	0.025
TAG42.1.FA16.0	26	-1.262	0.003	0.042
TAG42.1.FA18.1	28	-1.493	0.001	0.025
TAG44.0.FA14.0	31	-1.588	0.001	0.025
TAG44.0.FA16.0	31	-1.438	0.002	0.038
TAG44.0.FA18.0	30	-1.301	0.001	0.031
TAG44.1.FA12.0	31	-1.416	0.004	0.043
TAG44.1.FA14.0	31	-1.635	0.000	0.025
TAG44.1.FA14.1	18	-1.041	0.004	0.047
TAG44.1.FA16.0	31	-1.319	0.001	0.025
TAG44.1.FA16.1	31	-1.289	0.002	0.038
TAG44.1.FA18.1	31	-1.480	0.000	0.025
TAG44.2.FA12.0	20	-1.162	0.002	0.038
TAG44.2.FA16.0	31	-1.380	0.000	0.025
TAG44.2.FA16.1	21	-0.866	0.003	0.041
TAG44.2.FA18.1	29	-1.417	0.001	0.025
TAG44.2.FA18.2	31	-1.514	0.001	0.025
TAG44.3.FA18.2	14	-0.915	0.001	0.025
TAG45.0.FA14.0	29	-1.320	0.001	0.025
TAG45.0.FA15.0	27	-1.311	0.000	0.025
TAG45.0.FA16.0	31	-1.472	0.000	0.025
TAG45.1.FA15.0	18	-1.139	0.000	0.025
TAG45.1.FA16.0	24	-0.945	0.001	0.027
TAG45.1.FA18.1	23	-0.983	0.001	0.028
TAG46.0.FA14.0	31	-1.171	0.002	0.040
TAG46.0.FA16.0	31	-1.140	0.004	0.043
TAG46.0.FA18.0	31	-1.232	0.003	0.040
TAG46.1.FA14.1	31	-1.167	0.003	0.042
TAG46.1.FA18.0	30	-1.301	0.001	0.025
TAG46.1.FA18.1	31	-1.180	0.003	0.042
TAG46.2.FA14.0	31	-1.273	0.001	0.025
TAG46.2.FA18.1	31	-1.209	0.001	0.027
TAG46.2.FA18.2	31	-1.188	0.005	0.050
TAG46.3.FA14.0	20	-1.148	0.002	0.038
TAG46.3.FA14.1	16	-0.802	0.004	0.047
TAG46.3.FA16.0	26	-0.900	0.004	0.047
TAG46.3.FA16.1	26	-0.844	0.003	0.041
TAG46.3.FA18.1	30	-1.222	0.001	0.028
TAG46.3.FA18.2	30	-1.196	0.001	0.027
TAG46.4.FA18.2	16	-1.056	0.000	0.025
TAG47.0.FA14.0	29	-0.968	0.004	0.047
TAG47.0.FA15.0	31	-1.103	0.002	0.038
TAG47.0.FA16.0	31	-1.166	0.002	0.038
TAG47.0.FA17.0	31	-0.787	0.001	0.031
TAG47.1.FA14.0	31	-1.106	0.004	0.043
TAG47.1.FA15.0	31	-1.116	0.002	0.040
TAG47.1.FA16.0	31	-1.094	0.003	0.040
TAG47.1.FA16.1	31	-0.839	0.002	0.038

TAG47.1.FA17.0	24	-0.910	0.002	0.038
TAG47.1.FA18.1	31	-1.257	0.001	0.025
TAG47.2.FA14.0	26	-1.026	0.001	0.025
TAG47.2.FA15.0	29	-1.091	0.000	0.025
TAG47.2.FA16.1	30	-1.222	0.000	0.025
TAG47.2.FA18.1	30	-0.954	0.001	0.027
TAG47.2.FA18.2	29	-1.042	0.001	0.025
TAG48.0.FA18.0	31	-0.932	0.005	0.050
TAG48.2.FA18.2	31	-0.962	0.003	0.040
TAG48.3.FA16.0	31	-0.976	0.005	0.048
TAG49.0.FA15.0	31	-0.836	0.004	0.047
TAG49.2.FA18.2	31	-0.977	0.003	0.041
TAG49.3.FA18.3	25	-0.871	0.002	0.040
TAG50.2.FA18.0	31	-0.722	0.003	0.042
TAG50.4.FA14.1	31	-0.737	0.003	0.040

Analysis was performed by a multivariate logistic regression model. Statistical significance was determined using the Benjamini-Hochberg correction procedure with a false discovery rate < 0.05. TG lipid species are arranged from top to bottom in order of increasing number of carbons in the TG structure. Only TG with significantly adjusted P values (<0.05) are shown (n=66).

Adj P: adjusted P value, FC: fold-change, n: number of data analyzed, P: P value.

3.1.10.1 Additional data

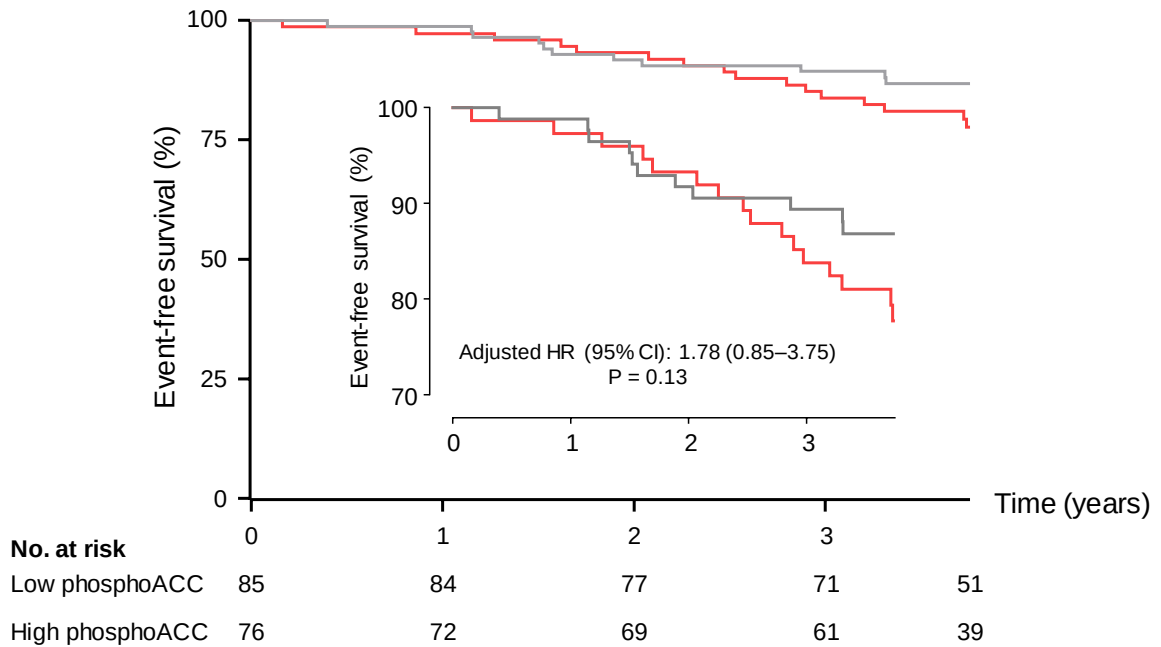
A. Diabetes and chronic kidney disease, two important parameters for risk assessment in CAD patients, have also been added to the univariable and multivariable models of factors associated with ACS (presented in section 3.1.5.2, Table 2).

Additional Table . Univariable and multivariable models of factors associated with ACS				
Variable	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		
Diabetes	2,35 (1,15-4,79)	0.019		
CKD	0.63 (0.22-1.82)	0.39		
D-dimer (log-transformed)	3.14 (1.16-8.51)	0.024	5.69 (1.82-17.76)	0.003
Non-HDL (mg/dl)	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 $p < 0.05$. OR: odds ratio, CI: confidence interval, ASCVD score: 10 year risk of atherosclerotic cardiovascular disease (2013 ACC/AHA Guideline on the Assessment of Cardiovascular Risk), BMI: body mass index, CKD: chronic kidney disease, hsCRP: high sensitivity C-reactive protein, TG/HDL-C ratio: triglyceride/high-density lipoprotein cholesterol ratio.

B. Kaplan-Meier event-free survival curves for the analysis of ischemic events have also been performed on the CAD cohort (excluding the N-CAD population), considering that recurrence of events occurs only in this population (related to Figure 4, section 3.1.5.2). Patients with high platelet phosphoACC

(above 0.5 A.U) show a trend increase in event rates compared to low phosphoACC levels (Log-rank test, $p=0.13$).



Additional Figure. Kaplan-Meier curves for ischemic events according to platelet phosphoACC levels in the CAD cohort.

Ischemic events include cardiovascular death, recurrent myocardial infarction and revascularization procedures. Low and high phosphoACC defined according to threshold of 0.5 AU.

CHAPTER IV – RESULTS (PART 2)

4.1 AMPK-ACC signaling modulates platelet phospholipids content and potentiates platelet function and thrombus formation

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4.1.1 Abstract

AMP-activated protein kinase (AMPK) α 1 is activated in platelets upon thrombin or collagen stimulation, and as consequence phosphorylates and inhibits acetyl-CoA carboxylase (ACC). Since 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-signalling 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 signalling 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 signalling 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.

4.1.2 Introduction

A growing body of evidence shows that lipids are essential in regulating platelet functions. Indeed, the platelet exhibits a complex array of over 5000 distinct lipid species with over 700 responding to thrombin activation(279). 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 (292). ACC catalyzes acetyl-CoA carboxylation to form malonyl-CoA. Its two isoforms, ACC1 and ACC2, have distinct cellular distributions (319). ACC1 is present in the cytosol and synthesizes malonyl-CoA for *de novo* lipogenesis (281), while ACC2 is localized on the outer mitochondrial membrane and generates malonyl-CoA which inhibits fatty acid transport into mitochondria for oxidation (280,320). 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 (33,298,299). AMPK phosphorylates ACC1/2 on serine residues (Ser79/212), leading to suppression of ACC activity (321,322). We have previously reported that AMPK α 1 is activated in platelets upon thrombin stimulation, and increases the phosphorylation of myosin regulatory light chains (MLCs), cofilin, and vasodilator-stimulated phosphoprotein (VASP) (33). These cytoskeletal proteins are critical for triggering platelet shape change and the centralization of secretory granules during platelet activation (323). However, the impact of AMPK-mediated ACC phosphorylation on platelet function has never been investigated.

Fatty acids fulfil at least three main roles: structural, signalling and energy storage. Phospholipids (PL) are the major structural lipids in platelets. Upon platelet

activation, reorganization of the plasma membrane PL facilitates shape change, filopodia and lamellipodia formation (324) as well as granule secretion (258) 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 (279). These secondary mediators are crucial for the tight regulation of platelet activation (325-327).

Finally, lipids contribute to platelet energy metabolism. In the basal state, platelets are more oxidative than glycolytic (261,266) and lipid oxidation contributes to at least one-third of the total oxygen consumed by mitochondria (261,272,328). Mitochondrial lipid oxidation increases upon thrombin stimulation, to cope with the energetic demands of platelet activation (267,272), a process which is supported by the enhanced availability of eicosanoids released from the membrane PL through the action of Ca^{2+} -dependent cytosolic phospholipase A₂ (cPLA₂) (279).

Clearly, the signalling 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 (292).

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 to wild type (WT), increasing thromboxane A₂ (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 generate key mediators of platelet activation.

4.1.3 Materiel and Methods

4.1.3.1 Mice

ACC1/2 DKI mice have been described previously (292). WT mice served as controls. Eight- to sixteen- 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 US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

4.1.3.2 Platelet preparation

Murine platelet Mice were bled under ketamine and xylazine anesthesia from the retro-orbital plexus. Blood was collected in 1/6 citrate-dextrose (ACD) solution with apyrase 1 U/ml. PRP was obtained by centrifugation at 800 g for 5 s, followed by 5 min at 100 g. It was washed by adding 2 volumes of ACD with apyrase 1 U/ml. The platelets were pelleted by centrifugation at 400 g for 5 min and re-suspended to a density of $2.5 \times 10^5/\mu\text{l}$ (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 .

4.1.3.3 Flow chamber assay

Blood was collected from the retro-orbital plexus into 48 μM D-Phenylalanyl-prolyl-arginyl Chloromethyl Ketone (PPACK), 5 U/ml heparin and 40 U/ml fragmin. Samples of 400 μl were flowed over type I collagen-coated (100 $\mu\text{g/ml}$), vWF-binding protein (BP) (12.5 $\mu\text{g/ml}$) and laminin (50 $\mu\text{g/ml}$)- or vWF-BP (12.5 $\mu\text{g/ml}$), laminin (50 $\mu\text{g/ml}$) and rhodocytin (250 $\mu\text{g/ml}$)-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 $1,000 \text{ s}^{-1}$ for 3.5 min, as described (329). Alternatively, samples were pre-incubated with 20 μM Ticagrelor for 10 min or the corresponding vehicle (DMSO) and flowed over type I collagen-coated coverslips, as described above. Activated platelets in thrombi were post-stained with FITC-labeled anti-P-selectin 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 min (stasis), after which unbound Abs were removed by perfusion with Tyrode's buffer. Brightfield phase-contrast and fluorescence images were recorded by a non-confocal 2-camera system. Surface coverage was analyzed by ImagePro software (Media Cybernetics) (1).

4.1.3.4 Ferric chloride-induced thrombosis

Carotid arteries were injured in anesthetized mice by topical application of 10% ferric chloride (FeCl_3) for 5 min, as described previously (330). Briefly, exogenous Carboxyfluorescein Succinimidyl Ester (CFSE)-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 min for a total of 24 min.

4.1.3.5 Untargeted lipidomics

Lipidomics analysis was carried out on the commercial Lipidyzer platform, according to the manufacturer's instructions (Sciex) (318). 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 (318), 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 Lipidyzer 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 (RSD) of quality control samples were below 15% for all lipid classes, except for sphingomyelin where a RSD of 25% was noted.

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

4.1.4 Results

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

ACC WT and ACC DKI mice have comparable erythrocyte, leukocyte and platelet counts in whole blood (Table S1). Expression levels of the major platelet surface receptors, $\alpha\text{IIb}\beta 3$, GPIb α , the collagen receptor GPVI, and the Protease-Activated Receptor-3 (PAR-3) and PAR-4, were equivalent in ACC WT and ACC DKI platelets (Figures S1A and S1B). ACC1, but not ACC2, was detected in both human and murine platelets (Figure 1A). Consistent with previous observations (33), 0.2 U/ml thrombin (Figure 1B) or 5 $\mu\text{g/ml}$ collagen (Figure 1C) induced phosphorylation on Ser79 of ACC in platelets from WT mice with maximal effects after 1 min and 5 min, respectively. As expected, no Ser79 ACC phosphorylation was detected in either basal or thrombin- or collagen-treated ACC DKI platelets (Figures 1B and 1C). ACC1 mutation did not influence AMPK activation, reflected by Thr172 phosphorylation (Figures 1D and 1E). In addition, thrombin- or collagen- induced phosphorylation of known AMPK cytoskeletal targets, MLC, cofilin and VASP, were similar between ACC DKI and ACC WT platelets (Figures 1D and 1E), as well as platelet-spreading and lamellipodia/filopodia formation after platelet immobilization on fibrinogen-coated coverslips (Figures S1 C-H). These data confirm that, although ACC phosphorylation was impaired, AMPK signalling was unaltered in ACC DKI platelets.

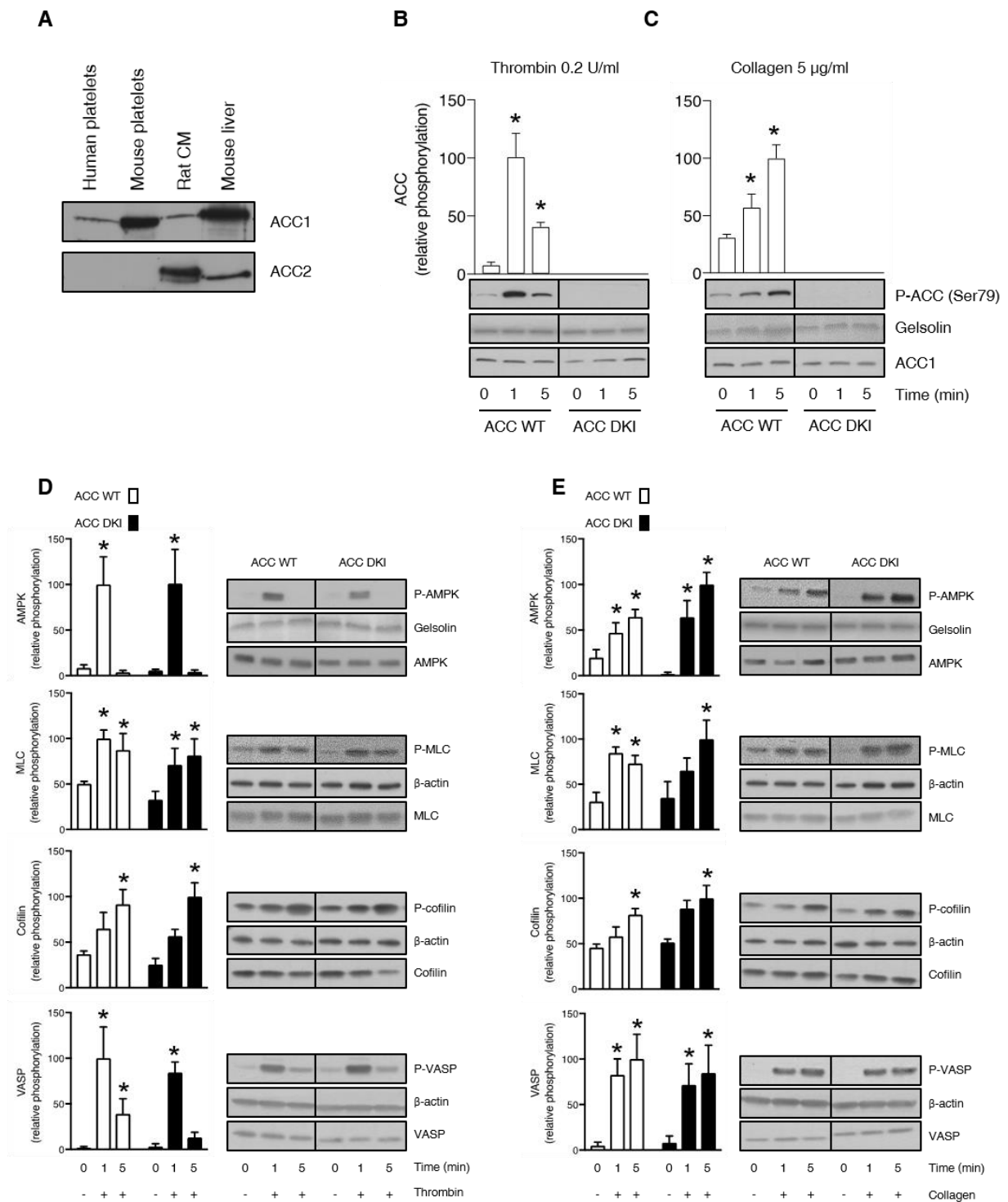


Figure 1. Lack of AMPK-ACC phosphorylation does not impact AMPK signalling or phosphorylation of cytoskeletal proteins. (A) Washed murine and human platelets were lysed and subjected to Western blotting for ACC1 or ACC2 isoform expression analysis. Isolated rat cardiomyocytes (CM) and mouse liver extracts 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 time points. 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 but were not contiguous. The results are expressed as means ± SEM (at least 3 experiments for each condition). *indicates values statistically different from respective untreated platelets, P≤0.05. Analysis was performed by 2-way ANOVA. See also Figure S1 and Table S1.

4.1.4.2 ACC DKI mice display enhanced primary haemostasis and thrombosis

The impact of AMPK-mediated ACC phosphorylation on haemostasis 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 s vs. ACC WT: 188 ± 51 s, P<0.05) (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 (Figures 2B and 2C). The rate of thrombus growth was significantly increased in ACC DKI mice compared to the WT animals. The fold change increase in thrombus growth over a 20-min period of time 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 signalling is a key mechanism regulating primary haemostasis and arterial thrombosis *in vivo*.

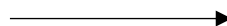
4.1.4.3 Lack of AMPK-ACC signalling favours thrombus formation during perfusion on collagen in flow conditions

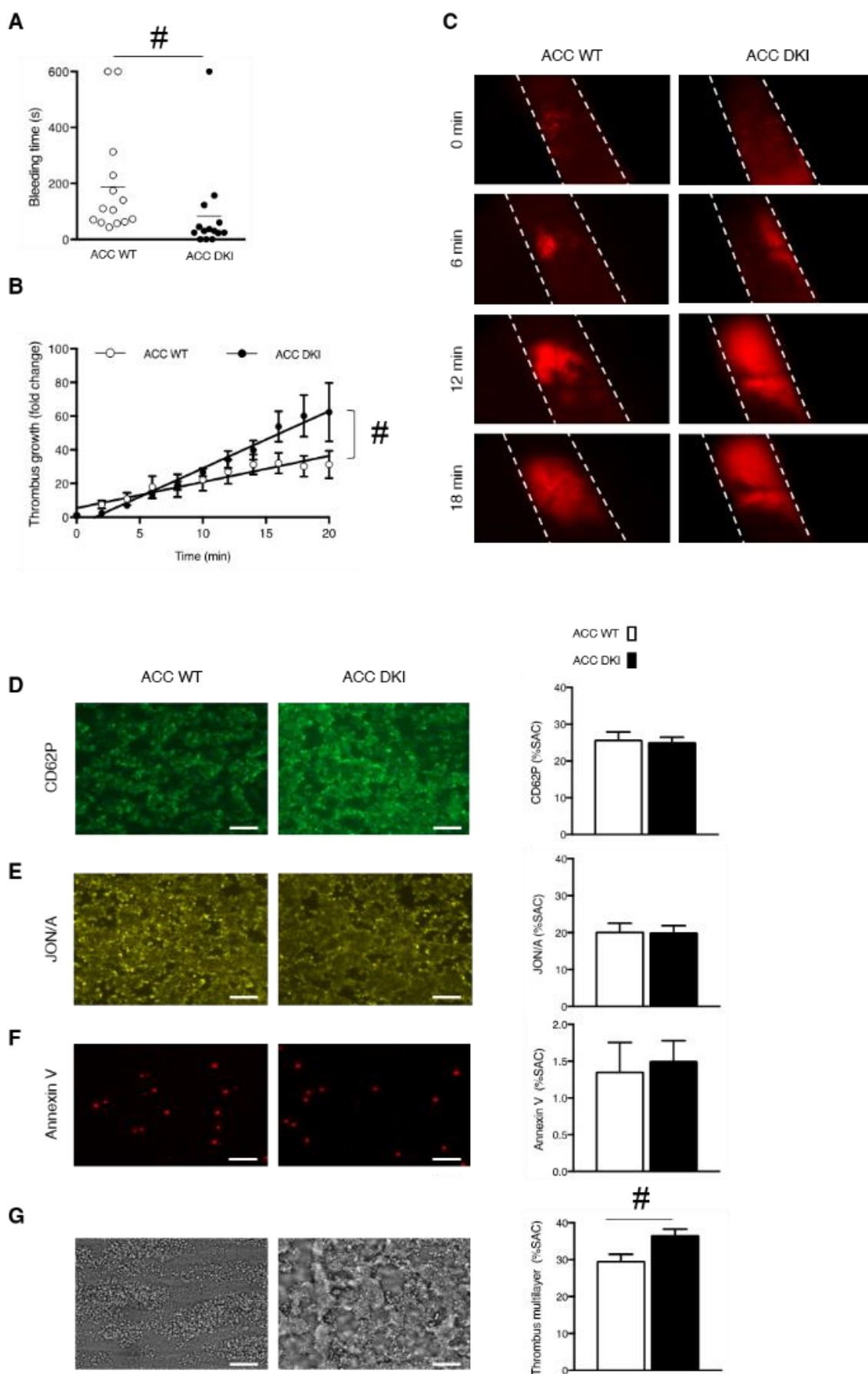
We next examined thrombus formation *ex vivo*, using a flow chamber system. Whole blood from ACC WT and DKI mice was perfused at an intermediate shear rate of 1,000

s⁻¹, on 3 different coated surfaces containing either collagen, laminin or vWF-binding peptide (vWF-BP) in the absence or presence of rhodocytin(1). Brightfield images were captured to assess overall platelet adhesion and thrombus formation. Platelet activation was simultaneously monitored by infusing fluorescently-labelled anti-CD62P antibody (Ab) as a marker of α -granule secretion, JON/A Ab to measure α IIb β 3 activation, and annexin V to analyze phosphatidylserine (PS) externalization, reflecting platelet pro-coagulant activity.

On collagen-coated coverslips, the surface area covered (SAC) by adherent platelets and their activation state (Figures 2D-F) were similar between the 2 genotypes. However, buildup of multilayered platelet thrombi was increased with ACC DKI blood compared to WT (Figure 2G), indicating that AMPK-ACC signalling 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-BP, in the absence or presence of rhodocytin (data not shown).

Figure 2. ACC DKI mice display enhanced primary haemostasis 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≤0.05. Data were analyzed by the Mann-Whitney 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 min. (B) 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 point at which the thrombus first reaches 100 μ m. Thrombus growth is expressed as means $\pm$ SEM per group at different time points with fitted regression lines (at least 6 mice/group). Slopes are statistically different on the basis of significant interaction ([#]P≤0.05) between slope and group in a linear model, with group, time and their interaction as covariates. (C) Representative fluorescence microscopy images at 0, 6, 12 and 18 min after FeCl₃ application. (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 1,000 s⁻¹. Exposure of P-selectin was evaluated by staining with CD62P Ab (D), α IIb 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 min 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 IIb 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≤0.05. Data were analyzed by the Mann-Whitney test.





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

To further decipher the mechanisms responsible for the gain-of-function phenotype of ACC DKI mice on thrombus formation, we studied the impact of a lack of AMPK-mediated ACC phosphorylation on washed platelet reactivity. First, we investigated the effect of thrombin or collagen on $\alpha\text{IIb}\beta\text{3}$ inside-out activation by analyzing platelet aggregation. ACC DKI platelets aggregated normally at low and high concentrations of thrombin or collagen (Figures 3A and S2A). Accordingly, thrombin- or collagen-induced activation of $\alpha\text{IIb}\beta\text{3}$, detected by JON/A Ab, was normal (Figure 3B). Integrin $\alpha\text{IIb}\beta\text{3}$ -mediated clot retraction consistently showed no differences between ACC WT and ACC DKI platelets after thrombin stimulation (Figures S2B and S2C).

Besides $\alpha\text{IIb}\beta\text{3}$ activation, TXA_2 generation and ADP released from dense granules are important players in collagen-induced platelet aggregate formation under flow (47,48) and in thrombus formation *in vivo* (39,331). Interestingly, the lack of AMPK-ACC signalling amplified dense granule release, specifically in response to collagen. Indeed, a significant 30% increase of ATP (Figures 3C and 3D) and serotonin secretion (Figure 3E) was detected after collagen stimulation in ACC DKI platelets compared to WT, but not in response to thrombin (Figures 3C and 3E). 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 and 3G). Regarding α -granule secretion, P-selectin surface exposure was similar between ACC WT and ACC DKI platelets (Figure S3A). However, platelet factor 4 (PF4) secretion was potentiated upon collagen stimulation in ACC

DKI platelets (Figure S3B), while total PF4 levels in α -granules showed no change (Figure S3C), indicating that the ACC DKI phenotype seems also associated with increased α -granule secretion, at least those containing PF4.

Lack of AMPK-ACC signalling potentiated TXA₂ generation in response to collagen (Figure 3H), independently of any change in basal cyclooxygenase 1 (COX-1) expression or activity (Figure S4A and S4B). To evaluate the connection between ACC DKI-related increased TXA₂ generation and dense granule secretion, collagen-stimulated ACC DKI platelets were pre-treated with 1 mM aspirin (ASA) before assessment of serotonin secretion. As expected, COX-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 to 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).

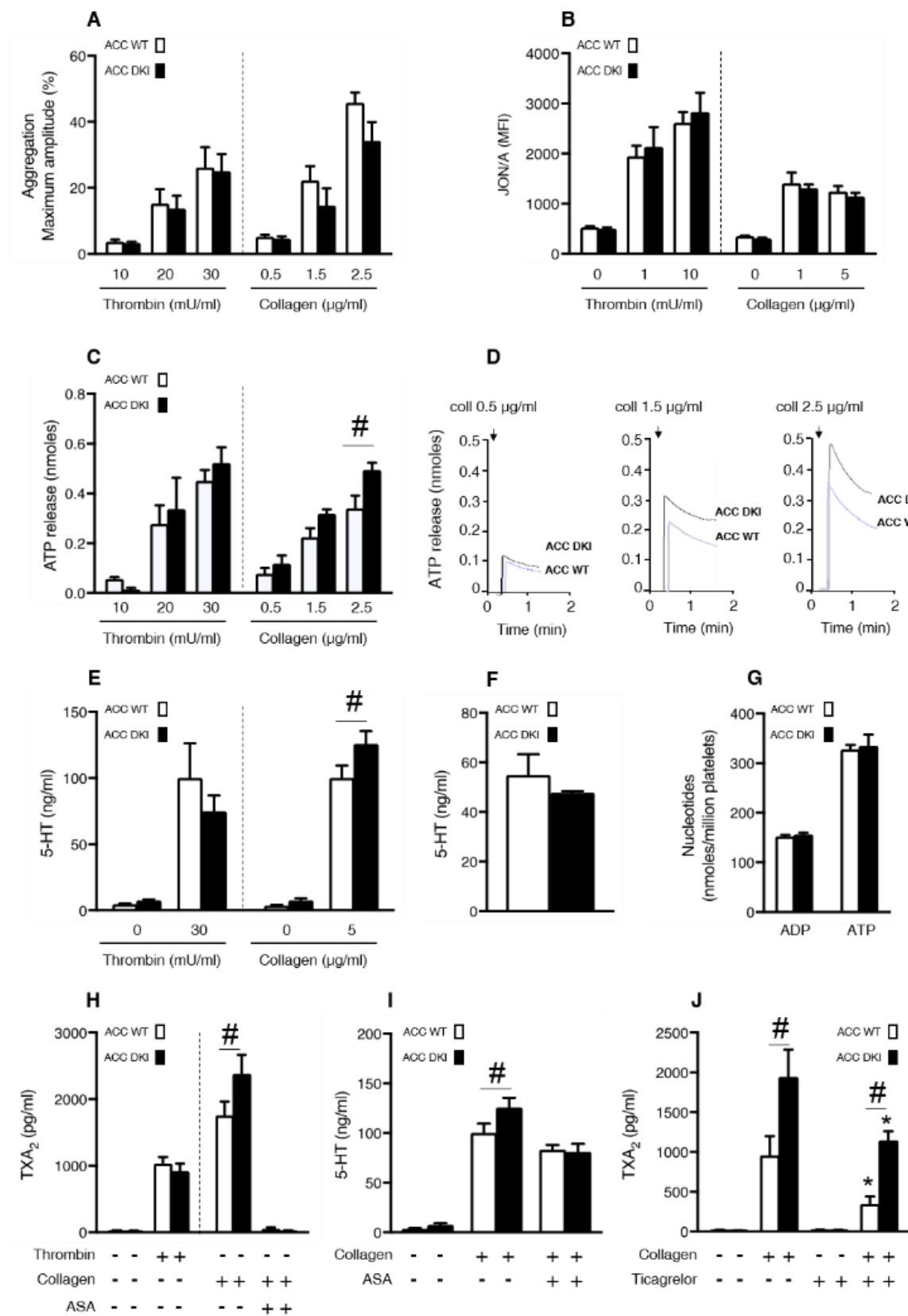


Figure 3. ACC DKI platelets display increased dense granule secretion and thromboxane generation upon collagen stimulation. (A) Washed platelets were stimulated with thrombin or collagen at the indicated concentrations, and light transmission was measured (Chrono-Log). Aggregation is expressed as the 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 min or collagen for 30 min 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 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). $^{*}P \leq 0.05$ 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 ($30 \times 10^3/\mu$ l) were stimulated with 30 mU/ml thrombin or 5 μ g/ml collagen for 5 min, 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). $^{*}P \leq 0.05$ between ACC WT and DKI platelets. The data underwent 2-way ANOVA. (F) Washed platelets ($7.5 \times 10^3/\mu$ 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 (HPLC). Results are expressed as means $\pm$ SEM (n=3). (H) Washed platelets were stimulated with 100 mU/ml thrombin alone or pre-incubated or not for 45 min 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). $^{*}P \leq 0.05$ between ACC WT and DKI platelets. The data underwent 2-way ANOVA. (I) Washed platelets were pre-incubated or not for 45 min 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). $^{*}P \leq 0.05$ between ACC WT and DKI platelets. The data were assessed by 2-way ANOVA. (J) Washed platelets were pre-incubated with 30 μ M Ticagrelor or the corresponding vehicle (DMSO) for 30 min and stimulated with 5 μ g/ml collagen for 5 min. TXA₂ was measured in the supernatant by ELISA. The results are expressed as means $\pm$ SEM (n=4). $^{*}P \leq 0.05$ relative to respective untreated platelets, $^{*}P \leq 0.05$ between ACC WT and ACC DKI platelets. The data underwent 2-way ANOVA. See also Figures S2-S5.

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 to WT, demonstrating the key implication of dense granule secretion in the enhanced thrombus formation (Figures 4A and 4B).

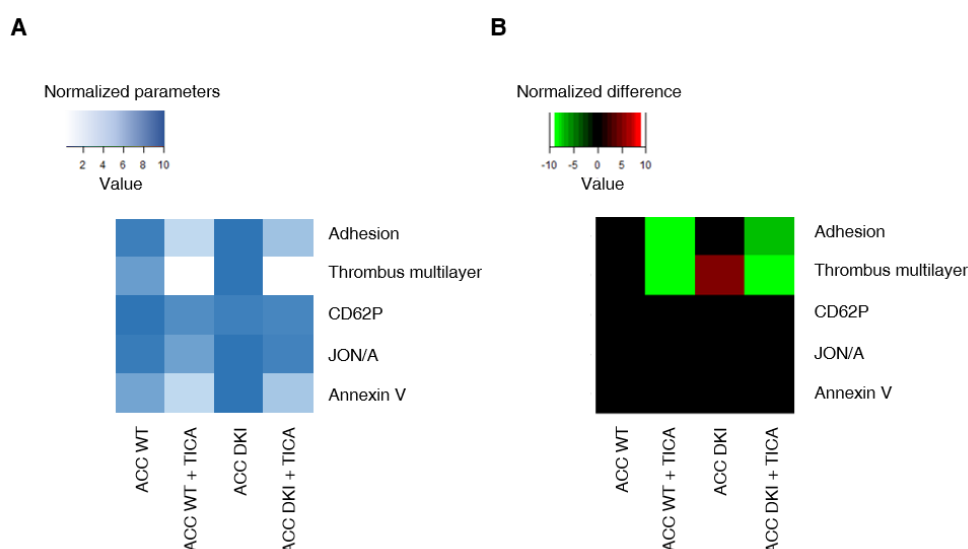


Figure 4. Increased thrombus formation in ACC DKI mice is prevented by P2Y₁₂ inhibition. (A-B) Whole-blood flow perfusion experiments were performed with collagen type I as platelet-adhesive substrate. Where indicated, autocrine effects were blocked by pre-incubation with 20 μ M Ticagrelor (TICA) or vehicle (DMSO) for 10 min. Microscopic images were analyzed for indicated parameters, and per parameter normalized on scale 0-10 (1). (A) Heatmap of normalized parameters. (B) Subtraction heatmap of normalized differences compared to WT mice, filtered for changes with $P \leq 0.05$.

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 like receptor-2, or collagen-related peptide (CRP), a specific GPVI agonist. For all agonists and concentrations tested, ACC DKI platelets aggregated normally (Figures S5A and S5B) and there was no difference in ATP released from dense granules (Figure S5C). These data reinforce the conclusion that the impact of AMPK-ACC signalling 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 $\alpha 2\beta 1$ integrin, which is compatible with the enhancing role of this integrin in GPVI-dependent thrombus stability via TXA₂ production (332).

Taken together these results indicate that the absence of AMPK-ACC signalling 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 $\alpha\text{IIb}\beta\text{3}$ activation.

4.1.4.5 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 (272,279). As expected, thrombin stimulated OCR (Figures 5A and 5B), which has been shown to be due to increased mitochondrial lipid oxidation (272,279). 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 to WT platelets. Carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), a proton-ionophore, then induced maximal OCR and a corresponding decrease in maximal respiration and reserve capacity upon thrombin stimulation, owing to the mobilization of endogenous metabolic substrates to cope with increased energetic demands. This occurred to a similar extent in platelets from both genotypes (Figures 5A and 5B). Finally, antimycin A and rotenone, complex III/I inhibitors totally inhibited mitochondrial-induced OCR. Proton leak was unchanged in all groups (Figures 5A and 5B). It has not been previously reported that collagen also

stimulates mitochondrial function using this approach. As shown in Figures 5C and 5D, collagen stimulates OCR with similar characteristics to thrombin. Taken together, these data indicate that in platelets, AMPK-ACC signalling 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 (Figures 5E and 5F).

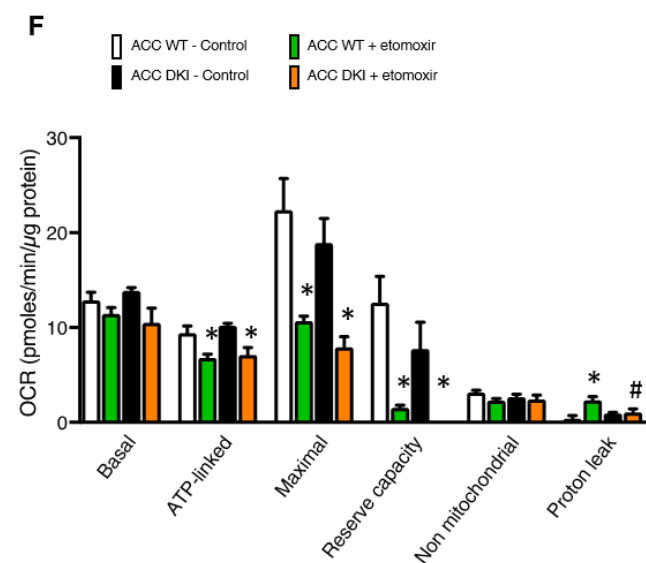
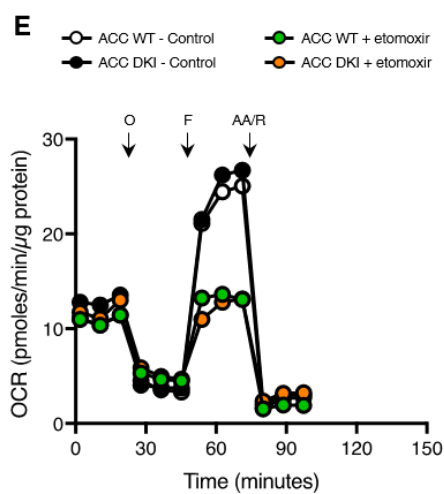
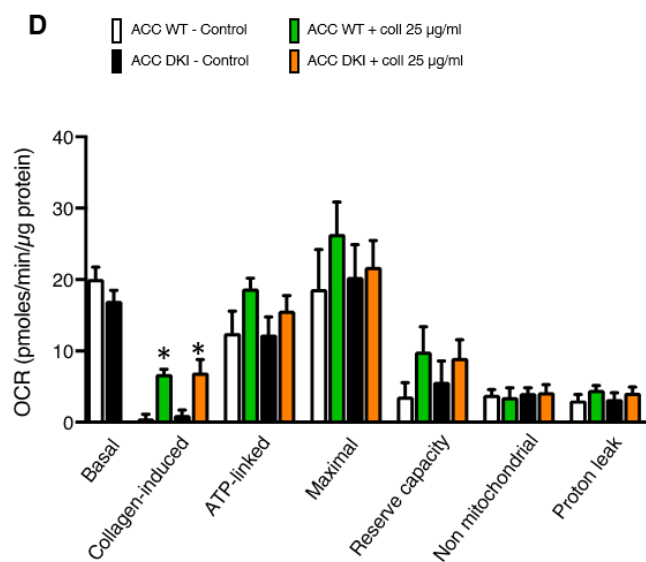
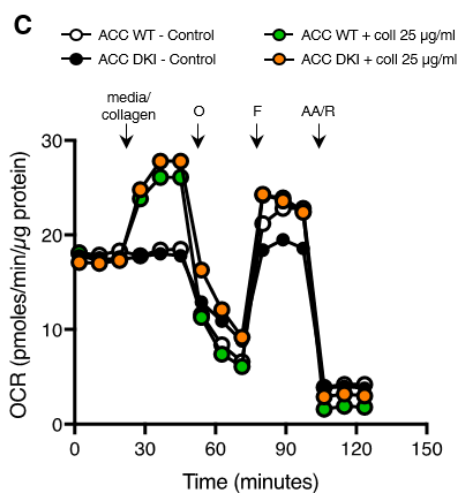
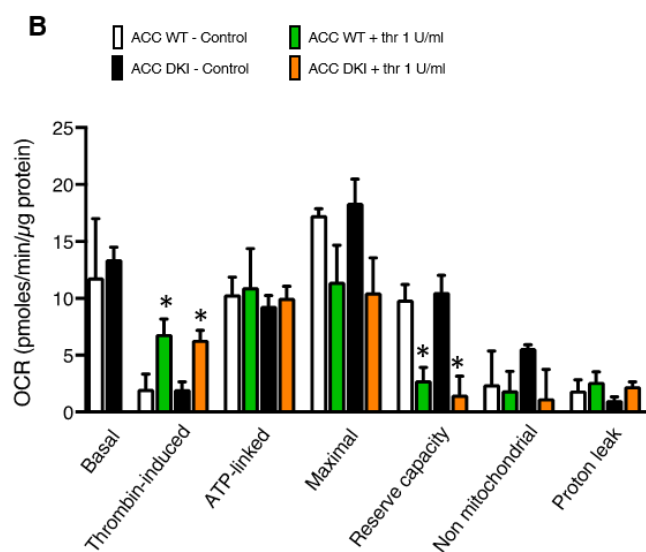
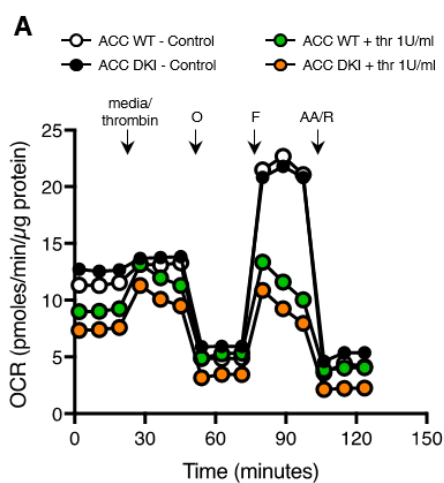


Figure 5. Lack of ACC phosphorylation does not impact oxidative metabolism. (A-F) Oxygen consumption rate (OCR) was measured in washed platelets pre-treated or not with Etomoxir (25 μ M) (E, F) for 1 h prior to bioenergetic measurements. OCR was assessed under basal conditions, after injection of media alone or 1 U/ml thrombin (A, B) or 0.25 μ g/ml collagen (C, D) or followed by treatment with 1 μ M oligomycin (O), 0.1 μ M FCCP (F) and a mix of 1 μ M antimycin A (AA) and 1 μ M 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 (B)- or collagen (D)-induced, ATP-linked, maximal, non-mitochondrial, 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). * $P \leq 0.05$ relative to respective untreated platelets, # $P \leq 0.05$ between ACC WT and ACC DKI platelets. The data underwent 2-way ANOVA.

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 (phosphatidylethanolamine [PE], PEP, plasmeryl phosphatidylethanolamine [PEO], phosphatidylcholine [PC], lysophosphatidylethanolamine [LPE], lysophosphatidylcholine [LPC]), sphingomyelin [SM], cholesteryl esters [CE], free fatty acids [FFA], DAG and triglycerides [TAG] (333).

In agreement with previous data (334), class enrichment analysis indicated that collagen significantly up-regulated lipid species among FFA (31%), LPC (57%), LPE (38%) and DAG (21%) lipid classes, in both ACC WT and ACC DKI platelets (Figure 6A, Table S2). Enrichment of FFA and DAG was also observed in response to thrombin alone (Figure S6A) and to a combination of thrombin and collagen (Figure S6B). More importantly, loss of AMPK-ACC signalling resulted in enrichment of upregulated lipid species among PEP lipid classes (28%), independently of the basal or simulated condition (Figure 6B, Table S3). Indeed, an increase in lipid species among PEP was strongly associated with lack of ACC phosphorylation (OR: 48 [14-217], Figure 6B). ACC DKI also displayed an increment of down-regulated lipid species among CE (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, up-regulated AA-containing PEP in ACC DKI platelets clearly supports the enhanced TXA₂ formation observed in this condition. Of note, the AMPK-ACC signalling did not influence platelet palmitate uptake (Figure S7A). Moreover, collagen-induced TXA₂ production was not modulated by extracellular fatty acids availability in ACC WT or ACC DKI platelets (Figure S7B).

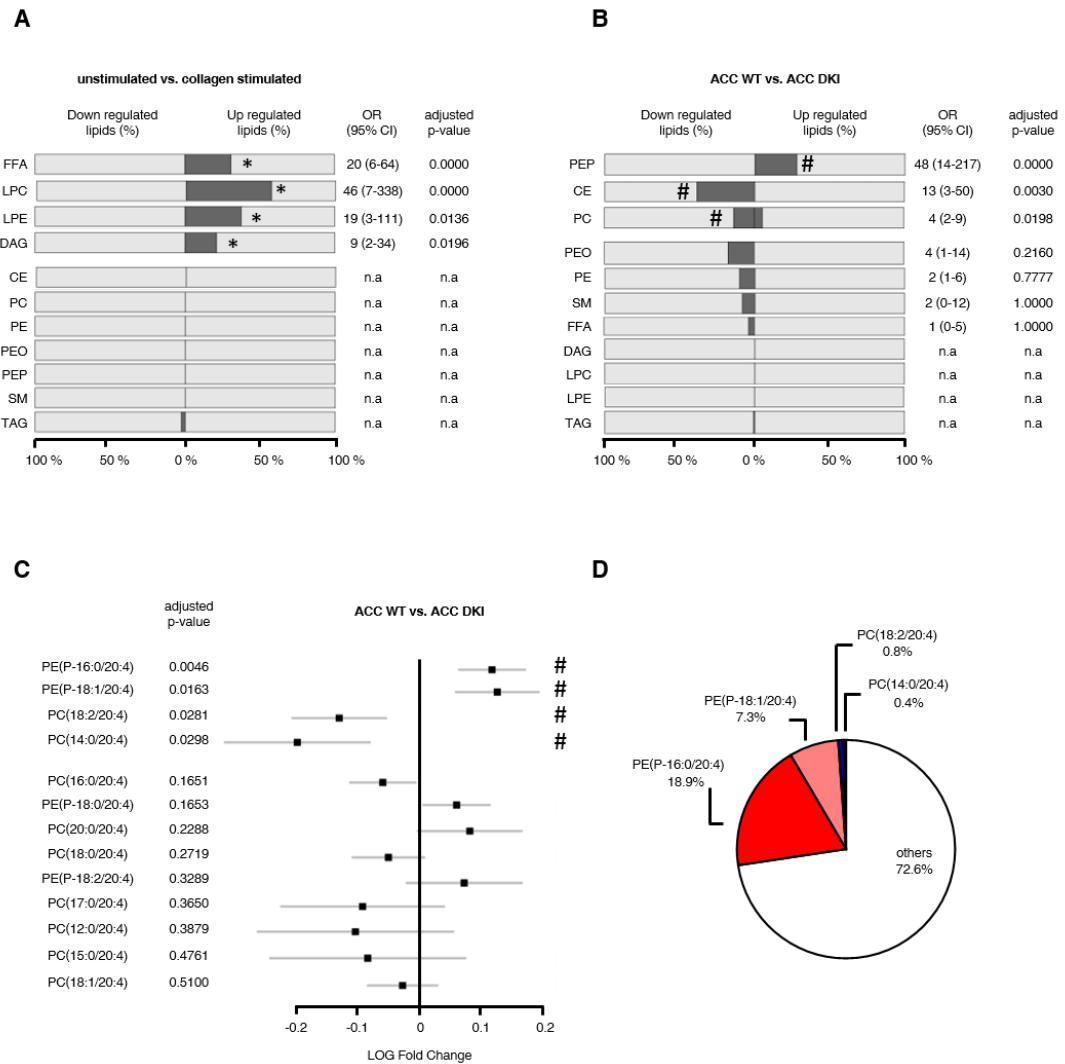


Figure 6. Lack of AMPK-ACC signaling results in an increased AA-containing PL pool. (A, B) The percentage (dark grey bar) of down- or up-regulated lipid species relative to the whole lipid class was calculated in collagen-stimulated platelets relative to unstimulated platelets (A) or ACC DKI relative to ACC WT platelets (B). The different lipid classes are arranged from top down in order of increasing adjusted P values. (A) *Adjusted $P \leq 0.05$ between collagen-stimulated platelets and unstimulated platelets ($n=3$). (B) # $P \leq 0.05$ between ACC DKI and WT platelets ($n=3$). Multivariate regression analysis was performed. OR: odds ratio, CI: confidence interval, n.a.: not applicable (if no change or if change is OR < 1). (C) Comparison of arachidonic acid (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% CI (horizontal lines). Statistical significance was based on false discovery rate (FDR) 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. # $P \leq 0.05$ 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 Figures S6 and S7, Tables S2 and S3.

4.1.5 Discussion

Lipids play fundamental roles in platelets, but little is known about the impact 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 (292). 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 (296,297). 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 (272), is observed between ACC WT and ACC DKI platelets under both basal conditions and upon 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 (335).

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 signalling 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 haemostasis, a concept which 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 (336).

Thrombosis induced by FeCl₃ in the carotid artery is a widely used model which can provide valuable information about the impact 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 (337-340). Recently, several studies reported that plasma proteins and blood cells, including platelets, aggregate because their negatively charged proteins bind to positively charged iron species (341,342). 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 (279). 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 (47,48). In addition, the relationship between TXA₂ and dense granule secretion has been clearly established (255,325,343). 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 $\alpha\text{IIb}\beta\text{3}$ -dependent platelet aggregation. This disconnection has been recently reported (336) and supports the notion that additional cell surface molecules participate in platelet/platelet interactions and support thrombus growth (336,344,345). Therefore, based on the core-shell thrombosis model of (346,347), we postulate that lack of AMPK-ACC signaling might potentiate platelet recruitment to the shell structure through increased TXA_2 and ADP secretion, augmenting thrombosis through mechanisms independent of $\alpha\text{IIb}\beta\text{3}$. In addition, increased PF4 secretion might possibly contribute to a rise in thrombus growth in ACC DKI mice.

Interestingly, the impact of AMPK-ACC signaling on TXA_2 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, while AA release by thrombin is dependent on cPLA_2 (279,348), collagen-induced AA generation involves cPLA_2 , the low-molecular-weight secreted PLA_2 (sPLA_2) and the Ca^{2+} -independent cytosolic PLA_2 (iPLA_2) (349). In line with these observations, thrombin and collagen differentially degrade AA-containing PL, depending on the nature of the PL (336,350). For instance, thrombin is more potent at cleaving AA-containing PI and PS than collagen, while the latter is 2 times more efficient towards AA-containing PEP (350). These data reinforce our observations and help us to understand the specific response(s) 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 (33). 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 impacts lipid composition in resting platelets, prior to 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 NCT03034148). How and whether such a pathway is involved in regulating platelet function in this context remain to be determined.

4.1.6 Supplemental Materials

Supplemental Methods

4.1.6.1 Reagents

Thrombin from bovine plasma (#T6634), citrate-dextrose solution (ACD) (#C3821), apyrase (#A6132), fibrinogen (#F4129), fluorescein isothiocyanate (FITC)-conjugated phalloidin (#P5282), Dulbecco's Modified Eagle's Medium (DMEM, #D5030), acetylsalicylic acid (#A5376), Etomoxir (#E1905), palmitic acid (#P0500), oleic acid (#O1008) and anti-phospho-MLC (Ser19) (#M6068), horse radish peroxidase (HRP)-conjugated anti-rabbit (#A0545) antibodies (Abs) were purchased from Sigma. Eptifibatide was obtained from GlaxoSmithKline. Bovine serum albumin (BSA) (#8076) was purchased from Roth, fatty acid-free BSA (#126575) and ADP (#1171) were from Millipore. Anti-phospho-VASP (Thr278) Ab (#VP2781) was sourced from ECM Bioscience. Collagen (equine tendon type I) came from ABP (#ABP-COL-1) for all experiments except for flow chamber assays, in which case it derived from Nycomed (#10345787). Carboxyfluorescein succinimidyl ester (CFSE) (#C34554), Alexa Fluor 647-conjugated annexin A5 (#A23204), BODIPY-palmitate (#D3821), anti-VASP (#MA5-14982) Ab were purchased from Thermo-Fisher Scientific. cOmplete, Mini Protease Inhibitor Cocktail (#11836153001) was obtained from Roche. Platelet factor 4 (PF4) ELISA Kit (#ab100735) was from Abcam. 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). Serotonin ELISA kit (#ADI-900-175) and thromboxane (TXA2) ELISA kit (#ADI-900-002) came from Enzo Life Science. COX activity assay kit (#700200-2) and

Ticagrelor (#15425) were from Sanbio. Anti-phospho-ACC (Ser79) (#3661), anti-ACC1 (#4190), anti-ACC2 (#8578), anti-phospho-AMPK (Thr172) (#2535), anti-AMPK α (#2532), anti-phospho-cofilin (Ser3) (#3313), anti-cofilin (#3312), anti-MLC2 (#3672), anti-COX-1 (#4841) and anti-gelsolin (#12953) Abs were obtained from Cell Signaling. Phycoerythrin (PE)- conjugated anti- α IIb β 3 (#M025-2), FITC-conjugated anti-GPVI (#M011-1), PE- conjugated anti-GPIb α (#M040-2) and PE-conjugated anti-active α IIb β 3 (clone JON/A) (#M023-2) Abs were from Emfret Analytics. FITC-conjugated anti-CD62P (P- selectin) (#553744) and HRP-conjugated anti-mouse (#554002) Abs were purchased from BD Bioscience. Anti- β -actin (#sc-69879) and anti-protease-activated receptor-3 (PAR-3) (#sc-5598) Abs were obtained from Santa Cruz Biotechnologies. Anti- protease-activated receptor-4 (PAR-4) (#APR-034) Ab was from Alomone Labs. U46619 (#1932) was purchased from Tocris. Rhodocytin and collagen-related peptide (CRP) were kind gifts from J. Heemskerk (Maastricht, the Netherlands) and C. Oury (Liège, Belgium), respectively. Palmitate and oleate coupled to BSA were made by dissolution of the fatty acid into a solution containing in 0.01 N NaOH, 0.9 % NaCl and 12.25 % fatty acid-free BSA, pH 7.4.

4.1.6.2 Human platelet preparation

Blood was collected from consenting healthy adults and drawn from the vein with a 21-gauge butterfly needle into tubes containing one-tenth volume of citrated solution (citrate, phosphate, dextrose, adenine). The platelet-rich plasma (PRP) was collected by centrifugation at 330 g for 20 min at 22 °C. After addition of eptifibatide (4 μ g/ml) and apyrase (1 U/ml), PRP was subsequently centrifuged at 800 g for 10 min at 22 °C. The platelets pellet was washed 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). Platelets were finally isolated by centrifugation at 1000 g for 10 min at 22 °C, re-suspended in modified Tyrode's buffer, their concentration counted with Cell-DYN Emerald (Abbott Diagnostics) and adjusted to 2.5×10^5 platelets/µl.

4.1.6.3 Western blotting

For lysate preparation, platelets were centrifuged and the pellet lysed in SDS-Laemmli sample buffer 1x (25 mM Tris-HCl, pH 6.8, 12.5 % (w/v) glycerol, 3 % (w/v) SDS, 0.01 % (w/v) Bromophenol blue, 6.25 % (v/v) β-mercaptoethanol). Whole platelet lysates were subjected to Western blotting. Abs dilutions in 5 % BSA were 1:1,000 for all primary Abs except for anti-gelsolin (1:50,000) and anti-β-actin (1:50,000) Abs. HRP-conjugated anti-rabbit (1:20,000) and HRP-conjugated anti-mouse (1:30,000) Abs were diluted in 5 % BSA. Band intensities were quantified by Image J, and those obtained with anti-phospho Abs were normalized to those of loading controls on the same gel. Respective total protein analyses were performed on different gels.

4.1.6.4 Platelet spreading

Coverslips were coated overnight at 4 °C with either 100 µg/ml fibrinogen or 100 µg/ml collagen and blocked with 5 mg/ml phosphate buffer saline (PBS)-BSA for 1 h at room temperature. After washing with PBS, washed platelets ($20 \times 10^3/\mu\text{l}$) were stimulated or not ("resting") with 0.5 U/ml thrombin in the presence of 2 mM CaCl_2 and were immediately placed on coverslips for 45 min at 37°C. After rinsing with PBS, adherent platelets were fixed with 1 % formaldehyde for 30 min at room temperature. After washing formaldehyde with PBS, the platelets were permeabilized with 0.1 % Triton and stained with FITC-conjugated phalloidin

(1:300) for 45 min at room temperature. After washing with PBS, platelets were visualized with a Zeiss (x 100 oil immersion) fluorescence microscope (Carl Zeiss) equipped with ApoTome (AxioImager). The area covered by adherent platelets and the number of adherent platelets were quantified by Image J. Platelet surface area represents the surface covered by adherent platelets divided by their number.

4.1.6.5 Tail bleeding time

Mice were anesthetized with isoflurane, and 3 mm of tail tip was cut with scalpel. The tail was immediately immersed in 0.9 % NaCl (37 °C). Time until cessation of bleeding (no blood flow for 1 min) was recorded. Bleeding was quantified for a maximum of 10 min.

4.1.6.6 Platelet aggregation and ATP secretion

Light transmission during aggregation of washed platelets was recorded at 37 °C, with constant stirring (1,200 rpm), in an aggregometer (Chrono-Log). ATP secretion was monitored in parallel to platelet aggregation by adding Luciferase-Luciferin reagent and comparing the luminescence generated by platelet ATP release with an ATP standard (Chrono-Lume, Stago, The Netherlands).

4.1.6.7 Flow cytometry analysis

For CD62P and JON/A analysis, washed platelets (6.25×10^6), re-suspended in modified Tyrode's buffer supplemented with 1.5 % BSA, were incubated with respective Abs at saturating concentrations (1:12.5) for 15 min at room temperature. Platelets were stimulated with thrombin or collagen at 37 °C at the indicated concentrations and time points. To stop the reaction, platelets were fixed in 4 % formaldehyde supplemented with 5 % BSA. For platelet receptor expression,

washed platelets (10^6) were incubated with respective Abs (1:40) for 15 min at room temperature and diluted with 0.1 % BSA-0.1 % Azide- PBS before analysis.

Samples were analyzed by BD Canto II flow cytometer, and 10,000 events were recorded. When necessary, appropriate compensation controls were included.

For analysis of palmitate uptake, washed platelets (6.25×10^6) were re-suspended in modified Tyrode's buffer supplemented with 1 % fatty acid-free BSA, incubated with 10 μ M BODIPY-palmitate or the corresponding vehicle (DMSO) and either left untreated or stimulated with 5 μ g/ml collagen for 10, 20, 40 or 60 min at 37 °C. To stop the reaction, platelets were diluted in 1.5 ml PBS and fluorescence was immediately analyzed by the BD Calibur flow cytometer (10,000 events were recorded).

4.1.6.8 Serotonin and TXA₂ assays

Serotonin and TXA₂ were assessed by enzyme immunoassay kit (see Reagents and materials). Washed platelets ($30 \times 10^3/\mu$ l) were stimulated with 5 μ g/ml collagen, 30 mU/ml or 100 mU/ml thrombin for 5 min at 37 °C. For some conditions, platelets were pre-incubated with 1 mM aspirin for 45 min, 200 μ M BSA-oleate/palmitate for 1 h or 30 μ M Ticagrelor for 30 min. After the samples were centrifuged for 15 s at 11,700 g, the supernatant was removed and stored at -80 °C for serotonin and TXA₂ analysis according to the manufacturer's instructions. Serotonin content was also determined in platelet lysates ($7.5 \times 10^3/\mu$ l) after 4 freeze-thaw cycles.

4.1.6.9 PF4 assay

Washed platelets ($60 \times 10^3/\mu\text{l}$), re-suspended in modified Tyrode's buffer not supplemented with BSA, were stimulated with 2.5, 5 or 10 $\mu\text{g/ml}$ collagen for 5 min at 37 °C. To stop the reaction, samples were centrifuged for 15 s at 11,700 g, the supernatant was removed and stored at -80 °C. Supernatant was diluted 30 x and PF4 assayed according to the manufacturer's instructions. Total PF4 content was also determined after platelet ($180 \times 10^3/\mu\text{l}$) lysis in 0.1 M Tris-HCl, pH 7.5, in the presence of protease inhibitor cocktail (cOmplete, mini) and sonication 3 x 10 sec. Supernatant was diluted 240 x before analysis.

4.1.6.10 COX activity

Washed murine platelets ($700 \times 10^3/\mu\text{l}$), re-suspended in modified Tyrode's buffer not supplemented with BSA, were lysed in 0.1 M Tris-HCl, pH 7.5, in the presence of protease inhibitor cocktail (cOmplete, mini) and sonicated 3 x 10 sec. Samples were cleared by centrifugation (16,000 g for 10 min at 4 °C) and supernatants pre-incubated or not with 1 mM ASA for 45 min. COX activity was assayed by a fluorescent assay kit according to the manufacturer's instructions.

4.1.6.11 Measurements of ADP and ATP

Washed platelets (100×10^6) were lysed in ice-cold 10 % (v/v) perchloric acid, 25 mM EDTA for 20 min before sonication for 30 sec. Samples were centrifuged at 10,000 g for 10 min at 4 °C and supernatant neutralized with 2 N KOH and 0.3 M MOPS. After centrifugation at 10,000 g for 10 min at 4 °C, ATP and ADP were analyzed in the supernatant by reverse-phase high performance liquid chromatography (HPLC), as already described ¹.

4.1.6.12 Clot retraction

Washed platelets ($500 \times 10^3/\mu\text{l}$) were incubated with 2 mg/ml fibrinogen, 2 mM CaCl_2 and 1 U/ml thrombin in the presence of 1.5 μl of erythrocytes. Subsequent clot retraction was monitored at 37 °C under non-stirring conditions, and pictures were taken at different time points by digital camera for up to 240 min. Clot surface area was quantified by Image J, and retraction was expressed as percentages of total surface area.

4.1.6.13 Extracellular flux analysis of mitochondrial respiration

Mitochondrial respiration was assessed by monitoring the real-time oxygen consumption rate (OCR) of individual platelets, with the Mito Stress Test Kit, and the Seahorse extracellular flux analyzer (Seahorse Bioscience), according to the manufacturer's instructions. Washed platelets were re-suspended in DMEM (supplemented with 5 mM glucose, 1 mM pyruvate, 4 mM glutamine and 20 mM HEPES for collagen stimulation). Platelets ($30 \times 10^6/\text{well}$) were then loaded into each well of a XF96 microplate pre-coated with Cell Tak (150 $\mu\text{g}/\text{ml}$).

Plates were subsequently centrifuged (1 min at 148 g and 1 min at 213 g), DMEM was replaced by fresh medium and incubated in a CO_2 -free incubator at 37 °C for 1 h in the absence or presence of Etomoxir 25 μM . OCR was measured in the presence of DMEM alone and after consecutive treatments with thrombin (1 U/ml) or collagen (25 $\mu\text{g}/\text{ml}$), oligomycin (1 μM), FCCP (0.45 μM) and a mix of rotenone (1 μM) and antimycin A (1 μM). 3 basal measurements, followed by 3 assessments after each injection, were recorded per assay. Mitochondrial functional parameters were assessed via (1) basal OCR, (2) agonist-induced OCR, estimated by subtracting basal OCR from OCR after thrombin or collagen addition, (3) ATP-linked OCR, calculated by subtracting OCR after oligomycin from baseline, (4) maximal

OCR, (5) reserve capacity, evaluated by subtracting basal OCR from OCR after FCCP, (6) non-mitochondrial respiration, and (7) proton leak, computed by subtracting OCR after antimycin A/rotenone from OCR after oligomycin.

4.1.6.14 Lipid extraction

Lipids were extracted according to the methyl-tert-butyl ether (MTBE) method 2,3. 250 x 10⁶ platelets were transferred to 2-ml Eppendorf vials, followed by the addition of 100 µl water and 100 µl of Internal Standard Mix (Sciex, Nieuwekerk aan den IJssel, the Netherlands). Next, 160 µl of methanol and 500 µl of MTBE were added. The extracts were then shaken at room temperature for 30 min. Subsequently, 200 µl of water was added, after which the samples were vortexed briefly and then centrifuged at 16,100 g for 3 min.

The upper organic layer (400 µl) was transferred to fresh glass vials. 500 µl MTBE, 100 µl methanol and 100 µl water were added to the original Eppendorf vials, and extraction was repeated. Combined organic extracts were concentrated under a gentle nitrogen stream and reconstituted in 250 µl of running buffer (10 mM ammonium acetate in 50:50 dichloromethane:methanol).

4.1.6.15 Lipidomics statistical analysis

Lipid species concentrations were analyzed using R.3.4.2 according to the following bioinformatics pipeline. Missing data were imputed by a probabilistic principal component analysis (PPCA) approach implemented in the `pcaMethods` Bioconductor package. Data were normalized with total lipid abundance, and log₂ transformation was applied on normalized concentrations.

The Limma Bioconductor package 4 was used to build a multivariate regression model for each lipid species with 2 predictors, including ACC DKI status (i.e., ACC WT vs ACC DKI) and treatment group (control, collagen, thrombin, collagen and thrombin). Fold-change estimates and corresponding P values were derived from regression models for each lipid species and for each predictor. To control for multiple testing, all P values were further adjusted for the Benjamini- Hochberg false discovery rate (FDR) 5, and $FDR \leq 0.05$ was considered as significant. As 865 lipid species belong to 11 lipid classes (CE, DAG, FFA, LPC, LPE, PC, PE, PEO, PEP, SM, TAG), enrichment analysis was performed with Fisher's exact tests to find lipid classes involving a high proportion of differentially-regulated lipid species.

4.1.6.16 Statistical analysis

All values are expressed as means $\pm$ SEM. Statistical analyses were performed by the Mann-Whitney test for the comparison of 2 different experimental groups, or 2-way ANOVA followed by Sidak's multiple comparison post hoc test for multiple testing comparisons. $P \leq 0.05$ values were considered to be statistically significant. All statistical analyses were performed using GraphPad Prism (GraphPad Software).

4.1.7 Supplemental Figures and Tables

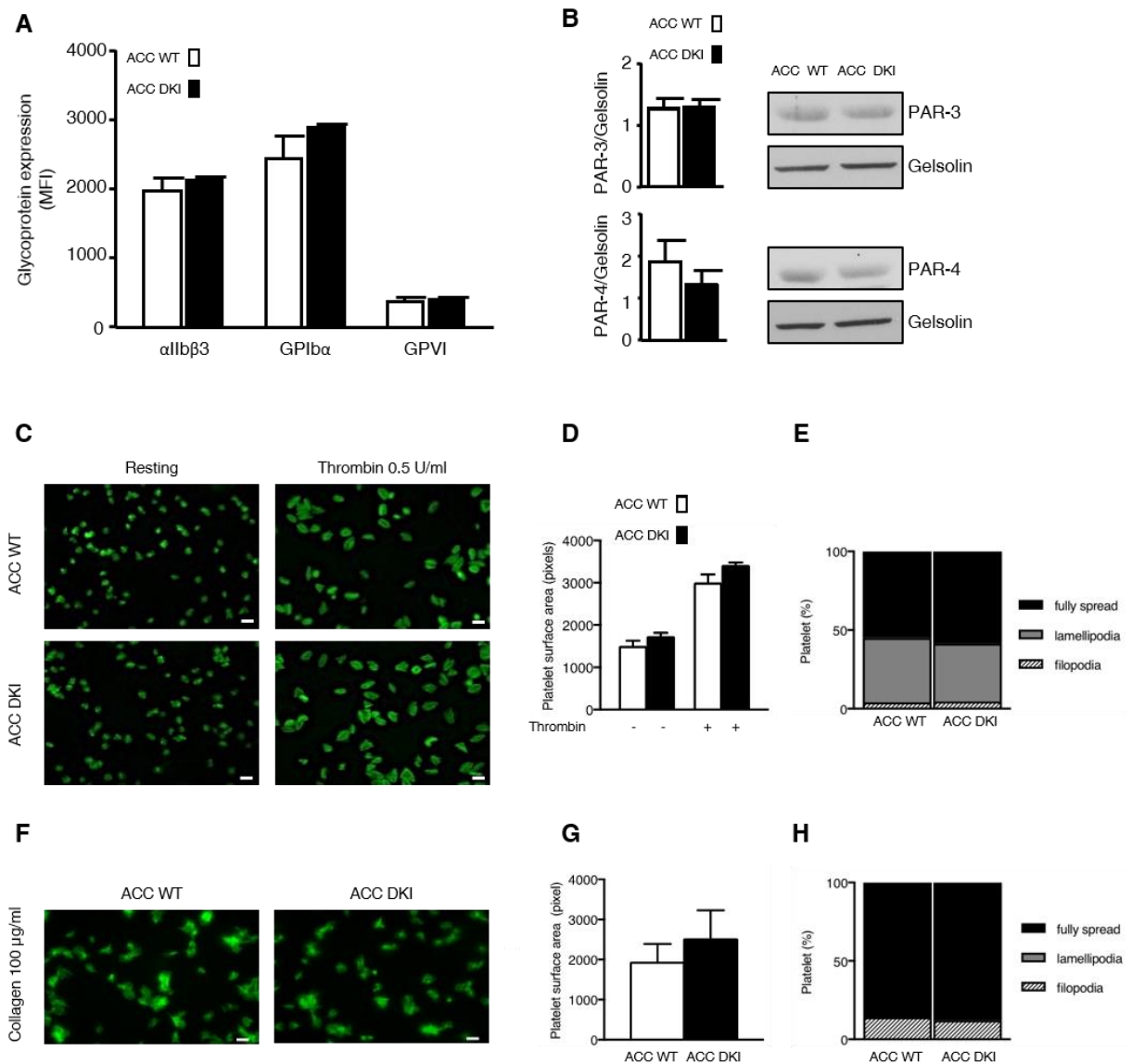


Figure S1. Lack of ACC phosphorylation does not impact platelet surface receptor expression, spreading, filopodia and lamellipodia formation on fibrinogen or collagen. Related to Figure 1. (A) Surface expression of glycoproteins on ACC WT or DKI platelets was analyzed by flow cytometry. The results are expressed as mean fluorescence intensity (MFI) $\pm$ SEM (n=3). (B) ACC WT and DKI platelets were lysed and subjected to Western blotting for PAR-3 and PAR-4 expression analysis. Gelsolin was used as a loading control. Representative Western blotting of PAR-3 and PAR-4 (right panel) and corresponding quantification (left panel) are shown. The results are expressed as means $\pm$ SEM (n=3). (C-H) Washed ACC WT and DKI platelets were allowed to spread for 45 min on fibrinogen (100 μ g/ml) in the absence (resting) or presence of 0.5 U/ml thrombin (C-E) or on collagen (100 μ g/ml) (F-H) and were stained with FITC-phalloidin. Representative fluorescence microscopy images of platelets are shown on fibrinogen (C) and collagen (F): scale bars represent 5 μ m. Quantification of platelet surface area on fibrinogen (D) and collagen (G): the results are expressed as means $\pm$ SEM (n=4). Evaluation of percentages of platelet spread on fibrinogen (E) or collagen (H) at different spreading stages: filopodia, lamellipodia and totally spread platelets (n=4).

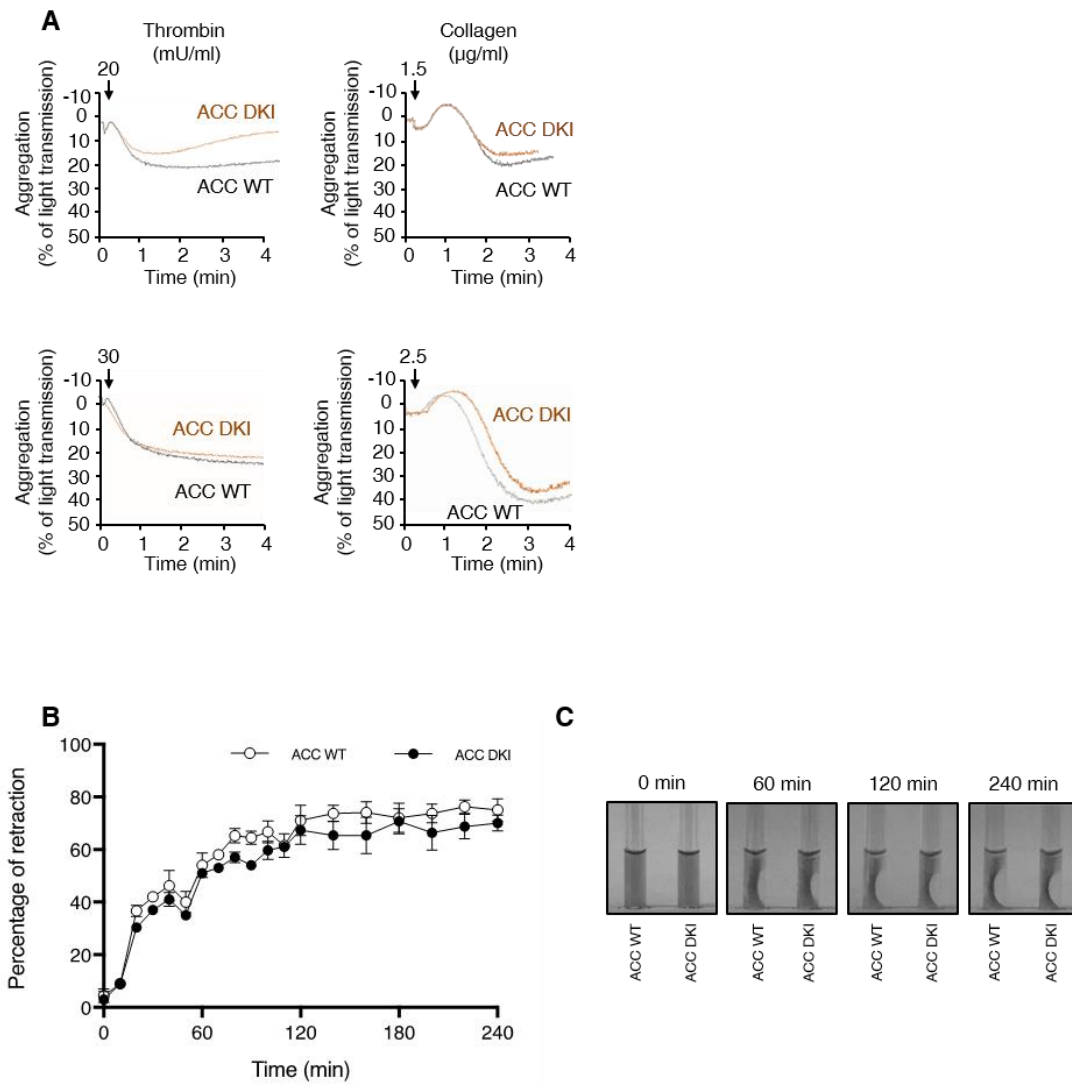


Figure S2. Normal $\alpha\text{IIb}\beta 3$ signalling in ACC DKI platelets. Related to Figure 3. (A) Representative aggregation traces induced by 20 mU/ml or 30 mU/ml thrombin and 1.5 $\mu\text{g/ml}$ or 2.5 $\mu\text{g/ml}$ collagen. Arrows indicate addition of the respective agonist. (B, C) Clot retraction was induced in washed ACC WT and DKI platelets by adding 2 mg/ml fibrinogen, 2 mM CaCl_2 and 1 U/ml thrombin. Pictures of the clot were taken at different time points, and the extent of clot retraction was quantified by measuring clot area. (B) Quantification of time-dependent clot retraction. The results are expressed as means $\pm$ SEM ($n=3$). (C) Representative pictures of clots at 0, 60, 120 and 240 min after thrombin addition.

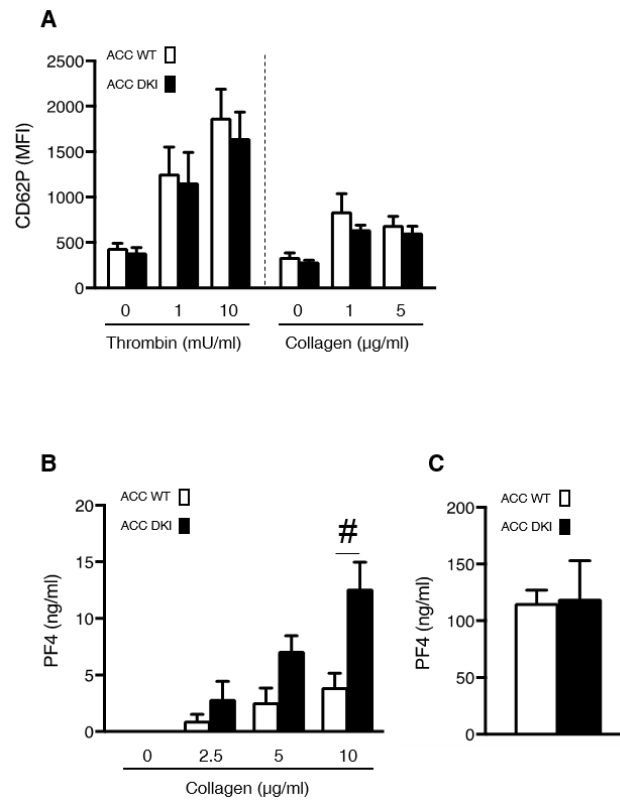


Figure S3. Increased PF4 secretion in response to collagen in ACC DKI platelets. Related to Figure 3. (A) P-selectin (CD62P) exposure was analyzed by flow cytometry in washed ACC WT and DKI platelets stimulated with thrombin for 8 min or collagen for 30 min at the indicated concentrations at 37 °C. The dashed line represents separate analyses. The results are expressed as mean fluorescence intensity (MFI) $\pm$ SEM (at least $n=4$). (B) Washed platelets were stimulated with the indicated concentrations of collagen for 5 min, and PF4 was measured in the supernatant by ELISA. Results are expressed as means $\pm$ SEM ($n=4$). [#] $P \leq 0.05$ between ACC WT and DKI platelets. The data were analyzed by 2-way ANOVA. (C) Washed platelets were centrifuged, the pellet was lysed, and total PF4 assayed. Results are expressed as means $\pm$ SEM ($n=3$).

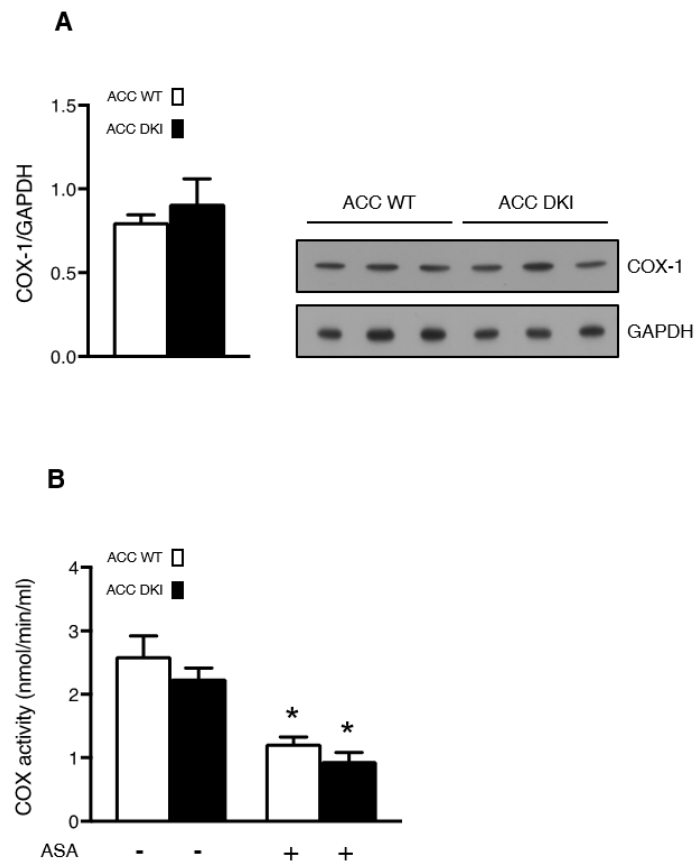


Figure S4. COX activity is similar between ACC WT and ACC DKI platelets. Related to Figure 3. (A) Washed murine platelets were lysed and subjected to Western blotting for cyclooxygenase-1 (COX-1) expression. GAPDH was used as a loading control. Quantification and Western blotting are shown. The results are expressed as means $\pm$ SEM (n=3). (B) COX activity was measured by a fluorescent assay kit in platelets lysates pre-incubated or not with 1 mM aspirin (ASA) for 45 min. The results are expressed as means $\pm$ SEM (n=4). *indicates values statistically different from respective untreated platelets, $P \leq 0.05$. Analysis was performed by 2-way ANOVA.

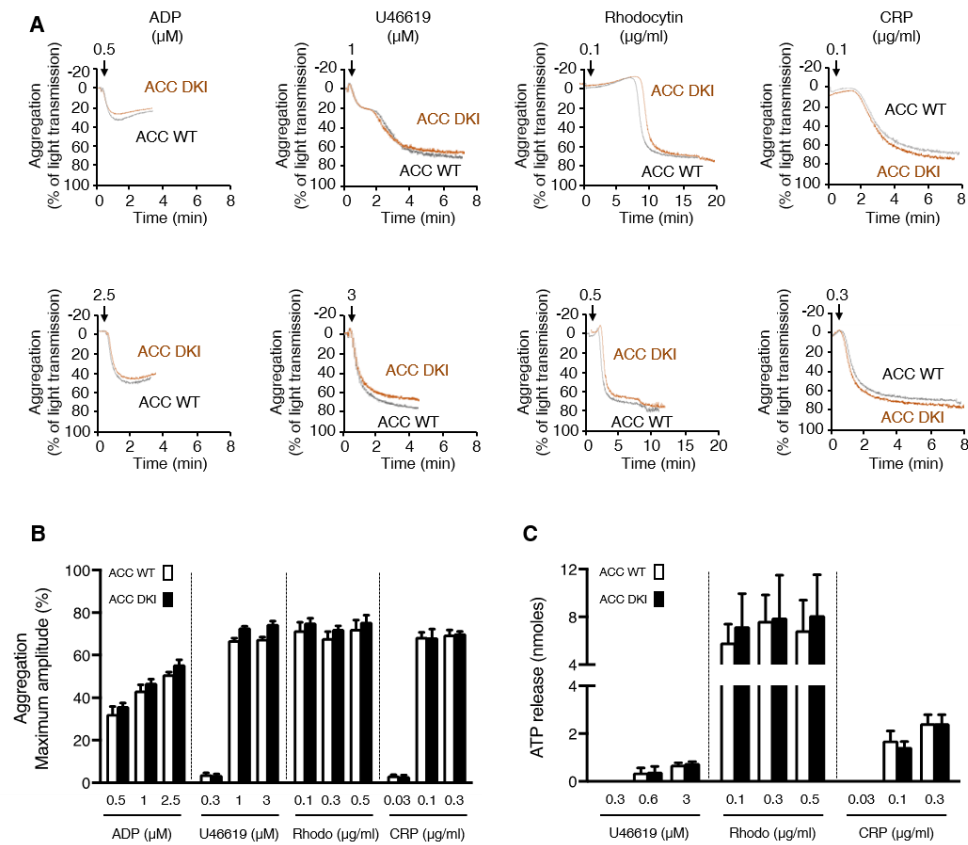


Figure S5. ACC WT and ACC DKI platelets display similar aggregation and dense granules secretion in response to ADP, U46619, rhodocytin or CRP. Related to Figure 3. (A-C) Washed platelets were stimulated with ADP, U46619 (TXA₂ analogue), rhodocytin or collagen-related peptide (CRP) at the indicated concentrations in the presence of Luciferase-Luciferin reagent, and light transmission was measured (Chrono-Log). (A) Representative aggregation profiles are represented. Arrows indicate addition of the respective agonist. (B) Aggregation expressed as the maximal percentage of light transmitted, and (C) quantification results of ATP release. The dashed lines represent separate analyses. Results are expressed as means $\pm$ SEM (n=3).

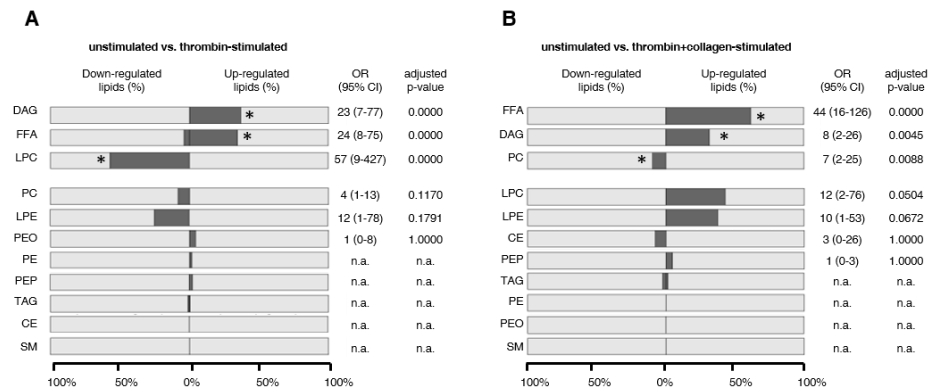


Figure S6. Analysis of lipid change in response to thrombin alone or to thrombin and collagen. Related to Figure 6. (A, B) Washed platelets were stimulated for 15 min with thrombin alone (0.3 U/ml) (A) or thrombin (0.3 U/ml) and collagen (25 µg/ml) (B), and the percentages (dark grey bar) of down- or up-regulated lipid species in both ACC WT and DKI platelets relative to unstimulated platelets are represented for each lipid class. The different lipid classes are arranged from top down in order of increasing P values. *P≤0.05 between stimulated and unstimulated conditions (n=3). Multivariate regression analysis was performed. OR: odds ratio, CI: confidence interval, n.a.: not applicable (if no change or if change is OR <1).

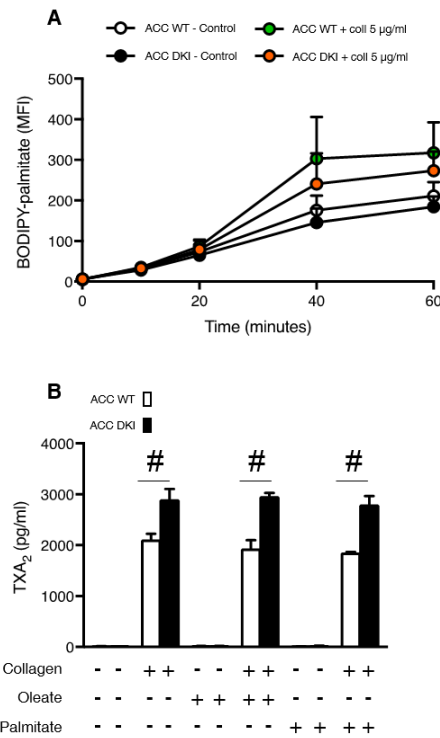


Figure S7. Extracellular fatty acids availability does not influence intra-platelet lipid transport and collagen-induced TXA₂ production in ACC DKI platelets. Related to Figure 6. (A) Murine platelets were incubated with 10 µM BODIPY-palmitate and either left untreated (Control) or stimulated with 5 µg/ml collagen for the indicated time points and fluorescence was immediately analyzed. Results are expressed as means ± SEM (n=3). (B) Washed platelets were pre-incubated for 1 h with 200 µM BSA-oleate, 200 µM BSA-palmitate or the corresponding vehicle (BSA) and stimulated with 5 µg/ml collagen for 5 min. Thromboxane A₂ (TXA₂) was measured in the supernatant by ELISA. Results are expressed as means ± SEM (n=3). #P≤0.05 between ACC WT and DKI platelets. The data were analyzed by 2-way ANOVA.

	ACC WT	ACC DKI	P
Erythrocytes, 10 ⁶ /μl	6.73 ± 0.15	6.98 ± 0.08	n.s.
Leukocytes, 10 ³ /μl	6.78 ± 0.29	7.41 ± 1.09	n.s.
Platelets, 10 ⁶ /μl	0.62 ± 0.03	0.69 ± 0.03	n.s.
Mean platelet volume, fl	4.72 ± 0.05	4.83 ± 0.04	n.s.

Table S1. ACC DKI mice show normal hematologic parameters. Related to Figure 1. Platelets as well as their mean volume, leukocytes and erythrocytes counts were analyzed by blood cell counter (Cell Dyn Emerald). fl.: femtoliter; n.s.: non-significant (n=10).

	n	log fc	Adjusted P value
FFA(22:2)	24	1.520	6.12E-14
FFA(20:5)	24	1.078	8.07E-11
DAG(16:0/20:4)	24	1.595	8.07E-11
LPC(16:0)	24	0.836	8.07E-11
LPE(16:0)	24	0.868	1.44E-10
LPE(18:0)	24	0.950	4.25E-10
LPC(18:0)	24	0.948	6.86E-09
LPE(18:1)	24	0.745	2.81E-07
FFA(22:4)	24	0.512	1.77E-06
LPC(18:1)	24	0.649	1.94E-06
FFA(20:4)	24	0.719	0.00001
FFA(18:2)	24	0.485	0.00013
LPC(18:2)	24	0.622	0.00030
DAG(16:0/18:2)	24	0.477	0.00139
TAG(48:0/FA16:0)	24	-0.715	0.00159
FFA(18:0)	24	0.368	0.00278
TAG(50:0/FA16:0)	24	-0.522	0.00278
TAG(56:8/FA18:3)	24	-0.482	0.00386
TAG(52:0/FA16:0)	24	-0.398	0.00404
TAG(50:0/FA18:0)	24	-0.555	0.00983
FFA(20:1)	24	0.309	0.01365
FFA(20:3)	24	0.295	0.01614
DAG(16:0/20:3)	17	0.537	0.02556
TAG(55:7/FA15:0)	24	-0.372	0.02687
DAG(18:1/20:4)	20	0.938	0.04050
TAG(54:0/FA16:0)	23	-0.441	0.04499

Table S2. Complete list of significantly modified lipids in collagen stimulated platelets relative to unstimulated platelets. Related to Figure 4. Differentially regulated lipids between collagen stimulated platelets and unstimulated platelets. Statistical significance was evaluated based on the false discovery rate (FDR) P values (adjusted P value). The different lipid species are arranged from top down in order of increasing adjusted P values and only those lipids having an adjusted P values ≤ 0.05 are represented (n=3). Analysis was performed by multivariate regression model. n: number of condition analysed, fc: fold change.

	n	Log fc	Adjusted P value
PE(P-16:0/18:2)	24	0.261	0.00017
PC(16:0/14:0)	24	-0.221	0.00027
PC(14:0/18:2)	24	-0.212	0.00027
PE(O-18:0/22:6)	24	-0.223	0.00032
PE(P-16:0/18:1)	24	0.227	0.00032
PC(20:0/18:1)	24	0.273	0.00040
PC(16:0/20:2)	24	-0.194	0.00114
PE(P-18:1/18:2)	24	0.271	0.00166
CE(18:0)	22	-0.426	0.00166
PE(16:0/18:3)	24	-0.329	0.00166
PE(O-16:0/22:4)	24	-0.242	0.00193
PC(18:2/18:2)	24	-0.234	0.00225
PE(P-18:0/20:5)	24	0.189	0.00244
PE(P-16:0/20:5)	24	0.235	0.00247
PE(P-18:1/18:1)	24	0.224	0.00298
PC(18:0/20:5)	24	0.246	0.00307
PC(16:0/20:5)	24	0.167	0.00308
PE(P-18:0/18:2)	24	0.181	0.00313
PE(P-16:0/20:4)	24	0.119	0.00454
PE(O-18:0/22:4)	24	-0.248	0.00483
PC(18:0/18:1)	24	0.104	0.00499
PC(18:0/14:0)	24	-0.252	0.00772
CE(18:1)	24	-0.546	0.00799
SM(16:0)	24	-0.089	0.00971
PE(18:0/22:4)	24	-0.168	0.01165
PC(18:2/20:2)	24	-0.257	0.01304
PE(O-16:0/22:6)	24	-0.140	0.01454
PE(P-18:1/16:0)	24	0.161	0.01605
PE(P-18:1/20:4)	24	0.126	0.01625
PE(P-16:0/16:0)	24	0.185	0.01632
CE(18:3)	24	-0.455	0.01686
PC(16:0/22:4)	24	-0.231	0.01859
CE(16:0)	24	-0.480	0.02026
TAG(54:6/FA18:3)	24	-0.345	0.02071
PE(18:0/18:3)	24	-0.227	0.02123
PC(18:2/20:4)	24	-0.131	0.02812
PE(P-16:0/22:5)	24	0.150	0.02911
PC(14:0/20:4)	24	-0.200	0.02976
PE(16:0/22:4)	24	-0.209	0.03196
CE(20:4)	24	-0.349	0.03461
PE(18:1/22:4)	24	-0.164	0.04307
PC(18:1/18:2)	24	-0.100	0.04870
FFA(18:2)	24	-0.175	0.04920
PE(18:2/18:2)	24	-0.143	0.04920
TAG(58:9/FA22:6)	24	-0.215	0.04942

Table S3. Complete list of significantly modified lipids in ACC DKI platelets relative to WT platelets. Related to Figure 4. Differentially regulated lipids between ACC DKI and WT platelets. Statistical significance was evaluated based on the false discovery rate (FDR) P values (adjusted P value). The different lipid species are arranged from top down in order of increasing adjusted P values and only those lipids having an adjusted P value ≤ 0.05 are represented (n=3). Analysis was performed by multivariate regression model. n: number of condition analysed, fc: fold change.

CHAPTER V – GENERAL DISCUSSION AND PERSPECTIVES

5.1 Residual risk and Platelet-Lipid Interplay

Given the role of ACC in regulating lipid metabolism and the impact of hyperlipidemic environment on platelet biology and function, we explored the activation of the AMPK-ACC signaling in the setting of CAD (ACCTHEROMA Clinical Trial [NCT03034148](#)). PhosphoACC was significantly increased in circulating platelets of CAD patients and highly associated with acute coronary events on admission, despite statins and aspirin intake. We identified a clear relationship between increased phosphoACC and TG/HDL-C ratio, a well-known atherogenic marker, suggesting interplay between lipids and platelets. Lipidomic profiling of circulating platelets confirm this interaction between plasma lipids and platelets: increased plasma TG levels were associated with an up-regulation of these lipid species in platelets. Interestingly, a comparison between CAD platelets from the ACCTHEROMA cohort and platelets from healthy volunteers showed drastic increase in TG and cholesterol esters content in platelets from diseased patients (unpublished results). Similar results have been reported by another group, showing an altered lipidomic profile in platelets of CAD patients (88). These platelets exhibit increased oxidized phospholipids, cholesteryl esters, TG, sphingomyelin, ceramides and acylcarnitines, accounting for an enhanced prothrombotic phenotype. Therefore, despite statin therapy with achieved LDLc goals, circulating lipids may influence thrombotic risk and relapsing ischemic events by interacting with platelets. PhosphoACC is a metabolic signature of this interplay and a promising risk marker that warrants further investigation in prospective multicenter studies.

High PCSK9 levels has been shown to predict cardiovascular ischemic events in patients presenting with ACS (351). Interestingly, beyond regulating LDL receptor metabolism, PCSK9 may exhibit pleiotropic effects. The correlation observed

between PCSK9 levels, urinary TxB₂ levels and platelet reactivity, suggest an impact of PCSK9 on platelet activation (242,351). Whether PCSK9 exhibit its effects via oxLDL (243) or directly on platelets remains unclear. This hypothesis has been tested recently. Pre-treatment of platelets with human recombinant PCSK9 prior to stimulation with low doses of epinephrine enhances platelet activation and aggregation *in vitro*. Furthermore, loss of PCSK9, in PCSK9 null mice submitted to a thrombotic challenge (carotid injury by FeCl₃), reduced the formation and stability of arterial thrombus and platelet prothrombotic functions (352). Therefore, risk reduction of cardiovascular events with PCSK9 inhibitors on top of statin therapy in the Fourier trial (98) can be, at least partly, due to its (in)direct impact on platelet activation status. Similarly, several studies have reported an association between elevated fasting TG levels and recurrent ischemic events in CAD patients (118-120). Recently, TG reduction by high dose icosapent ethyl, a highly purified eicosapentaenoic acid ethyl ester, on top of statin therapy, has been shown to reduce risk of ischemic events in the REDUCE-IT trial (353). Elevated circulating TG levels, induced either by a specific meal in human volunteers (354) or by a “western” diet in rats (355), has been shown to affect platelet function (enhanced P-selectin expression (354,355), platelet aggregation and TXA₂ secretion (355)). Therefore, given the impact of such proatherogenic environment on platelet functions, it would be interesting to assess evolution of platelet phosphoACC signal over time under more aggressive lipid management strategies. It is tempting to speculate that high dose icosapent ethyl, by reducing TG levels and TG/HDL-C ratio, affects the atherogenic lipid profile which may in turn influence platelet phosphoACC and platelet functions.

5.2 ACC phosphorylation: a counter-regulatory mechanism limiting platelet reactivity?

In our study, we showed that an increase in platelet phosphoACC (in CAD patients) was associated with a down-regulation of TG containing C14:0 (myristic acid) fatty acid chains. These results suggest that ACC phosphorylation and inhibition put a brake on platelet endogenous lipogenesis and contributes to the regulation of TG lipid species in CAD patients. Similar findings were reported in hepatocytes, where liver-specific pharmacological inhibition of ACC lowered lipogenesis, significantly reduced liver TGs and also enhanced fatty acid oxidation which might be beneficial for the treatment hepatic steatosis (311). Furthermore, ACC inhibition in cancer cells decreases *de novo* fatty acid synthesis, leading to a reduction in fatty acids containing 14 to 18 carbons, thereby limiting tumor growth (252).

Actually, a major perspective of our lab is to investigate how ACC modulates the interaction between plasma and intraplatelet lipids. Our hypothesis is that, in CAD patients, platelet ACC phosphorylation may impact the interaction between circulating lipids and intraplatelet lipidome by decreasing the synthesis of some specific lipid species and/or of their incorporated covalently bound-fatty acids. This platelet ACC-dependent effect can therefore influence platelet reactivity within a proatherogenic environment, and consequently impact the outcome of atherothrombotic disease. The hypothesis will be tested in a new prospective cohort. The plasma and intraplatelet lipid profiling will be systematically compared using an untargeted lipidomic analysis in subjects displaying high or low levels of phosphoACC. The deliverables include plasma candidate metabolite biomarkers that are correlated with disease severity and adverse cardiovascular events, potentially guiding therapies in the future. Lipidomics study will also improve our

knowledge on how more aggressive lipid lowering strategies (high dose icosapent ethyl, PCSK9 inhibitors) may affect plasma and platelet lipidome.

In the second part of our study, we showed that impaired AMPK-ACC signaling, in DKI mice where the AMPK phosphorylation sites Ser79 on ACC1 and Ser212 on ACC2 were mutated to prevent AMPK-signaling to ACC, promoted injury-induced arterial thrombosis *in vivo* and enhanced thrombus growth *ex vivo*. The underlying mechanisms involve elevated levels of arachidonic acid-containing phosphatidylethanolamine plasmalogen lipids in ACC DKI platelets, compared to wild type, increasing TxA₂ generation and granule secretion after platelet stimulation. These findings highlight a novel metabolic regulatory pathway in platelets that influences thrombus formation by modulating the content of specific phospholipids which generate key mediators of platelet activation (303). However, even if ACC affects platelet prothrombotic phenotype by regulating phospholipid content, its impact within a proatherogenic environment has to be investigated.

A second perspective is to study the impact of ACC phosphorylation/inhibition on platelet lipid content and platelet functions/reactivity in atherosclerotic mice (a hyperlipidemic context). We have previously demonstrated that, similar to platelets from CAD patients, phosphoACC levels drastically increase with the severity of atherosclerotic plaque burden in SR-B1^{flox/flox} ApoE^{-/-} mice developing spontaneous atherosclerosis, enhanced by a Western diet. In order to assess the impact of platelet phosphoACC on lipid content and atherosclerosis-associated thrombosis, SR-B1^{flox/flox} ApoE^{-/-} mice will be used for an adoptive bone marrow transfer coming from ACC DKI mice. SR-B1^{flox/flox} ApoE^{-/-} mice will be lethally irradiated (11Gy) and rescued 24 hours later by intravenous injection of 5x10⁶ bone marrow cells harvested from ACC DKI mice versus WT mice. After successful bone marrow

reconstitution, mice will be placed on a standard mouse chow diet or western diet for 20 weeks, prior to platelet function testing and untargeted lipidomic profiling.

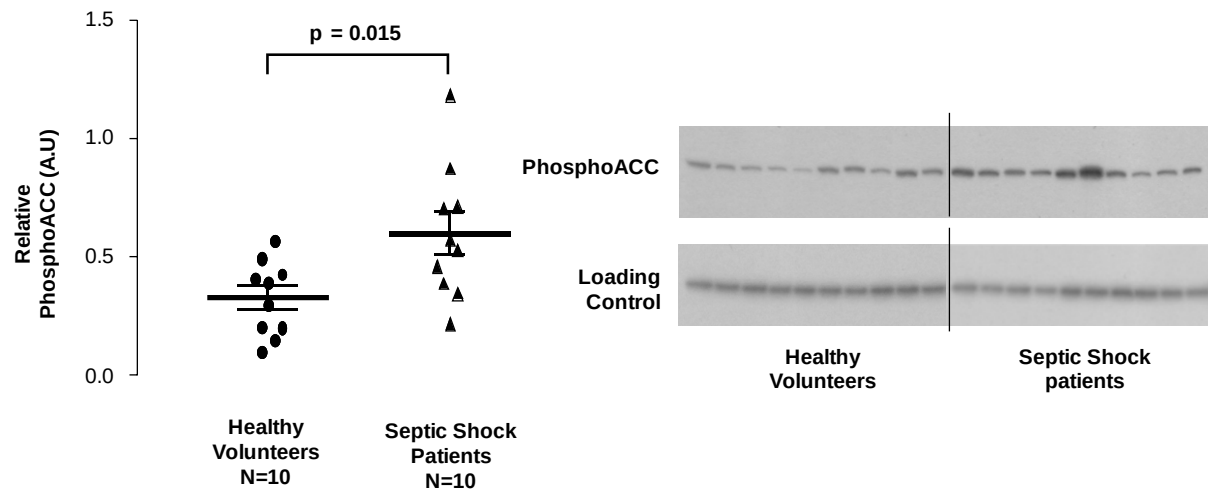
5.3 Protein Acetylation, another mechanism by which ACC regulates platelet function?

Beyond lipid metabolism, elevated cytosolic acetyl-CoA, consequence of ACC phosphorylation/inhibition, can be associated with an increase in protein acetylation. Intracellular levels of acetyl-CoA influence the acetylation of several proteins (356). Protein acetylation is a dynamic process involving lysine acetyl transferase (KAT) P300 and histone deacetylase HDAC6 for acetylation and deacetylation of proteins respectively (357). Proteomics analyses of the acetylome have revealed that several proteins with well-defined roles in platelet function are covalently modified by lysine acetylation, including cytoskeletal proteins (such as actin or tubulin) and metabolic enzymes (358). Accordingly, modification of acetylation levels has been shown to influence platelet functions, namely spreading, cytosolic Ca^{2+} levels, granules secretion and aggregation (358,359). Tubulin cytoskeleton plays a key role in maintaining the characteristic quiescent discoid shape of resting platelets. Tubulin is acetylated in platelets under basal state and deacetylated by HDAC6 when activated. HDAC6 inhibitors have been shown to inhibit platelet aggregation by blocking cytoskeletal changes and platelet signaling pathways (Rho GTPase activation) (360). Given the association between ACC activity, protein acetylation and platelet functions, one can speculate that besides regulating platelet lipid content, phosphoACC impacts protein acetylation thereby constituting an additional mechanism by which ACC controls platelet functions. The influence of ACC on this post-translational regulatory mechanism offers new avenues for exploration of platelet function.

5.4 Activation of AMPK-ACC signaling in other clinical settings

While thrombin, and less importantly collagen, are the major agonists leading to AMPK activation in platelets *in vitro*, we showed that in the setting of atherosclerosis, AMPK is activated by lipids from the pro-atherogenic environment. Therefore, other factors besides classical platelet agonists can affect AMPK-ACC signaling.

In macrophages, AMPK activation plays a key role in the inhibition of inflammatory pathways. There is a clear relationship between reduced fatty acid oxidation and inflammation, consequence of the reduction in ACC activity following AMPK activation. Of note, similar to platelets, macrophages express only the ACC1 isoform (361). Recently, preliminary data in our lab showed that platelet phosphoACC is increased in patients with septic shock (severe infection). Factors driving activation of AMPK-ACC signaling remain to be determined, but thrombin generation induced by severe inflammation during sepsis might be an important contributor. How and whether ACC phosphorylation/inhibition modifies platelet lipid metabolism and composition thereby affecting platelet prothrombotic phenotype, systemic pro-coagulant state and inflammation, are the objectives of our next study. Given the relationship between sepsis and thrombosis, regulation of platelet metabolism by AMPK-ACC axis can be a key mechanism controlling thrombosis in this pathological setting.



Washed platelets from healthy volunteers and septic shock patients were lysed and subjected to western blot analysis. Platelet lysates were probed with Ser79 phosphorylated ACC antibody. Quantification and representative western blot are shown. * denotes statistical significance, $p < 0.05$

5.5 Limitations in the assessment of platelet phosphoACC

Detection of phosphoACC was performed in purified platelet lysates in our study. Even if quantitative assessment by the ECLIA method confirmed phosphoACC increase in CAD patients obtained by western blot, its routine clinical evaluation remains challenging. Platelets isolation and purification, lysates to obtain protein extracts and protein denaturation for phosphoACC detection are time consuming. A future perspective of our lab is to analyse platelet phosphoACC in whole blood by flow cytometric assays. After fixing and permeabilising platelets, phosphoACC can be detected by the Rabbit-anti-mouse-pACC (Ser79) monoclonal antibody, Clone D7D11, Cell Signalling ref # 11818, followed by staining with Alexa Fluor 647-conjugated goat-anti-rabbit IgG Invitrogen ref # A-21244. Development of platelet phosphoACC flow cytometric assays will improve its detection for future clinical studies. Furthermore, increased platelet turnover, generating immature young platelets (Reticulated platelets - RPs) with increased mean platelet volume, has been shown to be associated with short-term major adverse cardiovascular events after PCI for ACS (362-364). RPs count is also a good candidate to assess platelet response to dual antiplatelet therapy (365). Given the heterogeneity of circulating platelets (312), flow cytometry provides the exciting possibility to detect different platelet populations. Whether phosphoACC can be detected in RPs and its role in regulating RPs functions remains to be determined.

CHAPTER VI - References

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ANNEXES

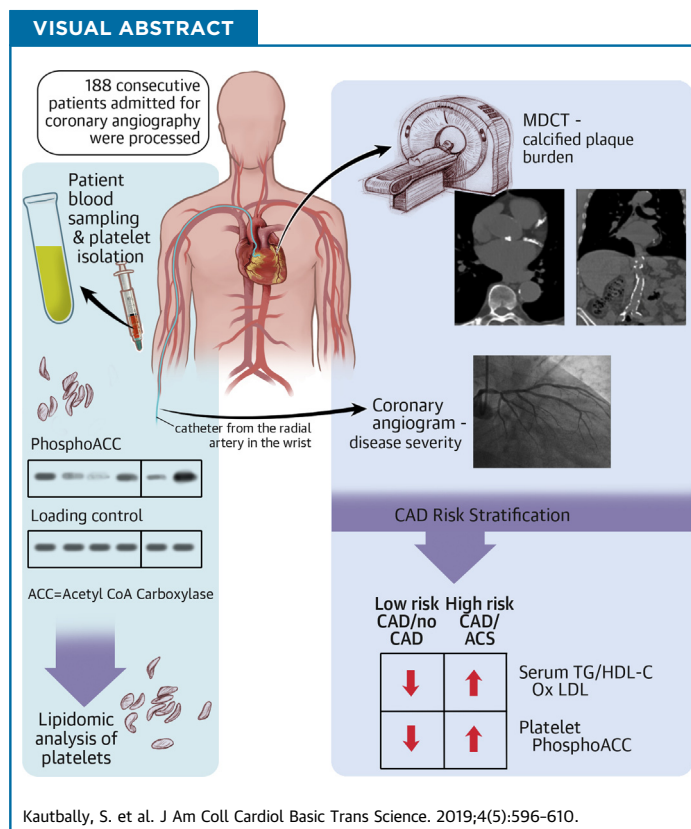
PRECLINICAL RESEARCH

Platelet Acetyl-CoA Carboxylase Phosphorylation



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

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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.

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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

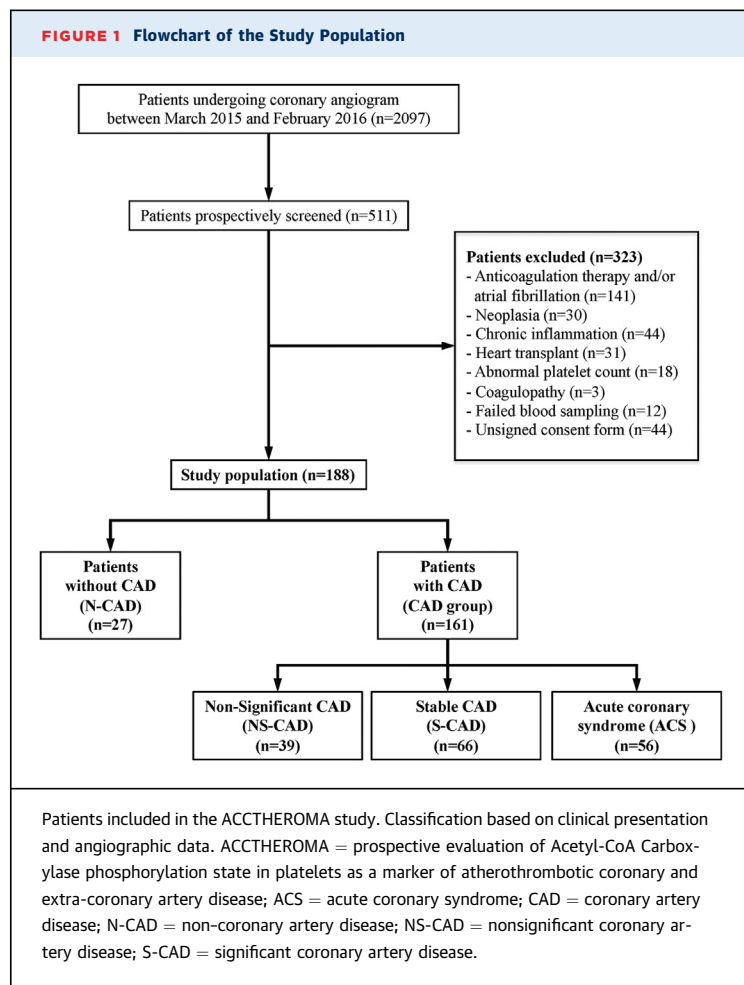
phosphoACC = acetyl-CoA carboxylase phosphorylation on serine 79

S-CAD = stable coronary artery disease

TG = triglyceride

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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](#).

FIGURE 1 Flowchart of the Study Population

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 Lipidzyer, 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 (*R*_p) or Spearman (*R*_s) 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 (Lipidzyer) 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 ± 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/m²	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 (×10³)/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 N-CAD. §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.

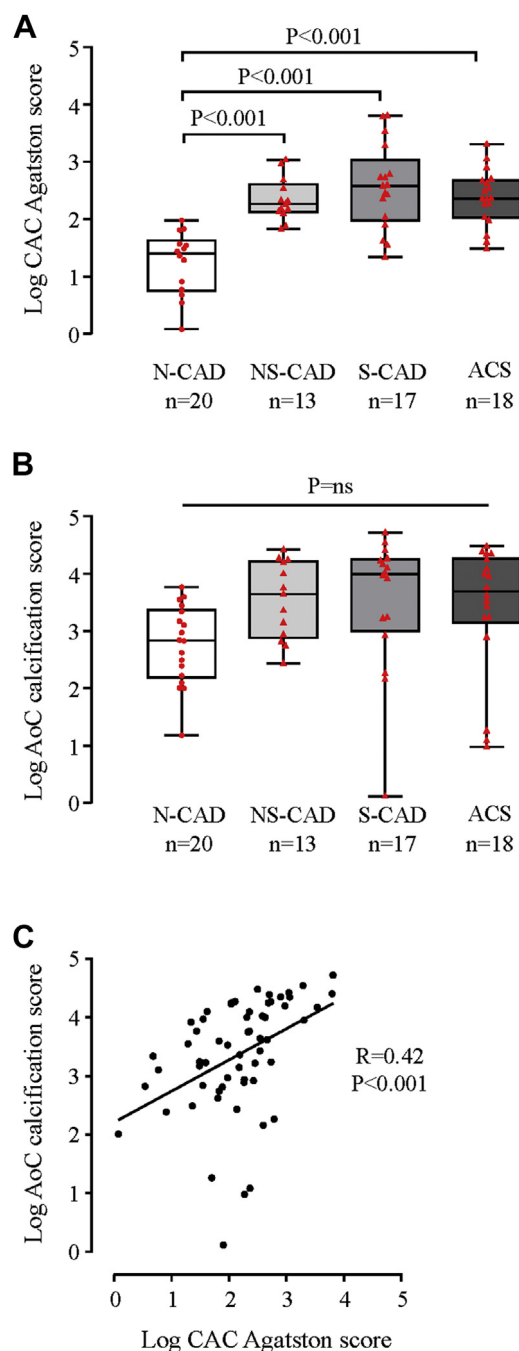
Thoracoabdominal MDCT with prospective electrocardiography gating aimed to evaluate calcified plaque burden, including coronary (CAC Agatston) and extracoronary (AoC) calcification scores. We confirmed that N-CAD patient (reference population) classification based on angiographic data was consistent 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).

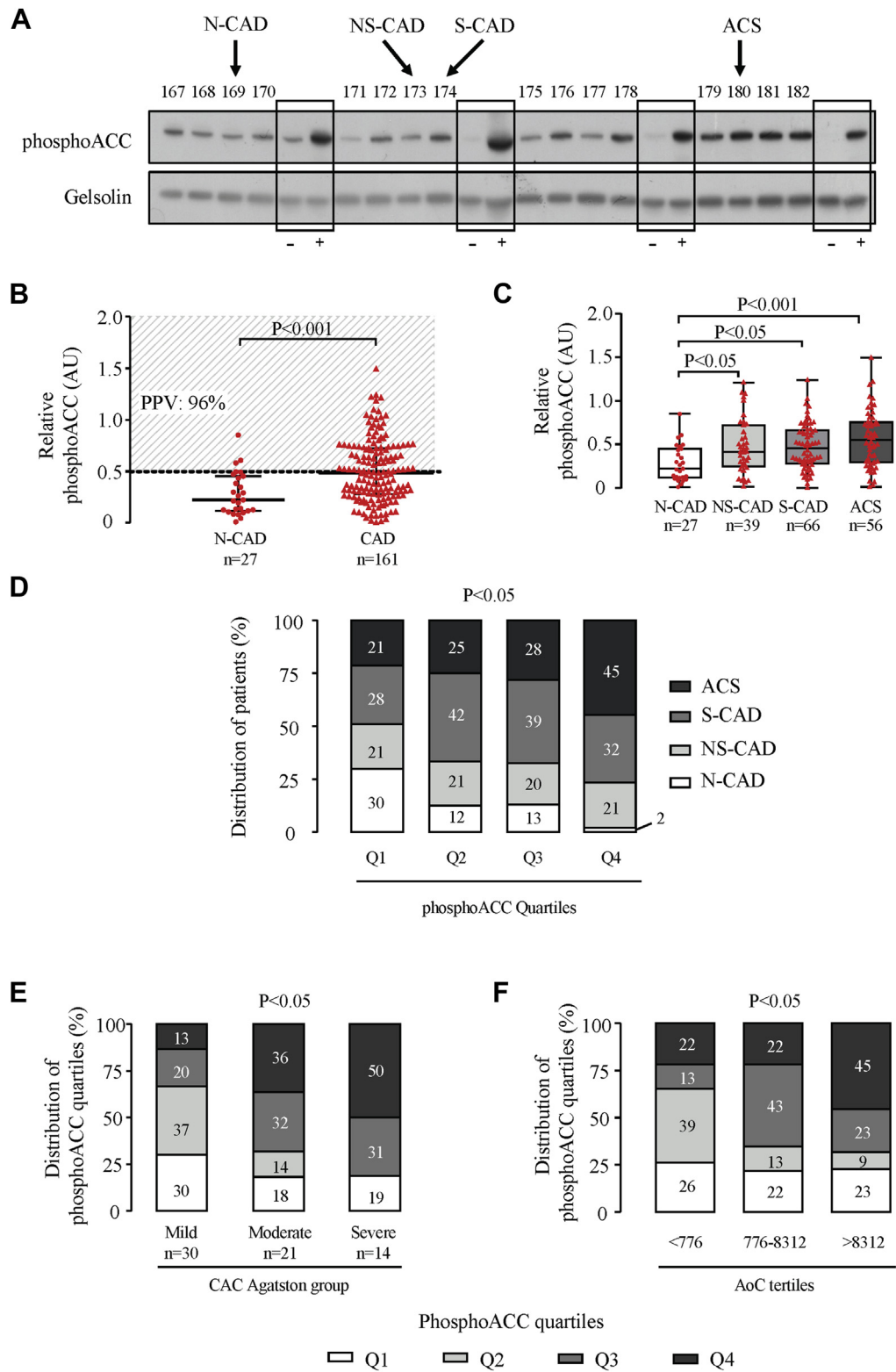
Among the CAD subgroups, the highest platelet phosphoACC level was found in ACS patients (median 0.55 [IQR: 0.29 to 0.76]) (Figure 3C). Indeed, quartile analysis of platelet phosphoACC demonstrated that the fourth quartile included 98% of CAD patients, with

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.

FIGURE 3 PhosphoACC Correlates With Calcified Plaque Severity and Identifies High-Risk ACS Patients



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

FIGURE 3 Continued

(A) Representative Western blot of platelet acetyl-CoA carboxylase phosphorylation on serine 79 (phosphoACC) in 16 consecutive patients from the ACCTHEROMA trial, with negative control (-) corresponding to washed unstimulated platelets from healthy volunteers and positive control (+) corresponding to washed platelets from healthy volunteers stimulated with thrombin (0.5 U/ml) for 2 min. Four different controls are shown on the Western blot. (B) PhosphoACC quantification in N-CAD and CAD patients. Dotted line represents the threshold value of 0.5 arbitrary units (AU) estimated from receiver-operating characteristic curve analysis for discriminating between N-CAD and CAD patients. Positive predictive values (PPVs) of this threshold for CAD are indicated on the graph. Red dots (N-CAD, reference population) and triangles (CAD patients) represent individual values. Medians with interquartile range are presented. (C) Boxplot representation of platelet phosphoACC quantifications in N-CAD and CAD subgroups. (D) Clinical and angiographic characteristics of patients across the different quartiles of platelet phosphoACC. Distribution of platelet phosphoACC quartiles across (E) CAC Agatston score groups and (F) AoC score tertiles. The statistical differences between the groups were determined using the Mann-Whitney U test in B, Kruskal-Wallis test in C, and chi-square test in D to F. ACCTHEROMA = prospective evaluation of Acetyl-CoA Carboxylase phosphorylation state in platelets as a marker of atherothrombotic coronary and extra-coronary artery disease; M = molecular weight marker; Q = quartile; other abbreviations as in Figures 1 and 2.

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).

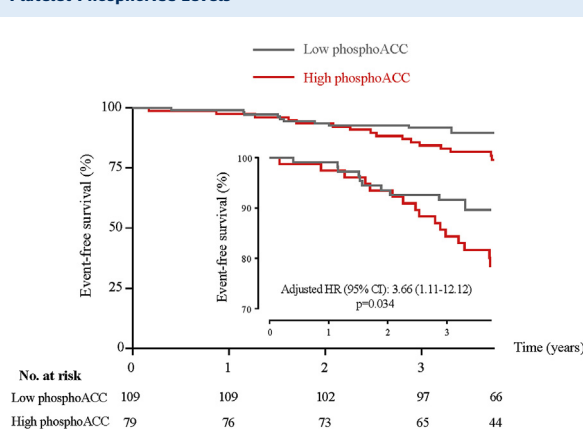
We continued our analysis to identify biological mediators of increased platelet phosphoACC in the CAD group (above 0.5 AU) (Figure 3B). Whereas high-sensitivity C-reactive protein and ThG markers were not associated with increased phosphoACC, we identified a striking positive association between TG/HDL-C 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 INTRAPATELET 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.

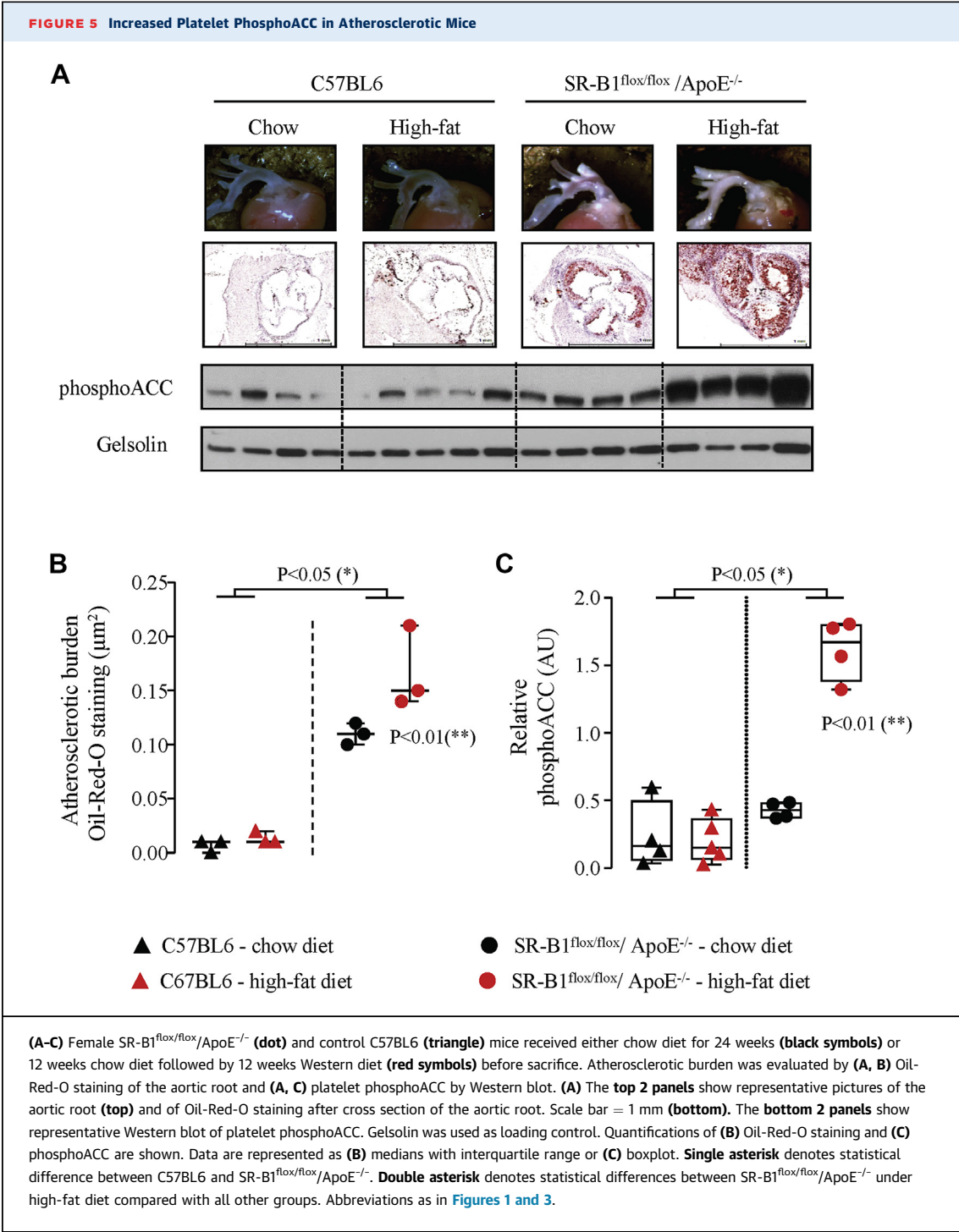
FIGURE 4 Kaplan-Meier Curves for Ischemic Events According to Platelet PhosphoACC Levels



Ischemic events include cardiovascular death, recurrent myocardial infarction and revascularization procedures. Low and high phosphoACC defined according to threshold of 0.5 AU. CI = confidence interval; HR = hazard ratio; other abbreviations as in Figures 1 and 3.

DISCUSSION

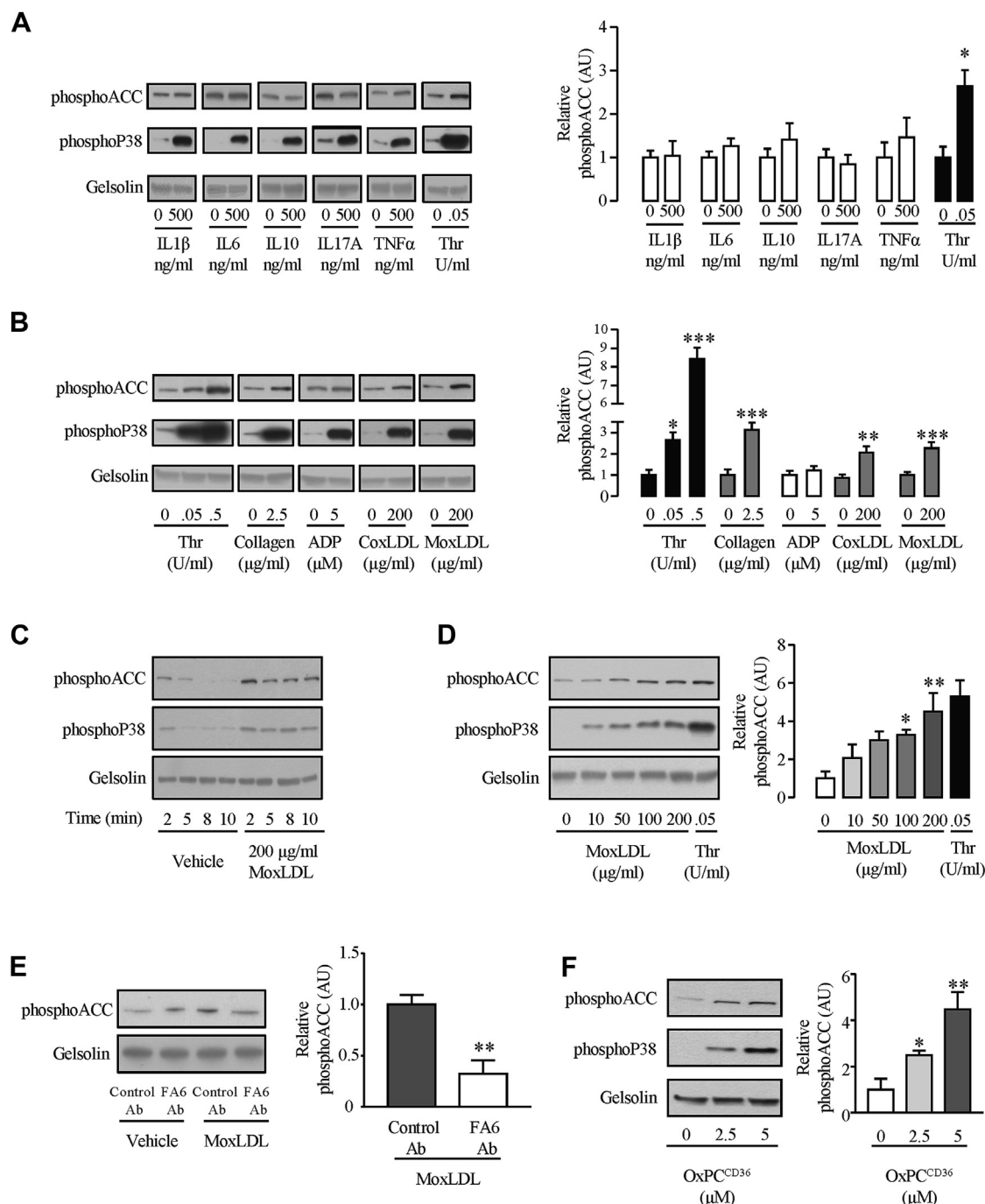
This study highlights that platelet phosphoACC is a risk marker in patients with suspected CAD and



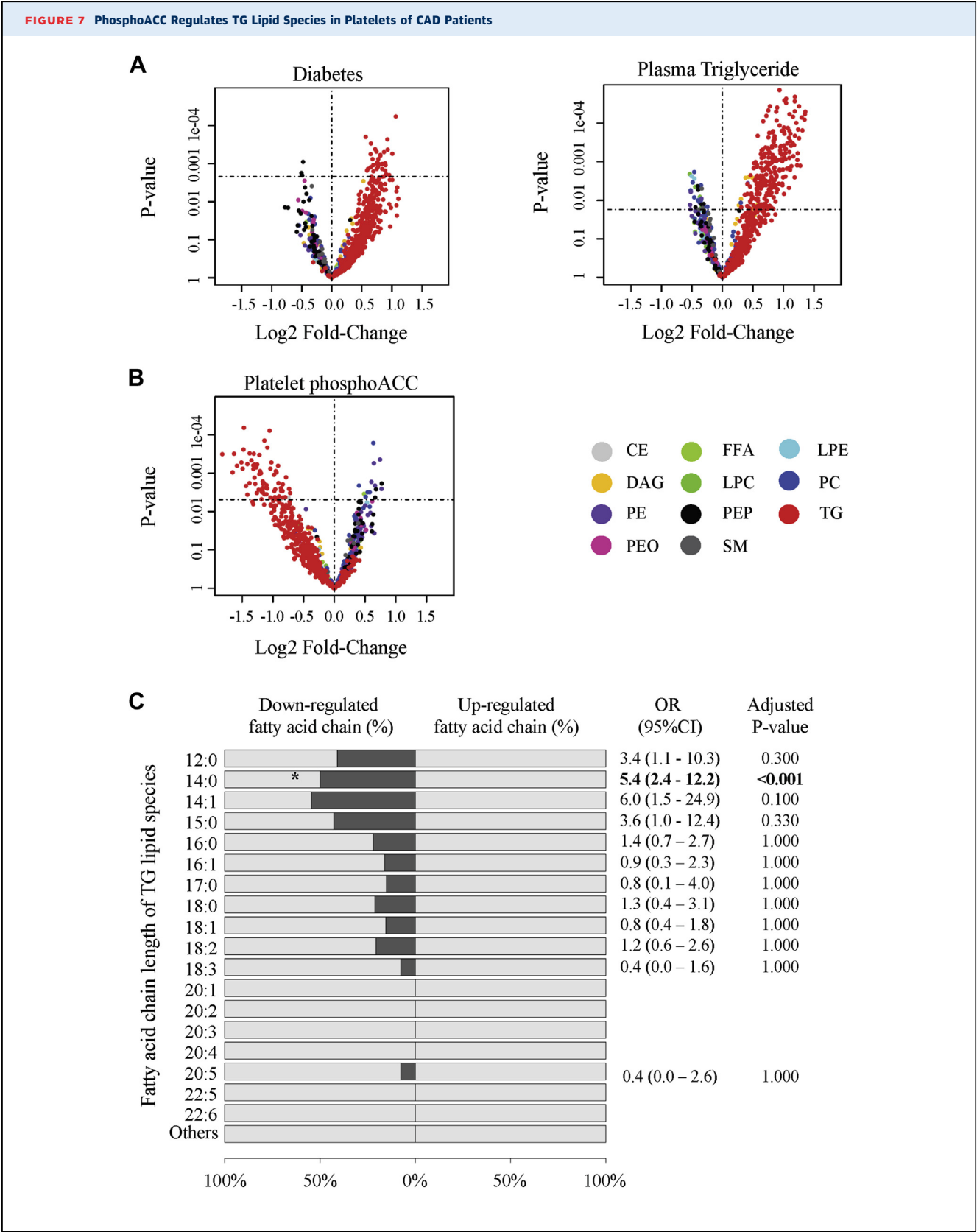
provides additional evidence of an interplay between platelets and lipids in atherosclerosis. We demonstrate a clear relationship between platelet phosphoACC and CAC Agatston score severity, and acute coronary events in CAD. In a molecular point of view, oxLDL participates in increasing phosphoACC in

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

FIGURE 6 Oxidized LDLs Induce Platelet phosphoACC in a CD36-Dependent Manner



Washed platelets (4.0×10^8 /ml) from healthy volunteers were **(A)** treated with the following selected cytokines (interleukin-1beta [IL1β], IL-6, IL-10, IL-17A, and tumor necrosis factor alpha [TNFα]) and **(B)** stimulated with thrombin (Thr), collagen, adenosine diphosphate (ADP), copper-oxidized low-density lipoprotein (coxLDL) or myeloperoxidase-oxidized low-density lipoprotein (moxLDL) before lysis. **(C)** Time course and **(D)** dose-response curve of effect of moxLDL on phosphoACC. **(E)** Platelets were pretreated with 0.2 U/ml anti-CD36 antibody (FA6-152) (FA6 Ab) or an isotype control (control Ab) for 15 min before stimulation with moxLDL. **(F)** Platelets were stimulated with varying concentrations of a specific CD36 ligand (OxPC^{CD36}) for 5 min before lysis. All experiments were carried out at least 4 times (biological replicates). Thr-stimulated platelets were used as a positive control. Gelsolin was the loading control. Representative Western blots are shown, with quantification of Western blots represented in the right-hand panels. Data are expressed as mean $\pm$ SEM. Significance was determined by **(A, B, E)** 2-tailed Student's *t*-test or **(B [Thr], D, F)** 1-way analysis of variance with Bonferroni post hoc analysis. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 compared with unstimulated platelets. Ab = antibody; OxPC^{CD36} = oxidized choline glycerophospholipids; other abbreviations as in Figure 3.



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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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.