ROLE OF MICROPARTICLES IN ATHEROTHROMBOSIS

Rosa Suades Soler

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DIRECTORA DE LA TESI

Prof. Lina Badimon Maestro (Institut Català de Ciències Cardiovasculars)

CO-DIRECTORA DE LA TESI

Dr. Teresa Padró Capmany (Institut Català de Ciències Cardiovasculars)

TUTOR DE LA TESI

Dr. Josep-Eladi Baños Díez

Departament de Ciències Experimentals i de la Salut

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A la meva família

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fem del passat i del present el repte
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tot s'acaba i, alhora, tot comença.

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SUMMARY

Summary

Cardiovascular disease and, specifically, atherothrombosis is a global health problem with huge devastating consequences. While cardiovascular research has progressed rapidly over the last years, there is still a need for clinically applicable tools for risk prediction, diagnosis, or therapeutic interventions; not only in order to improve earlier identification of cardiac diseases and the prediction of specific therapies avoiding invasive diagnostic procedures and unnecessary treatments, but also to further amplify the understanding of basic mechanisms responsible for their pathogenesis. This thesis mainly focuses on the role of cell-derived microparticles in atherothrombosis, showing their direct effect in the context of arterial thrombosis and investigating their association to preclinical atherosclerosis as a form to exploit them as potential biological markers of disease. The combination of functional, molecular, proteomic and genomic approaches allowed the elucidation of different important aspects of the microparticles both as an interesting therapeutic target and as a novel promising biomarker of silent atherothrombotic disease.

Resumen

La enfermedad cardiovascular y, específicamente, la aterotrombosis es un problema de salud global con enormes consecuencias devastadoras. Aunque la investigación cardiovascular ha progresado rápidamente durante los últimos años, aún se requieren herramientas aplicables a la clínica para la predicción del riesgo, el diagnóstico o la intervención terapéutica, con el objetivo no solo de mejorar la identificación precoz de las enfermedades cardíacas y la elección de terapias específicas, evitando procedimientos diagnósticos invasivos y tratamientos innecesarios, sino también para ampliar el conocimiento de los mecanismos básicos responsables de su patogenia. La presente tesis se centra principalmente en el papel de las micropartículas celulares en la aterotrombosis, poniendo en evidencia su participación directa en el contexto de la trombosis arterial y asociación con la aterosclerosis preclínica, con el fin de utilizarlas como potenciales marcadores biológicos. El desarrollo combinado de ensayos funcionales y aproximaciones moleculares, proteómicas y genómicas ha llevado a elucidar aspectos relevantes de las micropartículas, siendo de interés su uso como dianas terapéuticas así como nuevos prometedores biomarcadores de la enfermedad aterotrombótica silente.

Resum

La malaltia cardiovascular i, específicament, l'aterotrombosi és un problema de salut global amb grans conseqüències devastadores. Malgrat que la recerca cardiovascular ha progressat ràpidament durant els últims anys, encara es necessiten eines aplicables a la clínica per a la predicció del risc, el diagnòstic o la intervenció terapèutica, amb l'objectiu no només de millorar la identificació precoç de les malalties cardíaques i l'elecció de teràpies específiques, evitant procediments invasius i tractaments innecessaris, sinó també d'ampliar el coneixement dels mecanismes bàsics responsables de las seva patogènia. La present tesis es centra principalment en el paper de les micropartícules cel·lulars en l'aterotrombosi, demostrant la seva participació directa en el context de la trombosi arterial i associació amb l'aterosclerosi preclínica, amb l'objectiu d'utilitzar-les com a potencials marcadors biològics. El desenvolupament conjunt d'assaigs funcionals i aproximacions moleculars, proteòmiques i genòmiques ha permès elucidar aspectes fonamentals de les micropartícules, tant com a dianes terapèutiques com a nous i prometedors biomarcadors de la malaltia aterotrombòtica silent.

PREFACE

Atherothrombosis is a complex pathology that dramatically changes its course when complicated by thrombosis. It is the major cause of acute coronary syndromes and cardiovascular mortality in the western world with devastating socioeconomic implications. The atherothrombotic disease is characterized by atherosclerotic lesion disruption with superimposed thrombus formation. Atherosclerosis is asymptomatic during a long period and, therefore, there is a major effort in conducting outstanding cardiovascular biology research to early identify affected subjects and to improve current therapies in order to overcome this widespread disease.

The deep understanding of the atherothrombotic pathogenesis and its treatment has suffered a huge advance during the last decades with key discoveries. Since the initial establishment of the Virchow's triad, there have been breakthrough findings such as the role of tissue factor in coagulation, the dissection of the functional involvement of platelets and the new view of inflammation in the whole pathophysiological process and, finally, the recent development of non-invasive imaging modalities for detecting early atherosclerotic lesions. Despite these and other great improvements, there is still a high atherosclerotic plaque burden in industrialised countries.

Cell-derived microparticles have emerged as a potent organizing paradigm in biology and medicine. The awareness of cell-derived microparticles (MPs) has evolved rapidly during the past 10 years. The research on all types of microvesicles (microparticles, membrane blebs, exosomes, etc.) has increased considerably as demonstrated by a growing publishing rate in the last 15 year (Web of Science). Meanwhile, the International Society of Extracellular Vesicles and the Journal of Extracellular Vesicles have recently been founded. Now, the emergence of new detection technologies and the simultaneous increase in research are quickly reshaping our understanding of microparticles. While considerable challenges undoubtedly exist, MPs represent a fascinating and a potentially useful new window into the pathogenesis of atherothrombotic disease.

Besides, microRNAs are also currently being explored for their potential as biomarkers of cardiovascular disease because of their stability in the circulation and the ease by which they can be quantitatively detected. Nevertheless, there is a still great deal to be learned about circulating microRNAs.

With this background, the scope of the present thesis, entitled "Role of microparticles in atherothrombosis", involves the study of MPs both as potential disease biomarkers (either diagnostic or prognostic information), and as novel mechanistic mediators of atherothrombotic disease being likely candidate targets for therapeutic interventions.

The present thesis has been carried out in Professor Badimon's research group at Institut Català de Ciències Cardiovasculars (ICCC), a group with a high translational research experience, specifically focusing on the understanding of mechanisms involved in the initiation, progression and complication of atherosclerosis and ischemic diseases.

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ABBREVIATIONS

ACC	American College of Cardiology
ACD	acid citrate dextrose
ACS	acute coronary syndrome
ACEI	angiotensin-converting enzyme inhibitor
ADP	adenosine diphosphate
AFM	atomic force microscopy
Ago2	argonaute 2
AHA	American Heart Association
ANOVA	analysis of variance
ApoB	apoliprotein B
Apo2L	apoptosis ligand 2
ARF6	ADP-ribosylation factor 6
ATP	adenosine triphosphate
ATPIII	Adult Treatment Panel III
AUC	area under the curve
AV	annexin V
BCA	bicinchoninic acid
BMI	body mass index
Ca^{2+}	calcium
CAC	coronary artery calcification
CAD	coronary artery disease
cAMP	cyclic adenosine monophosphate
CD	cluster of differentiation
CD40L	CD40 ligand
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CHD	coronary heart disease
cMP	circulating microparticle
COX-2	ciclooxigenase-2
CRP	C-reactive protein
CT	clotting time
cTn	cardiac troponin
cv	coefficient of variation
CV	cardiovascular
CVD	cardiovascular disease
CVE	cardiovascular event
DLS	dynamic light scattering
DM	diabetes mellitus
DNA	deoxyribonucleic acid
dsRNA	double stranded RNA
DTT	dithiothreitol
EC	endothelial cell
ECM	extracellular matrix
ED	endothelial dysfunction
EDTA	ethylenediaminetetraacetic acid

ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
eMP	endothelial cell-derived microparticle
ErMP	erythrocyte-derived microparticle
FC	flow cytometry
FERMT3	fermitin family homolog 3
FH	familial hypercholesterolemia
FITC	fluorescein isothiocyanate
FPP	farnesyl pyrophosphate
FRS	Framingham Risk Score
FSC	forward scatter
GO	gene ontology
GP	glycoprotein
gp140	membrane glycoprotein 140
GTP	hydrolyze guanosine triphosphate
H_2O_2	hydrogen peroxide
HCMV	human cytomegalovirus
HCVR	high cardiovascular risk
HDL	high-density lipoprotein
HMG-CoA	3-hydroxy-3methylglutaryl-coenzyme A
HPLC	high-performance liquid chromatography
hsCRP	high-sensitivity C-reactive protein
HTB	HEPES-Tyrode's buffer
IABP	intra-aortic balloon pump
ICAM	intercellular adhesion molecule
IEF	isoelectrofocusing
Ig	immunoglobulin
IHF	immunohistofluorescence
IL	interleukin
IPA	Ingenuity Pathway Analysis
ISADE	Invitrox Surface Antigen Detection and Enumeration
ISTH	International Society on Thrombosis and Haemostasis
LDL	low-density lipoprotein
LLT	lipid-lowering therapy
LMP	leukocyte-derived microparticle
ℓMP	lymphocyte-derived microparticle
LPS	lipopolysaccharide
LTA	light transmission aggregometry
LV	left ventricular
MALDI-TOF	matrix-assisted laser desorption/ionization time-of-flight
MAPK	mitogen-activated protein kinases
MAXV-t	maximum clot formation velocity time

MCF	maximum clot firmness
mCRP	monomeric C-reactive protein
MI	myocardial infarction
miRNA	microRNA
MLC	myosin light chain
MMP	matrix metalloproteinase
mMP	monocyte-derived microparticle
MP	microparticle
MRI	magnetic resonance imaging
mRNA	messenger RNA
MV	microvesicle
MVB	multivesicular body
NCEP	National Cholesterol Education Program
ncRNA	non-coding RNA
NF-κB	nuclear factor kappa light chain
NHLBI	National Heart, Lung, and Blood Institute
NO	nitric oxide
NSTEMI	non-ST-segment elevation myocardial infarction
nt	nucleotide
NTA	nanoparticle tracking analysis
oxLDL	oxidized LDL
PA	plasminogen
PAF	platelet activating factor
PAI	plasminogen activator inhibitor
PAR	protease activated receptor
PBS	phosphate buffered saline
PC	phosphatidylcholine
PCI	percutaneous coronary intervention
PDI	protein disulfide isomerase
PDIA3	protein disulfide isomerase A3
PE	phosphatidylethanolamine
PECAM-1	platelet endothelial cell adhesion molecule-1
PFA-100	Platelet Function Analyzer-100
PFP	platelet free plasma
PG	prostaglandin
PGI_2	prostanglandin
PI3K	phosphatidylinositol 3-kinase
PMN	polymorphonuclear leukocyte
pMP	platelet-derived microparticle
PPAR	peroxisome proliferator-activator receptor
pPCI	primary percutaneous coronary intervention
PPP	platelet poor plasma
pre-miRNA	precursor microRNA
pri-miRNA	primary microRNA
PS	phosphatidylserine

PSGL-1	P-selectin glycoprotein ligand-1
PT	perfusion time
RANTES	regulated on activation, normal T-cells expressed and secreted
RBC	red blood cell
REGICOR	Registre Gironí del Cor
RF	risk factor
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
ROC	receiver operating characteristic
ROCK	Rho-associated coiled-coil-containing protein kinase
ROS	reactive oxygen species
RPS	resistive pulse sensing
RT	room temperature
RT-qPCR	reverse transcription quantitative polymerase chain reaction
SAFEHEART	SpAnish Familial hypErcHolEsterolemia cohoRT
SAXS	small-angle X-ray scattering
SCD	sickle cell disease
SDS-PAGE	sodium dodecyl sulfatepolyacrylamide
SE	standard error
SNARE	soluble NSF attachment protein receptor
SSC	side scatter
STEMI	ST-segment elevation myocardial infarction
TEM	thromboelastometry
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TG	triglyceride
TGF	transforming growth factor
TLR	toll-like receptor
TNFα	tumor necrosis factor alpha
TnI	Troponin I
TnT	Troponin T
TRAIL	tumour necrosis factor–related apoptosis-inducing ligand
TRAP	thrombin-receptor agonist peptide
tRNA	transfer RNA
TSP	thrombospondin
TXA_2	thromboxane A_2
UA	unstable angina
VCAM	vascular cell adhesion molecule
VLDL	very low-density lipoprotein
VSMC	vascular smooth muscle cell
vWF	von Willebrand factor
WB	whole blood

I. INTRODUCTION

1. Cardiovascular disease

1.1. Background

Cardiovascular disease (CVD) is the leading cause of death worldwide and, consequently, a global health problem.[1] The number of people, who die from CVDs is expected to reach 23.3 million by 2030 and, thus, CVDs are projected to remain the leading cause of death,[2] with a significant negative impact on socioeconomics and quality of life in our society.[3] Coronary artery disease (CAD) and stroke constitute the two leading causes of death in the world.[4] In Spain, CVD is also the main cause of death accounting for 29% of all-cause mortality, being atherosclerosis its major cause.[5]

CVD, a generalised and diffused pathology, refers to all the diseases of the heart and the circulatory system. Depending on the affected area, CVD will have distinct clinical manifestations, including: coronary heart disease (acute coronary syndromes [ACS] or aortic stenosis), cerebrovascular disease (stroke), peripheral arterial disease, other heart diseases (heart failure or arrhythmia), congenital heart disease, and deep vein thrombosis and pulmonary embolism. Among them, CAD is the most common type of CVD. Indeed, ACS are mainly caused by coronary atherosclerosis since ACS due to other causes, such as coronary dissection, thromboembolism, or arteritis, without obvious CAD, are very rarely.[6] Atherothrombosis underlies the majority of CV events independently of the specific vascular bed in which they occur.[7] Indeed, a high percentage of atherothrombotic diseases occur in more than one area of the vasculature and, therefore, are classified as coronary, cerebrovascular or peripheral arterial disease (Figure 1).[8]

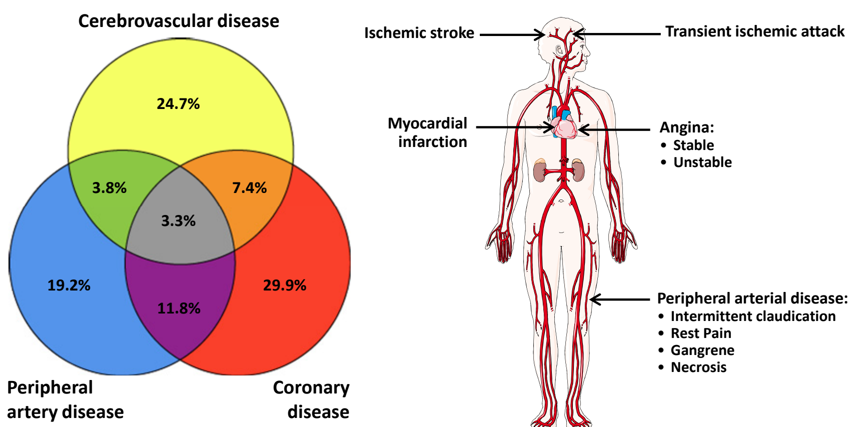

Figure 1. Cardiovascular disease presentation. Percentage of atherothrombotic events depending on the vascular territories affected. Data obtained from CAPRIE trial.[8]

Current diagnostic methods do not precisely identify early changes of the vasculature. Thus, there is a need to discover new intermediate diagnostic measures that may reflect and predict adverse changes before they become clinically apparent. In advanced atherosclerosis, thrombotic complications depend on the interplay between blood components and arterial plaque. Among thrombogenic blood factors, circulating microparticles (cMPs), small membrane vesicles released mainly by activated cells, have emerged as potential bioactive effectors of CVD. Several reports have suggested that the role of platelets in atherothrombosis is mediated, in part, by local secretion of platelet-derived microparticles (pMPs).[9] Indeed, high concentrations of circulating cMPs have been reported in patients with atherosclerosis, acute vascular syndromes and diabetes mellitus,[10-12] suggesting a potential correlation between the quantity of microparticles and the clinical severity of atherosclerotic disease. It is therefore necessary to deepen in understanding of cMPs to find new therapeutic targets and to evaluate their specificity as clinically useful biomarkers of atherothrombosis.

1.2. Atherothrombosis

Atherosclerosis is a chronic inflammatory disease of the vascular wall, produced by lipid infiltration, foam macrophage accumulation on the inner wall, and subsequent focal thickening of the intimal layer. When the lesion ruptures leading to local thrombus formation, arterial occlusion, and tissue ischemia, it becomes life threatening. The atherosclerotic and thrombotic processes with its clinical complications appear interdependent and, therefore, are integrated under the term *atherothrombosis* (Figure 2).

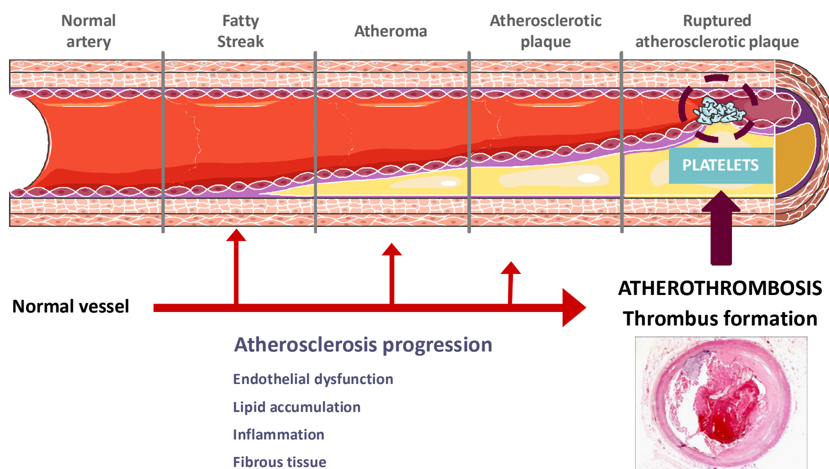

Figure 2. Atherothrombotic disease progression. Atherothrombosis chronically develop from early fatty streak to atheromatous plaque formation that by unpredictable disruption leads to platelet activation and thrombus formation.[13-15]

1.2.1. Arterial wall composition

The arterial vascular wall is a dynamic tissue that is able to adapt and reorganize itself under both physiologic and pathologic mechanic stimuli. All arterial vessels except capillaries are composed of three concentric layers with distinct cell and interstitial composition (Figure 3):

- **Intima**, the innermost layer, is in contact with the flowing blood. The intima layer is composed by a monolayer of endothelial cells (EC), a very thin basal lamina and a subendothelial layer formed by collagen and elastic fibrils.
- **Media**, the middle layer of the vascular wall, is composed by vascular smooth muscle cells (VSMC), collagen and a network of elastic fibrils. It is separated from the intima and the adventitia by the internal and external elastic lamina, respectively.
- **Adventitia**, the external layer of the vascular wall, consists of elastic fibers, fibroblast, collagen, nerves, and small blood vessels (*vasa vasorum*). Its thickness varies considerably depending on the type and location of the vessel.

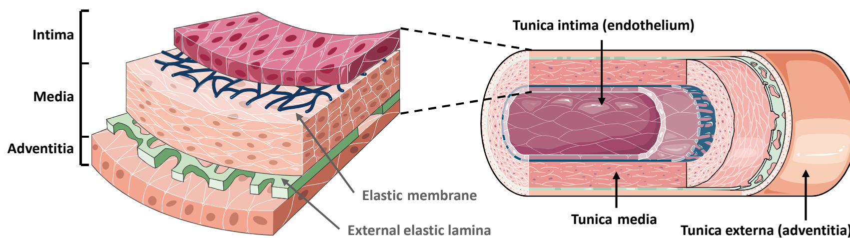

Figure 3. Arterial wall structure. The artery wall consists of three concentric layers, called tunica intima, media and adventitia, separated by elastic membranes.

1.2.2. Pathophysiology

Atherothrombosis is a systemic progressive arterial disease originally involving the intima (with secondary involvement of the media and adventitia) of large- and medium-sized arteries including the carotid, aorta, coronary, and peripheral leg arteries. Atherosclerosis begins with the development of fatty streaks (early lesions) in childhood and adolescents.[16] Indeed, young adults have already early atherosclerotic lesions in the coronary arteries. It is often silent and slow progressing and, then, atherosclerotic process advances through lipid core expansion and macrophage accumulation at the edges of the plaque, leading to fibrous cap rupture. Atherothrombosis is characterized by direct interaction between atherosclerotic plaque and arterial thrombosis (Figure 2).

The complex interaction between disease processes (explained below) in the development of atherothrombosis has been a matter of debate. A well-established histological classification of atherosclerotic lesions was provided by the American Heart Association (AHA), which relates morphologic characteristics and phases of coronary atherosclerosis progression (Figure 4).

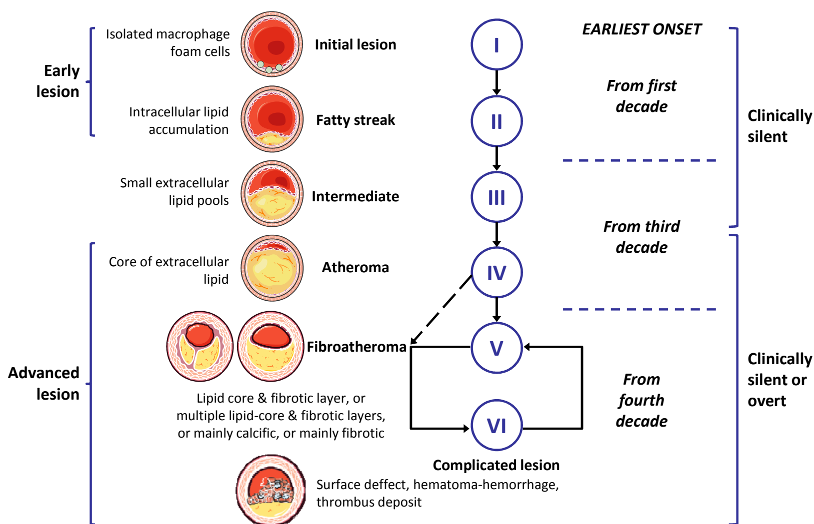

Figure 4. AHA atherosclerotic lesion classification. Adapted from Stary et al.[17]

A simple modification of this classification providing a link to clinical findings has emerged[18] as follows:

- Adaptative intimal thickening (*AHA Type I lesion*), which consists of VSMC and extracellular matrix within the intima.
- Fatty streak or intimal xanthomas (*AHA Type II lesion*) corresponding to the accumulation of macrophage foam cells interspersed within a VSMC and proteoglycan-rich intima, which at this stage is a reversible process.
- Pathological intima thickening (*AHA Type III lesion*). Composed of layers of VSMC in a proteoglycan-collagen matrix that is aggregated near the lumen with an underlying extracellular lipid pool consisting of an acellular area, rich in hyaluronan and proteoglycans with lipid insudation.
- Fibroatheroma (*AHA Type IV-V lesion*). Consists of an acellular necrotic core, covered by a thick fibrous cap consisting of VSMC in a proteoglycan-collagen matrix.
- Thin-cap fibroatheroma and/or fibrocalcific plaque (*AHA Type VI lesion*) that occurs when the necrotic core and surrounding tissue may eventually be calcified.

Advanced lesion types (fibroatheromas and fibrocalcific plaques) may evolve simultaneously and interrelated and their distinction is difficult. Nevertheless, 'complicated' lesions may lead to the formation of either a mural thrombus causing angina or an occlusive thrombus causing an ACS (unstable angina [UA], myocardial infarction [MI] or ischemic sudden death).

1.2.2.1. Initial lesion formation and progression

Specifically, under pathological conditions, such as risk factors or mechanical injury, the endothelium becomes dysfunctional leading to a proatherogenic environment. The vascular endothelium is a semi-permeable barrier that controls the diffusion of plasma proteins. Endothelial dysfunction is characterized by the loss of the ability to regulate vascular tone, inflammation and prevent thrombus formation due to changes in the pattern of synthesis and secretion of different substances by the endothelium (from the antiaggregant and vasodilatant nitric oxide (NO) and prostacyclin to the proaggregant and vasoconstrictor thromboxane), with three major consequences (Figure 5):

1. Exposure of adhesion proteins (selectin, intercellular adhesion molecule [ICAM], and vascular cell adhesion molecule [VCAM], among others) and chemotactic molecules that facilitate the activation of leukocytes (monocytes and lymphocytes) and their adhesion to the dysfunctional area and transmigration across EC surface into the arterial wall.

2. Enhancement of platelet activation and aggregation. Platelets at this stage act as inflammatory mediators by releasing the content of their α-granules, expressing various receptors and interacting with leukocytes and activated endothelium, which in turn facilitates the homing and internalization of the circulating monocytes to the subendothelial space, where they become macrophages.

3. Infiltration and accumulation of circulating lipids into the intimal layer plays a central role in atherogenesis. Low-density lipoproteins (LDLs) penetrate through the arterial endothelium into the intima. LDLs bind to the proteoglycans at the subendothelial space, where they undergo modifications (such as oxidative process), become more atherogenic triggering a cascade of proinflammatory reactions,[19] and consequently, are phagocytised by the vessel wall macrophages and VSMCs. Macrophages with internalized LDL become foam cells, which are separated from the blood by VSMC and collagen and constitute the lesion core of atherosclerotic plaques.

This leads to necrotic core and fibrous cap formation evolving into advanced atherosclerosis (atheroma), a key process in the progression of the atherosclerotic plaques and their evolution to unstable plaques with a high risk of rupture (Figure 5).

Of note, cMPs may have a role in initial stages of atherosclerotic process, as they can facilitate cell communication and adhesion processes between blood and vessel wall.[20]

1.2.2.2. Complication of advanced lesions

After atherosclerotic plaque rupture or erosion, the subendothelial space (containing tissue factor [TF], collagen, and von Willebrand factor [vWF]) is exposed to the blood flow (Figure 5). Specifically, exposed collagen triggers adhesion and activation of platelets through platelet glycoprotein VI. Besides, under conditions of high shear stress, as those found close to a significant stenosis, vWF plays a critical role in mediating platelet adhesion via glycoprotein Ibα. After collagen-induced platelet adhesion, platelets activate and undergo a remarkably complex series of morphological and biochemical changes, leading to the generation and release of soluble mediators, including thromboxane A_2 (TXA_2) , thrombin, adenosine diphosphate (ADP), and serotonin, which in turn cooperatively promote further activation, recruitment of additional platelets from the circulation and amplification of the signal for thrombus formation; and also upregulation of $\alpha_{IIb}\beta_3$-integrin (glycoprotein [GP] IIb/IIIa), which is capable of binding multiple ligands, including vWF, fibrinogen, fibrin, and fibronectin, and is fundamental for the formation of stable platelet aggregates.[21]

In addition to the formation of the initial haemostatic plug, vessel wall-bound platelets can recruit leukocytes via interaction of platelet P-selectin with its receptor P-selectin glycoprotein ligand 1 (PSGL-1) on leukocytes; this crosstalk interaction is important for the propagation of inflammation at the site of vascular injury, as well as for sustaining thrombus growth.[6] Indeed, pMPs have shown to induce leukocyte aggregation and recruitment via P-selectin/PSGL-1-dependent interactions.[22] Platelets also possess a procoagulant function, as they provide a catalytic surface for the optimal assembly of coagulation factors. TF interacts with circulating factor VIIa, which in turn activates factor IX and X, resulting in the conversion of the inactive zymogen prothrombin into the active enzyme thrombin.[23,24] cMPs also possess procoagulant properties that lead to thrombin generation.[25] Thrombin, on the other hand, not only has the ability to generate fibrin polymers, but is also the most potent platelet activator by binding platelet protease-activated receptors (PAR-1 and -4).

The thrombogenicity of blood can be partially explained by the fact that TF is not only present in the subendothelium, but also in a circulating state in the blood.[26] TF is associated with macrophages/monocytes, platelets and cMPs, the latter represent an important source of the so-called blood-borne TF. Although TF cell origin is still controversial, it has been shown that platelet-associated TF enhances platelet reactivity and thrombin generation with flowing blood.[27] Increased TF-positive procoagulant MPs are present in the circulating blood of patients under pathophysiologic conditions;[28] however, their cellular origin has not been established yet. TF molecules located on the cell surface have low activity because of encryption.

Phosphatidylserine (PS) exposure in response to various stimuli is a potent inducer of TF decryption, which together with coagulation factors, amplify the coagulation cascade. TF also mediate coagulation-independent biological effects, including angiogenesis, monocyte adhesion to the endothelium and proliferation of cells inside the atherosclerotic plaque. Finally, inflammation may also enhance prothrombotic phenotype by inducing functional TF on VSMC and ECs. Indeed, lipopolysaccharide (LPS) increases MP-associated TF procoagulant activity.[29]

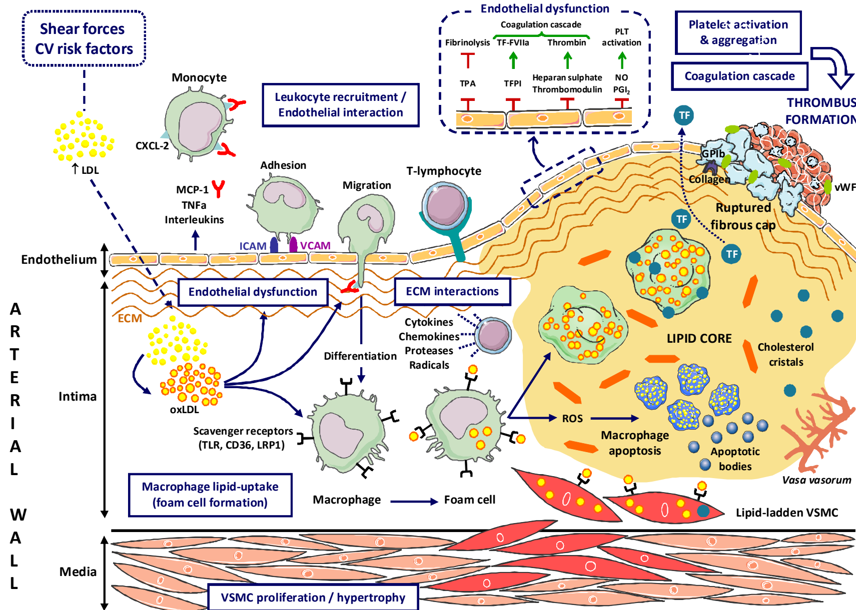

Figure 5. Pathophysiology of atherothrombosis. Under pathogenic stimuli, endothelial dysfunction causes the recruitment and chemotaxis of inflammatory cells. Modified LDLs trigger a cascade of proinflammatory reactions via different mediators and are internalized by macrophages that become foam cells. Apoptotic death of macrophages induces the release of cholesterol and inflammatory substances such as cytokines, reactive oxygen species (ROS), growth factors, tissue factor (TF), and matrix metalloproteases creating a thinner plaque cap prone to rupture and thrombus formation. Under these atherogenic stimuli, VSMCs change into an actively proliferative and migrating phenotype, by which alter extracellular matrix (ECM) composition and synthesis, leading to vascular remodelling (fibrosis) and, consequently, vasa vasorum proliferation in the inner layers of the vessel wall.

Atherosclerosis progression that may remain clinically silent for many years involves two distinct processes: a large one with slow luminal narrow, and a short one that causes rapid luminal obstruction. The mechanisms responsible for plaque growth and instability are different and multiple. Accordingly, there is more than one type of culprit coronary plaque which can lead to distinct clinical symptomatology or event presentation:

Ruptured plaques – Plaques are characterized by an enlarged and soft lipid-rich necrotic core covered by a thin cap, containing inflamed fibrous cap (activated macrophages and T cells), apoptotic macrophages, few VSMCs, neovascularisation from increased number of *vasa vasorum*, and more frequent intraplaque haemorrhage, adventitial/perivascular inflammation, positive remodelling mitigating luminal obstruction (mild stenosis) and a "spotty" pattern of calcifications.[18,30] The great majority of fatal coronary thrombi (73%) develop on top of a ruptured atherosclerotic plaque; indeed, plaque rupture with mural thrombi is the main cause of coronary thrombosis regardless of the clinical presentation[31] and appears to be a common cause of asymptomatic progression to severe stenosis.[32] Among ruptured plaques, those exposing high contents of collagen with small amounts of TF, provide the most procoagulant and, thus, occlusive combination.[33]

Healed plaque rupture – Plaque progression and luminal narrowing can occur secondary to repeated clinically silent plaque rupture in less severely narrowed arteries. They are composed of breaks in the fibrous cap with a proteoglycan-rich mass with collagen.

Eroded plaque – Plaque erosion is characterized by inducing thrombosis without plaque rupture.[34] Typically the endothelium is missing and the exposed intima consists predominantly of VSMC and proteoglycans, but the blood does not come into contact with the lipid-rich necrotic core. Apoptosis of ECs also contribute to desquamation due to oxidative stress and apoptotic cells are able to synthesize and release the procoagulant TF, propagating EC loss and local thrombosis in coronary arteries.[35]

Calcified nodule – A rare type of coronary thrombosis that occurs in highly calcified arteries and is related to disruptive nodular calcifications protruding into the lumen, surrounded by fibrin and with small luminal thrombus.[18] The PROSPECT trial has found that calcified nodules, although being associated to higher plaque volume were unlikely to cause coronary events, probably because they were also associated with more thick-cap fibroatheroma.[36]
There are two forms of atherosclerotic calcification: in the intima and in the media, the latter associated to advanced age, diabetes, chronic kidney disease, and arterial stiffness.[37] Quantification of coronary artery calcification (CAC) has been widely proposed as a marker of CAD.[38,39] Despite this, its utility is controversial since evidence suggests that it may be protective against development of ACS.[40] The majority of ACS-related plaques showed spotty calcification, low plaque density, and positive remodelling whereas stable angina plaques had large calcification and infrequent remodelling.[41,42] Indeed, heavily calcified plaque is significantly less likely to develop a thrombus than uncalcified or mixed plaque.[43] In line, autopsy studies have identified less calcification in rupture or vulnerable plaques as compared to stable plaques in sudden coronary death victims. Therefore, calcium may be a marker of plaque burden rather than of plaque instability.[44]

Vulnerable atherosclerotic plaques (high-risk plaques) are usually those plaques prone to rupture but the term of vulnerability is sometimes used for plaques at high-risk of thrombosis by any mechanism (rupture, erosion). Thickness of the fibrous cap, macrophage infiltration and necrotic core are the main discriminators of plaque vulnerability. In view of the mechanisms of coronary instability, Crea et al[45] have very recently proposed a new classification of ACS patients based on pathology: (a) patients with obstructive atherosclerosis and systemic inflammation, (b) patients with obstructive atherosclerosis and without systemic inflammation and, (c) patients without obstructive atherosclerosis.

Plaque rupture and thrombus formation do not always lead to coronary events but favour plaque progression and the development of lumen stenosis. Therefore, ACS is not a necessary consequence of coronary plaque rupture. Since plaque morphology is dynamic, identifying the presence of vulnerable plaques may confer only some increase in coronary event risk. Thus, other factors than the atherosclerotic lesion *per se* are involved in ACS. Indeed, the other determinants of the classic triad of Virchow[46] (blood rheology and systemic factors of the circulating blood) may also influence the magnitude and stability of the resulting thrombus and thus, the severity of ACS. Among blood thrombogenicity and systemic procoagulant activity components (such as metabolic and hormonal factors and plasma variables of haemostasis), cMPs may play a key functional role.

1.2.3. Main participant cells

1.2.3.1. Endothelial cells

Endothelial cells are the main component of the endothelium and, in turn, of the tunica intima, which consists in a monolayer of ECs. The endothelium controls the vascular tone, maintains the balance between thrombosis and fibrinolysis and regulates the recruitment of inflammatory cells into the vascular wall. These processes can be hampered by atherogenic or mechanic stimuli causing endothelial dysfunction, a hallmark of the atherosclerotic process. Injured or inflamed ECs downregulate antiplatelet prostaglandins and express molecules such as fibronectin, ICAM-1, endothelial P-selectin, E-selectin, β_2-integrin, and vWF which promote leukocyte and platelet adhesion,[47] which further induce atherosclerosis development.

cMPs derived from ECs (eMPs) have been related to vascular proinflammatory activity and thromboembolic complications.[48] Interestingly, eMPs are found increased in ACS,[49] CAD,[50] severe hypertension,[51] end-stage renal failure,[52] and pulmonary hypertension.[53,54] eMPs seem to be markers of inflammation,[50] endothelial injury[52] and endothelial function.[54]

1.2.3.2. Leukocytes

Atherosclerosis is not a mere lipid deposition process in the vessel wall and a luminal stenosis due to VSMC proliferation. Indeed, inflammation is a key regulatory process that links multiple risks factors of atherosclerosis with altered arterial biology. Inflammation, both a defence mechanism against infection and/or tissular injury and a repair mechanism of damaged tissues, regulates the fragility of the fibrous cap as well as the thrombogenic potential of the plaque. The inflammatory response, driven by white blood cells, is highly complex and can be divided in two types: innate and acquired, both of them important in the pathophysiology of atherothrombosis.

Leukocytes-derived cMPs (LMPs) may provide a link between inflammation and thrombosis.[55] Indeed, atherosclerotic plaques contain LMPs.[56,57] As such LMPs have been associated to cytokine release,[58,59] expression of EC adhesion molecules and functional TF,[59] PS exposure, and monocyte adhesiveness,[60] leading to proinflammatory and procoagulant activity.

– <u>Innate immunity</u> –

Monocytes are the main cells implicated in innate immunity and it is largely accepted that are active participants in the progression of atherosclerosis. Monocytes can migrate from blood into vascular tissue in response to signals and differentiate to dendritic cells, macrophages and foam cells.[61] Moreover, monocytes are proinflammatory by releasing myeloperoxidase[62] and also expressing other proinflammatory molecules (tumour necrosis factor [TNF] α, matrix metalloproteinases [MMPs], transforming growth factor [TGF] β, interleukins [ILs], cathepsins).[24] Different subsets of monocytes according to their surface expression of LPS receptor (cluster of differentiation [CD] 14) and low-affinity Fcγ-III receptor (CD16) have been characterized, as classic ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$) and non-classical ($CD14^{+}CD16^{++}$), being $CD14^{++}/16^{+}$ independent predictors of CV events.[63]

Macrophages present a crucial contribution to atherogenesis related to reverse cholesterol, inflammation and MMPs. Plaque resident macrophages differentiate from monocytes recruited from circulating blood. Two different macrophage subtypes have been reported due to polarization: M1 and M2. M1 macrophages release proinflammatory substances while M2 macrophages have a reparative or modulator role. In general, M1 associates to a more vulnerable plaque phenotype, consisting of greater intraplaque haemorrhage, oxidative stress, inflammation and apoptosis of VSMCs and macrophages.[64]

Neutrophils have recently been suggested to additionally contribute to both initiation of atherosclerosis and plaque vulnerability. Mast cells release proinflammatory molecules and vasoactive mediators, favouring atherosclerosis development.[65-67] Neutrophil-derived MPs have recently been shown to induce myeloperoxidase-mediated damage of vascular ECs.[68]

– Adaptative immunity –

Cellular response T-cells (*T-lymphocytes*) interact with dendritic cells, antigen-presenting cells that induce T-cell proliferation and amplification of the immune response, by producing cytokines and triggering inflammation, and thus aggravating atherosclerosis.[69] Similarly, lymphocyte-derived MPs (ℓMPs) were shown to increase the production of TNFα and IL-1β.[70]

Humoral response B-cells (*B-lymphocytes*) are suggested that attenuate atherosclerosis in contrast to T-cells increasing oxidized LDL (oxLDL)-reactive immunoglobulin M (IgM) levels, which are associated with atheroprotection, by a B1 subset-specific effect.[71]

1.2.3.3. Platelets

Platelets are key players in the pathogenesis of atherothrombotic processes.[72] Circulating platelets do not normally interact with the vessel wall and the endothelium is able to inhibit platelet reactivity by producing several local active substances, including NO and prostacyclin. However, the clustering of CV risk factors results in the aforementioned endothelial dysfunction, characterized by a decrease in NO bioavailability.[73] NO is able to activate cyclic guanosine monophosphate and/or adenosine monophosphate (cAMP)-related kinases and subsequently vasodilatation and/or inhibition of platelet aggregation, respectively. For instance, enhanced cAMP induces the phosphorylation of vasodilator-stimulated phosphoprotein and subsequent platelet inactivation.[73] Thus, reduction in endothelial-related antithrombotic properties together with high ROS and the local increase in prothrombotic and proinflammatory mediators contribute to platelet activation in the onset of atherosclerosis.[73]

In the early stages of atherosclerosis, platelet *rolling* in the activated endothelium is primarily mediated by P-selectin and followed by firm adhesion through integrin binding.[74] Platelet adhesion to intact but dysfunctional or activated EC layer may also be initiated by interaction of GPIbα and $\alpha_{IIb}\beta_3$-integrin with endothelial P-selectin and vWF.[74] Activated platelets, in addition to selectin and integrin expression, release several mediators retained within their granules that result in cell adhesion, survival and proliferation, coagulation and proteolysis, and synthesis of chemokines and proinflammatory cytokines, all of which accelerate and enhance the inflammatory process promoting plaque development.[75] In fact, platelet-related secretory effectors mediate the leukocyte-endothelium interactions.[76] Platelet-leukocyte interactions also occur via P-selectin/PSGL-1, which facilitates firm leukocyte adhesion to endothelial-adhered platelets or directly to the endothelium supporting plaque formation, as shown in Figure 6.

The exposure of the thrombogenic substrates to circulating platelets challenges platelet recruitment to the injured vessel wall by initial contact and attachment onto the exposed subendothelium, which promotes the recruitment and activation of additional platelets through the local release of major platelet agonists, and finally, leads to the stabilisation of the platelet aggregates (Figure 6).[77] In advanced disrupted plaques, platelet adhesion varies according to the shear rate.[78] Under *low shear rate* conditions, platelet attachment mainly occurs through the collagen receptor (by binding to GPIa/IIa). Fibrinogen, laminin, vitronectin and thrombospondin also contribute to platelet adhesion by binding to GPIc-IIa ($\alpha_V\beta_1$-integrin), vitronectin receptor ($\alpha_V\beta_3$-integrin), and to GPIV (CD36), respectively. Binding of platelet GPVI receptor to collagen promotes firm platelet adhesion and mediates platelet activation and aggregation. At *high shear rates* platelet adhesion is mainly driven by the interaction of circulating vWF (via its A3 domain) with exposed collagen through platelet GPIbα, despite being an unstable association. Again, GPVI receptor promotes firm adhesion and further activation and aggregation. It activates GPIIb/IIIa ($\alpha_{IIb}\beta_3$-integrin) and GPIa/IIa ($\alpha_{II}\beta_1$-integrin), which promote subsequent firm, irreversible and stable platelet arrest on the endothelial surface, by binding both to collagen and to the vWF.[78] As stated, P-selectin also mediates platelet adhesion to the damaged vessel. It is located in the platelet α-granules and EC Weibel-Palade bodies, and upon cell activation is translocated to the cell surface and bind to their multiple ligands on platelets, ECs or leukocytes.[79]

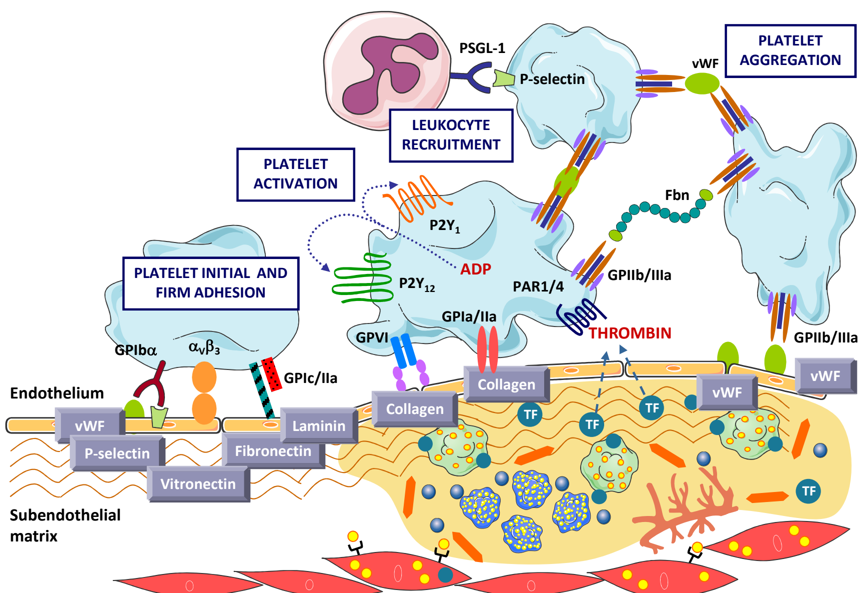

Figure 6. Platelet-vessel wall adhesive interactions at sites of vascular injury. Key platelet-vessel mechanisms indispensable for platelets to adhere, activate, form stable aggregates with other activated platelets, and promote thrombus formation. Adapted from Badimon et al.[78]

Besides collagen, circulating agonists such as epinephrine, serotonin, ADP and thrombin also activate platelets via specific surface receptors (Figure 7).[80] For instance, serotonin enhances interaction of platelets with TF^{+}-MPs, increasing platelet activation and their overall procoagulant activity.[81]

Once activated, platelets undergo shape change, calcium translocation and the subsequent release of platelet granule components: ADP/adenosine triphosphate (ATP), serotonin, calcium, adhesion proteins (fibrinogen, fibronectin, vWF, thrombospondin, vitronectin, P-selectin, GPIIb/IIIa), and coagulation factors (factor V, XI, plasminogen activator inhibitor (PAI)-1, plasminogen, protein S).[82] Platelet activation also leads to PS exposure in the cell membrane and release of membrane MPs (pMPs) conferring binding capacity for coagulation factors and thrombin generation,[83-85] which contribute to perpetuate and amplify the thrombotic response. Platelets store proinflammatory cytokines and mediators in their α-granules that are exteriorized in their membrane such as CD40 ligand (CD40L), which mediates platelet adhesion to the endothelium, or myeloid-related proteins,[86] which bind to the monocytes[87] and also induce EC apoptosis.[88] Platelets also generate lipid mediators like TXA_2 that activate different signalling cascades depending on the trigger.

Figure 7. Major agonists and receptors involved in platelet function. Schematic diagram of platelet surface receptors and ligands responsible of platelet adhesion, activation, secretion and further aggregation processes. ADP indicates adenosine diphosphate; ATP, adenosine triphosphate; PAF, platelet activating factor; PAR, protease-activated–receptor; NO, nitric oxide; PGI_2, prostanglandin; TXA_2, thromboxane A_2; vWF, von Willebrand factor.

Finally, platelet aggregation is regulated by activation of the platelet heterodimer GPIIb/IIIa ($\alpha_{IIb}\beta_3$-integrin) receptor, the most abundant protein on the platelet surface. Fibrinogen ligands not only bind to these receptors growing the thrombus and favouring platelet aggregation, but also trigger an "inside-outside" signalling, causing amplification of the initial signal and further platelet activation. In the final phase of thrombus formation, fibrinogen is converted to fibrin by thrombin, leading to the stabilization of platelet aggregates, with more platelets and blood cells, contributing to thrombus growth. pMP dissemination and exposure of their procoagulant surface into the extracellular matrix[89] at the sites of endothelial injury or onto the forming fibrin[90,91] enable adhesive functions and thrombin generation, which further stimulates aggregation.[92-94]

1.2.3.4. Erythrocytes

Erythrocytes are the main components of the "red thrombus", which also contains fibrin and preferentially forms in the low flow recirculation zones on the downstream margin of the developing thrombus. It has been shown that red blood cells (RBC) can promote platelet reactivity and, consequently, impair aspirin inhibition.[95] Thus, erythrocytes can actively contribute as signalling cells to platelet-driven thrombogenesis and microvascular occlusion.[96] Erythrocyte MPs (ErMPs) are rarest in blood and up to now mainly studied in relation to disorders involving red blood cells, such as haemolytic anemias, sickle-cell disease, and thallassemias.[97]

1.3. Risk factors in cardiovascular disease

Many risk factors have been associated with coronary heart disease (CHD) and stroke. The major established risk factors meet three criteria: a high prevalence in many populations; a significant independent impact on the risk of coronary heart disease or stroke; and their treatment and control result in reduced risk. Risk factors for CVD can be classified as modifiable or non-modifiable risk factors (Table 1). Approximately 75% of CVD can be attributed to conventional risk factors, which are deeply involved in the aforementioned blood thrombogenicity. When some of these factors are clustered, especially those related to metabolic or biochemical processes, the risk of heart disease increases enormously.

1.3.1. Major modifiable risk factors

In the developed countries, at least one-third of all CVD is attributable to five major modifiable risk factors, named also classical cardiovascular risk factors: dyslipidemia, diabetes, hypertension, obesity, and smoking. Other modifiable risk factors exist (Table 1), and all of them increase the risk of cardiovascular disease. Together, known modifiable risk factors explain >90% of the occurrence of MI.[98]

CVD risk factors		
Modifiables	Dyslipidemia *	Abnormal lipid levels. ⇒ *CHD, stroke.*
	Hypertension *	High blood pressure. ⇒ *CHD, stroke.*
	Diabetes *	Type-2 diabetes. ⇒ *CHD, stroke.*
	Smoking *	Active or passive smoking, chewing tobacco. ⇒ *CVD.*
	Obesity *	Predisposes to diabetes. ⇒ *CHD.*
	Physical inactivity	⇒ *CHD, stroke.*
	Chronic kidney disease	End-stage renal disease. ⇒ *CHD, CVD mortality.*
	Socioeconomic status	Being poor. ⇒ *CHD, stroke.*
	Mental ill health	Chronically stressful life, social isolation, anxiety, depression. ⇒ *CHD, stroke.*
	Alcohol abuse	>2 alcohol drinks a day. ⇒ *Heart muscle damage.*
	Use of certain medication	Like hormone replacement therapy. ⇒ *CHD.*
	Lipoprotein (a)	Especially in presence of high LDL-c. ⇒ *Heart attacks.*
	LV hypertrophy	⇒ *CVD mortality.*
	Unhealthy diets	A diet high in saturated fat. ⇒ *Heart disease, stroke.*
	Radiation therapy	Long-term close to the heart.
Non-modifiables	Age	Getting old, especially > 55 years ⇒ *CVD, stroke.*
	Gender	Greater for man than pre-menopausal woman. ⇒ *CHD.*
	Family history	1st degree blood relative (<55 years males, <65 years females)
	Ethnicity or race	African or Asian ancestry higher risk than other races. ⇒ *CHD, stroke.*

Table 1. Cardiovascular risk factors classified as modifiable and non-modifiable. * Major cardiovascular risk factors. CHD indicates chronic heart disease; CVD, cardiovascular disease; LDL-c, low-density lipoprotein cholesterol; LV, left ventricular.

1.3.1.1. Dyslipidemia

High blood cholesterol has been considered as one of the most important modifiable risk factors associated with CHD.[99] Cholesterol is an essential component of cell membranes and hormones that is found in all the body's cells and bloodstream. However, an excess of plasma cholesterol leads to its accumulation in the artery wall promoting atherosclerosis. Their levels are maintained through a tightly regulated and complex mechanism that includes *de novo* biosynthesis, internalization of exogenous cholesterol (acquired by animal-derived diet), and efflux of its excessive levels. Cholesterol is carried through the blood by micelle-like particles called lipoproteins (Figure 8). There are three major classes of lipoproteins carrying cholesterol: low-density lipoproteins (LDL), high-density lipoproteins (HDL), and very low-density lipoproteins (VLDL), which typically constitute 60-70%, 20-30%, and 10-15% of the total cholesterol, respectively. While high levels of LDL-cholesterol lead to atherosclerosis, HDL-cholesterol reduces the risk of CVD due to their role in the reverse cholesterol transport. A part from cholesterol, the most common type of fat in the body is triglyceride (TG). High levels of TG combined with high levels of LDL speed up atherosclerosis increasing the risk for ACS. Therefore, abnormal blood lipids such as high total cholesterol, LDL-c and TG levels, and low levels of HDL-c increase risk of CHD and ischemic stroke.

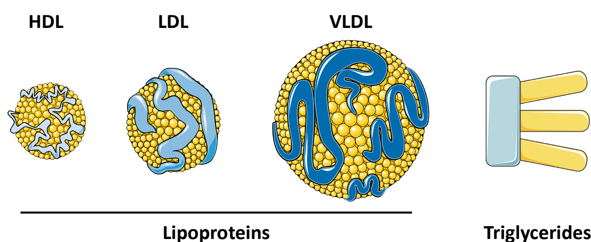

Figure 8. Main types of lipids in the circulation. Major classes of lipoproteins and triglycerides. HDL indicates high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very low-density lipoprotein.

LDL has been considered as the primary target of cholesterol lowering effort and, hence, the cholesterol-lowering drugs statins dramatically reduce heart attacks, CHD deaths, and overall mortality rates.[100] Most widely used clinical recommendation guidelines are from the National Cholesterol Education Program–Adult Treatment Panel III (NCEP/ATPIII), summarised in Table 2. Recently, the American College of Cardiology (ACC) and the AHA, in collaboration with the National Heart, Lung, and Blood Institute (NHLBI), released new guidelines for the prevention of CVD and management of elevated blood cholesterol, which differ in several respects from previous guidelines since they recommend the use of statins more aggressively, using a newly developed risk prediction algorithm based only on high quality randomized clinical trials data.[101]

Lipid parameter	Optimal goal		
	Risk category	**Target**	**Risk factors**
Total cholesterol	General	<190 mg/dL	
	High	<175 mg/dL	
LDL-cholesterol	Low	<160 mg/dL	≤ 1 RF
	Moderate	<130 mg/dL	≥ 2 RF, FRS <10%
	Moderately high	<130 mg/dL	≥ 2 RF, FRS 10-20%
	High	<100 mg/dL	≥ 2 RF, FRS >20%
	Very high	<70 mg/dL	CVD
Non-HDL-cholesterol	Low risk	<190 mg/dL	≤ 1 RF
	Moderate	<160 mg/dL	≥ 2 RF, FRS <10%
	Moderately high	<160 mg/dL	≥ 2 RF, FRS 10-20%
	High	<130 mg/dL	≥ 2 RF, FRS >20%
	Very high	<100 mg/dL	CVD
HDL-cholesterol	Males	>45 mg/dL	
	Females	>55 mg/dL	
Triglycerides	-	<150 mg/dL	
ApoB	Moderate	<90 mg/dL	DM
	High	<80 mg/dL	CVD / DM + ≥1 RF

Table 2. Clinical lipid management guidelines based on lipid targets. Clinical guidelines from NCEP Adult Treatment Panel III Guidelines (ATP III) 2004. Apo B indicates apoliprotein B; CVD, cardiovascular disease; DM, diabetes mellitus; FRS, Framingham Risk Score; RF, risk factor.

Types of dyslipidemias

There are many inherited conditions where plasma lipids are abnormal and CHD risk is altered: familial hypercholesterolemia, familial combined hyperlipidemia, and familial high-density lipoprotein deficiency syndromes. The most common of these diseases is:

Familial hypercholesterolemia (FH)

FH is a very frequently autosomal dominant inherited disorder that affects 1 in 400-500 subjects in the general population.[102] This common monogenic hereditary condition is mainly caused by mutations in the LDL receptor gene, leading to increased LDL levels in plasma, early development of atherosclerosis and premature CVD. Life expectancy is shortened and fatal coronary events are the principal causes of death.[103,104] Because of their high CHD risk, patients with FH are usually treated with statins at a young age in conjunction with lifestyle. Despite the use of lipid-lowering therapies, high number of patients remain at risk for CVD,[105] as seen in FH patients with presence of aortic plaque burden detected by magnetic resonance imaging (MRI).[106] However, CVD event presentation in affected subjects varies considerably across cohorts and individuals, suggesting that other factors contribute to the atherosclerotic burden in these patients.[107]

1.3.1.2. Other major atherosclerotic risk factors

Diabetes mellitus (DM), a dysfunction in glucose metabolism, is a major risk for CHD and stroke. Patients with diabetes, especially those with poorly controlled diabetes, have increased blood thrombenicity.[26] Platelets from diabetic patients have increased reactivity and hyper-aggregability and expose a variety of activation-dependent adhesion proteins,[108] leading to increased accumulation of platelet on the altered vessel wall.[109] Haemoglobin A1c, a useful indicator of diabetes that reflects average blood glucose levels, has been shown to be a predictor of CV events. As such, major guidelines recommend classifying diabetes as a CHD equivalent.

Hypertension is a major risk for heart attack and the most important risk factor for stroke. Arterial hypertension is the main source of combined mortality and morbidity, followed by obesity, hyperglycemia, hypercholesterolemia, and physical inactivity.[110] The association of high blood pressure levels with cardiovascular events seems to be continuous.

Obesity, defined by a body mass index of 30 kg/m^2 or greater, constitutes a major risk for coronary heart disease and diabetes, together with overweight.

Cigarette smoking. Tobacco use increases catecholamine release, potentiating platelet activation[111] and increasing fibrinogen levels.[112]

1.4. Biomarkers in cardiovascular disease

In primary cardiovascular risk prevention, the existence of established clinical risk assessment models such as Framingham risk score means that novel biomarkers should provide incremental power to existing algorithms. Beyond the more conventional and generally accepted biomarkers, new candidates have been proposed for atherosclerosis and its atherothrombotic complications during the last years, as shown in Table 3. However, up to now it is unclear whether these new biomarkers are useful predictors of future CV events. Thus, it remains essential to continue to explore new biomarkers with greater discriminatory power for the distinct types of CVD.

An ideal biomarker should display the following characteristics: (a) aid clinician in the diagnosis, prognosis and treatment of pathogenic processes, with accuracy –ability to identify individuals at risk–, reliability –stability of results when repeated–, and known specificity and sensitivity; (b) be readily available and adequately tested and have established reference value compared to a 'gold standard'; (c) have a rapid turnaround time and not be costly; and (d) have therapeutic impact with early intervention.

1.4.1. Inflammation biomarkers

Since inflammation is primary involved in the development and progression of atherosclerosis, its detection constitutes a potential indicator of atherosclerosis. Principal biomarkers of systemic inflammation in evaluation of increased risk of CAD and potential ACS are *interleukins* (such as IL-6,[113] IL-18[114,115] and IL-10[116]) and *C-reactive protein* (CRP), which is used as a marker of worst outcomes and mortality in ACS.[117] The main drawback of CRP is the lack of specificity as it is elevated in many disease states. Thus, it is better to use it as prognostic marker once diagnosis has been established. Besides, there are leukocyte-derived enzymes linked with the presence of coronary disease and stroke like *lipoprotein-associated phospholipase A2*[118] and *myeloperoxidase* that may have implications for early atherosclerosis and risk assessment.[119] Additionally, plaque-derived biomarkers may be useful for predicting the risk of new vascular complications such as *osteopontin*[120] and *matrix metalloproteinases* (MMP-9, MMP-11),[121] but until now they have only been proved useful for aortic dissection. Other inflammatory biomarkers include *oxidized LDL* that highly correlated with CV events[122] and acute-phase proteins *serum amyloid A*, *pentraxin 3* and *growth differentiation factor 15*.[123]

1.4.2. Biomarkers of blood vulnerability

Key elements in the pathophysiology of ACS from atherogenesis to plaque destabilization and thrombus formation may have potential for detection of disease and risk stratification. Nevertheless, further studies are needed to validate the preliminary evidence of these potential biomarkers, which include *growth factors*,[124] *adhesion molecules*,[125] *soluble CD40 ligand*,[126] *von Willebrand Factor*,[127] *d-dimer*[128] and *fibrinogen*,[129] and platelet markers, such as *platelet count*,[130] *mean platelet volume*,[131] and *reticulated platelet fraction*.[132]

1.4.3. Biomarkers of ischemia

Until very recently, only *heart-type free-fatty acid binding protein* and *ischemia-modified albumin* had been studied without much success. However, a new molecule has showed promising results. *Apoliprotein J*, an HDL-related glycoprotein with anti-inflammatory properties, has recently shown to be post-translationally modified by glycosilation after MI ischemic process.[133]

1.4.4. Biomarkers of neuroendocrine activation and left ventricular function

Brain natriuretic peptide[134] and *mid regional-proadenomedullin*,[135] may represent clinically useful markers of heart failure and prognosis after MI, respectively. *Troponins*, structural and regulatory proteins specific of skeletal and cardiac muscle cells, form a complex of three subunits termed troponin C, I, and T. Cardiac troponin T (cTnT) and I (cTnI) are the subunits usually assayed for ACS diagnosis.[136] Their detection indicates myocardial cell necrosis,[137] being superior compared other biomarkers of myocardial necrosis, such as *creative kinase muscle and brain*, *creative phosphokinase*, *aspartate transaminase* and *myoglobin*.[138] Unfortunately and despite high sensitivity cTn assays,[139] cTns are not specific markers of ACS[140] and they cannot detect MI in the early hours after symptom onset, due to the delayed increase of circulating levels.[141] Finally, *copeptin* or *C-terminal pro-vasopressin* is a potential diagnostic and prognostic marker for heart failure[142] and for ACS[143] due to its stability and pattern of rapid secretion; however, it has failed to distinguish patients with UA from non-ischemic patients.[144] Since accurate ACS diagnosis is not achieved by current strategies, useful prognostic markers are needed.

1.4.5. Others biomarkers

Other considered markers for CVD are related to family history and genetic biomarkers such as *single nucleotide polymorphisms*. Finally, in the last decade imaging and hemodynamic biomarkers have emerged as a non-invasive biomarker alternative. Among them, the most important are *aortic and carotid plaques* by MRI, *myocardial perfusion* imaging, *CAC score* measured by computed tomography, *carotid intima-media thickness* determined by ultrasound, *ankle-brachial index*, *flow-mediated dilatation* by functional flow reserve evaluation, *pulse wave velocity*, and others with coronary angiography, cardiac radionuclide perfusion imaging and positron emission tomography scanning in addition to the well-known electrocardiogram and echocardiogram.

In view of the ideal biomarker criteria, many characteristics argue for the potential of circulating microparticles and microRNAs (miRNAs), which are properly discussed in the next sections, as a promising source of new biomarkers in CVD. cMPs are indeed identifiable, isolatable, non-invasive and disease-specific. Since their purification results in biomarkers enrichment relative to total secretome, small changes in their content can be detected. Similarly, miRNAs may be potential circulating biomarkers for diagnosis or prognosis of various human diseases including CVD,[145] due to their time-course expression, stability in circulation and post-isolation, and that can be specifically detected at very low levels. Either cMPs or miRNAs could selectively mark different stages of atherothrombotic disease.

CVD BIOMARKERS	Associated disease
Endothelial dysfunction	
Adhesion molecules (ICAM-1, VCAM-1)[125]	CAD
E-selectin[125]	ACS
Atherogenesis	
Matrix metalloproteinases (MMP-3, MMP-11)[121]	ACS
Pregnancy-associated plasma protein A (PAPP-A)	ACS
Cathepsin S or K	CAD
Free-fatty acids (FFA) / Pentraxin 3 (PTX3)[123]	CAD
Growth factors[124]	ACS, CAD
Lipoproteins (TC, LDL, HDL, VLDL, Lp(a))[122]	CAD
Apoliproteins (ApoAI, ApoB, ApoE, ApoJ)	ACS, CAD, MI
Paraoxonase-1 / Follistatin / Osteopontin (OPN)[120]	ACS
Plasminogen activator inhibitor 1 (PAI-1)	ACS
Inflammation	
High sensitivity C-reactive protein (hs-CRP)[117]	ACS
Chemotactic molecules	CAD, ACS
Homocysteine / Cystatin C	ACS
Myeloperoxidase (MPO)[119] / Neopterin	ACS, CAD, CHF
Interleukins (IL-1β, IL-1Ra, IL-6, IL-10; IL-18)[113-116]	ACS, CAD, CHF
Myeloid-related proteins (MRP-8, MRP-14)	ACS
Growth differentiation factor 15 (GDF-15)[123]	ACS, CAD
Lp-associated phospholipase A_2 (Lp-PLA_2)[118]	ACS, CAD
Galectin-3 / Serum amyloid A[123]	CHF
Osteoprogenterin (OPG) / Fetuin-A	CAD
Thrombosis	
Von Willebrand Factor (vWF)[127]	ACS, CAD
Fibrinogen (Fbn)[129] / Soluble CD40 ligand (sCD40L)[126]	ACS
Prothrombin fragment 1.2 / Fibrinopeptide A	ACS, CAD
D-dimer[128]	ACS, PE
Neurohormonal activation	
Prohormone brain natriuretic peptide (proBNP)[134]	CHF
Mid regional proadenomedullin (MR-proADM)[135]	CHF, MI
Copeptin[143]	ACS, CHF
Ischemia / Necrosis	
Ischemia-modified albumin (IMA)	CAD
Heart type-fatty acid binding protein (H-FABP)	ACS
Cardiac troponins (cTnT, cTnI)[136]	CAD, CHF, MI
Myoglobin[138]	ACS

Table 3. Main known and novel CVD biomarkers. Biomarkers are classified based on their pathophysiological implication. ACS indicates acute coronary syndrome; CAD, coronary artery disease; CHF, chronic heart failure; ICAM-1, intercellular adhesion molecule 1; MI, myocardial infarction; PE, pulmonary embolism; VCAM-1, vascular cell adhesion molecule-1.

1.5. Prevention and treatment

1.5.1. Non-pharmacological interventions

Non-pharmacological interventions lie in overcoming and control CVD risk factors by lifestyle modifications, such as exercise and dietary interventions, and bariatric surgery for obesity. In primary prevention, a recent clinical trial showed the benefit of the adherence to the Mediterranean diet in patients at high cardiovascular risk in relation to the incidence of severe cardiovascular events.[146] Higher impact in reducing the risk of CVD is achieved by implementing policies that affect population at large, such as reductions in sodium in the food supply, smoke-free legislation, or develop walking / biking trails that promote low-volume physical exercise.

1.5.2. Pharmacological interventions

Since hypercholesterolemia is considered the primary risk for CVD, among pharmacological prevention of CVD, effective lipid management stands out as a key strategy to control vascular risk and reduce morbidity and mortality among patients with cardiovascular risk. Thus, lipid-lowering therapy (LLT) can be achieved with the use of different agents:

- Statins, 3-hydroxy-3methylglutaryl-coenzyme A (HMG-CoA) inhibitors, are the drug of choice for controlling lipid levels (Figure 9). Statins have been proven effectively in both primary and secondary prevention of CVD events for subjects at moderate to high risk or for low-risk individuals whose LDL-c levels are very high.[147] Currently, different classes of statins are clinically used. Atorvastatin, pravastatin, and simvastatin have been used for both primary and secondary prevention in a wide range of ages and cardiovascular risk factors. In contrast, rosuvastatin is especially indicated for primary prevention in high risk populations. Other available statins are fluvastatin, lovastatin, and the most-recently approved, pitavastatin.

  Importantly, beyond lipid-lowering effects, statins have additional beneficial effects. Statins are able to exert many pleiotropic functions, which involve anti-atherogenic, anti-inflammatory and anti-thrombotic properties.[148,149] Interestingly, they have shown to reduce atherosclerotic plaque progression rate, plaque regression and stabilization, and reduction of myocardial ischemia/reperfusion injury. Statins reduce thrombosis by inhibiting platelet activation and reducing pathologic expression of the procoagulant protein TF. Many of the pleiotropic effects are likely attributed to the inhibition of G protein prenylation, as

shown in Figure 9, and effects on other signalling molecules. Specifically, the Rho family of GTPases including RhoA, is an extremely important class of signalling molecules that need prenylation to function properly.

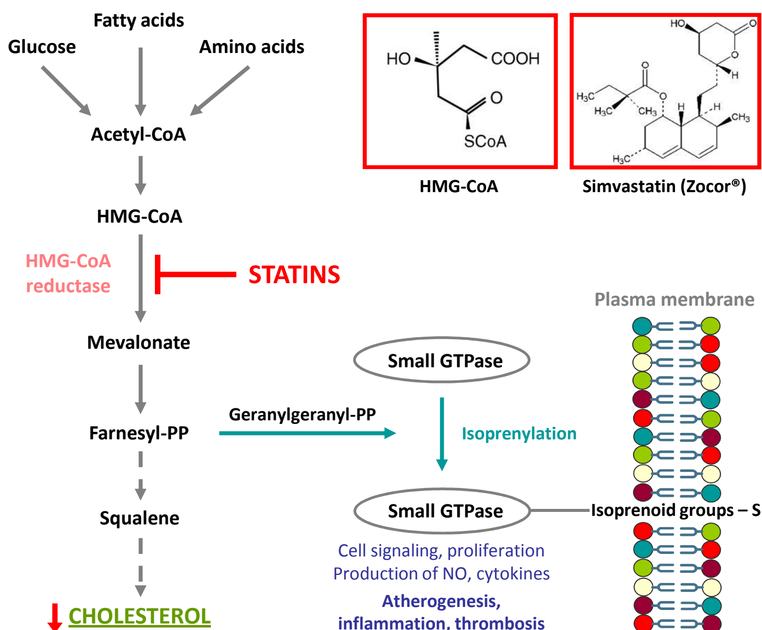

Figure 9. Mechanism of action of statin-mediated reduction of cholesterol and pleiotropic effects. Statins inhibit the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA), which initiates the first step in cholesterol synthesis. A plausible mechanism for the lipid-independent effects of statins is that they also reduce levels of farnesyl pyrophosphate (FPP), which is required for protein prenylation that is essential for binding of signalling proteins to the cell membrane.

Despite being first-line drug therapy for LDL-lowering, for patients in whom statin monotherapy does not reduce LDL-c concentrations to recommended levels, combination therapy with other lipid-regulating agents can further reduce the levels of LDL-c and other lipid fractions.

- Ezetimibe acts at the intestinal level, by selectively inhibiting cholesterol absorption. Combined therapy with statins reduces both intestinal and hepatic sources of cholesterol.
- Fibrates reduce plasma triglycerides and increase HDL-c levels, which is very convenient for mixed dyslipidemia when combined with statins.
- Bile acid sequestrants act by decreasing intrahepatic cholesterol and upregulating LDL receptor activity, thereby increasing lipoprotein removal from circulation. With long-term use, its efficacy is reduced.

- Nicotinic acid (niacin) has been traditionally used to lower LDL-c and TG levels and, until very recent, it is known that also raises HDL-c. Its main drawbacks are high dose requirements and poor compliance in clinical practice. It was used mainly in United States of America.

Investigational agents that reduce LDL-c by different mechanisms than conventional ones might provide additional strategies for LDL-c reduction. These include: (a) inhibition of proprotein convertase subtilisin/kexin type 9, which decreases the degradation of hepatocyte LDL receptors (phase-2 studies); (b) HDL-related therapies, which have recently emerged and are still quite controversial, like torcetrapib or dalcetrapib (cholesteryl ester transfer protein inhibitor); and (c) others such as enhancement of cholesterol efflux, antisense oligonucleotides to apoB, microsomal triglyceride transfer protein inhibitors, or thyromimetics.

In the clinical setting of acute CVD presentation, the vast majority of pharmacologic agents have been designed to specifically target one or more factors within the coagulation cascade. These agents are effective in reducing the risk on thrombotic complications in various conditions; however, they also increase the risk of bleeding complications, as they affect both thrombosis and haemostasis. These interventional drugs can be classified as anti-platelet therapies such as aspirin, ADP antagonists (ticlopidine, clopidrogrel, prasugrel, ticagrelor, cangrelor and elinogrel), GPIIb/IIIa antagonists (abciximab), phosphodiestarase inhibitors, PAR-1 antagonists (vorapaxar), and other novel investigational approaches (GPIV antagonists, $\alpha_2\beta_1$-integrin antagonist, seratonin receptor antagonist, NO-releasing variant of aspirin, antagonists of P-selectin and PSGL-1, GPIb antagonists, TXA_2 receptor antagonists, antagonism of the prostaglandin (PG) E receptor 3 for PGE_2 and antagonism of the β isoform of phosphatidylinositol 3-kinase (PI3K).[150] Additionally, anticoagulants (unfractionated heparin, bivalirudin, otamixaban, warfarin) and novel oral anticoagulants (rivaroxaban, dabigatran, apixaban, betrixaban, darexaban) are used in certain conditions. Also β-blockers, angiotensin converting enzyme inhibitors, calcium channel blockers, and diuretics are used as needed. Finally, novel oral agents are under study such as monoclonal antibodies, stem cell therapy and microparticle-based delivery systems (further expanded in the next section).

Besides pharmacological therapy, intervention strategy for ACS patients includes reperfusion therapy either with fibrinolysis or primary percutaneous coronary intervention (pPCI). Standard guidelines recommend a door-to-balloon time from first medical contact to pPCI of less than 90 minutes for patients presenting with ST elevation MI (STEMI). Finally, intra-aortic balloon pump counterpulsation is used to support high-risk PCI, especially after PCI in the clinical setting of cardiogenic shock (fatal MI complication).

2. Cell-derived microparticles

2.1. Background

2.1.1. Clinical significance

Microparticles (MPs) are vesicles that bud off from cells, lack a nucleus, contain a membrane skeleton and are defined by their size and expression on their surface of antigens specific of parental cells.[151] These membrane fragments are shed by cells activated by a variety of stimuli and are found in circulating blood at relative concentrations determined by the pathophysiological context.[152] Indeed, MP levels show gender-specific differences[153,154] and changes are observed with age,[155] during pregnancy,[156] after exercise[157-159] and after a high-fat meal.[160] Levels of circulating microparticles have been shown to correlate with disease progression and severity.[161] Overproduction of MPs have been related to various physiological and pathophysiological conditions such as diabetes, cardiovascular diseases, cancer, infections, inflammatory disorders as well as normal and pathological pregnancy,[161] indicating that they may play roles in a variety of processes such as cell adhesion, apoptosis, immune response, vascular function, vascular remodelling and angiogenesis, and haemostasis and thrombosis.

2.1.1.1. Microparticles in cardiovascular pathophysiology

Although cMPs are released in health under basal conditions,[92] various clinical disorders have been associated with increased numbers of MPs. Among them, cardiovascular diseases are of special interest. Indeed, MPs likely play a significant role in CVD and CV risk factors. Studies have shown increased cMP levels in patients with diabetes mellitus and hypertension.[51,162] Elevated microparticle levels have been correlated with a higher calculated 10-year Framingham CAD risk.[163] Increases in microparticle levels have also been correlated to the degree of CAD –myocardial infarction>unstable angina>stable angina–[10,164] and to clinical parameters, such as coronary artery endothelial function in CAD patients.[50] Not only the number of MPs but also the type of MP, based on their cell origin and activity, has been found altered. Under steady-state conditions, MPs carrying platelet or megakaryocyte-derived surface markers are the most common constituting ~70 to 90% of all MPs in circulation.[165] However, this proportion may change in different disease situations. Table 4 summarises the state of the art knowledge about specific changes in the levels of cMPs in distinct pathological conditions, related to thrombosis and vascular dysfunction. Thus, MPs might be promising markers of CVD, despite the specificity of individual MP populations for specific disease states remains unclear.

Clinical condition	MP subtype change	References
CARDIOVASCULAR DISEASE		
Cardiovascular risk factors		
Type-2 diabetes mellitus	↑ eMP, ↑ pMP, ↑ LMP	166-168
Severe hypertension	↑ pMP, ↑ eMP, ↑ LMP	51,169
Metabolic syndrome	↑ eMP, ↑ ErMP, ↑ pMP, ↑ LMP	170-173
Obesity	↑ pMP	172,174
Hypertriglyceridemia	↑ eMP	175
Smoking	↑ eMP	172,176
Endothelial dysfunction (ED)		
Acute endothelial dysfunction	↑ eMPs	176
Chronic renal failure with ED	↑ eMP	52
Atherosclerosis		
Subclinical atherosclerosis	↑ LMP	177
Coronary calcification	↑ eMP, ↑ pMP	178
Acute coronary syndrome	↑ eMP, ↑ pMP	10,49,164,179,180
Stable coronary artery disease	↑ eMPs	164
Coronary heart disease	↑ pMP	163,181
Peripheral artery disease	↑ pMP	155,182
Heart failure & vascular disease		
Congestive heart failure	↑ eMP	181,183
Cyanotic congenital heart disease	↑ pMP	184
Cardiopulmonary bypass	↑ PS^+-MP	185
Coronary stents	↑ pMP	186
Heart transplantation	↑ eMP	187
Heart transplant rejection	↑ eMP	188
Severe aortic stenosis	↑ eMP, ↑ LMP	189
Pulmonary hypertension	↑ eMP, ↑ pMP, ↑ LMP	48
Acute pulmonary embolism	↑ pMP	190
Valvular atrial fibrillation	↑ pMP	191
Deep vein thrombosis and venous thromboembolism	↑ eMP, ↑ pMP, ↑ LMP	192,193
Buerger's disease	↑ pMP	194
CEREBROVASCULAR DISEASE		
Acute ischemic stroke	↑ pMP, ↑ eMPs	195-197
Cerebral vasospasm	↑ eMP, ↑ pMP, ↑ LMP, ↑ ErMP	198
Lacunar infarcts	↑ pMP	195
Multi-infarct dementia	↑ pMP	195
Carotid atherosclerosis	↑ pMP	199
Cerebrovascular atherosclerosis	↑ eMPs	200

Table 4. Clinical conditions associated to circulating microparticles. Main changes in MPs among clinical pathologies. ↑ indicates increase, –, no change.

Clinical condition	MP subtype change	References
HEMATOLOGIC DISEASES		
Immune thrombocytopenia purpura	↑ pMP	201
Thrombotic thrombocytopenia purpura	↑ eMP, ↑ pMP	202,203
Paroxysmal nocturnal hemoglobinuria	↑ eMP, ↑ pMP	28,204
Heparin-induced thrombocytopenia	↑ pMP	205,206
Castaman syndrome	↑ pMP	207,208
INFECTIOUS DISEASES		
Sepsis	↑ eMP, ↑ gMP, ↑ pMP	209-212
Prion disease	↑ pMP	213
HIV-1 infection	↑ ℓMP	214
AUTOIMMUNE DISEASES		
Systemic lupus erythematosus	↑ eMP, ↑ pMP	215,216
Antiphospholipid syndrome	↑ eMP, ↑ pMP	217,218
Systemic sclerosis	↑ eMP, ↑ pMP, ↑ LMP	219,220
Rheumatoid arthritis	↑ pMP	216,221,222
Acute and systemic vasculitis	↑ eMP, ↑ pMP, ↑ LMP	223-225
Type-1 diabetes mellitus	↑ eMP, ↑ pMP	226
Multiple sclerosis	↑ eMP, ↑ pMP	227,228
Sjögren syndrome	↑ pMP	216
CANCER		
Gastric cancer	↑ pMP	229
Lung cancer	↑ PS^+-MP	230
Breast cancer	↑ pMP	231,232
Prostate cancer	↑ PS^+-MP	233,234
OTHER		
Obstructive sleep apnea	↑ eMP, ↑ pMP, ↑ LMP	235,236
Renal disease	↑ eMP, ↑ pMP	237,238
Decompression sickness	↑ pMP	239
Preeclampsia	↑ pMP, ↑ ℓMP, ↑ LMP, ↑ eMP	240-243
Spontaneous abortion	↑ pMP	244
Alzheimer disease	– pMP	245
Uremia	↑ pMP	246
Chron's disease	↑ PS^+-MP	247

Table 4 (*continued*).

2.1.2. Definition and nomenclature

Microparticles are defined as phospholipid microvesicles (MVs) containing certain membrane receptors as well as other protein and molecular components inherent to their parental cells.[248] To reliably define MPs, the terms microparticles, exosomes and apoptotic bodies need to be introduced. Cells release two heterogeneous pool of vesicles which include plasma membrane-derived microparticles and multivesicular body-derived exosomes.[249] Both vesicle types are generated upon cell activation and their distinction is complex due to an overlap in their molecular properties and sizes. Contrarily, apoptotic bodies are remnants of dead cells in the process of their shrinkage and elimination. Other designations of microparticles in the literature include microvesicles, ectosomes or exovesicles.

Microparticles, which directly originate from the membrane surface, are generally referred to be 100 to 1000 nm. It should be noted that MPs, with densities between 1.04-1.07 g/mL, are of irregular shape and very heterogeneous in size.[250] The minimal size of MPs was defined as 100 nm because commonly used flow cytometers are unable to distinguish between smaller particles and the electronic noise. The upper size of MPs was fixed just at 1 μm because a single bigger MP might be difficult to distinguish from MPs aggregates, platelets, or MPs-platelet aggregates.[165] In contrast, *exosomes* (20 to 100 nm) are cup-shaped vesicles released from multivesicular bodies and exocytosis of endocytic bodies, [251] with a density of 1.10-1.18 g/mL. Exosomes were first described in platelets[252] and in general form a more homogenous population than MPs, both by size and molecular content.[249] *Apoptotic bodies* tend to be larger than MPs, may contain different internal components (e.g. deoxyribonucleic acid [DNA], organelles) and do not present prothrombotic activity despite displaying phosphatidylserine.[253,254] They present a density of 1.24-1.28 g/mL and their role is still not clear. Apoptotic bodies might be an easier system for cellular clearance themselves due to small size or, alternatively, an active signal to promote cellular clearance of the remaining damaged cells.[255] Figure 10 depicts the size ranges of the different shedding vesicles.

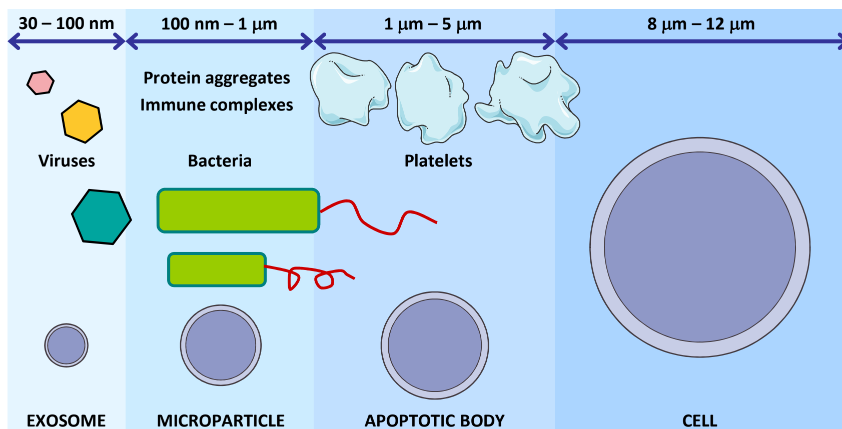

Figure 10. Size ranges of extracellular-released membrane vesicles. Schematic representations of major populations include exosomes, microparticles and apoptotic bodies. Exosomes share size range with viruses, while MPs overlap with bacteria and protein aggregates. Adapted from Williams et al[256] and Gyorgy et al.[257]

2.2. Modulation

2.2.1. Microparticle release

It is now widely accepted that MPs (and probably exosomes) are generated by all eukaryotic cells, including cells in the vasculature.[258] Therefore, MPs can originate from platelets, endothelial cells, leukocytes, monocytes, lymphocytes, granulocytes, erythrocytes (red blood cells) and other cell types (smooth muscle cells, cardiomyocytes).[254] Besides such vascular cellular source, epithelial cells and tumour cells are also capable of producing MPs.[259] Moreover, all extracellular body fluids contain microvesicles: plasma,[260] saliva,[261] urine,[262] breast milk,[260] bile,[263] tears, semen,[264] nasal mucus or secretions,[265] amniotic fluid,[266] cerebrospinal fluid,[267] bronchoalveolar lavage,[268] synovial fluid,[269] ascytes,[270] ocular effluent and aqueous humour,[271] and pleural effusions.[272]

Microparticle release is an active and highly controlled energy-dependent process.[250] Therefore, cells release MPs in response to a variety of stimuli, via (a) cell activation through multiple agonist-dependent signalling pathways,[161] (b) apoptotic cell blebbing or senescence[152,273] or, finally, (c) mechanic forces due to certain blood flow conditions such as high shear stress.[84] It is now well known that a number of biologic stress conditions (e.g., oxygen radicals [hydrogen peroxide], ultraviolet, serum deprivation, inflammatory mediators, ischemia) causing cell injury, cell differentiation, cell exposure to ATP and cell malignant transformation may also stimulate and activate vascular and

blood cells.[152,274-276] In platelets, MP release can be induced by many specific agonists. While most non-physiological agonists like calcium (Ca^{2+})-ionophore are the most potent inducers of microvesiculation, the order of potency of physiologic agonists is C5b-9 membrane attack complex > thrombin plus collagen > thrombin > collagen > adenosine diphosphate > epinephrine.[250,277] Table 5 summarizes a list of stimuli that have been described to promote MP formation from blood and vascular cells. It should be noted that many stimuli can be additive or even synergistic. In each one of these stimulus, signalling starts from the specific receptor(s), followed by the engagement of different combination of characteristic events universal for microvesiculation: calcium entry, cytoskeletal remodelling, calpain/caspase activity, plasma membrane blebbing and shedding of membrane fragments known as microparticles.[278,279] It is noteworthy that these MP formation-related events are regulated by distinct pathways depending on whether they are induced by agonists or apoptosis, which is a crucial factor for the determination of the properties of different MP species.[273]

2.2.2. Microparticle clearance

The mechanism of MP clearance from the circulation is not entirely known. Thus, platelets have a life span of about 10 days, contrasting with that of pMPs of which is about 30 minutes in mice,[280] or even less than 10 minutes in rabbits.[281] Recently, apheresis-derived pMPs were shown to circulate for more than 5 hours.[282] Discrepancy in the MP turnover may account for distinct 'eat-me' signals due to secretion process.[283,284]

MPs could be cleared from the circulating blood by phagocytosis, due to: (1) direct mechanisms such as PS exposure;[285] or (2) indirect mechanisms such as opsonization by proteins like growth arrest-specific gene 6 product, protein S, thrombospondin, complement and IgM[286-290] through, for instance, discriminating glycosilation patterns.[291] PS exposure is potentially important as interacts with several receptors on macrophages, resulting in phagocytosis of T-cell MPs[292] or removal of apoptotic cells.[293] Therefore, PS-negative MPs may evade this fate, and could serve as a distinct long-lasting 'surveillance reservoir' of MPs.[294-296] Endogenous lactadherin may also be an important 'tag', binding to PS on MPs and marking them for clearance.[283]

Alternatively, MPs may be cleared by the activity of circulating phospholipases.[297] Finally, MPs must be endocytosed by cells. In support of this, developmental endothelial locus-1, an extracellular matrix protein expressed by ECs, has been implicated in the process of MP uptake,[298] functioning as a bridging molecule between integrins and PS.

Cell type	Stimulus		Reference
Platelet	Proinflammatory	LPS, shiga toxin	299
		Soluble CD40 ligand	300
		Cytokines	301
	Procoagulant	Adenosine diphosphate	302
		Thrombin	303
		Collagen	304
		PAR agonists	305
		TRAP	306
	Other	Shear stress	307
		Epinephrine / noradrenaline	306
		Calcium ionophore	301
		Complement C5b-9	308
		Prolyl gallate	309
		Storage	310
		Dibucaine	311
Endothelial cell	Proinflammatory	Tumour Necrosis Factor α	312,313
		LPS	314
		Interleukin-1α	315
		C-reactive protein	316,317
	Procoagulant	Thrombin	318
		PAI1	313,319
		Activated protein C	320
	Uraemic toxins	p-cresyl sulphate	321
		Indoxyl sulphate	237
		Homocysteine	322
	Other	High glucose	303
		Angiotensin II	323
		Calcium ionophore	324
		Complement	325
		Camptothecin	326
		Growth factor deprivation	254
		Reactive oxygen species	327
		Shear stress	328
Leukocyte	Proinflammatory	Tumour Necrosis Factor α	70,329
		TLR-3 and TLR-4	330
		LPS, bacterial infection	331,332
	Other	Phytohemagglutinin	333
		Fas ligand	334,335
		Etoposide	303
		Staurosporin	276
		Actinomycin D	333,336
		Calcium ionophore	337
		Endotoxin	338
Erythrocyte	Other	Calcium ionophore	28
		Acid pH (5.4)	28
		Reactive oxygen species	339
		Diamide	340

Table 5. Main stimuli for MP formation depending on cell type. LPS indicates lipopolysaccharide; PAR, protease activated receptor; PAI-1, plasminogen activator inhibitor; TRAP, thrombin receptor activating peptide; TLR, toll-like receptor.

2.3. Microvesicle formation

Knowing the mechanisms of MP formation is essential for comprehending the MP pathophysiological implication in disease. The current knowledge on MP release is mainly obtained from *in vitro* experiments performed on isolated or cultured cells. However, the molecular basis of microparticle formation *in vivo* is not fully understood.

Microparticles and exosomes have normally distinct formation processes (Figure 11). In platelets, this differentiation is jumbled because of alpha-granules. Thus, multivesicular bodies, the source of exosomes, are also considered to be pre-stages of α-granules,[341] which may then liberate exosomes on fusion with the plasma membrane. However, several α-granule-derived molecules are also present on platelet-derived microparticles (pMPs). Moreover, the common exosomal marker tetraspanin (CD63) is not only enriched in the platelet-derived exosomes, but it is also present on pMPs[155,342] and *vice versa*, many common pMP proteins are detected on subsets of platelet exosomes.[249] Finally, apoptotic bodies are formed under less-controlled situations. Apoptotic bodies are originated from necrotic cells upon loss of membrane integrity and karyorrhexis (nuclear fragmentation), from mechanical destruction of cells following injury (cell shrinkage or collapse) or exclusively during the late stages of apoptosis.[343]

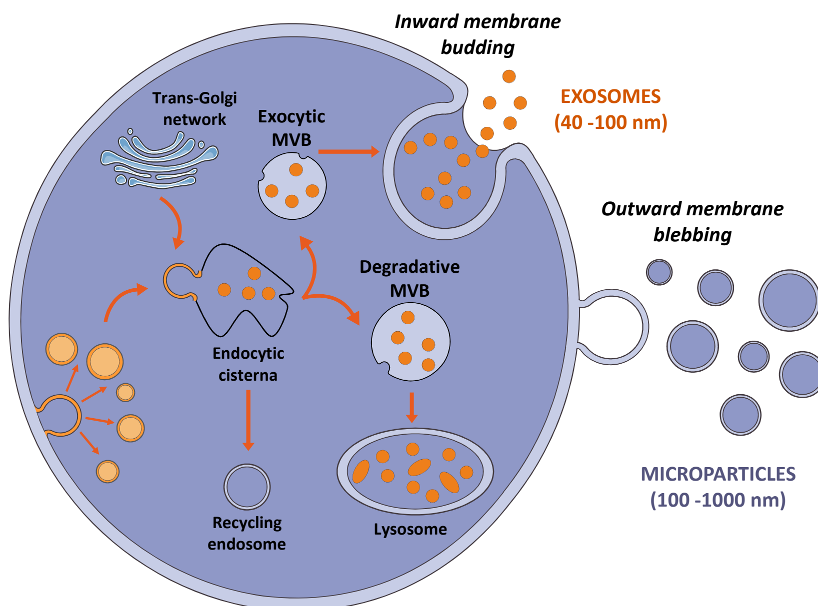

Figure 11. Microvesicle biogenesis. MPs bud directly off the plasma membrane whereas exosomes formed in early endosomes are released by fusion with plasma membrane. Arrows represent direction of transport between organelles and plasma membrane. Adapted from Cocucci et al [344]and Raposo et al.[345]

2.3.1. Microparticle formation

Microparticles emanate from cells through the outward blebbing of their plasma membranes (*ectocytosis)* by successive mechanisms of a complex nature that implicate various membrane (lipid transporters, receptors, and calcium channels) and cytoplasmatic (cytoskeleton, calpains) actors (Figure 12).[346]

1. Intracellular calcium release. With cell activation, the opening of endoplasmic reticulum and plasmatic membrane Ca^{2+} channels allows a cation influx to the cytosol. Elevation of intracellular calcium induces the loss of phospholipids asymmetry between the inner and the outer leaflets of quiescent cells that is maintained by the concerted activity of lipid transporter proteins.[347] In addition, calcium may interact with and activate specific proteins involved in MP formation, such as calpain[348] and caspases.[349]

2. Loss of phospholipid membrane asymmetry. The transverse migration of anionic phospholipids such as PS from the inner layer to the outer layer of the plasma membrane constitutes a key step of MP bebbling.[350] Under normal physiological conditions, most eukaryotic cells have an asymmetric composition and distribution of plasma membrane phospholipids, which is maintained by three enzymes: *flippase*, *floppase* and *scramblase*. The cooperative action of *flippase* and *floppase* controls membrane asymmetry in resting cells. Upon cell stimulation, which leads to the increase of intracellular Ca^{2+}, *flippase* is inhibited, but *scramblase* is activated.[351] *Scramblases* allow the phospholipids to move randomly between both leaflets. This bidirectional movement can lead within minutes to the collapse of lipid asymmetry. Several other enzymes are involved in plasma membrane remodelling, such as protein disulfide isomerase, which modulates *flippase* and *floppase* activities[352] and, acid sphingomyelinase, necessary for MP formation in glial cells.[353]

3. Cytoskeleton reorganization. Concomitant with plasma membrane remodelling, contacts between aminophospholipids and proteins of the cytoskeleton are then disrupted. Modification of the cell architecture with the disruption of cytoskeleton organization plays a key role in MP release. It has been shown that, in platelets, an actin depolymeriser, cytochalasin D, inhibited MP release from activated platelets.[354]

4. Microparticle bebbling. The movements between cytoskeletal proteins and their cleavage significantly affect the cell shape and plasma membrane mechanical stability, which tension and cause detachment of the cortical actin cytoskeleton, with the formation of blebs. During membrane budding, the cell membrane forms cytoplasmic protrusions, which can detach from the cell by fission of their membrane stalk with

the subsequent release of MPs enriched in phosphatidylethanolamine (PE) and PS exposed on their outer surface.[344]

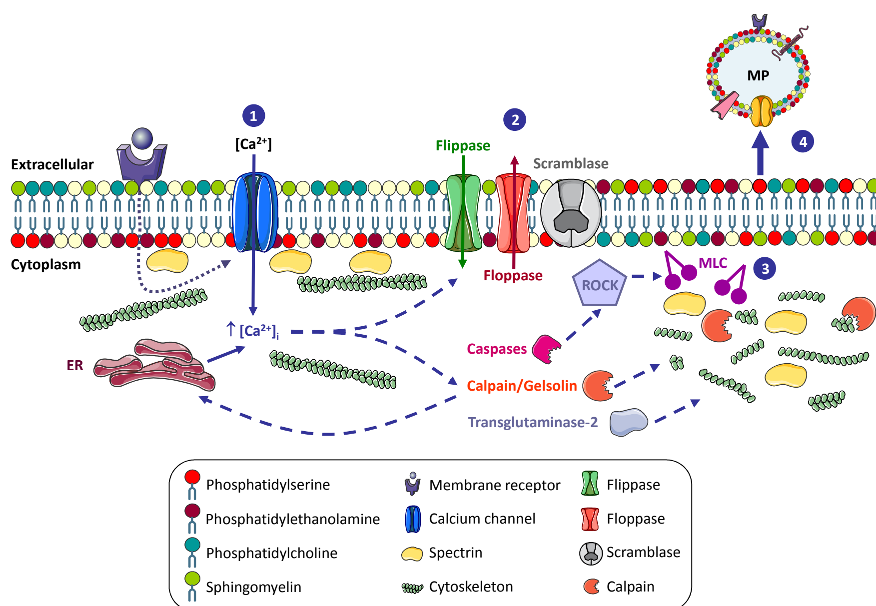

Figure 12. Microparticle formation. The mechanisms governing MP shedding begin with the activation of signalling systems that inducing **(1)** an increase in intracellular calcium, which activates flip-flop system causing **(2)** plasma membrane phospholipid redistribution; in parallel, there is a **(3)** cytoskeleton reorganization, by which skeletal proteins are cleaved resulting in **(4)** MP blebbing. MLC indicates myosin light chain; ROCK, Rho-associated coiled-coil-containing protein kinase.

2.3.1.1. Phosphatidylserine exposure

PS exposure is an early sign of either activation or apoptosis. The intensity and duration of PS egress during viable cell activation depends on cell type and agonists, whereas in apoptotic cells it constitutes a prerequisite for engulfment by phagocytes.[355] The release of MP is observed some hours after apoptosis induction,[214] whereas a swift MP release occur a few minutes after stimulation in platelets[356] as they have the highest scrambling rate in contrast to other vascular cell types.[357] In some cases, the processes of PS translocation and MP generation can be separated.[358] On one hand, PS exposure is not always followed by MP release. It seems that intracellular Ca^{2+} threshold required for MP formation to occur is higher than for PS exposure.[359] On the other hand, a portion of the MPs released from blood cells does not expose accessible PS on their surface.[204,360] The nature and the mechanisms of generation of these MPs are poorly understood, but could be due to cytoskeleton cleavage with maintenance of the asymmetric

phospholipid distribution in the plasma membrane. Alternatively, they can result from multiple fusion events between cell debris or small endosomal-secreted vesicles and the plasma components. Altogether these data suggest that PS translocation is not the only mechanism leading to MP release. Nevertheless, all these studies are based on a lack of detectable annexin V binding and, thus, it is unclear if these populations truly lack externalised PS or the level of externalization is simply below limits of detection.[277]

Furthermore, cellular plasmatic membranes also display lipid microdomains, named rafts, which are responsible of lateral mobility of proteins and recruitment of signalling molecules.[361] Recent data indicate that the lipid rafts, rich in cholesterol and sphingolipids, are essential for PS transmembrane redistribution.[362] Indeed, PS exposure was colocalized with membrane lipid rafts regions.[363] In addition, perturbation of lipid domains was associated with alterations in MP formation[364] and, similarly, disruption of lipid-rich domains impairs MP formation.[323] Interestingly, TF-rich microparticles have been found to arise from lipid rafts.[294] The raft origin of MPs is further supported by the fact that (a) in endothelial cells, the clustering of the platelet endothelial cell adhesion molecule 1 on the cell surface preceded the shedding of MPs enriched in this antigen[365] and (b) several proteins which localize to lipid rafts have been also identified in MPs (CD39, flotillin-2, caveolin-1).[366] However, the exact mechanism involving lipid rafts remains unknown.

2.3.1.2. Cytoskeletal involvement

The intracellular cytoskeleton, formed by a network of structural proteins, modulates the membrane asymmetry and cell stability via covalent protein-protein and protein-lipid interactions.[367] Plasma membrane-cytoskeleton adhesion is mediated at least in part by the binding of proteins of the membrane skeleton to specific phospholipids, such as phosphoinositol 4,5 biphosphate. Among the actin-binding proteins, <u>spectrin</u> is a submembrane skeletal protein that connects lipids with actin cytoskeleton through lateral and vertical connections. When membrane phospholipid translocation occurs, spectrin anchorage is abolished, contributing to plasma membrane budding and activation of actin regulatory proteins such as proteolytic calpains and lipid-binding gelsolins, which in turn induce calcium influx.

Beyond being effectors of apoptosis, <u>caspases</u> also contribute to the process of MP generation independently of cell death mainly through a Rho-associated coiled-coil-containing protein kinase (ROCK)-mediated pathway by *caspase-3*[349] and *caspase-2*.[368] *Caspase 1–like enzymes* also contribute to the externalization of membrane PS residues by hydrolysis of cytoskeletal proteins (moesin, filamin A).[369] Importantly, this PS exposure is a distinct process from the execution phase of apoptosis via caspase-3.

In addition to caspases, calcium release leads to activation of calpain and gelsolin, two enzymes also involved in cytoskeleton reorganization during MP generation. Calpain is a Ca^{2+}–dependent thiol protease that hydrolyzes actin-binding proteins decreasing the association of membrane skeleton proteins (spectrin, filamin, adducing, α-actinin, talin and actin) with membrane glycoproteins.[370] Additionally, gelsolin is involved in the cleavage of the actin capping proteins.[371] Rearrangements of cytoskeleton proteins and protein cleavage activate various receptors and proenzymes,[350] such as RhoA, a member of the Rho family, also involved in MP biogenesis.[372]

In addition, other potential calcium-independent mechanisms involving numerous signalling transduction pathways are involved in MP formation such as integrin $\alpha_{IIb}\beta_3$,[373] calmodulin activation,[374] p21-activated kinases,[375] p38-mitogen activated protein kinase,[376] TRAIL/Apo2L complex,[318] toll-like receptor 4[377] and others. Although participation of kinases[358] and phosphatase inhibition[374] may influence MP release, they are not essential. Finally, transglutaminase-2, an enzyme that catalyzes protein cross-linking and governs cytoskeletal reorganization, has recently been implicated in MP release from VSMC.[378]

2.3.2. Exosome formation

Exosomes differ from MPs in their formation and, consequently, in that they contain less exposed PS. Generation of exosomes occur through a multi-step endocytic-lysosomal process (Figure 11). The first step involves inward membrane budding and the formation of intracellular vesicles, called early endosomes, which are later transformed into more complex multivesicular bodies (MVB).[379] A secondary inward budding of endosomal membranes may lead to the formation of much smaller inner microvesicles (within MVBs) with the outside-out membrane orientation. MVBs are destined for several processes: (a) as storage sites; (b) recycling fate for reutilization;[380] (c) proteolytic degradation through their fusion with lysosomes (organelles that constitute, together with the MVB, the major cell site of protein and lipid degradation); and, (d) they can fuse with the plasma membrane, thereby releasing their vesicles into the extracellular milieu as *exosomes*.[381,382] The docking of the exosomal membranes with the target plasma membrane involves the energy-dependent formation of soluble NSF attachment protein receptor (SNARE) complexes regulated by Rab proteins.[383,384] A part from the canonical biogenesis, other mechanisms for exosome release have been proposed: (a) a role for the tetraspanin protein family;[382] (b) a lipid self-assembly process, consisting in a spontaneous secondary microvesiculation triggered by enrichment of larger artificial structures in ceramide;[353] and (c) an ARF6-regulated endosomal pathway.[385]

2.4. Structure and composition

MPs are not a random sample of the parental cellular content, but rather MPs are assembled through a highly selective process,[386] the nature of which remains unclear.

Different MP-inducing stimuli will influence the molecular content of the vesicles. Both plasma membrane remodelling and antigenic turnover associated to different signalling pathways participate in inclusive and exclusive cargo sorting. Hence, a given stimulus may influence MP composition and correlate with the spectrum of released MP phenotypes and their biological roles, as highlighted by proteomic studies.[387-389] For instance, pMPs activated by thrombin or collagen express glycoprotein IIb-IIIa complexes (leading to the binding of pMPs to fibrinogen) whereas those produced by platelets activated by complement do not.[85]

Microparticles can contain a spectrum of bioactive molecular effectors, messengers of cell activation and apoptosis, including lipids, proteins, and even different ribonucleic acid (RNA) species, depending on the type and temporospatial status of the cell origin,[254] as shown in Figure 13. MPs not only behave as a storage pool of bioactive molecules, but also possess a membranous skeleton and characteristically display procoagulant properties due to their cargo. Figure 13 summarizes the general composition of a canonical MP, with TF and PS as procoagulant effectors.

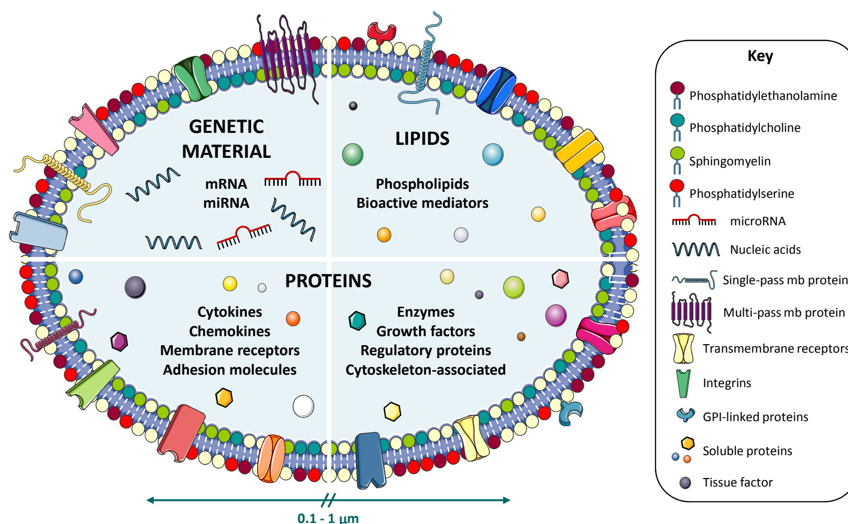

Figure 13. Microparticle structure and composition. Schematic representation of the repertoire of MP components. Adapted from Norling et al.[390]

2.4.1. Proteins

Microparticles bear both intracytoplasmic and membrane-bound protein effectors which allow them to act as carriers of biological and pathological messages. Protein cargo of MPs is highly variable depending on the cell of origin and the different stimulation conditions by which have been released.[391] To increase the complexity it has been shown that different size classes of pMPs contain different protein components.[392] Proteomic studies have reported that this protein content mainly consist of membrane receptors and fusion proteins, adhesion proteins (P-selectin), integrins (GPIIb/IIIa), cytoskeleton-associated proteins, enzymes and signalling proteins, proteins of the major histocompatibility complex, cytokines, chemokines, proteases, and growth factors. MP proteins have been involved in apoptosis regulation, targeting, adhesion, coagulation, cell communication and signal transduction, among other biological cell functions. Other components of MPs have been recently described, such as prions[213,393] or contractile proteins like thrombosthenin.[394]

Since MPs contain various proteins inherited from their parental cells, their origin can be identified by the presence of cell-specific surface antigens. Similarly, the presence of cell activation markers on MP surface might predict the degree or activation status of origin cells at the moment of MP shedding. However, the expression and proportion of these cell surface molecules on MPs can differ from their cells of origin.[395] Table 6 shows the most common markers depending on cell type.

2.4.2. Genetic material

MPs have the ability to contain and transport different RNA classes, including functional messenger RNA (mRNA) and microRNAs (miRNAs). Indeed, MPs contain a large number of non-coding RNAs (ncRNAs). Indeed, 1300 different mRNA transcripts have been identified in lipid vesicles.[386] Besides protein-coding RNAs and miRNAs, other types of regulatory RNA molecules, such as RNA transcripts overlapping with protein coding regions, repeat sequences, structural RNAs, transfer RNA (tRNA) fragments, vault RNA, Y RNA, small interfering RNA (siRNA), large intergenic ncRNA (lincRNA) and small nucleolar RNAs (snoRNA) have been found in microparticles.[396,397]

Cellular origin	Markers	Alternative name
Red blood cell	CD235a	Glycophorin-A
Leukocyte	CD11b CD45 CD62L CD64a CD162	Integrin alpha M Leukocyte common antigen L-selectin Immunoglobulin γ Fc Receptor I P-selectin glycoprotein ligand 1
Granulocyte	CD15 CD16b CD66b	Lewis X Immunoglobulin γ Fc Receptor IIIB Glycosylphosphatidylinositol-linked protein
Monocyte	CD14 Interleukin-1β Anti-oxLDL antibody	Lipopolysaccharide-receptor. Proinflammatory cytokine -
Lymphocyte	CD3 CD4 CD8 CD19 CD20 CD80 CD83 CD154	T3 T4 T8 B4 B1 B7 HB-15 CD40 ligand
Platelet	CD41 CD42a CD42b CD61 CD62P CD63 TSP1 PAF β-amyloid precursor Anticoagulant protein C/S Complement C5b-9	αIIb-integrin (glycoprotein IIb) Glycoprotein IX Glycoprotein Ibα β3-integrin (glycoprotein IIIa) P-selectin Lysosomal-associated membrane protein 3 Thrombospondin-1 Platelet-activating factor - - -
Endothelial cell	CD11a CD34 CD54 CD62E CD51 CD309 CD105 CD106 CD133 CD144 CD146	Integrin αL Hematopoietic progenitor cell antigen 1 Intercellular adhesion molecule-1 E-selectin α_V-integrin KDR or vascular endothelial growth factor 2 Endoglin Vascular cell adhesion molecule-1 Prominin 1 (also marker of stem cells) Cadherin-5 Melanoma-associated antigen MUC-18
Inflammatory & others markers	CD31 CD36 CD71 CD142 FasL	Platelet endothelial cell adhesion molecule 1 Scavenger receptor class B member 3 Transferrin Tissue factor Fas ligand

Table 6. Cell-derived markers for microparticle phenotyping.

2.4.2.1. Microparticle-associated microRNAs

Among all detected RNA species within MPs, microRNAs have become a focus of attention because of their potential as novel disease biomarkers as microvesicles might carry miRNA signatures that differ among health and disease. Moreover, in contrast to common RNA species like mRNA, rRNA, and tRNA, extracellular plasma miRNAs are remarkably stable even though the presence and activity of ribonucleases that destroy any freely circulating RNA,[398-400] which means that circulating miRNAs are selectively exported and packaged in order to avoid RNAses and prevent their degradation.[401] Indeed, miRNAs are protected against degradation not only (1) by their inclusion in protein and lipid microvesicles (exosomes and microparticles)[386,402] as well as in apoptotic bodies,[403] but also leaked into (2) RNA binding proteins (Argonaute 2 [Ago2]),[404] or associated with (3) lipoproteins (high density lipoproteins),[405-407] as shown in Figure 14. The proportion of miRNAs in the different locations is not yet established. Interestingly, some microvesicle-associated miRNAs were found at relatively higher levels in microvesicles than in their donor cells,[408] which implies that selected miRNAs are actively promoted toward exosomes or MPs. In addition, cells can select some miRNAs either in the mature miRNA or the precursor form (pre-miRNAs) for cellular release while others are retained.[409]

MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression at the posttranscriptional level by targeting the 3' untranslated region of mRNA transcripts. miRNAs constitute an epigenetic mechanism involved in regulating the cellular transcriptome and proteome by destabilizing mRNA and/or attenuating protein translation.[410] This regulation is dynamic; temporary, when miRNA release the mRNA or, permanent, causing mRNA strand degradation.[411] Thus, miRNAs have the ability to regulate a wide range of biological processes.[412] Indeed, bioinformatic predictions indicate that mammalian miRNAs can regulate ≈30% of all protein-coding genes.[413] As an example of its complexity, a single microRNA can regulate multiple mRNAs, and a single mRNA can be regulated by several distinct microRNAs.

Currently, there are 2042 discrete miRNA species identified in humans (miRBase), and approximately 300 are detectable in blood.[414] Importantly, stable regulatory non-coding RNA was detected not only in plasma or serum[398-400,415,416] but also, similarly to MPs, in other body fluids including blood plasma, urine, tears, breast milk, amniotic fluid, cerebrospinal fluid, saliva, and semen,[417-420] varying their concentration in each one. miRNAs are likely found in these compartments because they are in the process of degradation, reabsorption, or excretion.[421] However, their abundance and stability also implies a possible role in cell-cell communication.[421-423]

Moreover, the importance of epigenetics regulation in CV risk factors and diseases has raised due to the fact that RNA molecules can enter into the circulation through the diet[424] or other means (fungal or bacterial origins).[425] Epigenetic events are the sum of genetic risk factors and sustained environmental exposures that drive an organism towards adaptive responses to maintain homeostasis.[426] Therefore, miRNAs can also modulate the effects of environmental exposures through interactions gene-environment and, as a consequence, affect risks factors for CVD, such as miR-410 for lipoprotein lipase gene,[427] among others.[428]

a. microRNA biogenesis

All miRNAs are originally transcribed from DNA by RNA polymerase II in the cell nucleus as primary miRNAs (pri-miRNAs) transcripts,[429] which are cleaved and spliced into ≈70-100 nucleotide (nt) precursor miRNA (pre-miRNA) by Drosha complex.[430] Pre-miRNAs are actively exported from the nucleus into the cytoplasm, where they are further cleaved into ≈22-nt imperfect miRNA:miRNA duplex, double stranded RNA (dsRNA), by RNase III Dicer protein complex.[431] The maturity of miRNA depends on the separation of dsRNA by various helicases,[432] leaving one of the strands associated with an Ago2 protein, forming then the RNA-induced silence complex (RISC).[433] Through the formation of RISC, miRNAs can either cleave mature mRNA molecules or inhibit their translation by binding to target mRNA, in a sequence-specific manner, promoting their decay and inhibiting translation, which is an additional posttranscriptional stage of gene silence regulation (Figure 14).

Up to now, the mechanism by which miRNAs are targeted to be loaded to microvesicles, retained within the cell or exported as miRNA-protein complexes remains unknown. During RISC disassembling in the cytoplasm, miRNAs can be sorted into MVBs which by fusion with the plasma membrane form exosomes[434] or directly packed into microparticles.[344,385] Finally, miRNAs may also translocate across cell membrane, but the exact mechanism by which they are exported and whether the Ago2-miRNA complex in plasma is directly derived from intracellular intact complex or reassembles in the extracellular space remains to be determined. Similarly to these described forms of miRNA transport, how HDL is loaded with miRNAs is not entirely known, although it has been proposed that HDL binds miRNAs in the circulation.

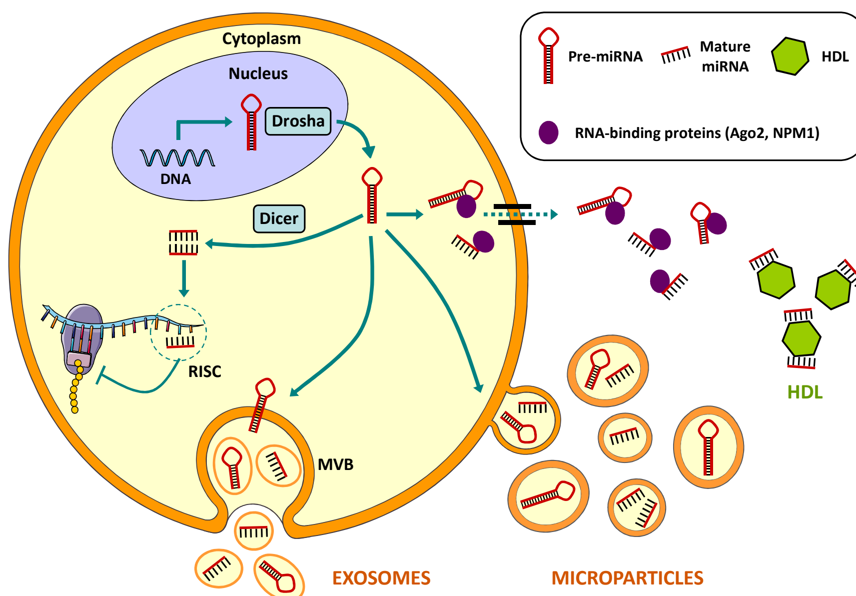

Figure 14. Extracellular microRNA biogenesis. In the cell nucleus, miRNA genes are transcribed by RNA polymerase II into primary miRNA (pri-miRNA) transcripts, which consist of a stem-loop structure. Pri-miRNAs are further processed to precursor miRNAs (pre-miRNA), which are exported to the cytoplasm and cleaved producing a double stranded RNA duplex. When the duplex is separated, the mature miRNA assemble into RNA-induced silence complex (RISC) and can inhibit protein translation of target mRNAs. In addition, miRNAs can be exported out of the cells through various carriers: membrane-derived vesicles (exosomes and microparticles), miRNA-binding protein complexes, or high-density lipoproteins (HDL).

b. MP-associated microRNA functions

The presence of miRNAs in microparticles suggests that miRNAs could have a function as modulators of cell-to-cell communication, being selectively targeted for secretion in one cell and taken up by a distant, target cell, possibly to regulate gene expression. [386,416,435] Indeed, the fact that cMV-miRNAs can be efficiently transferred to recipient cells and be functional has been demonstrated in endothelial cells,[403,436] fibroblasts,[437,438] peripheral blood mononuclear cells,[230] smooth muscle cells,[439] antigen-presenting cells[440] and cardiomyocytes,[441] as a paracrine mode of cell-cell signalling. Further research is needed to demonstrate to what extent this occurs under physiological and pathological conditions. HDL also can deliver miRNAs to cells and alter gene expression. But it remains unknown whether miRNA-Ago2 complexes can be taken up by distant recipient cells and whether it regulates gene expression.

c. microRNAs as biomarkers of CVD

Circulating miRNAs have been recently pointed out as potential biomarkers since they accomplish several criteria of being an ideal biomarker: (a) they are stable in body fluids and therefore easily accessible from patients, (b) can be measured with high sensitivity because they are amplifiable, and (c) some miRNAs and clusters of miRNAs have shown profiles associated with specific pathologies.[442-446] To this respect, different studies have reported on the association of miRNAs with CV risk factors[447] and clinical conditions, such as myocardial infarction,[448-457] heart failure,[458,459] and atherosclerosis,[460-462] suggesting a role in diagnosis and prognosis of CVD. Some of the microRNAs described in major CV risk factors and CVD are summarised in Table 7. However, their potential role as a prognostic tool to monitor therapeutic treatments is under discussion.[463] As circulating miRNAs in plasma have multiple origins and locations, the study of a subfraction such MP-associated miRNAs in disease states, could confer a high degree of specificity. This is a promising strategy that warrants further investigation.

Disease	Altered circulating microRNA
CARDIOVASCULAR RISK FACTORS	
Type-II diabetes mellitus	hsa-miR-126 / -15a / -29a/b / -223 / -28-3p / -375 / -20b / -21 / -24 / -197 / -320 / -486 / -150 / -9 / -30d / -34a / -124a / -146a / -144 / -132 / -222
Hypertension	hsa-miR-266-5p / -let 7e / HCMV-miR-UL112
Dyslipemia	
Dyslipemia and metabolic syndrome	hsa-miR-103 / -17 / -183 / -197 / -23a / -509-5p / -584 / -652
Atherogenic dyslipemia	hsa-miR-100 / -106b / -125 / -143 / -148a / -17 / -18a / -20a / -21 / -221 / -374a / -7 / -93 / -96
Dyslipemia and CAD	hsa-miR-122 / -370 / -34
CARDIOVASCULAR DISEASE	
Atherosclerosis	hsa-miR-130a/ -27b / -210
Coronary artery disease	hsa-miR-126 / -92a / -195 / -let-7b / -17 / -30e-5p / -17/92 cluster / -145 / -155 / -140 / -182 / -150 / -146a/b / -17 / -19a / -584 / -222 / -29a / -378 / -342 / -181d / -214 / [-135a / -147 (PMN)] / [-134 / -198 / -370 (UA)]
Acute myocardial infarction	hsa-miR-208a/b / -499 / -1 / -133a/b / -30c / -328 / -663b / -145 / -1291 / -126 / -197 / -223 / -1915 / -186
Heart failure	hsa-miR-423-5p / -675 / -18b / -126 / -129-5p / -1254 / -622 / -210
Aortic aneurysm	hsa-miR-29b / -124 / -155 / -223
Stroke	hsa-miR-125b / -27a / -422a / -488 / -627 / -145

Table 7. Circulating microRNA in cardiovascular disease. HCMV indicates human cytomegalovirus; PMN, polymorphonuclear leukocytes; UA, unstable angina.

2.4.3. Lipids

The plasma membrane of MPs is reorganized by the active externalization of PS and internalization of phosphatidylcholine (PC). Indeed, MPs are enriched in PE and PS exposed on their outer surface. Specifically, MPs isolated from blood (mainly derived from platelets) comprise 60% PC, 20% sphingomyelin, 9% PE, 5% PS and minor quantities of other.[464,465] Notwithstanding lipid composition depends on the vascular milieu and/or inflammatory environment[297] as well as the oxidation status of lipids.[466]

PS is an anionic phospholipid that serves as a catalytic surface for the assembling of blood coagulation factor complexes and, thereby, accelerates the activation of factor X and the subsequent generation of thrombin.[25,467,468] Additionally, PS enhances the procoagulant activity of TF, one of the main cellular promoters of blood coagulation.[469] As described that coagulation factors do not normally encounter PS, external PS acts as a major prothrombotic signal that ensures efficient recognition. Similar to proteins and miRNAs, PS is highly enriched in MPs compared to their parental cells. For instance, the surface of pMPs is approximately 50- to 100- fold more procoagulant than the surface of activated platelets.[470] The interaction of membrane-PS with coagulation factors is inhibited by its affinity ligand annexin V (AV) in presence of calcium.[471] Thus, this property of annexin V is used in analytical detection of MPs.

However, not all MPs exhibit PE and PS as lipid components on their surface. Thus, eMPs originating from activated ECs are different in their lipid composition than those derived from apoptotic ECs.[254] Although it is commonly accepted that MPs expose PS, some studies have identified vesicles expressing specific markers of cellular origin, in the size range of MPs but not binding AV.[277,472] Nevertheless, annexin V-negative MPs may be biologically distinct, as previously stated in the MP formation section.

2.4.4. Exosomes

Exosomes differed markedly from MPs in that contain different types of molecular cargo and less exposed PS.[353,473] As a consequence of their origin, exosomes contain endosome-associated proteins (e.g., Rab GTPase, SNAREs, annexins, and flotillin) and membrane proteins such as tetraspanins and those typically enriched in lipid rafts.[474,475] In terms of lipid content, exosomes are highly enriched in cholesterol, sphingomyelin, and hexosylceramides at the expense of PC and PE in comparison to plasma membrane. The database ExoCarta catalogs proteins, lipids, and RNAs identified in exosomes from different cell types.

2.5. Functional relevance

Besides their potential as markers of CVD, MPs might exert either beneficial or deleterious effects in disease states, depending on cellular origin, stimulus for production, and the clinical setting. Indeed, it is worth to stress the fact that the ultimate effect of a MP is likely to be dependent on the cellular milieu (both temporally and spatially), which may explain in part the contradictory functions of the same MPs. In the atherothrombotic context, MPs seem to contribute to vascular disease initiation, progression and its clinical complications (Figure 15).

2.5.1. MPs in intercellular communication

MPs have recently been considered as mediators of intercellular communication, true vectors in the transcellular exchange of biologic signals and information. MPs might be able to transfer part of their components and content to selected target cells by proposed mechanisms not precisely known that are explained in the next section. Because MPs circulate in the blood flow, they could serve as shuttle modules and signalling transducers not only to neighbouring cells (local environment) but also to cells at relative distance from their cell or site of origin, triggering cell activation, phenotypic modification, and reprogrammation of cell function. Thus, MPs are believed to complement the well-known methods of intercellular communication such as direct secretion of signalling molecules, physical interaction of membrane proteins and involvement of gap junctions.

2.5.2. MPs in homeostasis

Cells may release MPs to communicate or initiate signalling or cell contact. Thus, MP release may play a role in organ- as well as in cell-defence systems: stress response, inflammation, and tissue regeneration. Cells may also shed MPs as a self-defence mechanism such as release of PS^{+}-MPs to prevent PS-induced phagocytosis of the cell. Similarly, release of MPs seem to facilitate removal of other potentially harmful pathogens from the cell.[250]

Under normal physiologic conditions, MPs are involved in tightly controlled biologic functions. MPs possess both procoagulant and anticoagulant activities due to expression of PS and membrane-dependent enzymatic reactions. Procoagulant properties of MPs rely on the expression of anionic phospholipids on the membrane surface, especially PS. Due to its negative charge, PS can assemble calcium-dependent coagulation factors on the MP surface, forming tenase and/or prothrombinase complex followed by thrombin formation.[476]

Moreover, MPs have recently been considered as blood-borne TF reservoir. TF is a critical component of the early stages of the clotting system where it forms a complex with factor VII/VIIa, which in turn activates both FX and FIX, ultimately leading to the initiation of coagulation. Indeed, PS^+-MPs rich in TF constitute the MPs with highest level of procoagulant activity.[477]

Besides the procoagulant activity, MPs might additionally depict anticoagulant and fibrinolytic properties reflecting the status of their parental cells and contributing to regulate the haemostatic balance and vascular integrity. On the one hand, MPs can express down-regulators of thrombin generation such as tissue factor pathway inhibitor (TFPI), which regulates TF:FVIIa complex;[478,479] thrombomodulin, endothelial protein C receptor, and activated protein C.[480-482] Indeed, MPs have the ability to promote activation of protein C by thrombin, inactivation of procoagulant factors Va and VIIIa, and thereby downregulation of thrombin generation.[483] On the other hand, novel fibrinolytic properties have recently been assigned to MPs. MPs might bind urokinase (u-PA) and tissue-type plasminogen (t-PA) activators and thus be an efficient support for plasmin generation; however, this ability has only been demonstrated for endothelial- and leukocyte-derived MPs. [484-486]

2.5.3. MPs in atherosclerosis

2.5.3.1. Endothelial function and oxidative stress

MPs might directly contribute to endothelial dysfunction by regulating the production of nitric oxide and prostacyclin as well as reactive oxygen species. Endothelial cell-derived MPs have been shown to impair NO bioavailability[52,487] and increase endothelial permeability.[488] Besides, lymphocyte MPs from T cells increase ROS production and decrease NO synthesis in ECs.[489] Moreover, MPs isolated from metabolic syndrome[170] and CAD patients[490] reduce *in vitro* NO production and increase superoxide dismutase expression in ECs, impairing endothelium-dependent relaxation.

2.5.3.2. Inflammation

Microparticles could contribute to inflammatory responses in various ways. Generation of eMPs during oxidative stress has been found to result in biologically active oxidized phospholipids, which promote neutrophil activation and monocyte adherence to endothelial cells, an early key event in atherogenesis. eMPs might be able to activate leukocytes via CD11b surface expression[491] and enhance macrophage adhesion.[323] In addition, pMPs were also found to enhance monocyte adhesion to the vascular endothelium.[492] In

fact, MPs from stimulated platelets are able to activate monocytes through RANTES (regulated upon activation, normal T cell expressed and secreted) pathway, which in turn facilitate monocyte migration, tissue recruitment, and differentiation toward macrophage.[20,493,494] pMPs also could favour leukocyte-leukocyte interactions through P-selectin expression under flow conditions.[22] Furthermore, MPs from macrophages expressing CD40L may play essential roles in inflammation during plaque progression.[495] Lymphocyte-derived MPs can stimulate monocytes with cytokine production[70] and induce smooth muscle cell inflammation through nuclear factor kappa light chain (NF-κB) and ciclooxigenase-2 (COX-2) upregulation.[70,496] Leukocyte-derived MPs might also stimulate cytokine release by endothelial cells, leading to increased proinflammatory activity,[58,59,497] leukocyte recruitment and monocyte chemotaxis.[60]

When focusing on total cMPs, similar results have been observed. cMPs from patients with obstructive sleep apnoea or preeclampsia increased proinflammatory COX-2 expression in human ECs[498] and in mouse aorta.[499] Indeed, total MP levels from human atherosclerotic plaques were able to increase adhesion molecules, such as ICAM-1 levels in ECs and CD11a in monocytes, and stimulated endothelial-monocyte adhesion.[492] Thus, these MPs may be implicated in the recruitment of inflammatory cells and in the promotion of atherosclerotic plaque progression.[500]

2.5.3.3. Apoptosis

Some evidence suggests that MPs are able to promote apoptosis. First, monocyte-derived and T-cell-derived MPs were found to induce apoptosis in macrophages.[292,501] Other studies revealed the presence of caspases in MPs. MPs from endothelial cells and platelets may also contain active executive caspase-3.[315,502,503] And mMPs induce death of target cells by delivering caspase-1.[504] Interestingly, MPs isolated from hypertensive patients induce H_2O_2 production, cellular senescence and apoptosis.[505]

2.5.3.4. Angiogenesis

Microparticles could regulate endothelial proliferation and capillary tube formation. Platelet-derived microparticles have been shown to promote angiogenesis in a number of studies.[506-508] MPs from other cells may also influence angiogenesis. MPs from activated lymphocytes harbour Sonic hedgehog and induce angiogenesis.[509-511] MPs isolated from atherosclerotic plaques might be involved in neovessel formation and the progression of plaques to a vulnerable state prone to rupture.[495] Nevertheless, contradictory results exists[512,513] and it has been suggested a balance between pro- and anti-angiogenic activities of MPs of different cell origin present in human plasma.[56]

2.5.4. MPs in thrombosis

Besides a physiological role of MPs in normal haemostasis, cMPs might have a role in pathological thrombosis. MPs seem to actively participate in the coagulation process. MP-characteristic phosphatidylserine exposure on the surface provides binding sites for coagulation enzyme assembly,[92] enhances platelet adhesion to the endothelium, and is important for tissue factor function[294] and thrombin generation and clot formation.[25] TF-enriched MPs are taken up by platelets and induce platelet aggregation in the presence of factor VII.[514] Besides, the interaction between MP PSGL-1 and platelet P-selectin that favour fibrin formation[515] has been argued to play also a role in the recruitment of TF-rich monocyte-derived MPs to the site of thrombosis in wild-type mice.[295,516] MPs bearing active TF, may accumulate and allow for unregulated thrombus formation and fibrin generation[477] and thrombus propagation[517] predisposing to thrombotic complications.

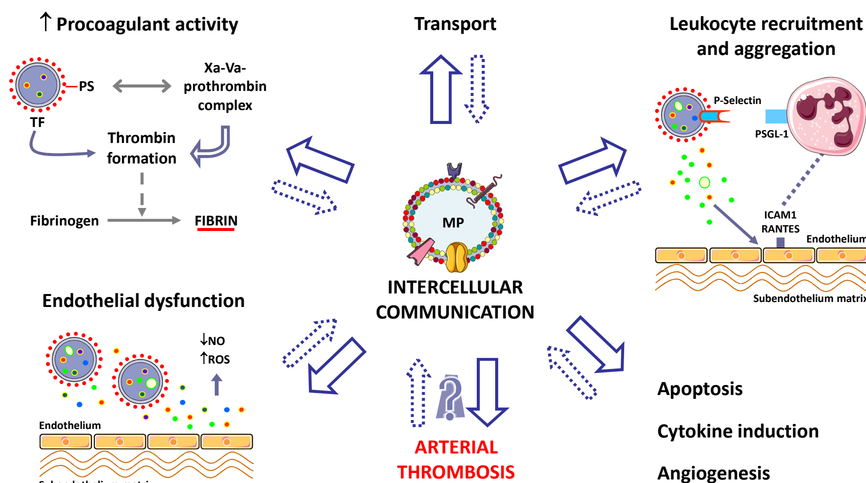

Figure 15. Biological functions of microparticles. Due to their phenotoypic composition and structure and through mechanisms of intercellular communication, MPs are able to exert a vast amount of diverse cellular functions related to haemostasis, atherosclerosis and thrombosis that include endothelial dysfunction, inflammation (leukocyte adhesion and cytokine release), apoptosis, angiogenesis, coagulation and thrombosis, although the latter is less well-known.

2.6. Mechanisms of action

Until now, MP-mediated molecular mechanisms for disease progression were poorly understood. However, the effects of MPs can be either (1) direct, mediated by the MP itself or, (2) indirect, mediated by the recipient cells, upon MP interaction or fusion, as shown in Figure 16.

a. Direct effects

   The main putative direct mechanisms of action of MP effects are:

   - A potential own *de novo* production of ROS.[518,519]
   - Effects on the extracellular matrix by proteolytic enzymes of MPs, which are able to cleave signalling molecules.[520,521]
   - Acting as a catalytic surface, especially on coagulation.

b. Indirect effects

   - Physical interaction with target cells.
     MP recognition is mediated by surface receptors or ligands. A close physical interaction between MP and target cell interaction results in juxtacrine signal transmission[303] as demonstrated by platelet,[522] endothelial,[303] smooth muscle[523] and neutrophil-derived MPs.[524]
   - Fusion or internalization and transfer of MP contents to target cells.
     MP-cell anchorage takes place through the expression of adhesive molecules on MP surface. MPs can be either fused with plasma membrane, named also *trogocytosis*[525] or internalized, through *endocytosis*, *phagocytosis* or *macropinocytosis*,[526] in a dose-dependent manner by macrophages,[527] endothelial cells,[528] and other cell types.[529] The endocytic uptake is differentially regulated in various cell types and mainly depends on the actin cytoskeleton, PI3K activity, and dynamin-2 function.[530,531] As a featured example of the internalization process, platelets possess mechanisms to capture and incorporate TF-rich vesicles.[532]

   MP fusion and internalization can lead to:

   - Transfer of their contents, including RNA,[533] bioactive lipids and proteins[500] into the recipient cell. For instance, MPs can transfer fully operational surface receptors onto the recipient cells, such as CXC4R and CD41 of pMPs.[527] Interestingly, recent studies suggest a scenario, whereby microvesicular transfer would encompass multiple effectors at once.[416]
   - Modulation of both functional and phenotypic characteristics and reprogramming of cell function of targets cells.

These indirect effects on recipient cells confer MPs the capacity for the recently discovered relevant biological role on cell-to-cell communication.

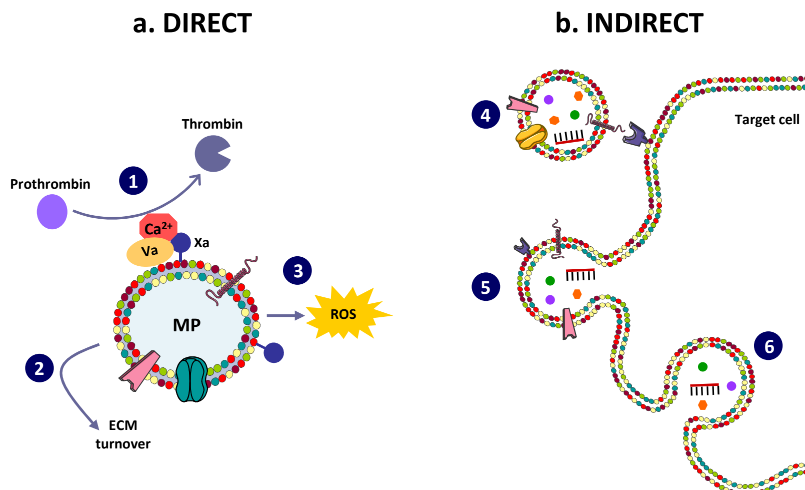

Figure 16. Main mechanisms of action of MP biological effects. (a) Direct effects are mediated by MP itself and include **(1)** acting as a catalytic surface enabling the binding of coagulation factors and the generation of thrombin, **(2)** modulation of extracellular matrix proteins by proteolysis, and **(3)** *de novo* ROS generation. **(b)** Indirect effects: MP can **(4)** physically interact with surface receptors of target cell inducing signal transduction. Bound MPs may either **(5)** fuse directly with plasma membrane or **(6)** be endocytosed, transferring their contents to the target cell.

2.7. Microparticles and therapeutics

Acquiring knowledge about the role of MPs in CVD may also have implications for their treatment. Since several therapeutic drugs seem to influence the levels of cMPs[534,535] or their composition,[536] the lowering of cMP load in the circulation may prove to be a novel therapeutic strategy for treatment.[174] Since several pathways are associated to MP release, until now distinct treatment options for CVD are under investigation. For instance, anti-platelet drugs such as GPIIb/IIIa inhibitors,[523,537,538] acetylsalicylic acid,[539] clopidogrel,[540] and ticlopidine[541,542] have shown to reduce MP levels. Similarly, anti-hypertensive drugs like angiotensin II receptor antagonists[543] and calcium channel blockers,[544,545] anti-oxidants,[546] and peroxisome proliferator-activator receptor (PPAR) activators[54,547] have also shown influence on MP shedding. Up to now, however, the cornerstone drugs for lipid-lowering statins, have demonstrated controversial results in this regard. While some authors reported that statins may stimulate MP release[548-550] others found that statin treatment promote MP inhibition.[551-553] Besides, there are currently under study a variety of inhibitors of MP release such as calpain inhibitors,[554-556] ROCK inhibitors,[557] TNFα inhibitor,[558] and pantethine and cystamine.[559] However, whether the beneficial effect of a pharmacological approach is associated to MP reduction and to a clinical improvement has not been sufficiently demonstrated.

In addition to pharmacological modulation, the therapeutic potential of progenitor cell-derived vesicles is promising, as they are a naturally occurring, efficient, therapeutic delivery vehicle that might be used to deliver drugs to specific cell type.[560] Furthermore, the therapeutic potential of MPs has also been pointed out by the use of synthetic MPs, mimicking natural ones, such as carboxylated polystyrene particles of 500 nm in diameter and negatively charged or MPs made of biodegradable material like poly(lactic-co-glycolic acid). MP administration could have a broad potential in several conditions such as myocardial infarction or inflammatory bowel disease. Indeed, a very recent study showed that infusion of synthetic MPs can dampen inflammation and alleviate symptoms in several mouse models of diseases like multiple sclerosis, MI, and kidney injury.[561] Nevertheless, further characterization of the biological effects of these MPs is warranted.

2.8. Methods for microparticle analysis

Methods of MP measurement in biological samples vary among laboratories and need standardization. To this aim, the International Society on Thrombosis and Haemostasis (ISTH) and the recently formed International Society for Extracellular Vesicles are putting great efforts in standardization that will drive MP research forward in terms of cMP quantification. It must be taken into account that blood collection and sample processing procedures have a strong impact on qualitative and quantitative MP analysis. The importance of using defined pre-analytical conditions is crucial for MP assays in order to be useful for clinical diagnostic or prognostic decisions.

2.8.1. Preanalytical stages for microparticle isolation

Major causes of MP variability in analysis of blood samples include:[151,562-564]

- Choice of anticoagulant. The Scientific Standardization Committee of ISTH has recommended the trisodium citrate in a concentration of 0.105 or 0.129 mol/L (3.2% or 3.8%), based on calcium chelation.[565]

- Venopuncture method (from cubital vein preferably). Platelets are susceptible to the activation and release of pMPs by physical forces associated with the blood draw procedure. Use of a 21-gauge needle or larger (up to 19G), slow-pull syringe or vacutaainer tubes, avoidance of butterfly systems and light tourniquet (prolonged used should be avoided) have been advised for venipuncture to minimize shear forces. The first several milliliters following venipuncture or the first tube should be discarded because of the activating effects of pressure and contamination by fibroblasts.

- Temperature. Sample kept at room temperature (RT) to avoid platelet lysis or activation leading to subsequent errors in MP quantification.

- Delay in processing (needle-to-analysis time). Freshly filled tubes may be inverted 10 times without shaking for proper mixing with anticoagulant. Blood samples should not be extensively shaken because shear stress may induce MP release from blood cells. Collected blood should be handled gently and processed rapidly within first two hours.

- Plasma *versus* serum. Plasma is the matrix of choice to avoid background noise due to MPs released during *in vitro* clotting process for serum generation, which accounts for over 50% of MPs in serum.[566] Analysis in platelet-free plasma (PFP) is recommended to avoid artefacts due to platelet activation.

- Centrifugation conditions. High differences in centrifugation conditions are a major cause of variability in MP analysis (Table 8).

- Storage. Considering fresh *versus* freezing samples, analysis of fresh samples minimizes the potential for fragmentation of residual cells during the freeze/thaw cycle. In multi-centre studies and prospective trials it is often inevitable to freeze and store the plasma samples before performing the assay. Current consensus seems to support storage at -80ºC. It has been reported resistance of MPs to freeze/thaw in the literature.[567] Nevertheless, samples should be freeze rapidly: first snap frozen in liquid nitrogen, for maximal preservation of morphology and function, prior to store at -80ºC. The best approach of defrosting, equally important to freezing, is thaw samples in melting ice, which also ensures the best possible preservation of MP structure and function.

- Washing steps. Washing steps before immunolabelling increase the specificity and minimize the formation of artefactual immunocomplexes. There is the potential risk for losing some MPs during several washing steps when not done carefully. Indeed, analysis of MPs obtained from PFP after a spin at ≈20000 ×g, which quantitatively sediments particles more than 0.2 μm in diameter, although it is time-consuming, is a good option, because a particle of this size is at the detection limit of the flow cytometer, so a more extensive ultracentrifugation is not needed. MPs could be phenotyped directly from plasma; however, the size of analyzed MP, the contribution of plasma soluble antigens, and the formation of immunocomplexes by different antibodies must be considered.

- Others. Diluents used (to avoid MP aggregation), filtration, vortex duration.

2.8.2. Methods for microparticle isolation

Differential centrifugation is the most widely used method for the isolation of microvesicles, despite being a critical step in the process (Table 8). In addition to centrifugation, other isolation methods are used for MP purification depending on the specific MP yield, such as size exclusion, immunoaffinity, polymeric precipitation and microfluidic devices.

Centrifugation protocol		Technique	References
Platelet free plasma	**Microparticles**		
1500 xg 15' 13000 xg 1'	100000 g x 90' 10ºC	FC, EM	325
1500 xg 20' 20ºC	17500 g x 20' 20ºC	FC	212
1500 xg 20' 20ºC	17570 g x 30' 20ºC	FC	209
1500 xg 20' 20ºC	17570 g x 30' 20ºC	FC	167
1500 xg 10' 13000 xg 2' 20ºC	-	FC	226
1500 xg 10' 20ºC 1300 xg 20' 20ºC	100000 g x 60' 2 times 20ºC	FC, ELISA, PCA	360
1500 xg 10'	-	FC	51
160 xg 10' 1000 xg 6'	-	FC	164
1550 xg 20' 20ºC	18000 g x 30' 20ºC	FC	568
2700 xg 2 times	19800 g x 10' 20ºC	FC	204
710 xg 15'	150000 g x 90' 4ºC	FC, Proteomics	388
3200 xg 30'	250000 g x 1h	FC, Proteomics	569
160 xg 10' 1000 xg 8'	-	FC	193
1550 xg 20' 20ºC	-	FC	160
13000 xg 2' 20ºC	50000 g x 45' 4ºC	FC, Functional assays	570
1500 xg 20' 20ºC	-	ELISA	172
13000 xg 2'	-	FC	50
1500 xg 20' 20ºC	17570 g x 30' 20ºC	FC	155
786 xg 15' 20ºC 13000 xg 2' 20ºC	18000 g x 45'	FC	571
710 xg 15' 20ºC	150000 g x 90' 10ºC	FC, Proteomics	572
500 xg 15' 9500 xg 5'	-	FC	176
1500 xg 20' 20ºC	-	FC	166

Table 8. Centrifugation conditions for MP obtention. Centrifugation step in the MP isolation protocol has been performed differentially among literature in terms of centrifugation fields (g-force and time of exposure). FC, flow cytometry; EM, electron microscopy; ELISA, enzyme-linked immunosorbent assay.

While some authors spun at 13000 xg 2 minutes, others have applied low speed during larger times. It must be assumed that centrifugation required to remove all platelets might also eliminate large MPs and that over centrifugation may lead to platelet activation with *ex vivo* MP generation, to a loss of vesicles or their aggregation, and the presence of contaminants like vesicle protein complexes and lipoprotein particles, which in turn may skew the cMP population to be analyzed. All samples to be studied should be spun at the same speed and with the same rotor type. Furthermore, during centrifugation the use of breaks should be avoided as much as possible.

2.8.3. Biophysical methods for microparticle detection and characterization

Detection methods are limited for MP low size and low refractive index as well as for their considerable heterogeneity. Most frequently used technical approaches used for MP characterization are displayed in Figure 17.

2.8.3.1. Standard methods

A variety of conventional techniques, generally available, are used to characterize and/or quantify microparticles:

- Flow cytometry (FC)[212,236,573] is the method of choice for cMP enumeration since is a simple, reproducible, and high-throughput method. However, the limited scattering properties of MPs make it not straightforward. FC is based on several criteria: size (lower than 1 µm), potential PS positivity (by binding of fluorescent labelled annexin V) and the presence of a cell-specific antigen or combination of antigens, which allows identification of their cellular origin. Particles with size inferior to the wavelength of the laser light used for detection may be undetectable. Indeed, commercial FC typically has a lower practical size limit of around 200 nm at which point the signal is indistinguishable from the baseline noise level. However, together with the usage of impedance-based flow cytometry, the development of digital-acquiring flow cytometers and thinner laser beam has improved the discrimination and characterization of MPs.[165] Moreover, recently there have been major improvements in the standardization of FC measurements of MPs, a key step for the clinical use. Besides sizewise, drawbacks like overlap of immune and protein complexes, viral particles, lipoproteins, and exosomes, may obscure detection, not only in FC but also in other technologies. Another limitation is that it does not provide information on MP functionality. Nevertheless, FC is the most widespread, convenient, and favoured and the gold standard method for MP characterization due to is available to most research and clinical facilities.

- Solid-phase capture assays, based on the principle of enzyme linked immunosorbent assay (ELISA), constitute another method used to detect MPs. The advantages of ELISA are, first, the analysis of small MPs, not detectable with flow cytometry, and, second, the possibility of higher throughput than with flow cytometry, allowing easy, rapid, low-cost and reproducible measurements in large cohorts. However, a particular attention should be paid to the MP preparation method, because ELISA does not include size as a criterion of measurement. ELISA thus does not allow discrimination of MPs from contaminating cells, exosomes or apoptotic bodies. Moreover, this method does not assess the concentration of MPs, but only quantifies single antigen content in the sample and quantification is done in bulk. Finally possible interference of soluble antigens can lead to specific MP underestimation.

- Functional assays, such as prothrombinase assay or procoagulant phospholipid-dependent clotting time assay.[570] A 96-well microplate is coated with annexin V or with an antibody of interest for MP detection. After washing, a mix containing prothrombin, factors Xa and Va, and calcium is introduced in the wells. PS on the MP surface allows activation of prothrombin to thrombin in the presence of factors Xa and Va. The generated amount of thrombin is measured with a specific chromogenic substrate. Another method involves TF exposure on MPs. After MP fixation on AV–coated plates and washing, MPs exposing TF are revealed using a TF antibody coupled with peroxidase. Similarly advantages and drawbacks of ELISA account to functional assays, since it is based on the same principle but applied to biological activity.

- Electron microscopy (EM)[250,574] provides direct evidence of vesicular structures and is valuable for assessments of morphology, size and the presence of markers (by immuno-EM). The technique, however, is of limited use for concentration measurements. EM is a useful research tool for studying microparticles but at the expense of capital running costs and extensive sample preparation which precludes side-by-side comparison of samples in larger batches. Indeed, centrifugation, dehydration and fixation for EM may alter the size and morphology of vesicles. Despite these concerns, EM is the only method by which the nature of the MP, its size and structure is determined at the same time.

- Confocal laser microscopy[325] has been used to characterize MPs and, interestingly, it also can be used to identify fusion particles. Prior to detection by confocal laser microscopy, MPs are stained with fluorescently labelled antibodies to enable the identification of subsets of MPs based on their antigen expression. Unfortunately it is not possible to use this method directly in plasma because of the high background caused by the interference of plasma proteins. Like other optical

methods that are based on the detection of a fluorescence signal, the results obtained by confocal laser microscopy also depend on the specificity and affinity of the antibody to the target antigen and on the density of the antigens on the MP surface. Fluorescence confocal laser scan microscopy gives information on morphological features of MPs (e.g. size, membrane structure, and cytoskeleton). It requires several hours of operation; however, it is useful for visualizing MPs and for validation of other MP measurements.

- Immunohistology, western blotting and proteomics.[575] The protein content of MPs is usually ascertained by these techniques despite they require large numbers of MPs, limiting their utility for translational studies. In histological sections, for instance, recognition of MPs is quite limited by the resolving power of the light microscope, since MP diameter is usually below the limit of resolution.

- Others such as capillary electrophoresis[397] and high performance liquid chromatography[386] that is the method of choice for lipid content.

2.8.3.2. Novel methods

Trying to overcome the resolution limitations of flow cytometry, new detection methods have emerged in the past few years. However, these novel non-universal technologies are still in their infancy and are of limited used in the clinic due to scarcity of available instrumentation and the lack of molecular identification.

- New generation flow cytometers. As aforementioned, one major limit of FC is the detection of very small size MPs (less than 0.2 µm) not always feasible with the cytometers currently used. Recently, by applying other physical methods, novel generation cytometers (NAVIOS, Apogee A50) have allowed the detection of MPs with a higher sensitivity. In addition, flow cytometer-coupled imaging has also emerged (Image Stream X).[576]

- Dynamic light scattering (DLS).[577-579] DLS, also known as photon correlation spectroscopy, is a useful method for MV sizing. DLS determines the differential size distribution of particles ranging in diameter between 1 nm and 6 µm, by calculating the average size of relatively monodisperse populations of isolated particles through intensity fluctuations of scattered light within a laser beam. DLS is less suited for the analysis of heterogeneous MP populations (polydisperse analyzed system) and results may vary depending on analysis software. Therefore, DLS requires careful data interpretation and may be a useful method provided that the shape of the size distribution is known.

- Optical single particle tracking or nanoparticle tracking analysis (NTA)[564,580] is an optical particle tracking method for obtaining concentration and absolute size distribution of MP populations in the range of 50-1000nm. On the contrary of DLS that measures all the particles at the same time, NTA visualizes individual particle and counts them in real-time. A laser beam is scattered by particles in a liquid suspension sample, and the mean velocity of each particle is calculated by the Stokes-Einstein equation on the basis of Brownian motion recorded by a CCD camera. NTA does not detect biochemical composition or cellular origin of vesicles, but it can accurately size MPs, provide a high-resolution particle size distribution profile and concentration measurements. As major challenges, NTA is time-consuming and the detection of small particles is underestimated when larger particles are present. Although with standard light scatter NTA is difficult to extract biological information on surface markers, analysis of fluorescently labelled vesicles is also feasible, but requires optimization and as yet is not used routinely.

- Atomic force microscopy (AFM)[564,581] allows performing a nanoscale measurement of individual MPs. In AFM, a mechanical cantilever is passed over a surface, with deflections indicating the presence of surface structures. With the possibility of sub-nanometre resolution, AFM is particularly suited to assessments of MP morphology. However, AFM has some limitations when analyzing non-rigid particles: the z value (the height of the particle) seems to be much smaller than the x, y values (characteristic for the surface area).

- Resistive pulse sensing (RPS),[582] commercialized as the IZON qNano technique, is a novel alternative to NTA for concentration and size distribution measurements. RPS determines the absolute size distribution of vesicles in suspension ranging in diameter between ~50 nm and 10 μm. This technique detects individual MP by transient decrease of an ionic current caused by the transport of a vesicle through a nanopore in a membrane. Polydisperse systems, such as heterogeneous vesicle populations, often require the combination of results obtained using more than 1 nanopore membrane (each of which is used for detecting a limited size range of particles.

- Size fractionation yielding monodisperse samples and Invitrox Surface Antigen Detection and Enumeration (ISADE).

Furthermore, there are new four methods not commercially available (Roman microspectometry, micronuclear magnetic resonance, small-angle X-ray scattering [SAXS], and anomalous SAXS), which are currently being explored.[582]

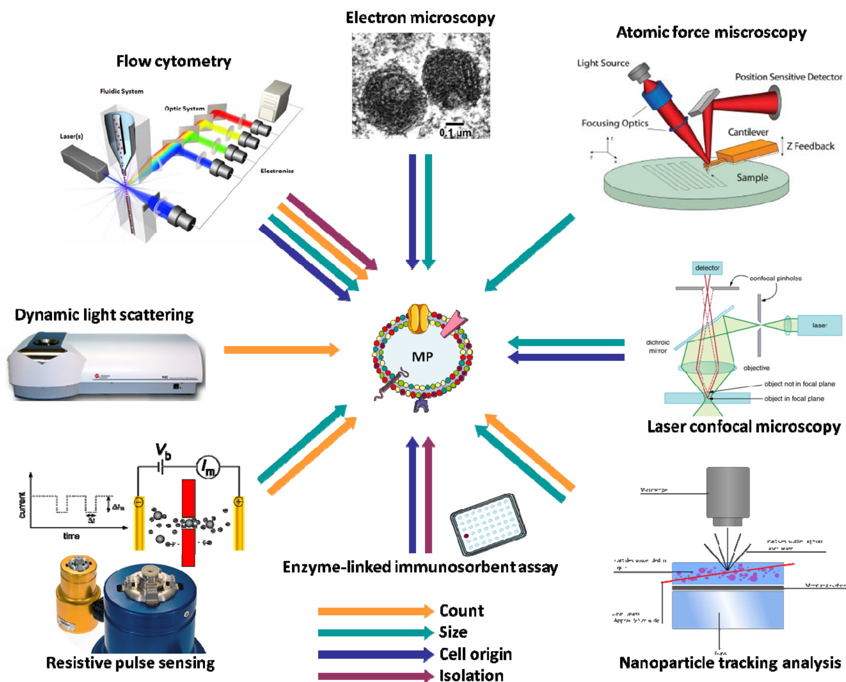

Figure 17. Microparticle detection methods. Major standard and novel technical approaches for microparticle investigation. Colour arrows show their main use for MP isolation, counting, size determination, and phenotypic characterization (cell origin). For vesicle characterization, these methods can be used in combination.

3. Concluding remarks and unresolved issues

Atherothrombosis is a complex multifactorial disease leading to arterial thrombosis and the clinical presentation of acute coronary syndromes, which is the result of the interplay of various factors encompassing the vessel wall, blood flow parameters and the thrombogenic potential of blood. Elevated LDL cholesterol levels increase blood thrombogenicity and growth of thrombus under defined rheology conditions. Understanding how the type and size of the thrombotic mass either mural or occlusive contribute to the clinical coronary event presentation has become crucial since similar atherosclerotic lesions can trigger different clinical event types. Therefore, unrevealing the contributing factors beyond the underlying atherosclerotic plaque is a major step in cardiovascular research.

Microparticles are subcellular membrane blebs shed from cells in response to various stimuli. It is now widely accepted that microparticles are generated by all eukaryotic cells, including cells in the vasculature. Notably, recent studies have shown that circulating MPs are increased in disease states, including in patients with CV risk factors and CVD. While the recognition of cMPs as potential biomarkers in atherothrombotic disease is growing, especially in well-studied conditions such as diabetes mellitus, much remains unknown regarding their prognostic value in subclinical atherosclerosis and as markers of atherosclerotic plaque burden.

Beyond biomarkers of cardiovascular disease, microparticles have emerged, due to their implicit role in cell-to-cell communication, as direct biological effectors acting at various stages of atherothrombotic disease. However, their specific role in arterial thrombus formation remains unknown. Furthermore, accumulating progress has been made in the scenario of considering cMPs as novel intercellular communicators; indeed, MPs might carry a battery of signalling molecules and nucleic acids, such as microRNAs, depending on the pathophysiological context, by which they may transport and deliver proinflammatory and prothrombotic signals. However, we are just beginning to understand how MPs are selectively released and targeted to exert their various biological and pathologic functions.

Finally, although it is not completely elucidated, MPs might be susceptible targets for pharmacological modulation. In this regard, MPs offer new options for therapies specifically focused on lowering MP levels.

II. HYPOTHESIS AND OBJECTIVES

One of the major challenges in cardiovascular research is the identification of atherosclerotic lesions that progress to vulnerable plaques finally causing devastating thrombotic complications. The existing imaging techniques mainly detect the end stage of atherosclerotic disease. Thereby, there is a need to find reliable biomarkers of atherosclerosis progression that can be measured routinely in easily accessible samples, such as plasma. Circulating microparticles have been reported to be increased in patients with coronary artery disease and with clustering of cardiovascular risk factors. However, whether individual cMP phenotypes may be markers of subclinical atherosclerosis remains poorly unknown.

State-of-the-art knowledge unrevealing thrombus formation on disrupted or eroded atherosclerotic plaques has evolved in the last decade from the arterial wall view to the new concept of vulnerable blood. Among all systemic factors contributing to atherothrombosis, microparticles have emerged as potential relevant targets with procoagulant and cell communication properties.

Taken together, we hypothesize that **cMPs due to their protein and acid nucleic cargo possess an atherothrombogenic potential, being novel candidates for innovative diagnostic, prognostic and therapeutic biomarker discovery in cardiovascular disease**.

In order to prove this hypothesis, the overall objectives of the present thesis were:

1. **Characterize the circulating microparticle phenotype of high-risk atherosclerotic patients with different degree of disease evolution.**

   The specific aims were:

   a. To set up a flow cytometric method for measurement of plasma-derived microparticles, including pre-analytical sample procedure.

   b. To analyze quantitatively and qualitatively circulating microparticles in patients at high cardiovascular risk with (i) familial or secondary hypercholesterolemia; (ii) clinical manifestation of cardiovascular disease (acute ST segment elevation myocardial infarction); and (iii) healthy individuals.

   c. To determine the association between cMP phenotype and atherosclerotic lesion type.

   d. To investigate the effect of lipid-lowering treatment with statins on circulating microparticle shedding.

2. **Investigate whether circulating and, specifically, platelet-derived microparticles contribute to blood thrombogenicity on areas of arterial damage.**

   The specific aims were:

   a. To evaluate the effect of high concentration of microparticles on coagulation, platelet function, and platelet deposition under flow conditions.

   b. To dissect the mechanisms by which microparticles enhance thrombus formation.

   c. To characterize the phenotype of circulating microparticles released from the growing thrombi under flow conditions and validate it to patients with acute coronary syndromes.

3. **Analyze the molecular composition of microparticles in relation to thrombogenic effects.**

   The specific aims were:

   a. To investigate the prothrombotic proteome of platelet-derived microparticles.

   b. To study the composition in microRNAs of circulating microvesicles and their relation with cardiovascular event presentation.

III. MATERIALS AND METHODS

1. Study design

Involvement of microparticles in atherothrombosis was investigated at the functional, pathological and compositional level. To this aim, *in vitro* and *translational* studies were performed, mainly with microparticles derived from washed human platelets from healthy donors and with blood circulating microparticles from CV high-risk or atherosclerotic-diseased patients in different degree of severity, as well as from healthy subjects.

MP pathological levels and phenotypic characterization were carried out by flow cytometric analysis, while their pathophysiological effects were investigated by platelet and coagulation functional studies. Additionally, MP composition was also studied. Protein content was assessed by proteomic analyses and specific microRNA cargo was determined by real time-quantitative PCR. MP composition enables to explain and expand either functional and/or pathological characterization results. Figure 18 shows a flow-diagram of the experimental design used in this thesis.

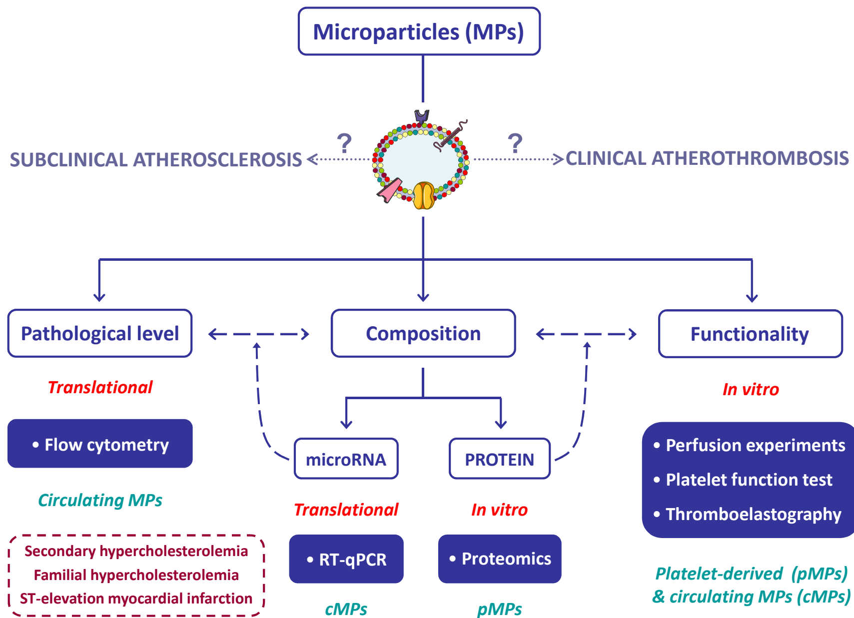

Figure 18. Schematic experimental design. cMPs indicates circulating microparticles; pMPs, platelet-derived microparticles; RT-qPCR, reverse transcription quantitative polymerase chain reaction.

In the present thesis, the commonly used term 'microparticles' will be used, since they are the main class of vesicles studied herein. Although this work is mainly focused on the study of circulating MPs, which are specifically addressed, both vesicles (MPs and exosomes) are treated as an entity, named also microvesicles, when derived from platelets (*Paper 7*). Finally, exosomes alone are also featured in some specific studies due to their recent growing relevance in the field (*Paper 8*).

2. Clinical study populations

The present thesis comprises studies performed in high cardiovascular risk (HCVR) populations with and without ischemic disease presentation, acute CHD patients, and control groups for comparative purposes. Importantly, neither patients nor control subjects had past history of cancer, inflammatory disorders, infection, and sepsis because these conditions are known to independently impair cMP number. A written informed consent was obtained from all participants prior to the studies. All study protocols were approved by the Clinical Research Committee of ICCC, Hospital Sant Pau and/or Fundación Jiménez Diaz and were conducted according to good clinical practice and to the Declaration of Helsinki for studies using human subjects. Patient's data were codified to guaranty anonymity. The results are presented in accordance with STROBE guidelines.

2.1. SAFEHEART cohort

The present thesis included three groups of subjects from the SpAnish Familial hypErcHolEsterolaemiA cohoRt STudy (SAFEHEART), which is an open, multicenter, long-term prospective ongoing cohort study in a well-molecularly defined Familial Hypercholesterolemia cohort population.[583] Specifically, the three groups of patients (n=37/group, Table 9) refer to:

(A) HCVR patients with clinical and genetic diagnosis of heterozygous FH, which had been previously characterized by MRI-imaging[106] (n=37; *Papers 3-4*). For cMP-microRNA study, FH patients who suffered an ischemic cardiovascular event (CVE) within approximately 3 years post-sampling after entering the cohort were included (n=42; *Paper 8*). Ischemic events included sudden death, fatal and non-fatal myocardial infarction, unstable angina, and cerebrovascular disease. All FH patients were receiving LLT according to clinical guidelines[584-587] and an 11% of the HCVR-FH patients achieved therapeutic LDL targets according to guidelines. Maximum statin dose were: simvastatin 40 mg/day, pravastatin 40mg/day, lovastatin 80 mg/day, fluvastatin 80 mg/day, atorvastatin 80 mg/day, rosuvastatin 20-40 mg/day.[588] All FH patients fulfilled the WHO criteria;

	HCVR-FH	Non-FH	Control
Gender (male/female)	19/18	14/23	18/19
Age (years)	48.4 ± 1.7	49.1 ± 2.5	47.2 ± 1.6
Body mass index (BMI; kg/m^2)	25.5 ± 0.7	27.0 ± 0.8	26.4 ± 0.8
Risk factors			
Hyperlipidemia (n, %)	37 (100%)	37 (100%)	0 (0%)
Diabetes mellitus (n, %)	0 (0%)	1 (2.7%)	0 (0%)
Systemic hypertension (n, %)	4 (10.8%)	7 (18.9%)	1 (2.7%)
Tobacco consumption (n, %)	13 (35.1%)	12 (32.4%)	10 (27%)
Obesity (BMI>30) (n, %)	5 (13.5%)	7 (18.9%)	0 (0%)
Hypothyroidism (n, %)	4 (10.8%)	1 (2.7%)	0 (0%)
Waist diameter (cm)	83.5 ± 2.1	82.4 ± 2.3	102.4 ± 1.3
Corneal arcus (n, %)	21 (56.8%)	12 (32.4%)	0 (0%)
Gene mutation*	(21/15/1)	-	-
Biochemical data			
Total cholesterol (mg/dL)	265.9 ± 16.7	216.2 ± 7.6	210.1 ± 7.4
Triglyceride (mg/dL)	108.4 ± 13.1	96.9 ± 7.1	113.7 ± 14.0
LDL-cholesterol (mg/dL)	196.1 ± 15.3	140.4 ± 7.1	131.4 ± 6.4
HDL-cholesterol (mg/dL)	47.2 ± 2.6	56.5 ± 2.1	56.0 ± 2.4
Non-HDL-c (mg/dL)	218.8 ± 16.9	159.7 ± 7.5	154.1 ± 7.1
Lp(a) (mg/dL)	38.9 ± 6.4	32.8 ± 4.8	24.1 ± 4.0
Total cholesterol/HDL-C ratio	6.4 ± 0.6	4.0 ± 0.2	3.9 ± 0.2
hsCRP	2.9 ± 0.6	2.3 ± 0.6	4.2 ± 2.0
Fasting glucose (mg/dL)	87.4 ± 3.2	86.7 ± 2.6	85.4 ± 1.8
Medication therapy (n, %)			
ACEI	3 (8.1%)	3 (8.1%)	0 (0%)
Angiotensin-II receptor blocker	1 (2.7%)	1 (2.7%)	0 (0%)
Anti-platelet drugs	4 (10.8%)	1 (2.7%)	0 (0%)
β-blockers	1 (2.7%)	0 (0%)	0 (0%)
Statins	37 (100%)	37 (100%)	0 (0%)
Ezetimibe	12 (32.4%)	4 (10.8%)	0 (0%)
LLT time (years)	11.3 ± 1.0	7.5 ± 1.0	-
Target LDL-cholesterol†	4 (10.8%)	17 (45.9%)	-

Table 9. Clinical characteristics of groups of patients from the SAFEHEART cohort. Values are given as mean ± SE. *Gene mutation (null/defective/unknown), †Target LDL-cholesterol (FH≤100mg/dL, non-FH≤130mg/dL). ACEI indicates angiotensin-converting-enzyme inhibitor, hsCRP, high-sensitive C-reactive protein.

(B) non-FH patients with adult secondary hypercholesterolemia and lipid-lowering treatment but with negative genetic testing, matched by age, gender, demographics, and treatment (*Papers 3-4*). For comparative purposes of cMP-microRNA study, a group of these non-FH patients that did not have an event within the same time frame (nCVE) were selected (*Paper 8*); and,

(C) control group of subjects from the same cohort with the same LDL-c levels that were not on LLT, matched by age, gender, and demographics (*Paper 2*) in order to study the effect of LLT on cMPs.

Sociodemographic data, lifestyle, medical and therapeutic data, current LLT and classical risk factors were obtained from all subjects using a standardized report form at the inclusion. Main clinical characteristics of the three groups are summarised in Table 9. Data related to LLT included statin, dose, time of treatment and compliance. Adherence to lipid-lowering treatment was assessed by indirect method with a single question.[589] Physical examination included weight, height, body mass index, waist circumference, and blood pressure. Patients were also classified depending on the known residual activity of the LDL receptor as null or defective mutations.

2.2. STEMI patients

A group of ST-elevation myocardial infarction (STEMI) patients (n=40) undergoing percutaneous coronary intervention (PCI) and thrombus aspiration at the Acute and Intensive Cardiac Care Unit in the Cardiology Department of Hospital Sant Pau were included for specific studies of cMP characterization of coronary and peripheral blood in patients with ongoing thrombosis (*Papers 5-6*). Primary PCI was performed according to guidelines.[590] All patients were admitted to the coronary unit with chest pain, persistent ST-segment elevation, cardiac troponin T (TnT) elevation, and/or regional wall motion abnormalities. All patients received standard heparin. Patients undergoing rescue PCI were excluded. Glycoprotein IIb/IIIa antagonist were administered at physician's discretion.

Peripheral blood from another group of post-STEMI patients at day 3 after admission (72 hours after the symptom onset) (n=20) from Santa Creu Sant Pau Hospital was also collected and compared to a group of healthy subjects without thrombosis (n=20). All groups were matched by age, gender, cardiovascular risk factors and, biochemical parameters. Similarly, pharmacological treatments have been also taken into account and matched when possible.

3. Blood sampling and clinical determinations

For microparticle analysis, venous blood was withdrawn from cubital vein without tourniquet using a 20-gauge needle after 10-14 hours of fasting into 3.8%-sodium citrate tubes, except when indicated. Samples have been stored deep-frozen (-80ºC) at the sample repository of the ICCC and maintained under continuous tracking and unthawed until they were used for analysis.

From the SAFEHEART cohort, information about baseline clinical characteristics were used for the analysis of the results and included:

- Genetic analysis of FH diagnosis with DNA-microarray (LIPOCHIP) in EDTA-samples.[591]

- Biochemical parameters in serum samples such as lipid profile (total cholesterol, HDL-c, triglycerides, by standardized enzymatic methods, and LDL-c calculated using Friedewäld formula)[592] and lipoprotein (a) using a turbidimetric method.

- Atherosclerotic plaque characterization by aortic MRI at the level of the descending thoracic aorta, determining plaque composition (lipidic and fibrocellular components and calcium deposits).[106] MRI has been widely used in the last decade to evaluate atherosclerosis burden in high-risk patients including FH patients.[593,594] Interestingly, the microparticle study was in blood collected from the FH-patients at the time of the MRI-analysis.

Clinical information about STEMI undergoing pPCI was also used including demographic, baseline characteristics and pharmacological treatment.

In addition, blood from non-smoking healthy voluntary donors, without any antiplatelet medication for 15 days prior to blood extraction, was drawn by a cubital venopuncture into tubes containing anticoagulant as needed and used for the flow cytometry characterization and functional experiments. Donors had given informed consent, and the study protocol was approved by the Clinical Research Committee of ICCC and was in accordance with the Declaration of Helsinki.

4. Microparticle isolation

4.1. Circulating microparticle isolation

Blood cells were removed from citrate-anticoagulated blood (except when indicated) by low-speed centrifugation (1258×g, 20 minutes [min] at RT) in order to avoid *in vitro* platelet activation. Platelet poor plasma (PPP) was carefully aspirated, leaving about 0.1 cm undisturbed layer on top of the cells. An equally second centrifugation step was made to ensure the complete removal of cells and obtain the PFP. All samples were processed identically and within 60 minutes after extraction. Samples were tested with a cell counter for the absence of residual cells after centrifugation.

For flow cytometric and functional experiments, the cMP-fraction was isolated and washed from PFP by a two-step high-speed centrifugation.[212,595,596] Briefly, frozen PFP aliquots were thawed on melting ice for 1 hour and centrifuged at 20000×g for 30 min to pellet cMPs. The supernatants were discarded and the cMP-enriched pellet was washed once with citrate-phosphate buffered saline solution (citrate-PBS; 1.4 mmol/L phosphate, 154 mmol/L NaCl, 10.9 mM trisodium citrate, pH 7.4) before a second equal centrifugation step was made. Finally, the remaining cMP-pellets were resuspended in citrate-PBS.

4.2. Platelet-derived microparticle isolation

Human platelets from fresh platelet concentrates were centrifuged (1200×g, 10 minutes, 20ºC), washed and resuspended in HEPES-Tyrode's buffer (HTB; containing 134 mM NaCl, 0.34 mM Na_2HPO_4, 2.9 mM KCl, 12 mM $NaHCO_3$, 1 mM $MgCl_2$, 5 mM $C_6H_{12}O_6$, 20 mM HEPES and 1 mM $CaCl_2$; pH 7.35). Platelet function was checked by optical aggregometry. Platelet count was adjusted to a final concentration of 4.0×10^6 platelets/µL (Medonic hematologic analyzer) and incubated at 37ºC for 5 minutes without stirring to allow spontaneous pMP release.[258]

Platelets were then separated by a double centrifugation step (3220×g, 10 minutes, 20ºC) and the final suspension contained only pMPs. Thereafter, pMPs from 10mL-aliquots were concentrated by ultracentrifugation (at 150000×g, for 90 minutes, 10ºC)[506] and resuspended in HTB containing 0.105M trisodium citrate (citrate-HTB).

Once obtained, aliquots of both MPs (cMPs and pMPs) were snap-frozen in liquid nitrogen and stored at -80ºC until experiments were performed.

5. Flow cytometry of circulating microparticles

Triple-label flow cytometric analysis was performed as described by Nieuwland et al with slight modifications.[212] A schematic figure of the procedure is shown in Figure 19. Briefly, washed cMP suspensions (5µL) diluted in 30µL PBS buffer containing 2.5mM $CaCl_2$ were incubated (20 min, RT, dark) with combinations of BD-horizon V450-conjugated annexin V (5µL) with two specific monoclonal antibodies (mAbs, 5µL each) labelled with fluorescein isothiocyanate and phycoerythrin, or with the isotype-matched control antibodies (Table 10) and, then, diluted with 2.5mM $CaCl_2$-PBS buffer before being analyzed on a Beckman Coulter EPICS XL flow cytometer with Expo32 ADC analysis software (*Papers 1,7*) or on a Becton Dickinson (BD)-FACSCantoII™ flow cytometer and with FACSDiva™ software (version 6.1.3) (*Papers 2-6*).

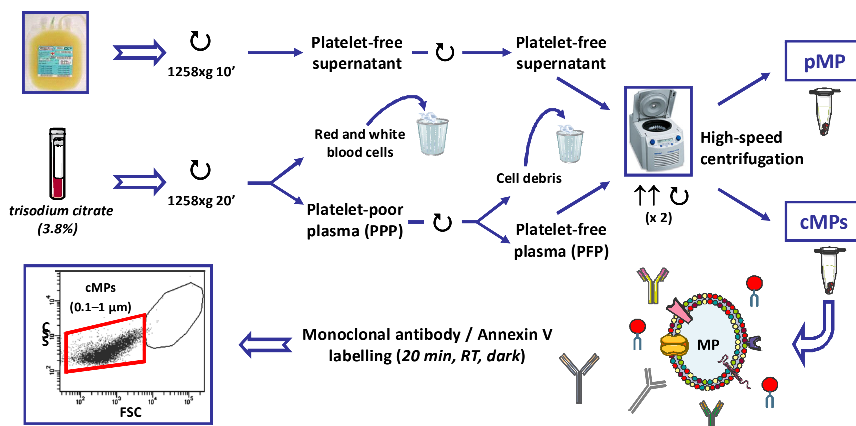

Figure 19. Workflow of microparticle flow cytometric analysis. Major steps for platelet-derived (pMP) and circulating microparticle (cMP) isolation and labelling in flow cytometry analysis. FSC indicates forward scatter; SSC, side scatter.

Acquisition was performed for 1 minute per sample. In flow cytometry technique, physical properties (size and internal complexity) and fluorescence characteristics of single particles are measured, as shown in Figure 20. Forward scatter (FSC), side scatters (SSC) and fluorescence data were obtained with gain settings in the logarithmic scale. cMPs were identified and quantified based on their FSC/SSC characteristics according to their size, binding to annexin V and reactivity to cell-specific mAb. Gate limits were established following two criteria: (1) calibration using a Flow Check YG Size Range Calibration Kit (Polysciences)[597] and (2) with an *in vitro* platelet-derived microparticle population as positive control,[342,596] since calibration beads have different properties of FSC/SSC compared with biologic MPs.[256,598] The lower detection limit was placed as a threshold above the electronic noise of our flow cytometer and a threshold was set at SSC parameter.

To identify positive marker events, thresholds were set based on samples incubated with the same final concentration of isotype-matched control antibodies after titration experiments. PS-positive cMPs were labelled using annexin V in the presence of 2.5mM $CaCl_2$, since calcium is essential for AV conjugation. Annexin V binding level was corrected for autofluorescence using fluorescence signals obtained with MPs in a calcium-free buffer.

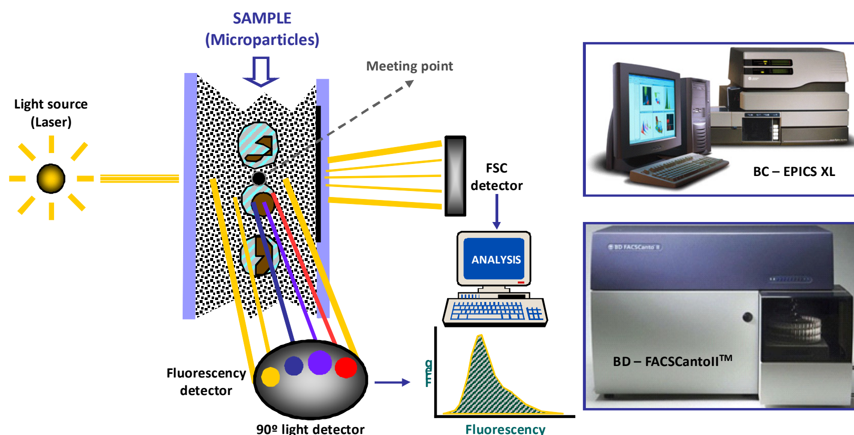

Figure 20. Flow cytometry principle. Detection of MPs is performed by passing them through the centre of a sheath flow. The combined flow is reduced in diameter, forcing one particle at a time into the centre of the stream and passing through one or several laser beams in the flow chamber. Forward-scattered (FSC) and side-scattered (SSC) lights and fluorescence signal emission were measured by detectors and photomultiplier tubes and digitized for computer analysis. Used flow cytometers were Beckman Coulter EPICS XL and Becton Dickinson FACSCantoII.

The MP concentration (number of cMPs per µL of plasma) was assessed by (1) comparison with calibrator FlowCount beads in a predetermined concentration (*Paper 1*) and (2) according to Nieuwland's procedure,[212] based on sample's volume, flow cytometer's flow rate and the number of fluorescence-positive events (N), as follows (*Papers 2-6*):

$$\left[\frac{cMPs}{\mu L}\right] = N \times \left(\frac{V_f}{V_a}\right) \times \left(\frac{V_t}{FR}\right) \times \left(\frac{1}{V_i}\right)$$

[V_f(µL) = final volume of washed cMP suspension, V_a(µL) = volume of washed cMP suspension used for each labelling analysis, V_t(µL) = total volume of cMP suspension before fluorescence-activated cell sorting analysis, FR(µL/min) = flow rate of the cytometer at low mode (the average volume of microparticle suspension analyzed in one minute), 1 is the µL unit of volume, and V_i(µL) = original volume of plasma used for MP isolation].

Flow rate was measured before each experiment. Intra-assay coefficient of variation (cv) of cMP counts was 3.1%, while inter-assay cv was 5.4%. To reduce background noise, buffers were prepared on the same day and filtered through 0.2µm pore size filters.

MPs mediate intercellular transfer of bioactive molecules such as surface receptors. The identification of cMP origins could be more complex if the resulting membrane fusion distributes cell-specific markers between MPs. We have overcome this complex interaction determining cMP cell source by triple-staining (with two different markers of the same parent cell and PS). Additionally, an overlapping biophysical interaction between protein complexes and MPs could affect the purity of isolated MP-preparations. However, we used direct immunolabelling and we centrifuged the antibodies before their use in order to avoid immune complex formation.

Marker	Expression	Conjugation	Clone	Paper
Annexin V	Widely expressed	BD Horizon V450 / PE	-	*1-6*
CD3	Lymphocytes	FITC	HIT3	*2,3,5,6*
CD11b	Neutrophils, monocytes	FITC	VIM12	*2,3,5,6*
CD14	Macrophages, monocytes	PE	M5E2	*2-6*
CD31	Platelets, ECs, leukocytes	PE	1F11	*1-3,5-6*
CD36	Widely expressed	PE	CB38	*1*
CD41	Platelets	FITC	SZ22	*1-6*
CD42b	Platelets	FITC	HIP1	*1,5*
CD45	Leukocytes	PE	Immu19.2	*1,2,3,5*
CD51	Platelets	FITC	AMF7	*1*
CD61	Platelets	PE	VI-PL2	*1-6*
CD62E	Activated ECs	PE	68-5H11	*6*
CD62P	Activated platelets	PE	AK-4	*1,2,4-6*
CD63	Widely expressed	PE	H5C6	*1*
CD66b	Granulocytes	FITC	G10F5	*6*
CD142	Widely expressed	FITC	VD8	*1,2,4,6*
CD146	Endothelial cells	FITC	P1H12	*2,3,5,6*
CD235a	Erythrocytes	FITC	11E4B-7-6	*5,6*
PAC1	Activated platelets	FITC	PAC1	*1,2,4,6*
TSP1	Platelets, megakaryocytes	PE	P10	*1,2,4,5*
IgG1γ	-	FITC / PE	X40	*1-6*
IgG1κ	-	FITC / PE	MPOC21	*1-6*

Table 10. Cell surface molecules for cMP identification and characterization. ECs indicates endothelial cells; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

6. Functional studies

6.1. Perfusion experiments

6.1.1. Experimental design

The effect of cMPs and pMPs on flow-induced platelet deposition was analyzed using the previously validated Badimon perfusion chamber[599,600] and the flat perfusion chamber.[601,602] The thrombogenicity of microparticles was assessed exposing: (a) porcine arterial wall to human blood enriched with cMPs/pMPs in the Badimon perfusion chamber with human ^{111}Indium–labelled platelets, (b) human arterial wall to human blood enriched with pMPs in the Badimon perfusion chamber by morphometric analysis, and (c) collagen type I surface to human blood enriched with pMPs in the flat perfusion chamber by confocal microscopy analysis.

For the perfusion experiments, blood was collected in sodium heparin (10 IU/mL),[600] kept at 20ºC, and used within 2 hours of collection. The hematologic parameters (platelet, leukocyte, and haematocrit counts) and platelet reactivity were very similar among donors and were within physiological ranges. Blood was then incubated at RT for 3 minutes with a suspension of MPs (adjusted to a final concentration of 6000/μL) or similar volume of citrate-HTB as a vehicle control. In the Badimon chamber perfusion experiments, platelets were labelled with ^{111}Indium-oxine, and in the flat chamber perfusion experiments, platelets were rendered fluorescent by the addition of (a) mepacrine (quinacrine dihydrochrolide, 10μM, Sigma) or (b) CellTracing Calcein green AM (1μg/mL, Invitrogen) to blood, before incubation with pMPs.

6.1.2. Radioactive labeling of human platelets

Platelets from healthy donors were isolated by differential centrifugations and labelled with ^{111}Indium-oxine (^{111}In) as described by Fernandez-Ortiz et al. with slight modifications.[603] Briefly, 17 mL of blood were collected into 3 mL of acid citrate dextrose (ACD) solution (38 mM citric acid, 85 mM trisodium citrate, 66.6 mM dextrose, pH 5.0). Platelets were isolated and washed by low speed centrifugation (400×g, 10 min), resuspended in ACD saline (14.4% ACD solution in saline, pH 6.50) and labelled with ^{111}In. An average of $1.34 \pm 0.26 \times 10^6$ ^{111}In-platelets were added to a final volume of 1 mL of autologous plasma and resuspended in 50 mL of fresh blood collected from the same donor. The average efficiency of the labeling procedure was 91.8±0.3% and the mean final activity was 5.9±0.07 μCi.

6.1.3. Badimon perfusion chamber

Badimon chamber is a perfusion system developed to investigate platelet interaction, platelet deposition and thrombus formation in flowing blood under controlled conditions. Specifically, it is a bioreactor that consists of a cylindrical flow channel (1 or 2-mm diameter, 2-cm length) that mimics the cylindrical shape typical of the vasculature and allows a broad range of pathophysiological flow conditions, ranging from laminar to nonparallel streamline flows, over an exposed thrombogenic surface, either biological or synthetic. Therefore, Badimon perfusion chamber enables to test the effect of blood elements (such as cholesterol and glucose levels) and rheology (different degrees of shear stress and stenosis) as well as atherosclerotic vessel components (e.g., collagen, fatty streaks, smooth muscle cells, etc) on thrombus formation in a controlled manner. Indeed, it also enables to evaluate the interaction of a given compound with the blood and vascular compartment such as the antiplatelet effects of antithrombotic drugs. In the present thesis we used Badimon perfusion chamber to investigate the potential effects of microparticles on platelet deposition and thrombus formation on atherosclerotic plaques under shear rate conditions encountered in the arteries and typical of areas of the atherosclerotic vessels.

Two types of aorta specimens were used in the studies. Pig aorta specimens were obtained fresh from local slaughterhouse and human aorta specimens from autopsy cases within 10-12h of death (unused tissues from an on-going study on sudden death), immediately washed in PBS, cleaned from adventitia, cut in long pieces and frozen at -80ºC unthawed until needed.

Before the experiments, the aortas were thawed in PBS at 4ºC, open longitudinally, and cut into 30x10mm segments. Segments of pig aorta were denuded (model of erosion). Human specimens composed of fatty streaks and atherosclerotic lesions (macroscopically characterized by raised yellow streaks and raised white or yellow-white plaques) were used as substrates for each experiment in a randomized fashion.

Aorta substrates were mounted in the previously characterized Badimon perfusion chamber (Figure 21).[77,600] After a preperfusion period of 60 seconds with PBS (37ºC), human blood was drawn into the chamber to perfuse over the human vessels at a constant flow rate of 10 mL/min to reach a shear rate of $1690s^{-1}$,[599] mimicking moderately stenotic coronary arteries. The selected flow rate gives theoretically a calculated average blood velocity of 21.2 cm/s and the shear conditions at the vessel wall were calculated from the expression for shear rate given for a Newtonian fluid in the tube flow. Perfusion period was 3 minutes, within the described time in which microparticles are cleared from circulation.[281] Finally, PBS was passed for 30 seconds to wash out the unattached cells.

The perfused segments were fixed in 4% paraformaldehyde and counted in a gamma counter for quantization of deposited platelets. After each sequence of perfusions, blood samples collected from each donor and experimental condition were evaluated for hematologic counts and platelet indium-release. The number of platelets deposited on each specimen was calculated from the indium activity on the perfusion area and normalised by blood ^{111}In activity, platelet counts in blood, and area of exposed surface.

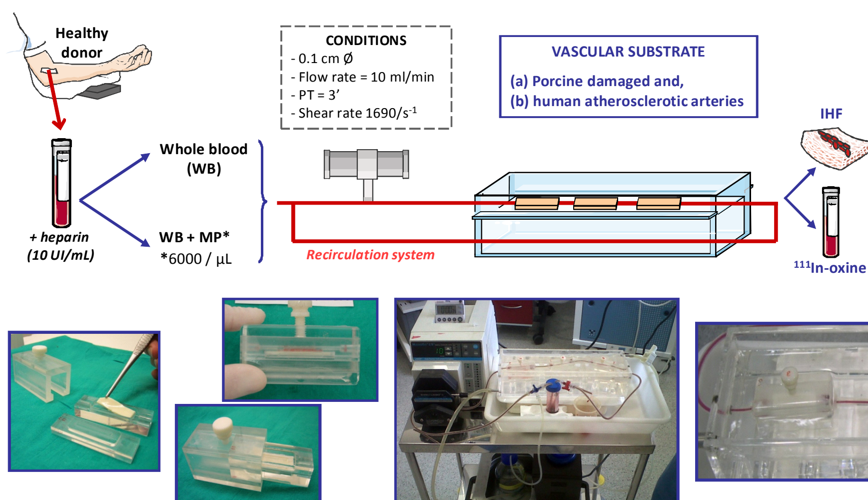

Figure 21. Badimon perfusion chamber. Schematic representation of Badimon perfusion experimental design and representative photographs of the substrate placement within the Badimon perfusion chamber and system. IHF indicates immunohistofluorescence; PT, perfusion time; WB, whole blood.

Immunohistology: Perfused and fixed aorta substrates were cryoprotected with 2.3M sucrose, frozen over dry ice and stored in OCT. 5µm-serial sections cut from the centreline of the vessel and longitudinal to the blood flow direction were analyzed. Atherosclerotic lesions were microscopically characterized by Masson's Trichromic staining. Figure 22 shows the double immunohistofluorescence analysis performed for platelet and fibrin identification in the aortic segments. Controls of antibody staining were used to test for non-specific binding.

Quantitative analysis: Fibrinogen and platelet deposition on the aortic segments were evaluated morphometrically by fluorescence microscopy (Olympus Vanox AHBT3) using fields at 20x magnification (Figure 22). Twenty serial images of the centreline segment of each substrate were taken with a digital Sony 3CCD (DXC-5500) camera and were systematically analyzed at 100-µm intervals using the Visilog 4.1.5 software. Platelet

interaction with the vessel wall was evaluated by both platelet adhesion and mean thrombus height.[604] Fibrin deposition was calculated by the thickness of the protein layer and the total area covering the substrate.

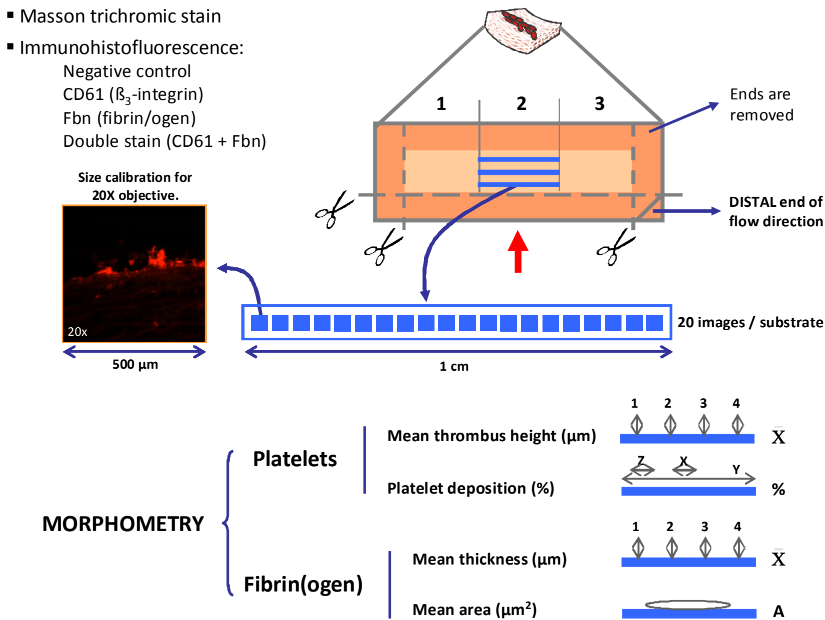

Figure 22. Immunohistofluorescence. Schematic representation of the immunohistofluorescence (IHF) and morphometric analysis of aortic substrates.

6.1.4. Flat perfusion chamber

Flat perfusion chamber allows the study of platelet deposition under well-defined shear stress in an exposed underlying extracellular matrix, which can be covered by distinct biomaterials, sprayed proteins or cell cultures. We performed additional flow chamber perfusion studies using collagen-coated plastic slides to assess the effect of platelet-derived microparticles on platelet-collagen interaction under high shear rate conditions.

For this purpose, glass slides were coated with type-I collagen (10 µg/mL, 4ºC, overnight) and placed in a flat chamber (Figure 23), as described.[601,602] Briefly, blood was circulated through the chamber at a constant shear rate (1500s^{-1}, 5 minutes). Then a wash was performed for one minute interval with HTB buffer. After blood perfusion, slides were carefully rinsed with PBS, fixed with 3.8% paraformaldehyde (15 minutes) and mounted on glass slides with Glycerol Mounting Medium and stored in the dark until analysis.

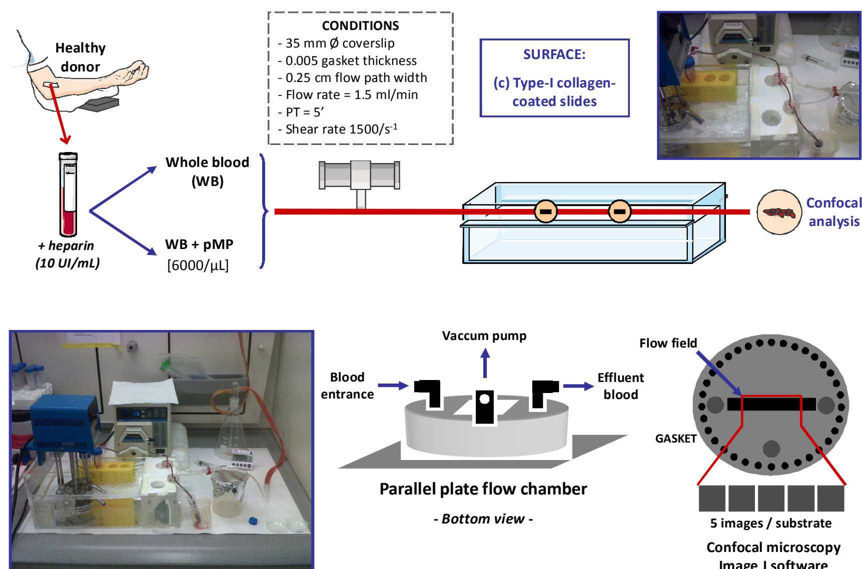

Figure 23. Flat perfusion chamber. Schematic representation of flat perfusion experimental design and platelet adhesion quantification using confocal microscopy and Image J software, and representative photographs of the technique. pMP indicates platelet-derived microparticles; PT, perfusion time; WB, whole blood.

<u>Imaging of platelet thrombi</u>: Platelet deposition on the collagen surface was analyzed with an inverted fluorescence confocal laser scanning microscope (Leica TCS SP2-AOBS).[601,602] 488nm-Ar-Kr and 633nm He-Ne lasers were used as light source. Platelets and pMPs were viewed with a HCX PL APO 20X/0.7 IMM CORR objective. Five fields along the adhesion surface were systematically acquired for total platelet deposition analysis, discarding the entrance and exit of the flow path. The surface covered by platelets was calculated using Image J and expressed as the area covered by platelets per analyzed field. Fluorescent images of platelet and pMPs were acquired in a scan format of 1024x1024 pixels in a spatial data set every 0.5 µm (xyz) and processed with the Leica Standard Software.[605] Thresholds were applied to distinguish adhered platelets and pMPs from the background, and the same values were used for analyzing all the stacks collected for a given experiment.

<u>Fluorescence labeling of platelet-derived microparticles</u>: For specific studies in the flat perfusion chamber in order to determine whether pMPs interfere with platelets during platelet adhesion, pMPs were labelled with 2.5 µmol/L fluorescence dye BODIPY™ 630/650-SE (Molecular Probes) for 20 minutes at RT in the dark, washed twice by centrifugation (20000×g, 30 minutes, RT), and then resuspended in citrate-HTB. Fluorescence-tagged pMPs were quantified by flow cytometry.

6.2. Thromboelastography (Whole blood clotting model)

Thrombus dynamics was analyzed in a thromboelastography system (Figure 24),[606] using extrinsically- and intrinsically-activated, and fibrin-based thromboelastometric assays (Ex-, In-, and Fib-TEM, respectively). The analyses were performed by adding 0.2M $CaCl_2$ to citrated whole blood in the presence of specific activators, depending on the test (rabbit brain thromboplastin as an activator for Ex-TEM assay, thromboplastin-phospholipid for In-TEM assay, and both extrinsic activation and addition of cytochalasin D to inhibit platelet contribution to the formation of the clot for Fib-TEM assay). All measurements were performed at 37ºC during 30 minutes directly after blood withdrawal. The following parameters were recorded for each test: clotting time (CT; seconds), maximum clot formation velocity time (MAXV-t; seconds), maximum clot firmness (MCF; mm), and the area under the velocity curve (AUC), as described by Sorensen et al.[607] Standard calibration of ROTEM device was performed using a control serum (ROTROL) according to the manufacturer's recommendation. All reagents were purchased from Pentapharm GmbH.

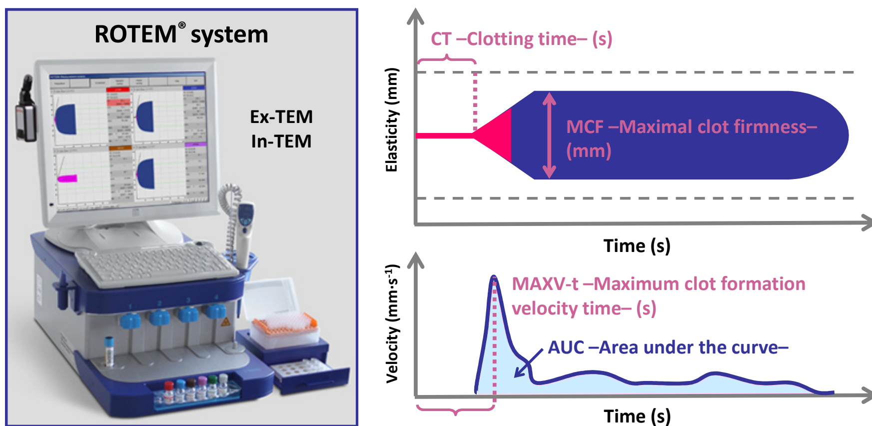

Figure 24. Thromboelastometry. ROTEM system and schematic representation of the thromboelastrograph tracing showing parameters of the dynamics of clot development recorded by thromboelastometry (TEM): clotting time (CT, seconds) corresponds to the time from the beginning of the reaction to an increase in amplitude of 2 mm; maximal clot firmness (MCF, mm) is the maximum amplitude reached; maximum clot formation velocity time (MAXV-t, seconds) is the time to reaction start to reach maximum velocity; and area under the curve (AUC) the area under the velocity curve (the first derivative of the clot curve ending at a time point that corresponds to MCF). CT indicates the initiation phase of the clotting process and, MCF and AUC quantify the maximum clot firmness of the established clot and correlate with the platelet count and function as well as with the concentration of fibrinogen.

6.3. Platelet function tests

The ability of various agonists to induce *in vitro* activation and platelet-to-platelet activation was measured by two methodologies: (1) platelet function analyser system and (2) platelet aggregation testing.

6.3.1. Capillary closure time

Platelet reactivity induced by (a) collagen-epinephrine and (b) collagen-adenosine diphosphate was determined using a Platelet Function Analyser (PFA-100) as shown in Figure 25. Briefly, citrate-anticoagulated blood was incubated with various concentrations of pMPs (0, 2500, 5000, 7500, and 10000 pMPs/µL) resuspended in citrate-HTB for 3 minutes, and with 2500 pMPs/µL at distinct time periods (3, 5, and 10 minutes), before closure time determination.

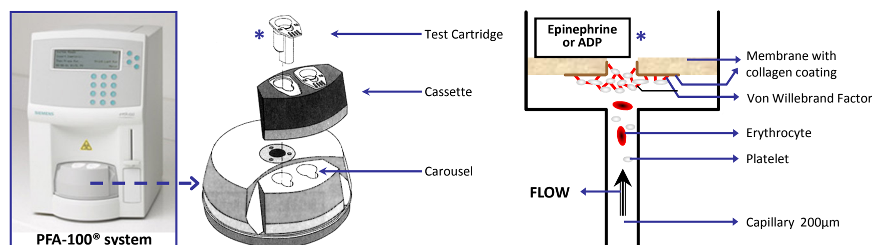

Figure 25. Platelet function analyzer-100 system. Capillary closure time was measured with PFA-100 system with collagen/epinephrine and collagen/ADP cartridges. Schemes adapted from Dade Behring Siemens.

6.3.2. Light transmission aggregometry

Platelet aggregation was analyzed in samples incubated with 6000 pMPs/µL for different periods of time and challenged with four agonists: thrombin (0.2, 0.3 uNIH/mL), collagen (1, 3 µg/mL), ADP (1, 3 µM), and epinephrine (3, 5 µM), as previously reported.[608] Maximal platelet aggregation was measured for 15 minutes after addition of the agonist in all cases. The extent of platelet aggregation was defined as the percentage change in optical density as measured by the optical aggregometer (Figure 26).

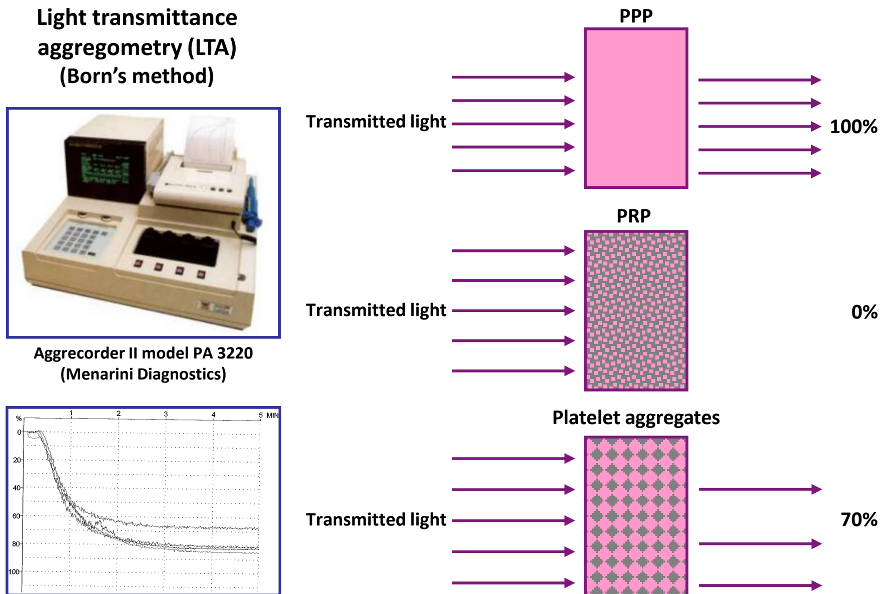

Figure 26. Light transmission aggregometry principle. The aggregometer works on the basic principle of light transmission (Born's method). As platelets aggregate, the light transmission of the sample increases. Classically aggregometry uses platelet rich plasma (PRP), which is stirred in a cuvette at 37°C and the cuvette sits between a light course and a photocell. The system tracks the changes in transparency of the PRP sample once an agonist is added to the tube. Platelet poor plasma (PPP) from each subject's own blood is used to set a reference value.

7. Differential proteomics studies

Analysis of differential protein expression patterns and protein identification of platelet-derived subfractions from healthy donors was performed by a proteomic approach. Platelet-derived microparticle (pMP), platelet *releasate* (SPN) and platelet membrane (Mbs) samples from thrombin-activated platelets were compared to samples from resting platelets using two-dimensional electrophoresis (2-DE), or one-dimensional electrophoresis (1-DE), and mass-spectrometry identification (*Paper 7*).

7.1. Platelet subfractionation

Human platelets from fresh healthy donor platelet concentrates were centrifuged (1200 ×g, 10 minutes, 20ºC), washed 3 times and resuspended in Ca^{2+}-free HTB. Platelet function was determined by optical aggregometry. Washed platelets were counted and adjusted to a final concentration of $4.0x10^6$ platelets/µL (Medonic CA530-16 hematologic analyzer). The platelet suspension was pretreated with simvastatin (100 µM –S–) or similar volume of its buffer for 15 minutes at 37ºC and, then, it was also activated with human thrombin (0.5 uNIH/mL –T–) or its buffer (controls –C–) for 3 minutes at 37ºC with constant slow stirring. Immediately thereafter, one aliquot of platelets was taken for flow cytometry analysis and 2.5 mM Gly-Pro-Arg-Pro was added to all activated aliquots in order to avoid platelet aggregation while preserving activation. Platelets were pelleted by a centrifugation step (3220 ×g, 10 minutes, 20ºC) and platelet supernatant was reserved for platelet secretion analysis.

Platelet subfractionation was performed as described[609,610] with slight modifications. Briefly, centrifuged platelets were resuspended in isolation medium (0.25 M sucrose, 10 mM Tris-chloride, 1mM EDTA, and protease inhibitor cocktail; pH 7.4). After homogenate sonication, differential centrifugation was performed: (1) 1000 ×g for 22 min at 4ºC to discard the large platelet fragments, (2) 12000 ×g for 20 min at 4ºC for mitochondria and granules pellet obtention, and (3) 100000 ×g for 60 min at 4ºC to isolate plasma and intracellular membrane pellet. Each mitochondria and granule pellet was resuspended in the sucrose isolation medium and further fractionated on a sucrose density step gradient (diluted in 20mM Hepes, 1mM EDTA, pH 7.2) that increased from 0.8 to 2.0M (in 0.2 M increments) by ultracentrifugation (100000 ×g, 60 minutes, 4ºC) with a SW41Ti rotor (Beckman Coulter). Six bands were obtained and subfractions of dense and α-granules were collected and further combined as granule-enriched fraction.

Supernatants were analyzed by flow cytometry for pMP characterization. Presence of residual platelets was excluded by centrifuging platelet supernatant once more (5000 ×g, 10 minutes, 20ºC). The final supernatant contained only MPs (particles less than approximately 1.0 µm). MPs were isolated by ultracentrifugation (150000 ×g, 90 minutes, 10ºC) with a 50.2Ti rotor (Beckman Coulter)[572] and the final supernatant (soluble *releasate*) was filtered and concentrated 1:30 with 10-kDa centrifugal filter devices (Millipore) and precipitated with seven volumes of acetone.

7.2. Sample preparation

Protein extraction: The final membrane-, granule-, pMP- and supernatant-pellets were resuspended in lysis buffer containing protease inhibitors (10mM Tris/HCl, 0.15M KCl, 0.1% Triton X-100, 2.9mM PMSF, 0.1mM DTT, 1μg/mL Leupeptin, 1μg/mL Aprotinin, pH 7.4) and aliquots were snap-frozen in liquid nitrogen and stored at -80ºC until western blotting and/or proteomic studies were performed.

High-abundant proteins and salt removal: pMP- and SPN-samples were subjected to IgG removal with protein G sepharose (GE Healthcare) in order to increase protein resolution and detection power. Then, all the samples were cleaned by centrifugation using 3kDa centrifugal filter devices (Millipore) and sample buffer was exchange to a urea denaturing buffer (8mol/L urea, 2mol/L thiourea, 2% w/v CHAPS). Prior protein separation, samples were desalted and decontaminated (from ionic detergents, nucleic acids, lipids, salts) by a commercial kit (ReadyPrep 2D-CleanUp Kit, Bio-Rad), following supplier's manual.

Protein quantification: Protein concentration was determined in triplicate using Quant Kit protein assay reagents (GE Healthcare).

7.3. Protein separation

7.3.1. One-dimension electrophoresis (1-DE)

For 1-DE experiments, proteins extracts were separated in 12% sodium dodecyl sulfatepolyacrylamide (SDS-PAGE) electrophoresis gels, as originally described by Laemmli.[611] Thereafter, gels were fixed with 40% ethanol, stained with a 0.5% Coomassie Brilliant Dye solution, scanned and visualized with a scanner for the visible spectrum (GS800, BioRad).

7.3.2. Bidimensional electrophoresis (2-DE)

For 2-DE analysis, analytical and preparative gels were prepared (Figure 27).[612] A protein load of 120 μg and 300 μg protein of the urea/chaps extracts were diluted in rehydratation solution (7mol/L urea, 2mol/L thiourea, 2% w/v CHAPS, 100 mmol/L DTT, and 0.2% carrier ampholytes) and applied to 17-cm dry strips (pH 3-10 linear range; Bio-Rad) by active rehydratation at 50 V during 16 hours. Proteins were separated according to their isoelectric point (pI) by electrofocusing (0.05 mA/strip, 70 kV/h at 20ºC) using the Protean-IEF cell (BioRad).

Once completed the strips were equilibrated with a reducing solution (50 mM Tris-HCl buffer, pH 8.8, containing 6 M urea, 2% SDS, 30% glycerol, and 2% DTT) and an alkylating solution (50 mM Tris-HCl buffer pH 8.8, 6 M urea, 2% SDS, 30% glycerol, and 2.5% iodoacetamide), for 15 minutes each. Second dimension was resolved in 12% SDS-PAGE electrophoresis gels using an Ettan Dalt Six system (GE Healthcare) at 17w/gel for analytical gels or a Protean system (Bio-Rad) at 40 mA/gel for preparative gels. For each independent experiment, 2-DE for protein extracts from samples derived from control and thrombin-activated and/or simvastatin-pretreated platelets were processed in parallel to guarantee a maximum of comparability. The gels were developed by fluorescent staining (Flamingo Fluorescent Gel Stain; Bio-Rad) using a Typhoon 9400 scanner (GE-HealthCare, Uppsala, Sweden), as shown in Figure 25.

7.4. Differential proteomic analysis

Analysis of differences in protein patterns was performed with between control, thrombin-stimulated and/or simvastatin-treated samples PD-Quest 8.0 software (BioRad) by the use of a single master that included all gels of each independent experiment (Figure 25).[612] Each spot was assigned a relative value that corresponded to the single spot volume compared with the volume of all spots in the gel, after background extraction and normalization between gels. Normalization between gels was based on local regression model (LOESS).

7.5. MS identification

Protein bands (1-DE) or spots of interest (2-DE) were excised from gels and analyzed by (a) LC/MS/MS using a nano-HPLC and a triple-quadrupole associated to a lineal-trap mass spectrometer (Q-TRAP3200) or (b) MALDI-TOF/TOF mass spectrometry for protein identification, respectively, after in-gel tryptic digestion and extraction of peptides from the gel pieces, as previously described (Figure 27).[612]

In order to identify proteins by matrix-assisted laser desorption/ionization time-of flight (MALDI-TOF) with an AutoFlex III Smartbeam MALDI-TOF/TOF (Bruker Daltonics), samples were applied to Prespotted AnchorChip plates surrounding the calibrants provided on the plates (Bruker Daltonics). Spectra were acquired with flexControl on reflector mode (mass range 850–4000 m/z, reflector 1: 21.06 kV; reflector 2: 9.77 kV; ion source 1 voltage: 19 kV; ion source 2: 16.5 kV; detection gain 2.373) with an average of 3500 added shots at a frequency of 200 Hz. Each sample was processed

with flexAnalysis (version 3.0, Bruker Daltonics) considering a signal-to-noise ratio over 3, applying statistical calibration, and eliminating background peaks.

For identification, peaks between 850 and 1000 m/z were not considered because in general only matrix peaks are visible on this mass range. After processing, spectra were sent to the interface BioTools (version 3.2; Bruker Daltonics) and MASCOT search on Swiss-Prot 57.15 database was done (taxonomy: homo sapiens, mass tolerance 50–100, up to 2 trypsin miss cleavages, global modification: carbamidomethyl [C], variable modification: oxidation [M]). Identification was accepted with a score greater than 56 by peptide mass fingerprint and confirmed by tandem mass spectrometry.

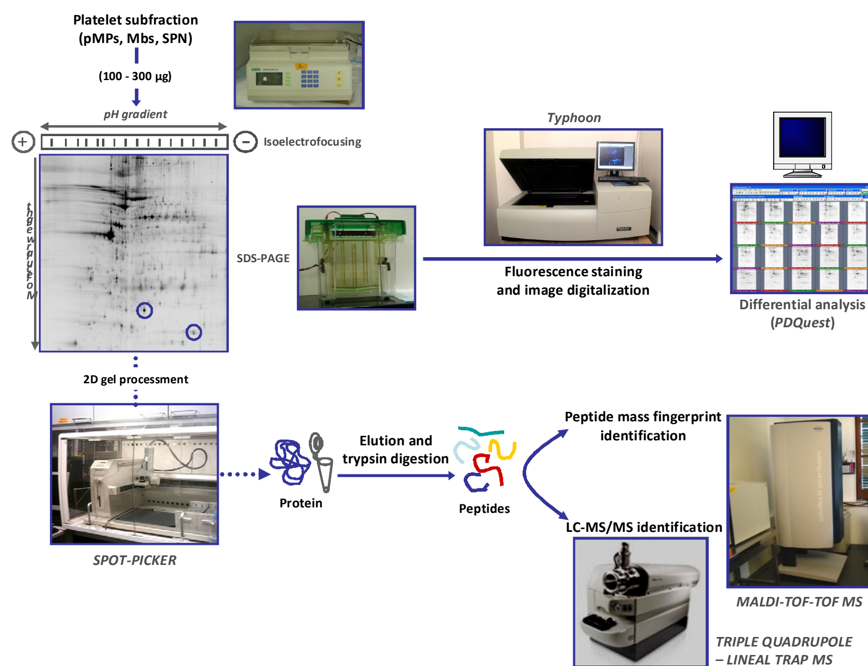

Figure 27. Proteomic analysis flowchart.[612] Detailed sequence of two-dimensional gel electrophoresis, protein differential analysis and protein identification by mass spectrometry.

8. microRNA analysis

The circulating microvesicle-associated miRNA signature profiling was studied in plasma samples of familial hypercholesterolemic patients.

8.1. Microvesicle isolation and RNA extraction

miRNAs were measured from total RNA fraction contained in microvesicles using the Exo-MiR extraction kit (Bioo Scientific), specifically designed to isolate RNA from the microvesicle (microparticle and exosome) fractions of the sample, according to the manufacturer. Briefly, microparticles and exosomes were obtained by size exclusion filtration from EDTA-plasma samples thawed in melting ice. Briefly, a clarifying pre-spin was previously performed in order to ensure cell and lipoprotein depletion (Figure 28-1). Then, plasma samples were diluted and pushed through two different sized filters provided in the kit (Figure 28-2,3): the first MP-specific sized filter (>200 nm) retains the larger microvesicles, while the second filter captures the smaller exosomes sized between 20-200nm (Figure 28-4). Total RNA was isolated and extracted from the filter-trapped MVs by eluting the sample off the filters with the lysis solution (Figure 28-5). RNA was obtained with an organic extraction with chloroform and (Figure 28-6,7) and isopropanol precipitation as described by the providers. Purification of RNA was performed by ethanol-based wash solutions and centrifugation steps and the final pellet was resuspended in RNAse-free water (Figure 28-8). Finally, total RNA was quantified with Nanodrop spectrophotometer and used for further analysis (Figure 28-9,10). All samples were spiked-in with 25 fmol/µL of *Caenorhabditis elegans* miR-39 prior to RNA extraction for normalization.

8.2. miRNA profiling

For microRNA profile analysis, total RNA from plasma MVs was analysed using the low-density TaqMan® Array Human MicroRNA A Card v2.0 according to the manufacturer's protocol. This array card set enables assaying 377 most relevant specific human microRNAs, aligned with Sanger miRBase v20 database. Seven control miRNAs were also included. Briefly, miRNA screening was performed by reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis from total RNA samples (input of 100 ng) that were reversely transcribed to cDNA with MegaPlex reverse transcription primers pool A followed by a pre-amplification step using MegaPlex PreAmp Primer Pool Set v2.0 under the thermal-cycling conditions provided in Table 11. Real-time PCR amplification of miRNAs using low-density TaqMan Arrays was performed on an Applied Biosystem 7900HT system using SDS software v2.4. Assays were median normalized.

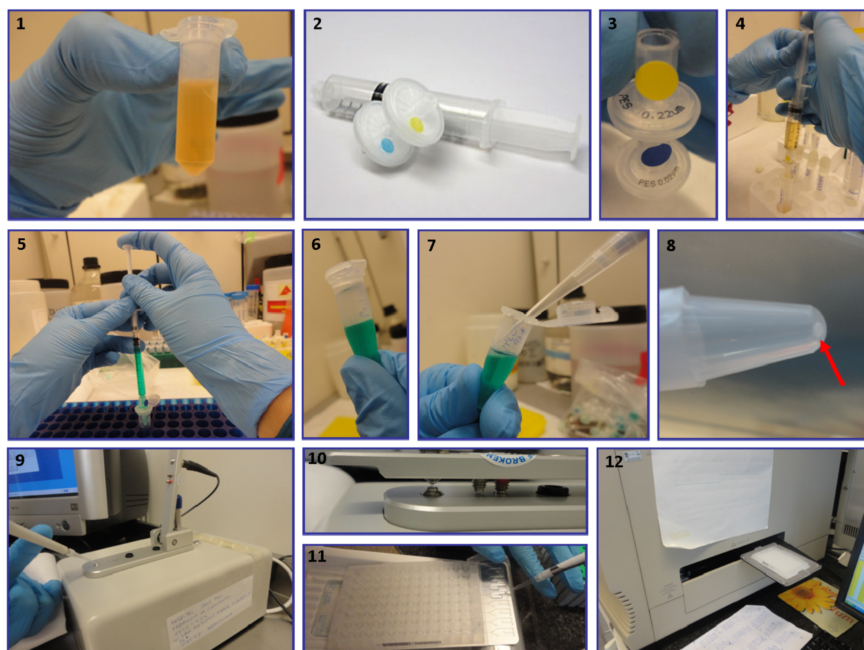

Figure 28. Microvesicle-associated miRNA extraction and analysis workflow. (1) Plasma centrifugation; **(2)(3)** Exo-MiR kit-based filters; **(4)** plasma passing through filters; **(5)** lysis buffer passing through filter-trapped MVs; **(6)(7)** aqueous phase of organic extraction; **(8)** RNA final pellet; **(9)(10)** RNA quantification with Nanodrop; **(11)** loading of diluted pre-amplified samples into TaqMan array miRNA cards; and **(12)** running them to Applied Biosystem 7900HT system.

	STEP	TIME	TEMPERATURE
Reverse transcription	Cycles (40 cycles)	2 min	16ºC
		1 min	42ºC
		1 sec	50ºC
	Hold	5 min	85ºC
	Hold	∞	4ºC
Preamplification	Hold	10 min	95ºC
	Hold	2 min	55ºC
	Hold	2 min	72ºC
	Cycles	15 sec	95ºC
	(12 cycles)	4 min	60ºC
	Hold*	10 min	99.9ºC
	Hold	∞	4ºC
PCR	Hold	10 min	95ºC
	Cycle	15 sec	95ºC
	(40 cycles)	60 sec	60ºC

Table 11. Thermal cycling conditions of preamplification and RT-qPCR of Megaplex TaqMan Array MicroRNA Cards. * Required for enzyme inactivation.

8.3. miRNA validation

For detection and quantification of selected miRNAs and cel-miR-39 spike-in, Custom TaqMan Array MicroRNA Cards as well as single Taqman microRNA assays (Applied Biosystems) were used. Single-stranded cDNA was synthesized using the multi-scribe reverse transcriptase kit and custom reverse transcription primer pool specific to the miRNAs being assessed. cDNA was preamplified with TaqMan PreAmp MasterMix, and then specific miRNAs were measured by quantitative PCR using either specific human TaqMan miRNA Assays or Custom TaqMan Array MicroRNA cards (Life Technologies) on an Applied Biosystem 7900HT system using SDS software v2.4 (Figure 28-11,12). All thermal-cycling conditions for these processes are listed in Table 12.

8.4. miRNA data analysis

A RQ Study was set up to review the amplification plots, analyze comparative cycle thresholds (Ct) and to adjust when necessary the baseline and the threshold settings using RQ Manager software v1.2.1. Ct values were normalized to cel-miR-39 by the formula $2^{-(Ct\ [miRNA]\ -\ Ct[cel\text{-}miR\text{-}39])}$ with DataAssist software v3.01. Ct values ≥ 35 were considered as undetermined. This spike-in method of normalisation was chosen because the frequently used normalisers are not expressed in cMVs.

	STEP	TIME	TEMPERATURE
Reverse transcription	Hold	30 min	16ºC
	Hold	30 min	42ºC
	Hold	5 min	85ºC
	Hold	∞	4ºC
Preamplification	Hold	10 min	95ºC
	Hold	2 min	55ºC
	Hold	2 min	72ºC
	Cycles	15 sec	95ºC
	(12 cycles)	4 min	60ºC
	Hold*	10 min	99.9ºC
	Hold	∞	4ºC
PCR	Hold	10 min	95ºC
	Cycle	15 sec	95ºC
	(40 cycles)	60 sec	60ºC

Table 12. Thermal cycling conditions of preamplification and RT-qPCR of custom TaqMan Array MicroRNA Cards. * Required for enzyme inactivation.

9. Validation techniques

9.1. MP-TF activity assay

TF-bearing microparticle procoagulant activity (PCA) was measured using a functional assay (Zymuphen MP-TF, Hyphen Biomed) with an automated microplate washer device (Revelation-Dsx 5.19, Dynex). Briefly, TF^{+}-cMPs from citrated-PFP were captured by a murine-MoAb directed against the extracellular domain of TF, as shown in Figure 29. Following overnight incubation and a washing step, FVIIa and FX were added into the reaction mixture. TF-FVIIa complexes form and convert FX into the active protease FXa in the presence of Ca^{2+}. Then a FXa-specific substrate was added and absorbance was recorded at 405nm. A lyophilized calibrator, containing recombinant relipidated TF with synthetic lyposomes, enabled the standardization of the assay. MP-TF concentration was established using an internal standard and expressed as TF antigen equivalent in pg/mL. Measurements were done in duplicate.

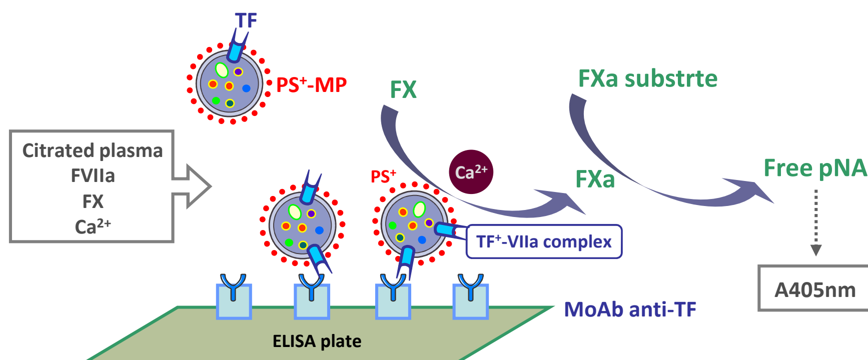

Figure 29. Microparticle-associated tissue factor activity.

9.2. Western Blot

Protein extracts were quantified by a protein assay kit based on bicinchoninic acid (BCA) (Pierce Protein Assay kit). Defined protein quantities (25µg) were separated under reduction and non-reduction conditions in SDS-PAGE. Briefly, of protein extract were mixed with loading buffer (0.25M Tris pH 6.8, 8% SDS, 40% Glycerol, 0.02% bromphenol blue with/without 400mM mercaptoethanol) and incubated at 95ºC for 5 minutes before being loaded into the SDS-polyacrylamide gels (4% stacking gel and 10% running gel). Separated proteins were transferred to nitrocellulose membranes using a semi-dry transfer system (BioRad). To confirm that proteins were correctly

transferred, membranes were stained with Ponceau solution (0.1M Tris, 1M NaCl, 0.05% Tween-20, pH 7.4). Membranes were blocked to avoid unspecific binding of antibodies. Primary monoclonal antibodies and horseradish peroxidise-conjugated secondary antibodies were used. Detection of protein bands was achieved by an enhanced-chemiluminescence system using a peroxidase enzymatic reaction (Supersignal, Pierce) and images were obtained with a ChemiDoc™ XRS system. Bands intensities were quantified by densitometry using Image Lab software (Bio-Rad). For normalization of results, total protein was used as loading control.

10. *In silico* bioinformatic analyses

Data mining

In the proteomic analysis, GO Slim / MGI (Mouse Genome Informatics) software was used for gene ontology (GO) assignments to identify proteins and determine significantly under-and-over-represented functional GO categories: on the basis of cellular component, molecular function and biological process categories. The annotations and analyses were made using the default MGI human database and the GO cell component, GO molecular function and GO biological process ontology. Signalling pathways were investigated using the Kyoto Encyclopedia of Genes and Genome (KEGG) database and with Phanter software. Group of pMP identified proteins was also compared to Vesiclepedia and ExoCarta databases in order to better define our microparticle fraction.

Ingenuity Pathway Analysis (IPA)

The statistically significant neural networks in which the identified proteins were involved were also generated through the use of ingenuity pathway analysis (Ingenuity Systems, www. ingenuity.com). The functional analysis of a network identified the biological functions and/or diseases that were most significant to the molecules in the network in the Ingenuity Knowledge Base.

miRNA-mRNA target databases

In the miRNA profiling, several web databases and algorithms of miRNA target prediction (PicTar, TargetScan, miRDB, DIANA–MicroT CDS, microRNA.org) were used for the search of miRNAs targeting specific genes and the genes that are targeted by specific miRNAs.

11. Statistical analyses

All data are presented as either median (interquartile range) or mean ± SE. For studies on the phenotypic characterization of cMPs from patients (*papers 2-6)*, sample size was calculated in basis of sample variability analysis to provide sufficient statistical power for group comparisons. In these studies, an initial descriptive analysis was provided using number of cases and percentages for qualitative variables and median and interquartile range for quantitative variables. For all studies, frequencies of qualitative variables (such as risk factors and medications) were compared between groups by using the Chi-square analysis. Mean values of quantitative variables were compared with two-sided parametric tests. The statistical significances for differences between two groups were determined with unpaired Student T-test and multiple comparisons by analysis of variance (uni- or multivariable ANOVA). In cases that Kolmogorov-Smirnov test showed that the data were not normally distributed, median values were compared with two-sided nonparametric tests. Then statistical significances between two groups were determined with U-Mann Whitney and multiple comparisons by Kruskal Wallis. When significant, Fisher's PSLD (parametric) or Bonferroni (non-parametric) post-hoc analysis were used to assess intergroup differences. Simple linear regression (parametric) or Spearman's rank correlation coefficients (non-parametric) were calculated to determine the strength of the association between continuous variables. The statistical significances between paired conditions such as intracoronary and peripheral blood (*paper 5-6*) and pre- and post-perfusion levels (*paper 5*) were determined with the non-parametric Wilcoxon Signed Rank Test. StatView (5.0.1, Abacus Concepts) was used for all statistical tests and a $P<0.05$ was considered statistically significant.

To evaluate the prognostic value provided by cMPs, an associated receiver operating characteristic (ROC) curve analysis for predicted probabilities was generated and the corresponding area under the curve (AUC) along with its 95% CI was calculated. Cut-off levels of MPs were determined with the shortest distance from upper left corner of the ROC curve (minimizing $[(1-\text{sensitivity})^2 + (1-\text{specificity})^2]$. To evaluate combination of prognostic markers, a binary logistic regression model with cMP levels was carried out to estimate the likelihood of a lipidic plaque by creating the predicted probabilities before ROC curve analyses. SPSS Statistics Version 21.0.0 (21.0.0, SPSS, Chicago) was used for c-statistics analyses and a $P<0.05$ was considered statistically significant.

Regarding the functional analysis of networks, the right-tailed Fisher exact test was used to calculate a P value determining the probability that each biological function assigned to that network is due to chance alone.

IV. RESULTS

Article 1

Circulating and platelet-derived microparticles in human blood enhance thrombosis on atherosclerotic plaques

Rosa Suades, Teresa Padró, Gemma Vilahur, Lina Badimon

Published – Thromb Haemost 2012; 108(6):1208-1219.

Objective

The aim of this study was to investigate whether an increased number of circulating microparticles and platelet-derived microparticles could functionally contribute to blood thrombogenicity on areas of arterial damage.

Highlights

- Circulating microparticles play key roles on blood thrombogenicity due to an enhancing effect on platelet function and aggregation, and coagulation.
- Increased number of circulating microparticles in human blood enhance platelet deposition and thrombus formation on arterial vessel wall with vascular injury under controlled flow conditions.
- Human blood platelet-derived microparticles increase platelet and fibrin deposition under high shear rate on human complex atherosclerotic lesions and to purified collagen surfaces, where they can bind and localize within the growin platelet thrombi.

Suades R, Padró T, Vilahur G, Badimon L. Circulating and platelet-derived microparticles in human blood enhance thrombosis on atherosclerotic plaques. Thromb Haemost. 2012; 108(6):1208-19.

doi: 10.1160/TH12-07-0486

Suades R, Padró T, Alonso R, Mata P, Badimon L. Lipid-lowering therapy with statins reduces microparticles shedding from endothelium, platelets and inflammatory cells. Thromb Haemost. 2013; 110 (2):366-77.

doi: 10.1160/TH13-03-0238

Suades R, Padró T, Alonso R, López-Miranda J, Mata P, Badimon L. Circulating $CD45^{+}/CD3^{+}$ lymphocyte-derived microparticles map lípido-rich atherosclerotic plaques in FH patients. Thromb Haemost. 2013; 111(1):111-21.

doi: 10.1160/TH13-07-0612

Article 4

High levels of circulating TSP1^{+}/CD142^{+} microparticles and low microparticle-packed miR-143 characterize young patients with high cardiovascular risk and subclinical atherosclerosis

Rosa Suades, Teresa Padró, Rodrigo Alonso, Pedro Mata, Lina Badimon

Manuscript under revision.

Objectives

The aim of this study was to investigate whether patients with high cardiovascular risk and lipid-rich atherosclerotic lesions display cMPs with a prothrombotic phenotype, regarding microRNA-143 and activated platelet and tissue factor protein cargos.

Highlights

- HCVR-patients with atherosclerotic plaque burden have a significantly higher number of cMPs carrying markers of cell activation and tissue factor that is biologically active than controls.
- Prothrombotic cMP numbers and their miRNA-143 content identify subclinical lipid-rich atherosclerotic lesions.
- Increased prothrombotic-cMP release and a decreased MP-miRNA-143 as biomarkers of silent atherothrombotic disease might help to predict cardiovascular risk.

Suades R, Padró T, Alonso R, Mata P, Badimon L. High levels of TSP1+/CD142+ platelet-derived microparticles characterise young patients with high cardiovascular risk and subclinical atherosclerosis. Thromb Haemost. 2015 Nov 25;114(6):1310-21. doi: 10.1160/TH15-04-0325.

Article 5

Shifts in circulating microparticles during arterial thrombosis: Increase in glycophorin A ($CD235a^+$)-rich microparticles in peripheral and coronary blood in STEMI patients

Rosa Suades, Teresa Padró, Gemma Vilahur, Victoria Martin-Yuste, Manel Sabaté, Jordi Sans-Roselló, Alessandro Sionis, Lina Badimon

Manuscript under revision.

Objectives

The aim of this study was to investigate whether high shear rate and the degree of thrombogenicity of vascular lesions trigger the release of a determined circulating microparticle phenotype and the relevance of these cMPs in patients with acute coronary thrombosis.

Highlights

- Total cMP shedding is increased after thrombosis elicited by high shear and thrombogenic lesions, conditions that mimic stenotic coronary blood flow on damaged vascular wall.
- The phenotype of microparticles released by thrombi growing on substrates with different thrombogenic potential at a high shear stress triggers change towards a high levels of erythrocyte-derived microparticles into the circulation.
- Measuring glycophorin A-rich erythrocyte MPs could be a method of detection of ongoing thrombosis.

Shifts in circulating microparticles during arterial thrombosis: Increase in glycophorin A ($CD235a^{+}$)-rich microparticles in peripheral and coronary blood in STEMI patients

Suades et al: Arterial thrombosis-induced microparticles

By:

Rosa Suades[1], Teresa Padró[1], Gemma Vilahur[1], Victoria Martin-Yuste[2], Manel Sabaté[2], Jordi Sans-Roselló[3], Alessandro Sionis[3], Lina Badimon[1,4]

[1] Cardiovascular Research Center, IIB-Sant Pau, Barcelona, Spain

[2] Department of Interventional Cardiology, Hospital Clinic, Barcelona, Spain

[3] Acute and Intensive Cardiac Care Unit, Cardiology Department, Hospital Santa Creu i Sant Pau, Biomedical Research Institute Sant Pau (IIB Sant Pau), Barcelona, Spain

[4] Cardiovascular Research Chair, UAB, Barcelona, Spain

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Correspondence to:

Prof. Lina Badimon
Cardiovascular Research Center,
c/Sant Antoni Mª Claret 167, 08025 Barcelona, Spain.
Phone: +34.935565880.
Fax: +34.935565559.
E-mail: lbadimon@csic-iccc.org

Abstract

Aims. The combined immediate effect of shear stress (SS) and type of exposed atherosclerotic vascular lesion regulates coronary thrombus formation. Activated cells of the attached thrombi release microparticles to the circulation (cMPs); however, their phenotype is unknown. Our goal was to investigate whether SS and the degree of thrombogenicity of vascular lesions triggers the release of a determined cMP phenotype.

Methods and Results. This study investigated flow- and arterial wall-induced thrombosis and platelet adhesion in well-characterized perfusion chambers. cMPs in resting blood before and immediately after substrate perfusion were characterized by triple-labelling flow cytometry. cMPs from blood collected in ACS-STEMI-patients were afterwards investigated to validate the *ex-vivo* results. Both culprit coronary artery and simultaneously collected peripheral blood were investigated. Additionally, peripheral blood from age-matched controls and STEMI-patients that had an acute event 72 hours before blood collection were investigated for comparative purposes. Levels of annexin V^+-cMPs were found increased in blood after both vascular wall and isolated collagen perfusions under characterized flow conditions ($P<0.05$). Effluent bloods contained significantly higher levels of erythrocyte-derived-cMPs (ErMPs) carrying glycophorin-A than pre-perfusion blood, indicating erythrocyte activation in the growing evolving thrombus. Total platelet-cMPs (pMPs) were increased whereas pMPs from activated platelets were decreased after perfusions indicating their retention into the growing thrombi. Interestingly, STEMI-patients had significantly higher concentrations of ErMPs than controls. The highest levels were found in the ACS-STEMI-patients compared to the post-STEMI-patients.

Conclusion. Flow and substrate, that regulate thrombus formation, modulate the phenotype of the released cMPs. Glycophorin-A-rich cMPs are markers of ongoing thrombosis.

Key words: cell-derived microparticles, erythrocytes, platelets, shear stress, thrombosis.

Translational perspective

The role of red blood cells in atherothrombosis has been largely overlooked. Here, we found that circulating plasma concentration of glycophorin A-rich erythrocyte-derived microparticles are increased in blood after a thrombotic event and propose that these CD235a^{+}-cMPs could be markers of ongoing mural thrombosis.

Introduction

Thrombus formation at sites of atherosclerotic plaque rupture or vessel injury leads to ischemic coronary artery and cerebrovascular disease.[1, 2] Platelets are key players in the atherothrombotic process by means of activation, adhesion and aggregation, which influence thrombus growth and, subsequently, the degree of life-threatening complications.[3, 4] Beyond platelets and blood components, other variables such as local hemodynamic parameters or atherosclerotic plaque components regulate arterial thrombosis.[5-7] Previous studies have demonstrated that high shear stress induces platelet deposition, aggregation and thrombus formation.[8, 9] Identifying additional factors that may determine the thrombotic mass, the likelihood of subsequent thrombosis and its influence on distal microvascular function is important and may lead to more effective strategies to better prevent the deadly manifestations of atherothrombosis.

Circulating microparticles (cMPs) are small plasma membrane vesicles (0.1–1 um diameter) released from cells undergoing activation or apoptosis.[10] The primary hallmark of microparticles is the membrane exposure of phosphatidylserine, which confers them prothrombinase activity and thus procoagulant activity.[11] cMPs are present in the blood of healthy individuals[12] but their numbers and cellular sources are altered in pathological states.[13-16] Recent research is showing that cMPs are not merely biomarkers of cell activation, but also active players in the development of atherothrombotic disease.[17] Indeed, we have recently reported that high levels of blood cMPs and, specifically platelet-derived microparticles (pMPs), increase platelet deposition on damaged arterial wall contributing to thrombus formation.[18] MPs generated at sites of vascular injury and growing thrombi may serve as distal messengers and play important roles in triggering further thrombosis or in the regulation

of distal microvascular function by interacting with circulating cells or the vessel wall.

High shear stress (SS), a mechanical force generated by circulating blood against the vessel wall conduit, stimulates platelet-derived microparticle formation,[19] which can be further enhanced by stenosis,[20] cytokines[21] or an activated endothelium.[22] Specifically, pMP generation in blood under the effect of SS was reported to be dependent on $\alpha_{IIb}\beta_3$-integrins,[23] on vWF-GPIbα interaction[24] or on both of them.[25] Furthermore, SS related to aortic valve stenosis,[26] strenuous exercise,[27] post-surgery in upper gastrointestinal malignancy[28] and cyanotic congenital heart disease[29] also contributes to the release of procoagulant pMPs. However, it is not known whether microparticles are released into the perfusing blood while the thrombus is formed nor whether occlusive coronary thrombi causing ST-elevation myocardial infarction (STEMI) may release microparticles of characteristic and determined phenotype.

It was our hypothesis that growing thrombi release cMPs that disseminate the prothrombotic message and affect the distal vasculature and the microcirculation. The aim of this study was to investigate: a) the phenotype of microparticles released by thrombi growing on substrates with different thrombogenic potential; and b) the relevance of these MPs in patients with acute coronary thrombosis.

Methods

For expanded Methods please refer to the supplemental file.

Experimental design

In the first part of the study, blood from non-smoking healthy voluntary donors was used for functional experiments and flow cytometry to characterize the effect of SS and lesion type on cMP release. In the second part, both culprit coronary (after the aspiration of the occluding thrombus) and peripheral blood of ST-elevation myocardial infarction (STEMI)-patients undergoing percutaneous coronary intervention (PCI) was analyzed (n=40). Additionally, peripheral blood of STEMI-patients 72 hours after the acute event (n=20) and from a control group (n=20), matched by age and cardiovascular risk factors, was also assessed. Demographics and clinical characteristics are summarised in Table 1. All patients and donors had given informed consent, and the study protocol was approved by the Clinical Research Committee of our Institution and was in accordance with the Declaration of Helsinki.

Flow experiments

Microparticle release from shear-induced growing thrombi was analyzed exposing blood to damaged arterial substrates using the previously validated Badimon perfusion chamber[5, 8] and to isolated type-I collagen-coated surfaces in a flat perfusion chamber.[30, 31] Blood before and after perfusion was collected and platelet-free plasma (PFP) obtained for cMP FACS analysis. In the Badimon perfusion chamber, fibrinogen and platelet deposition on the substrate were morphometrically evaluated by immunohistology and fluorescence microscopy as reported.[18] In the flat chamber, platelet deposition on the collagen surface was analyzed by measuring surface covered by platelets with a fluorescence confocal laser scanning microscope.[18, 31]

Flow cytometric analysis of circulating microparticles

Triple-label flow cytometric analysis was performed as previously described.[32] Briefly, washed cMP were incubated with combinations of annexin V with two specific monoclonal antibodies (mAbs), or the isotype-matched control antibodies. After incubation, samples were immediately analyzed on a FACSCantoII™ flow cytometer (BD). cMPs were identified and quantified based on their FSC/SSC characteristics according to their size, binding to annexin V and reactivity to cell-specific mAb (Table S1). Data were analyzed with FACSDiva™ software. The concentration was based on sample's volume, flow cytometer's flow rate and the number of fluorescence-positive events.

Statistical analysis

Results are reported as median (interquartile range [IQR]), except when indicated. Unpaired t-test was used to compare clinical characteristics between STEMI-patients and controls.

Frequencies for categorical data were compared with chi-square. Median values of quantitative variables were compared with two-sided non-parametric tests as Kolmogorov-Smirnov test showed that the data were not normally distributed. The statistical significances between pre- and post-perfusion conditions of each donor as well as peripheral and coronary blood were determined with the Wilcoxon Signed-Rank Test, and relationships between two studied parameters with Spearman correlation. To evaluate the prognostic value of ErMPs, receiver operating characteristic (ROC) curve analyses and the corresponding areas under the curve (AUC) along with their 95%CI were calculated. StatView-5.0.1 and SPSS-21.0.0 software were used for all tests and a $P<0.05$ was considered statistically significant.

Results

Release of microparticles by growing thrombi triggered by high SS and vascular wall components

Microparticles were analyzed by flow cytometry in blood taken immediately before and immediately after the growing thrombus (Fig. 1). Annexin V-positive cMP numbers were significantly increased after perfusions in both the Badimon chamber (343.8 [331.6-389.4] *vs* 600 [447.2-927.6] cMPs per μL of PFP, $P<0.05$, Fig. 1A-1) and the flat chamber (364.3 [327.4-389.7] *vs* 543.1 [453.9-566.2] cMPs per μL of PFP, $P<0.05$, Fig. 1A-2) by Wilcoxon Signed-Rank Test. Thus, both damaged atherosclerotic vessel wall and the less thrombogenic type-I collagen-triggered thrombi induced cMP release to perfusing blood. Indeed, mural thrombosis with platelet aggregation and deposition formed on vascular substrates triggered significantly higher number of cMPs than platelet adhesion produced on isolated type-I collagen surfaces ($P<0.05$, Fig. 1B).

Blood microparticle phenotype released by thrombi formed under high SS and vascular damaged wall conditions

Specific cell-derived markers were investigated in MPs in order to determine the parental cell shedding the microparticle during thrombosis (Table 2). Cytofluorimetric analysis revealed that circulating erythrocyte-derived microparticles (ErMPs) were significantly increased in the perfusion effluents (Fig. 1C) while other cell-derived microparticles did not drastically change. Platelet-derived microparticles (pMP) showed a non-significant increase (Table 2), but cMPs carrying the red blood cell protein glycophorin A, CD235a^{+}, significantly increased after perfusion of human vascular damage substrates (Badimon chamber) and after exposing human type-I collagen (flat chamber). Indeed, not only the number of cMPs (per μL of PFP) but also the percentage of annexin V-positive cMPs

(AV^+-cMPs) labelled with $CD235a^+$ increased after thrombus formation (Fig. 1C). $CD235a^+$-MP shedding was significantly higher after perfusing damaged vessel in the Badimon chamber (Fig. 1C-1) that induce a more complex thrombus than collagen type-I. In order to discriminate the effects of thrombus on $CD235a^+$-cMPs from the chamber circuit-associated effects, we tested the perfusion system without substrate and established a background threshold produced by the experimental extracorporeal circuit. Normalization of AV^+-ErMPs in post-perfusion experiments was performed correcting by this threshold value (calculated as the increase in $CD235a^+$/AV^+-ErMPs by testing the system). There was no significant effect of the circuit after adjustment, as AV^+-ErMPs ($CD235a^+$) were significantly increased in the perfusates of biological and isolated protein substrates (Table S2).

Shedding of microparticles bearing activation markers and adhesion surface receptors

Circulating microparticles bearing markers of cell activation and surface receptors involved in deposition / adhesion were analyzed in resting (pre-) and effluent (post-) perfused blood (Table 3, Figs. 2 and 3). cMPs carrying MAC-1 ($CD11b^+$, marker of activated leukocytes) and α_V-integrin ($CD51^+$, adhesion receptor for vitronectin) did not differ before and after perfusion (Table 3). cMPs bearing P-selectin ($CD62P^+$) and tissue factor ($CD142^+$) did not change either (Table 3). In contrast, percentage of AV^+-pMPs carrying glycoprotein-Ib ($CD42b^+$) and thrombospondin-1 ($TSP1^+$) were significantly reduced in blood after perfusion of severely damaged vessel ($P<0.05$, Figure 2AI-II) but not in the flat chamber after exposure to type-I collagen (Fig. 2BI-II).

We further studied circulating platelet-derived cMP subpopulation as percentage of AV^+-pMPs (Tables 2/3, Fig. 3). Specifically, the relative amount of total pMPs bearing β_3-integrin ($CD61^+$) was significantly

decreased in the blood exposed to damaged vascular wall while it did not change when blood was exposed to collagen (Fig. 3). The relative amount of pMPs carrying the platelet activation markers PECAM-1 ($CD31^+$) and activated $\alpha_{IIb}\beta_3$-integrin ($PAC1^+$) tended to decrease in the Badimon chamber exposing blood to vascular substrates (Fig. 3A) whereas they were significantly reduced in the flat chamber after exposing blood to collagen surfaces ($P<0.05$, Fig. 3B). Taken together, these data indicate that only circulating microparticles with the highest prothrombotic phenotype had been retained within the mural growing thrombus.

Erythrocyte-derived microparticles in STEMI-patients

We next investigated the systemic and intracoronary concentration of $CD235a^+$-cMPs in blood of the STEMI-patients (n=40). Peripheral blood was also analyzed at day 3 post-event (post-STEMI) in another group of hospitalized patients (n=20) and in a group of controls without thrombosis (n=20). ErMPs (as % of annexin V^+-cMPs) were significantly higher in peripheral blood of STEMI-patients and in patients 3 days post-STEMI than in controls (Fig. 4A). Interestingly, ErMPs were significantly lower 3 days after thrombus removal ($P<0.001$). There were no significant differences between intracoronary and peripheral levels (Fig. 4B); Thus, ErMPs can be useful to estimate coronary thrombosis in peripheral blood.

We performed a receiver operating characteristic (ROC) curve analysis to determine the potential of ErMPs as predictors of STEMI. The AUC of c-statistics measured with $CD235a^+$-cMPs from controls and ACS-STEMI patients was 0.950 (95%CI:0.889-1.000), suggesting that the percentage of RBC-derived MPs could signal for the thrombotic occlusion in the STEMI-patients. Additionally, a cut-off value of $CD235a^+$-cMPs for the prediction of total occlusion was obtained. A level of >26.3 $CD235a^+$-MPs (as % of AV^+-cMPs) predicted total occlusion of the culprit vessel with 85% sensitivity and 90% specificity.

Discussion

In the present study, we investigated the shift in cMPs in circulating blood induced by thrombus formation. Thrombosis was induced under controlled conditions of shear stress and exposure of atherosclerotic substrates using blood from healthy donors. Our results show that total cMP shedding is increased after thrombosis elicited by high shear and thrombogenic lesions, conditions that mimic stenotic coronary blood flow on damaged vascular wall. In these conditions there is an increase in circulating ErMPs. Circulating pMPs bearing epitopes involved in adhesion were reduced after perfusion in both chambers while pMPs carrying activation markers were found decreased after blood perfusion on collagen surfaces, that only support platelet adhesion and not on vascular wall that anchor growing thrombi.

The significant elevation of ErMPs after exposing human blood to damaged arterial substrates and collagen under high shear rate highlights the importance of red blood cells (RBCs) in arterial thrombosis, an aspect largely overlooked so far. Indeed, a very recent study reveals a previously unrecognised ability of RBCs to participate in thrombosis by mediating platelet adhesion to an intact endothelial surface in a $FeCl_3$-mediated thrombosis model.[33] RBC-MPs have also recently emerged as potential mediators of transfusion-related morbidity. However, the physiological role of ErMPs has not been fully elucidated. High levels of ErMPs have been detected in haematological disorders such as β-thalassemia,[34] paroxymal nocturnal haemoglobinuria,[35] sickle cell anemia,[36, 37] severe preeclampsia,[38] nephrotic syndrome,[39] Crohn's disease[40] and in G6PD-deificient patients.[41] Due to their haemoglobin content and phosphatidylserine exposure, ErMPs may account for nitric oxide scavenging and procoagulant activity enhancement.[42] In fact, ErMPs are able to support coagulation by decreasing prothrombin time[43] and,

together with pMPs, have demonstrated to trigger thrombin generation (TG) in a FXII-dependent/TF-independent manner[44] and their TG and coagulation activation involvement was corroborated in the setting of sickle cell disease (SCD).[45, 46] Recently, ErMPs have shown to induce endothelial injury and facilitate vaso-occlusive events in a murine model of SCD, connecting sickle cell anemia to vascular disease.[47] Therefore, elevated levels of ErMPs or a high ErMP-percentage of AV^{+}-cMPs triggered by incipient thrombus formation, may also play a pathogenic role in the thrombotic profile of patients with vascular diseases. Indeed, in blood from both systemic and coronary arteries during primary PCI interventions in STEMI-patients we have found a high amount of ErMPs. Of note, peripheral arterial blood presented a raised content of ErMPs similar to levels found in intracoronary blood, suggesting that circulating ErMPs may be a marker for an ongoing thrombotic event on the culprit coronary artery. Additionally, c-statistics revealed that ErMPs display a significant discrimination power for the prediction of STEMI with a cut-off value of 26.3% of annexin V^{+}-MPs. As expected, systemic blood of post-MI-patients at day 3 (72 h) after admission displayed lower percentage of AV^{+}-$CD235a^{+}$-cMPs compared to that at the acute phase because the activation trigger for ErMPs (occlusive forming thrombi) had disappeared, but still higher than controls probably due to a not yet completed thrombus resolution and clearance. Therefore, ErMPs could be considered as a novel independent hemorheological index to characterize arterial thrombosis and possibly microcirculatory disease with thrombotic complications.

Platelet-derived microparticles are of high importance in the pathogenesis of CVD.[49] pMPs provide the membrane surfaces necessary for assembly of the tenase and prothrombinase complexes. A number of studies have highlighted the fact that shear stress enhance their formation with a variety of stimulus and conditions.[19-29] We have recently reported

that cMPs and in particular pMPs enhance the deposition of platelets and fibrin to atherosclerotic vessel wall.[18] We also found a blunt decrease of pMPs with surface markers of adhesion and activation in the post-thrombus blood. Therefore, our data reinforce our previous results showing that pMPs bind to activated and adhered platelets under high shear rate conditions stimulating further platelet deposition and thrombus growth.[18] Altogether these results indicate that the presence of a high concentration of pMPs in blood promote platelet adhesion due to a high tendency to adhere, as previously reported[18] and support their clear implication in the atherothrombotic process.

In the context of atherothrombosis, vascular and inflammatory cells help to establish the atherosclerotic milieu. Erythrocytes by mechanical fluid dynamic forces push platelets to circulate in the boundary liquid layer over the surface of the injured vessel and platelets recruited at the site of thrombosis are activated and able to shed MPs which can disseminate a procoagulant state and provide a trigger for further thrombogenicity or distal microvascular dysfunction. Thus, in a deleterious vascular environment, the generation of cMPs, mainly originating from activated platelets, may further accelerate progression of disease by cross-talk with other blood cells, inducing their activation and amplifying arterial thrombus formation. It has been shown that P-selectin-containing MPs enhance leukocyte aggregation and accumulation on selectin-expressing substrates under high SS.[50] The specific cell-derived MPs shed could represent distinct biological vectors contributing to vascular disease.

Taken together, these data demonstrate that blood perfusing thrombogenic damaged atherosclerotic vessel wall at a high shear stress triggers platelet deposition and thrombus formation that induce a rapid cMP release into the circulation. The release of ErMPs has been shown to be an integral part of the thrombotic process. Our data on ErMP release at

sites of arterial thrombosis suggests that quantification of ErMPs could be a method of detection of ongoing thrombosis and/or serve as prognostic indicators for the risk of future thrombotic events. This experimental and proof of concept study will require validation in larger scale studies aiming to evaluate the value of ErMPs (CD235a^{+}-cMPs) in the diagnosis of ongoing silent thrombosis to initiate an early treatment in patients at risk.

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Table 1. Baseline clinical characteristics of study population.

	STEMI-patients		Controls	Statistics (*P*-value)		
	Acute-STEMI (n=40)	**Post-MI 72h (n=20)**	**(n=20)**	**Controls *vs* STEMI**	**Controls *vs* post-STEMI**	**STEMI *vs* post-STEMI**
Male/Female (n)	27/13	17/3	15/5	*P*=0.55	*P*=0.43	*P*=0.15
Age (years, mean±SE)	64.2±2.11	63.6±3.0	58.4±1.9	*P*=0.09	*P*=0.18	*P*=0.87
Risk factors (n, %)						
Smoking	22 (55%)	9 (45%)	7 (35%)	*P*=0.14	*P*=0.52	*P*=0.46
Dyslipidemia	24 (60%)	13 (55%)	12 (60%)	*P*=0.99	*P*=0.74	*P*=0.71
Diabetes mellitus	11 (27%)	5 (25%)	5 (25%)	*P*=0.84	*P*=0.99	*P*=0.84
Systemic hypertension	26 (65%)	11 (55%)	11 (55%)	*P*=0.45	*P*=0.99	*P*=0.45
Obesity	9 (22%)	3 (15%)	5 (25%)	*P*=0.83	*P*=0.43	*P*=0.49
Drugs of abuse	3 (7.5%)	0 (0%)	0 (0%)	*P*=0.21	*P*=0.99	*P*=0.21
STEMI-clinics (n, %)						
Antithrombotic therapy						
Clopidogrel	11 (27%)	18 (90%)	-	-	*P*<0.0001	-
Acetylsalicylic Acid	29 (72%)	20 (100%)	-	-	*P*=0.01	-
Anti-glycoprotein IIb/IIIa	35 (87%)	6 (30%)	-	-	*P*<0.0001	-
Heparin	24 (65%)	20 (100%)	-	-	*P*=0.001	-
TIMI flow grade 3						
Pre-PCI	3 (1%)	1 (5%)	-	-	*P*=0.71	-
Post-PCI	34 (85%)	17 (85%)	-	-	*P*=0.99	-

SE, standard error; STEMI, ST-segment elevation myocardial infarction; TIMI, thrombolysis in myocardial infarction.

Table 2. Shear stress effect on circulating microparticles depending on their cellular origin.

Cell origin	Circulating MPs		Vascular damage		Statistics	Type-I collagen		Statistics
			Preperfusion	Postperfusion		Preperfusion	Postperfusion	
ECs	eMP (CD31$^+$/CD41$^-$)	cMPs/μL	14 (11-26.5)	14 (12-32)	*P*=0.13	13 (10.5-20)	15 (10.5-18)	*P*=0.91
		% of AV$^+$	3.1 (1.7-5.6)	1.9 (1.1-3.4)	*P*=0.18	4.5 (3.5-5.6)	3.1 (2.1-4.0)	*P*=0.25
RBCs	ErMP (CD235a$^+$)	cMPs/μL	52 (40-119)	259 (95.5-446.5)	*P*=0.001	32 (17-51.5)	106 (91.5-123.5)	*P*=0.03
		% of AV$^+$	6.0 (3.9-13.8)	31.1 (16.3-36.8)	*P*=0.001	8.7 (4.8-13.6)	21.8 (17.8-27.7)	*P*=0.03
PLTs	pMP (CD41a$^+$/CD61$^+$)	cMPs/μL	453.5 (3345-465)	596 (360.5-724.5)	*P*=0.25	343 (255.5-384)	458 (411.5-471.8)	*P*=0.04
		% of AV$^+$	78.3 (58.3-99.8)	61.8 (50.9-71.6)	*P*=0.04	88.9 (74.7-103.0)	91.7 (82.0-99.2)	*P*=0.92

AV$^+$ indicates annexin V-positive; cMPs, circulating-derived microparticles; EC, endothelial cells; eMP, endothelial-derived microparticles; ErMP, erythrocyte-derived microparticles; PLTs, platelets; pMP, platelet-derived microparticles; RBCs, red blood cells.

Table 3. Shear stress effect on circulating microparticles carrying markers of cell activation.

Cell origin	Activation marker	cMPs	Vascular damage		Statistics	Type-I collagen		Statistics
			Preperfusion	Postperfusion		Preperfusion	Postperfusion	
PLTs	$\alpha_{IIb}\beta_3$-integrin (PAC1$^+$)	cMPs/µL	32 (25.5-53)	28 (24-46.5)	*P*=0.73	31 (25.5-36.5	23 (16.5-18)	*P*=0.50
		% of AV$^+$	3.5 (2-9.6)	3.5 (2-6.6)	*P*=0.64	8.7 (7.3-9.6)	4.0 (2.9-6.1)	*P*=0.03
	Glycoprotein Ib (CD42b$^+$)	cMPs/µL	22 (12-39)	14 (6.5-17)	*P*=0.02	12 (7.5-25.5)	5 (2.5-9)	*P*=0.34
		% of AV$^+$	3.4 (1.8-4.0)	1.0 (0.6-2.9)	*P*=0.005	3.4 (2.1-6.7)	0.9 (0.4-2.1)	*P*=0.25
Multiple	Thrombospondin-1 (TSP1$^+$)	cMPs/µL	64 (41.8-68)	64 (39.8-89.3)	*P*=0.11	47.5 (36.8-62.8)	53 (20.8-64.3)	*P*=0.60
		% of AV$^+$	10.7 (6.2-13.4)	6.4 (5.2-9.8)	*P*=0.02	12.5 (9.8-16.7)	9.5 (4.0-11.8)	*P*=0.60
	α_V-integrin (CD51$^+$)	cMPs/µL	16 (12-38)	19 (12.5-23.5)	*P*=0.27	13 (6-39.5)	12 (7-18.5)	*P*=0.53
		% of AV$^+$	2.9 (0.8-5.9)	1.7 (1.3-2.2)	*P*=0.14	4.9 (1.8-12.2)	2.2 (1.2-4.3)	*P*=0.07
	P-selectin (CD62P$^+$)	cMPs/µL	48 (33-51)	56 (43-98)	*P*=0.68	25 (15-36.5)	33 (15.5-52)	*P*=0.67
		% of AV$^+$	7.4 (6.5-10.3)	7.5 (5.5-12.3)	*P*=0.97	6.2 (4.4-9.3)	6.1 (3.2-9.0)	*P*=0.46
	Tissue factor –TF– (CD142$^+$)	cMPs/µL	437 (110.3-489.8)	477 (206.5-773.3)	*P*=0.05	114.5 (103-238.5)	184.5 (146-234.8)	*P*=0.35
		% of AV$^+$	37 (25.7-68.3)	41.2 (22.8-86.9)	*P*=0.25	30.8 (27.2-62.8)	36.0 (25.7-49.0)	*P*=0.60

AV$^+$ indicates annexin V-positive; cMPs, circulating-derived microparticles; PLTs, platelets.

Figure Legends

Figure 1. Blood rheology and vascular wall components effect on blood circulating microparticles. (A) Total annexin V-positive cMP numbers (AV^+-cMPs per μL of PFP) pre- and post-perfusion both in the **(A-1)** Badimon (n=14) and **(A-2)** flat (n=6) chambers. Box and whisker plots represent median (interquartile-range) values. *$P<0.05$, vs. pre-perfusion group (Wilcoxon Signed-Rank test). **(B)** Representative immunophotograph of platelet staining on perfused human vascular substrates in the Badimon chamber (green-mepacrine) **(B-1)** and confocal image of adhered platelets to type-I collagen surface in the flat chamber (green-CD61) **(B-2)**. Scale bar is 25 μm and 5 μm, respectively. **(C)** Box and whisker plots showing erythrocyte-derived cMPs (ErMPs, $CD235a^+$) before and after exposing blood to **(C-1)** arterial substrates in the Badimon chamber (n=14) and **(C-2)** collagen surfaces in the flat chamber (n=6) under high shear rate flow conditions. Data are expressed as percentage of AV^+-cMPs. *$P<0.05$, vs. pre-perfusion group (Wilcoxon Signed-Rank test).

Figure 2. Flow- and substrate-dependent release of adhesion epitopes-containing cMPs. Relative amount of cMPs carrying molecules related to adhesion markers [thrombospondin 1 ($TSP1^+$) and glycoprotein Ibα ($CD42b^+$)] before and after exposing blood to **(A)** arterial substrates in the Badimon chamber (n=14) and **(B)** collagen surfaces in the flat chamber (n=6) under high shear rate flow conditions. Box and whisker plots represent median (interquartile-range) values. Data are expressed as percentage of AV^+-cMPs. *$P<0.05$, vs. pre-perfusion group (Wilcoxon Signed-Rank test).

Figure 3. Flow- and substrate-dependent release of circulating microparticles carrying activation markers. Box and whisker plots showing relative amount of total pMPs (pMPs; $CD61^+$) and pMPs bearing markers of activation: PECAM-1 ($CD31^+$) and activated $\alpha_{IIb}\beta_3$-integrin ($PAC1^+$) before and after exposing blood to **(A)** arterial substrates in the Badimon chamber (n=14) and **(B)** collagen surfaces in the flat chamber (n=6). Data are expressed as percentage of AV^+-cMPs. *$P<0.05$, vs. pre-perfusion group (Wilcoxon Signed-Rank test).

Figure 4. Erythrocyte-derived microparticle levels in STEMI-patients. Box and whisker plots showing ErMPs from **(A)** peripheral blood of control subjects (n=20), acute-STEMI-patients at baseline (n=40) and STEMI-patients at day (72 hours) after admission, *$P<0.0001$ (Kruskal-Wallis), *$P<0.0001$ vs. controls and $^{\dagger}P<0.001$ (Mann-Whitney); and from **(B)** both peripheral and intracoronary blood of STEMI-patients (n=40) undergoing PCI at baseline (Wilcoxon Signed-Rank). **(C)** For the discrimination power of ErMPs in the prediction of STEMI, an associated ROC curve analysis was determined with $CD235a^+$-cMPs of controls and STEMI-patients (n=60).

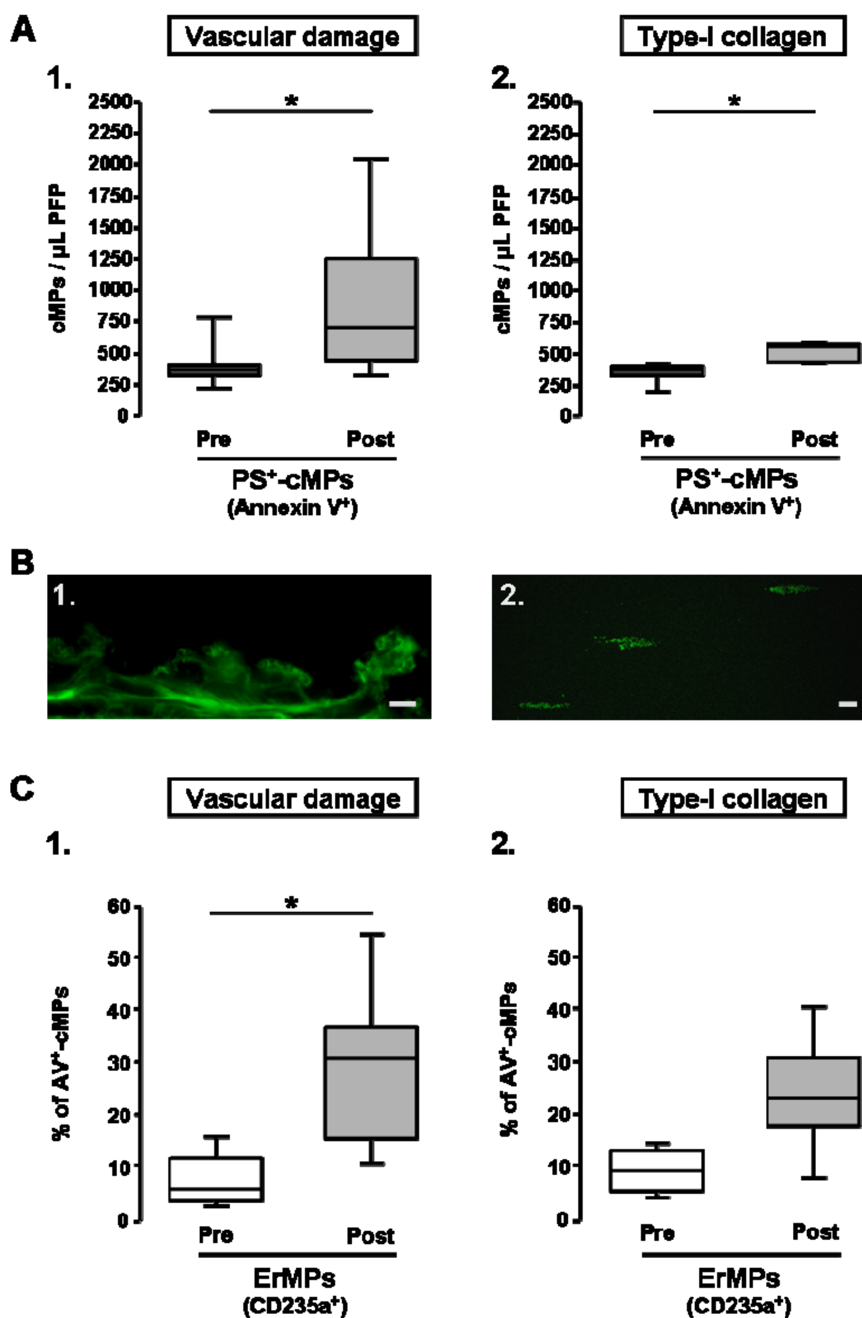

Figure 1

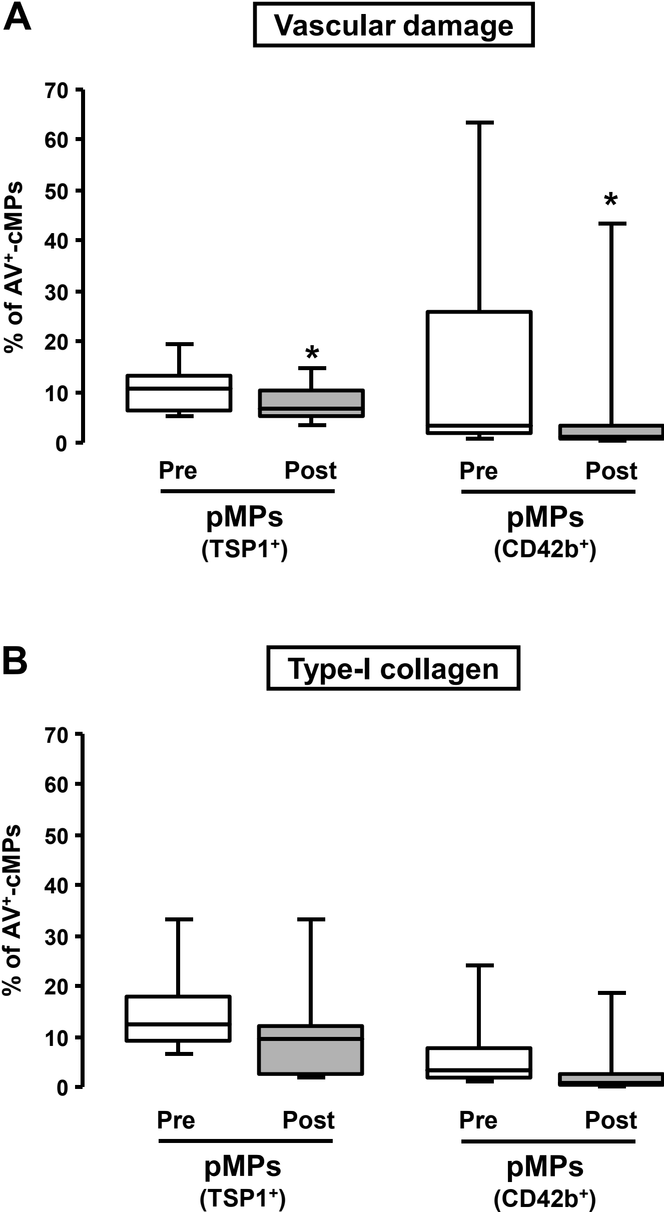

Figure 2

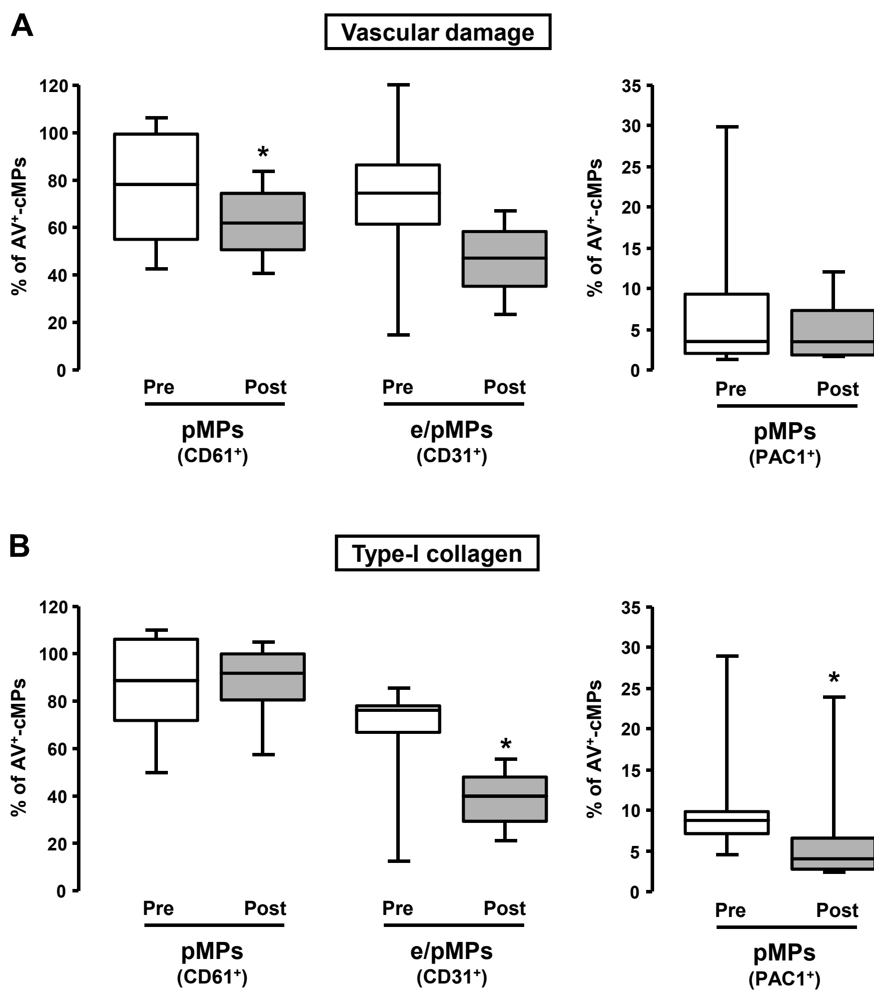

Figure 3

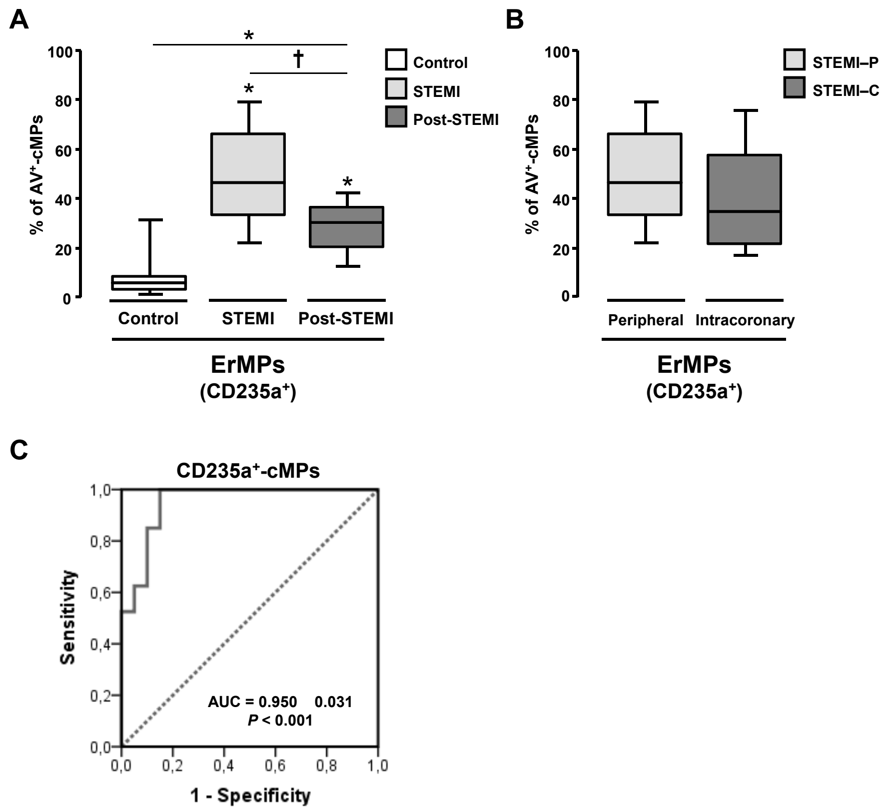

Figure 4

Supplementary Data

1) Supplemental Methods

Blood sampling

Blood from non-smoking healthy voluntary donors, without any antiplatelet medication for 15 days prior to blood extraction, was drawn by a cubital venopuncture into tubes containing anticoagulant as needed and used for the functional experiments and flow cytometry in the first part of the study to characterize the effect of SS and lesion type on cMP release. In the second part and as a proof-of-concept analysis, coronary (after thrombus aspiration) blood of ST-elevation myocardial infarction (STEMI) patients (n=40) undergoing percutaneous coronary intervention (PCI) was collected in EDTA-containing tubes, immediately processed and stored at -80ºC. Peripheral blood from the same patients (n=40) and from STEMI-patients at day 3 (72 hours) after admission (n=20) were also collected identically. Blood from a group of healthy subjects without thrombosis (n=20) was collected and processed similarly. All patients and donors had given informed consent, and the study protocol was approved by the Clinical Research Committee of our Institution and was in accordance with the Declaration of Helsinki.

Experimental design

The effect of high shear stress and platelet deposition on blood circulating microparticles was analyzed using the previously validated Badimon perfusion chamber[1] and a flat perfusion chamber, as previously described.[2, 3] The shear-induced thrombus formation was induced exposing blood to damaged arterial substrates (porcine and human) in the Badimon perfusion chamber and to isolated type-I collagen-coated

surfaces in the flat perfusion chamber. For the perfusion experiments, blood was collected in sodium heparin (10IU/ml),[4] kept at 20ºC, and used within 2h of collection. The haematological parameters (platelets, leukocyte and red blood cell counts) and platelet reactivity were very similar among donors and were within physiological ranges. In the flat chamber perfusion experiments, platelet were rendered fluorescent by the addition of mepacrine (quinacrine dihydrochrolide, 10M, Sigma) to blood after an aliquot was taken for flow cytometric analysis. Before and after all perfusion experiments, platelet-free plasma (PFP) of all effluent samples (trisodium-citrate anticoagulated) was obtained, frozen in liquid nitrogen and stored at -80ºC for FACS analysis of circulating microparticles.

Flow experiments in Badimon perfusion chamber

Pig aorta specimens were obtained fresh from local slaughterhouse, and human aorta specimens from autopsy cases within 10-12 h of death, immediately washed in PBS, cleaned from adventitia, cut in long pieces and frozen at -80ºC until needed. Before the experiments, the aortas were thawed in PBS at 4 ºC, opened longitudinally, and cut into 30x10 mm segments. Segments of pig aorta were denuded (model of erosion). Human specimens composed of fatty streaks and atherosclerotic lesions (macroscopically characterised by raised yellow streaks and yellow or yellow-white plaques) were used as substrates for each experiment in a randomised fashion. Aorta substrates were mounted in the previously characterized Badimon perfusion chamber,[1, 5] and placed in a water bath at 37 °C. After a preperfusion period of 60 seconds with PBS (37 ºC), human blood was drawn into the chamber to perfuse the human vessels at a constant flow rate of 10 mL/min for 3 minutes to reach a shear rate of 1690 s^{-1},[4] mimicking moderately stenotic coronary arteries. Finally, PBS was passed for 30 seconds through the chamber to wash out the

unattached cells. The perfused segments were fixed in 4% paraformaldehyde in PBS, cryoprotected with sucrose, frozen over dry ice and stored in OCT.[6] Fibrinogen and platelet deposition on the substrate were also morphometrically evaluated by immunohistology and fluorescence microscopy as reported.[6]

Flow experiments in Flat perfusion chamber

Glass slides were coated with type-I collagen (10µg/ml, 4 ºC, overnight) and placed in a parallel plate chamber.[2, 3, 6-8] After 1 min HEPES-Tyrode's buffer preperfusion, blood was circulated through the chamber at a constant shear rate (1500 s^{-1}, 5 minutes). Then, buffer was circulated for 1 minute through the chamber under identical flow conditions. Thereafter, slides were carefully removed from the system, rinsed with PBS, fixed with 3.8% paraformaldehyde (15 min) and mounted on glass slides with Glycerol Mounting Medium (Dako Cytomation). Platelet deposition on the collagen surface was analyzed with an inverted fluorescence confocal laser scanning microscope (Leica TCS SP2-AOBS), as previously described. The surface covered by platelets was calculated using Image J and expressed as the area covered by platelets per analyzed field.[3, 6]

Flow cytometric analysis of circulating microparticles

Triple-label flow cytometric analysis was performed as previously described.[9, 10] Briefly, washed cMP suspensions diluted in PBS buffer containing $CaCl_2$ were incubated with combinations of BD-horizon V450-conjugated annexin V with two specific monoclonal antibodies labelled with fluorescein isothiocyanate and phycoerythrin, or the isotype-matched control antibodies (Table S1). Samples were diluted with $CaCl_2$-PBS buffer before being immediately analyzed on a FACSCantoII™ flow cytometer. Acquisition was performed for 1 minute per sample. Forward scatter (FSC), side scatter (SSC) and fluorescence data were obtained with

gain settings in the logarithmic scale. cMPs were identified and quantified based on their FSC/SSC characteristics according to their size, binding to annexin V and reactivity to cell-specific mAb. Gate limits were established using a Flow-Check Size Range Calibration Kit (Polysciences) and an *in vitro* platelet-derived microparticle population as positive control. The lower detection limit was placed as a threshold above the electronic noise of our flow cytometer. To identify positive marker events, thresholds were also set based on samples incubated with the same final concentration of isotype-matched control antibodies after titration experiments. Phosphatidylserine-positive cMPs were labelled using annexin V in the presence of 2.5mM $CaCl_2$. Annexin V binding level was corrected for autofluorescence using fluorescence signals obtained with microparticles in a calcium-free buffer. Data were analyzed with FACSDiva™ software (BD). The concentration (number of cMPs per µL of plasma) was determined according to Nieuwland's procedure,[11] based on sample's volume, flow cytometer's flow rate and the number of fluorescence-positive events. Intra-assay CV of cMP counts was 3.1%, while inter-assay CV was 5.4%.

Statistical analysis

Results are reported as median (interquartile range [IQR]), except when indicated. Unpaired t-test was used to compare clinical characteristics between STEMI-patients and controls. Frequencies for categorical data were compared with chi-square. Median values of quantitative variables were compared with two-sided non-parametric tests as Kolmogorov-Smirnov test showed that the data were not normally distributed. The statistical significances between pre- and post-perfusion conditions of each donor as well as between peripheral and coronary blood were determined with the Wilcoxon Signed Rank Test, and relationships between two studied parameters with Spearman correlation. To evaluate

the prognostic value of ErMPs, receiver operating characteristic (ROC) curve analyses and the corresponding areas under the curve (AUC) along with their 95%CI were calculated. StatView-5.0.1 (Abacus Concepts) and SPSS-21.0.0 (SPSS Statistics) software were used for all tests and a $P<0.05$ was considered statistically significant.

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2) Supplemental Tables

Table S1. Cell surface molecules for circulating microparticle identification and characterization.

Marker	Alternative name	Expression	Conjugation	Clone
Annexin V	PS-binding protein	Widely expressed	BD Horizon V450	-
CD31	PECAM-1	PLTs, endothelial cells	PE	1F11
CD41	α_{IIb}-integrin	Platelets	FITC	SZ22
CD42b	Glycoprotein Ib	Platelets	FITC	HIP1
CD61	β_3-integrin	Platelets	PE	VI-PL2
CD62P	P-selectin	Activated platelets	PE	AK-4
CD146	MUC18	Endothelial cells	FITC	P1H12
CD235a	Glycophorin A	Erythrocytes	FITC	11E4B-7-6
PAC1	$\alpha_{IIb}\beta_3$-integrin	Activated platelets	FITC	PAC1
TSP1	THBS-1	PLTs, megakaryocytes	PE	P10
IgG1γ	-	-	FITC / PE	X40
IgG1κ	-	-	FITC / PE	MPOC21

FITC fluorescein isothiocyanate; MUC18, melanoma-associated antigen; PE, phycoerythrin; PECAM-1, PLT endothelial cell adhesion molecule-1; PLT, platelet; PS, phosphatidylserine; THBS1, thrombospondin 1.

Table S2. High SS and vascular wall components effect on blood erythrocyte-derived circulating microparticles normalized by system haemolysis threshold.

		ErMPs ($CD235a^+$)		
		Preperfusion	**Postperfusion**	**Statistics**
Vascular damage	cMPs/μL	48.0 (26.0-104.0)	225.3 (50.4-321.4)	P=0.001
	% of AV^+	6.0 (3.8-11.9)	22.8 (7.0-31.6)	P=0.02
Type-I collagen	cMPs/μL	24.8 (14.0-38.0)	82.9 (71.0-85.5)	P=0.03
	% of AV^+	8.75 (4.3-12.7)	12.35 (11.0-17.2)	P=0.17

AV^+ indicates annexin V-positive; cMPs, circulating-derived microparticles; ErMPs indicates erythrocyte-derived microparticles.

Article 6

Circulating microparticle signature in coronary and peripheral blood of ST elevation myocardial infarction patients

Rosa Suades, Teresa Padró, Javi Crespo, Ilaria Ramaiola, Victoria Martin-Yuste, Manel Sabaté, Jordi Sans-Roselló, Alessandro Sionis, Lina Badimon

Manuscript under revision.

Objectives

The aim of this study was to investigate circulating microparticle signature in relation to thrombotic occlusion time in the systemic and culprit coronary artery blood of ST-segment elevation myocardial infarction patients at the time of primary percutaneous coronary intervention as well as 72 hours after symptom onset.

Highlights

- Changes in prothrombotic, proinflammatory and endothelial dysfunction cMPs are found both at systemic and intracoronary level, reflecting the sensitivity of cMPs as markers of the ongoing thrombus formation.
- The procoagulant profile signature of cMPs at both systemic and intracoronary levels in STEMI patients undergoing pPCI associates to duration of pain-to-PPCI ischemic time.

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Circulating microparticle signature in coronary and peripheral blood of ST elevation myocardial infarction patients

Suades et al: Microparticle signature in STEMI

By:

Rosa Suades[1], Teresa Padró[1], Javi Crespo[1], Ilaria Ramaiola[1], Victoria Martin-Yuste[2], Manel Sabaté[2], Jordi Sans-Roselló[3], Alessandro Sionis[3], Lina Badimon[1,4]

[1] Cardiovascular Research Center, CSIC-ICCC, IIB-Sant Pau, Barcelona

[2] Department of Interventional Cardiology, Hospital Clinic, Barcelona

[3] Acute and Intensive Cardiac Care Unit, Cardiology Department, Hospital Santa Creu i Sant Pau, Biomedical Research Institute Sant Pau (IIB Sant Pau), Barcelona, Spain

[4] Cardiovascular Research Chair, UAB, Barcelona, Spain

Correspondence to:

Prof. Lina Badimon
Cardiovascular Research Center,
c/Sant Antoni Mª Claret 167, 08025 Barcelona, Spain.
Phone: +34.935565880.
Fax: +34.935565559.
E-mail: lbadimon@csic-iccc.org

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Abstract

Background. Circulating microparticles (cMPs) levels have been shown to increase in the acute phase of ST elevation myocardial infarction (STEMI)-patients and associate with microvascular obstruction in their coronary artery; however, their precise cell origin and activation level has not been fully elucidated. Thus, we aimed to study cMP phenotype in relation to thrombotic occlusion time in the systemic and culprit coronary artery blood at the time of primary percutaneous coronary intervention (PPCI).

Methods and Results. cMPs of STEMI-patients (n=40) in both coronary artery and peripheral blood were characterized by triple-labelling flow cytometry. Peripheral blood of age-matched post-STEMI-patients and healthy controls (n=20/group) was comparatively analyzed. Cell origin cMPs as well as those carrying markers of parental-cell activation and tissue factor displayed a significantly different profile in STEMI-patients compared to controls. After 72 hours of the ischemic event cMP profile was still different from controls. Specific cMPs were in higher proportion in the culprit coronary artery than in peripheral blood in STEMI-patients, especially in the shorter thrombus occlusion times. Additionally, cMPs in coronary blood were inversely related to duration of thrombotic occlusion (more abundant in thrombi with pain-to-PPCI time shorter than 3 hours). Peripheral blood cMPs were positively associated to multivessel disease.

Conclusion. Changes in cMP signature in the culprit coronary artery of STEMI-patients reveal their sensitivity to detect the thrombotic occlusion homeostatic effect and its impact at the systemic level. cMPs in the circulating blood may be sensitive markers of the thrombo-occlusive vascular process developing in the coronary arteries of STEMI-patients.

Key words: circulating microparticles, revascularization, myocardial infarction, thrombus.

Acute coronary syndromes (ACS) are the leading causes of mortality and morbidity worldwide.[1, 2] Among ACS, the majority of deaths are attributable to myocardial infarction (MI). Most cases of persistent ST-segment elevation MI (STEMI) are caused by atherosclerotic plaque rupture and thrombosis which ultimately leads to occlusion of a major coronary artery.[3-5] Primary percutaneous coronary intervention (PPCI) represents the reperfusion strategy of choice in patients with STEMI, provided it is delivered in a timely fashion and being most beneficial when given within the first 2 h after symptom onset.[6]

Despite being complex, the mechanism unrevealing thrombus formation on disrupted or eroded atherosclerotic plaques has partly been elucidated, evolving from the arterial wall view to the new concept of vulnerable blood.[7, 8] Indeed, we have recently demonstrated that circulating and, specifically, platelet-derived microparticles increase the thrombogenic potential of underlying triggering atherosclerotic plaques by modifying the composition of the growing thrombus, contributing to thrombosis.[9, 10]

Circulating microparticles (cMPs), small phospholipid microvesicles shed into blood from activated or apoptotic cells of the vascular compartment as a way of cell communication,[11] have emerged as new players in atherothrombosis.[12] High levels of procoagulant microparticle subpopulations have been reported in the circulation of patients with ACS,[13] including STEMI,[14] even when compared to stable angina,[15] reflecting both platelet and cell activation status.[16, 17] Circulating MPs also correlate to the size of myocardium at risk in STEMI patients,[18] indicating their involvement in disease severity. Local elevation of procoagulant MPs has also been detected within the occluded coronary artery of patients with STEMI[19, 20] showing again that they might play a role in the formation of intracoronary thrombi and on microembolization.[21]

Interestingly, several studies have investigated cMPs as novel biomarkers at different stages of cardiovascular disease progression.[22-24] cMPs have been proposed as potential biomarkers of ongoing thrombosis in the coronaries, but this has never been demonstrated by a multi-panel procoagulant Annexin V^+-cMP approach during the temporal evolution of STEMI.

The present study aimed to investigate the cMP signature, in terms of parental cell origin and activation status, in the (1) peripheral blood of acute STEMI patients compared to healthy controls as well as post-STEMI patients (72 hours after symptom onset), (2) systemic and local (culprit coronary artery) blood of patients with acute STEMI undergoing primary PPCI, in relation to pain-to-PPCI evolved time; and, (3) whether cMP signature correlates with other clinical parameters in the STEMI patients.

Methods

Clinical population

Forty STEMI patients treated by primary PPCI with thrombus aspiration were included. pPCI was performed according to guidelines.[25] All patients were treated with unfractionated heparin while glycoprotein IIb/IIIa antagonist was administered at physician's discretion. To investigate the effect of onset-of-pain-to-PPCI time on dynamic evolution of cMP-signature, patients were categorized in two groups: ≤3 hours (n=24) or >3 hours (n=16). Main clinical characteristics of these groups are listed in Table 1. A control group of healthy subjects (n=20) matched by age, gender, risk factors and pharmacological treatments were also included for comparative purposes. Demographic and clinical characteristics of the STEMI and control groups are provided in Supplemental Table I. Pharmacological treatment of both STEMI and post-MI patients is summarised in Supplemental Table II. Local ethics committee approved the study and all patients signed informed consent. All procedures were followed in accordance with ethical standards and Declaration of Helsinki. Exclusion criteria for this study were presence of sepsis, infectious disease, cancer or pregnancy, since these conditions may condition MP measurement. Patients undergoing rescue PCI were not included in the study. The results are presented in accordance with STROBE guidelines.

Blood sampling

Blood was withdrawn from peripheral and culprit coronary arteries of the STEMI patients. Peripheral artery blood was obtained from healthy controls and from post-MI patients. Blood was collected in EDTA tubes for cMP characterization. Blood cells were removed by low-speed centrifugation to obtain the platelet free plasma (PFP), which was snap-

frozen in liquid nitrogen and stored at -80ºC until flow cytometric studies were performed, as we have previously described.[23, 26]

Flow cytometric analysis of circulating microparticles

The cMP-fraction was washed and isolated from PFP by a two-step high-speed centrifugation. Triple-label flow cytometric analysis was performed as previously described.[23, 26] Briefly, washed cMP were incubated with combinations of annexin V with two specific monoclonal antibodies (mAbs), or the isotype-matched control antibodies. After incubation, samples were immediately analyzed on a FACSCantoII™ flow cytometer (BD). cMPs were identified and quantified based on their FSC/SSC characteristics according to their size, binding to annexin V and reactivity to cell-specific mAb (Supplemental Table III). Data were analyzed with FACSDiva™ software. The concentration was based on sample's volume, flow cytometer's flow rate and the number of fluorescence-positive events.

Statistical analysis

Results are reported as median (interquartile range [IQR]), except when indicated. Unpaired t-test was used to compare clinical characteristics between STEMI-patients and controls.

Frequencies for categorical data were compared with chi-square. Median values of quantitative variables were compared with two-sided non-parametric tests (Mann Whitney and Kruskal Wallis) as Kolmogorov-Smirnov test showed that the data were not normally distributed. The statistical significances between peripheral and coronary blood were determined with the Wilcoxon Signed-Rank Test, and relationships between two studied parameters with Spearman correlation. StatView software was used for all statistical tests and a $P<0.05$ was considered statistically significant.

Results

Clinical characteristics

Baseline clinical characteristics of STEMI-patients undergoing PPCI are summarized in Table 1. The majority of patients were in Killip's class I at admission (92.5%) and about 22.5% presented with a total ischemic time longer than six hours. Optimal PPCI results (TIMI 3) were obtained in 85% of patients. Eleven patients (27.5%) had multivessel coronary artery disease. There were significant differences in gender and major adverse cardiac events (MACE) incidence between STEMI-patients with an ischemic time ≤3 hours and >3 hours. Clinical characteristics of STEMI-patients compared to 72 h post-STEMI patients and healthy controls are listed in Supplemental Table I. Baseline characteristics were not different between the two groups.

Cell origin-specific circulating microparticles in STEMI and post-STEMI-patients

All data here presented are expressed as percentage of annexin V-positive circulating microparticles (AV^+-cMPs). The pattern of distribution of annexin V^+-cMPs in STEMI-patients differed from healthy controls (Figure 1 and 2). Specifically, AV^+-cMPs derived from leukocytes (LMPs) (including monocytes [mMPs] and lymphocytes [ℓMPs]) and endothelial cells (eMPs) were significantly increased in STEMI-patients compared to healthy controls (Figure 1A-B). Erythrocyte-derived MPs (ErMPs) were also significantly higher in STEMI-patients than in controls (46.6 [32.8] vs. 5.5 [5.3] % of AV^+-cMPs, median [IQR]). On the contrary, pMPs were reduced in STEMI patients (Figure 1C). In order to characterize the dynamics of MP shedding in STEMI, we evaluated cMP cell origin profile in a group of patients at 72 hours (day 3). After 72h

there was a lower number of mMPs, ℓMPs, and eMPs in peripheral blood of STEMI patients (Figure 1A-B) and ErMPs were significantly reduced after 72h of the acute event but without reaching the level of healthy controls (29.9 [16.4] % of AV^+-cMPs). In contrast, LMPs remained increased after 72h of MI compared to healthy controls (Figure 1A). Finally, pMPs remained in reduced amounts of after 72 h of MI (Figure 1C). To further investigate the potential role of cMPs as biomarkers of ongoing thrombus formation, subsequent ROC analyses were performed in healthy controls and STEMI-patients data (n=20 C, n=40 STEMI). A selected panel of cMPs with parental cell markers panel composed of ℓMPs ($CD45^+$/$CD3^+$), mMPs ($CD14^+$), eMPs ($CD146^+$), and pMPs ($CD41^+$) showed an AUC of 0.974±0.017 [95%CI:0.941-1.000] (P<0.0001), indicating a higher discriminatory power than each cMP type alone (Figure 1D).

Activated cell-specific circulating microparticles in STEMI and post-STEMI-patients

Not only annexin V^+-cMPs bearing parental cell origin markers significantly changed but also those AV^+-cMPs carrying markers of parental cell activation (Figure 2). Indeed, percentage of AV^+-cMPs from activated white blood cells with markers of granulocyte activation ($CD66b^+$-gMPs; Figure 2A) was increased compared to healthy controls while pan-activated-leukocytes ($CD11b^+$-LMPs; Figure 2A) did not change. Similarly AV^+-cMPs from endothelial cells (ECs) bearing E-selectin ($CD62E^+$-eMPs; Figure 2B) and from activated platelets carrying activated $\alpha_{IIb}\beta_3$-integrin ($PAC1^+$-pMPs; Figure 2D) was significantly higher in STEMI-patients. Contrarily, fraction of AV^+-cMPs carrying $CD31^+$ (from platelets and ECs) (PECAM-1, $CD31^+$-pMPs; Figure 2C) were decreased ($P<0.05$). Interestingly, annexin V^+-cMPs rich in tissue

factor (TF; $CD142^+$) were also significantly higher in STEMI-patients than healthy controls (Figure 2E). When evaluating the cMPs after 72 hours of MI/thrombotic occlusion nMPs were at comparable levels to healthy controls (Figure 2A); the rest of cMP at 72h post-MI showed lower levels than in the acute MI phase but higher than in control patients (Figure 2B-D). Only the activated LMPs ($CD11b^+$-cMPs) were significantly higher after 72h of MI than in both healthy controls and acute STEMI patients (Figure 2A). To evaluate the discriminatory applicability of activated-cMPs, a ROC analysis was performed with the combination of gMPs ($CD66b^+$), LMPs ($CD11b^+$), eMPs ($CD62E^+$), and TF^+-cMPs ($CD142^+$) in healthy controls and STEMI-patients (n=20 C, n=40 STEMI) and an AUC of 0.975 ±0.019 [95%CI:0.937-1.000] (P<0.0001) was obtained.

Circulating microparticles in peripheral and intracoronary blood

When comparing cell-derived AV^+-cMPs between systemic and intracoronary blood, only monocyte-derived ($CD14^+$)-mMPs and EC-derived ($CD146^+$)-eMPs were significantly raised in intracoronary blood (Figure 3A-B). Other cell-derived MP types from leukocytes ($CD45^+$-LMPs), lymphocytes ($CD3^+$-ℓMPs), platelets ($CD61^+$-pMPs), and erythrocytes ($CD235a^+$-ErMPs) were similar in the two compartments (Figure 3A, C-D). As shown in Figure 4, AV^+-cMPs from activated cells were significantly higher in intracoronary blood than in peripheral blood. Specifically, AV^+-cMPs from activated monocytes ($CD14^+/CD11b^+$; mMPs; Figure 4A), activated platelets ($PAC1^+$ and $PAC1^+/CD62P^+$; pMPs; Figure 4B) and activated ECs ($CD62E^+$; eMPs; Figure 4C) were significantly increased in intracoronary blood. In addition, TF-bearing AV^+-cMPs were also higher within culprit coronary artery as compared to peripheral blood: total ($CD142^+$; TF^+-cMPs) and endothelial-derived ($CD62E^+/CD142^+$; TF^+-eMPs; Figure 4D; $P < 0.05$ in both cases).

Circulating microparticles and time of ischemia (pain-to-PPCI) in peripheral intracoronary blood

Intracoronary AV^+-cMPs were significantly related to the duration of the ischemic time (IT; time from the onset of chest pain to PPCI). STEMI-patients were divided into two groups based on IT≤3h and IT >3h. AV^+-cMPs derived from monocytes ($CD14^+$-mMPs) and activated monocytes ($CD14^+/CD11b^+$-mMPs) (Figure 5A), endothelial cells ($CD146^+$-eMPs; Figure 5B), and TF-rich monocytes and platelets ($CD142^+/CD14^+$-TF^+-mMPs and $CD142^+/CD61^+$-TF^+-pMPs; Figure 5C) were significantly higher in patients with an IT≤3h than in those with IT >3h. In contrast, systemic MPs were not related to IT. In the peripheral blood, AV^+-cMPs in IT ≤3h group of STEMI-patients were similar to those observed in IT >3h group (Figure 6).

In STEMI-patients the changes in MPs were quite different in coronary and peripheral blood depending on IT≤3h (n=24) or IT>3h (n=16). In the IT ≤3h group, monocyte-derived AV^+-mMPs ($CD14^+$ and $CD14^+/CD11b^+$; Figure 6A), endothelial-derived AV^+-eMPs ($CD146^+$ and $CD146^+/CD31^+$; Figure 6B), activated EC-derived AV^+-eMPs ($CD62E^+$ and $CD62E^+/CD142^+$; Figure 6C), and activated platelet-derived AV^+-pMPs ($PAC1^+$ and $PAC1^+/CD62P^+$; Figure 6D) were significantly elevated in culprit coronary artery compared to peripheral artery. In contrast, no significant differences, in terms of all type of analyzed AV^+-cMPs among peripheral versus coronary blood were observed in >3h, except for AV^+-cMPs derived from activated platelets carrying the activated $\alpha_{IIb}\beta_3$-integrin ($PAC1^+$-pMPs; Figure 6D), which were also elevated in the culprit intracoronary blood in IT >3h group. Interestingly, intracoronary and peripheral levels were related to each other in the case of AV^+-mMPs ($CD14^+$) and TF^+-mMPs ($CD14^+/CD142^+$) (R^2:0.580, $P < 0.05$ for $CD14^+$ and R:0.605, $P < 0.05$ for $CD14^+/CD142^+$; Supplemental Figure 1). Again, when STEMI-patients were divided in two groups based

on IT, the direct and positive association between systemic and coronary proportion was maintained except for TF^+-mMPs ($CD14^+/CD142^+$) in the IT >3h group.

Impact of clinical parameters on circulating microparticles

Multivessel diseases affected cMPs. Interestingly, peripheral blood AV^+-eMPs ($CD62E^+$) and TF^+-eMPs ($CD62E^+/CD142^+$) were higher in patients with multivessel atherosclerotic disease (Figure 7A). Additionally, AV^+-cMPs were also correlated with specific therapeutic intervention parameters. Thus, peripheral lymphocyte- and neutrophil-derived AV^+-cMPs ($CD3^+$-ℓMPs and $CD66b^+$-nMPs, respectively) were significantly higher in those patients with a drug-eluting stent (DES), specifically an everolimus-eluting stent (EES, Xience), than in those carrying a bare metal stent (BMS) (Figure 7B).

Discussion

The present study demonstrates a significant shift in the profile of cMPs in STEMI patients undergoing primary PPCI compared to healthy controls and patients 72 hours post-STEMI (day 3). Changes in prothrombotic, proinflammatory and endothelial dysfunction cMPs can be found both at systemic and intracoronary level, reflecting the sensitivity of cMPs as markers of the ongoing thrombus formation. Indeed, when the procoagulant cMP phenotype was investigated as a function of ischemic time, cMPs were significantly altered in blood samples collected from the culprit coronary artery in the first 3 hours after symptom onset but not at later times, suggesting their local release and contribution to intracoronary thrombus.

High levels of cMPs in STEMI patients have been well-described.[13-17] Upon coronary plaque rupture, extracellular microvesicles are released from activated platelets and apoptotic endothelial cells, which are associated to the area at risk during ST-elevation myocardial infarction.[18, 27] Similarly, we detected changes in the pattern of distribution between controls and STEMI-patients. Higher levels of proinflammatory-, endothelium-, activated platelet-, and erythrocyte-derived as well as tissue factor-rich cMPs were found in the acute phase of STEMI. After 3 days we observed a global decrease in cMPs, in accordance with the high rate of successful revascularization of the culprit artery. Nevertheless, post-STEMI patients maintained the same levels of activated pMPs and eMPs, tissue factor-rich MPs and nearly similar ErMPs. Surprisingly, pan-LMPs increased even after acute STEMI, likely due to the inflammatory burst occurred at STEMI onset. Indeed, not all subpopulations of LMPs remained elevated, post-STEMI cMPs from monocytes return to proportions of healthy controls. These results seem to indicate a selective targeting of leukocyte subsets to the damaged myocardium, likely

reflecting that specific monocyte types exert reparative roles in MI[28] while others participate in the cross-talk platelet-monocytes, which indicates a complex interplay of these cells in the post-MI state. The absence of concomitant decrease in a-LMPs and eMPs after 3 days of the ischemic event, suggest that atheromatous core of the plaque or residual thrombotic material may act as a reservoir of procoagulant MPs within the coronary artery. Additionally, variations in cMP concentrations during STEMI evolution could predict thrombus persistence or relapse, emerging as a potential marker of increased risk of ischemic events.

It has been noted that cMPs from endothelium, monocytes, activated platelets and rich in tissue factor are in a significantly higher proportion in blood samples collected from the culprit coronary artery than in samples from the peripheral artery of the same patients, suggesting a local shedding of activated cells in the leading edge of growing thrombus. Likewise, intracoronary cMPs are directly related to ischemic time, being more prominent at the earlier time after symptom onset, and peripheral cMPs also associated to the number of diseased vessels.

Indeed, the main MPs that are changed in STEMI are from circulating cells known to play a direct effect on atherothrombosis. In particular, endothelial-derived MPs have a role in endothelial dysfunction;[29] $CD31^+/AV^+$-cMPs are an independent predictor of CV events in stable CAD patients[22] which may be useful for risk stratification. Higher numbers of pMPs are found in STEMI patients that have exacerbated platelet activation and thrombus formation;[9] and, pMPs carrying activation epitopes were higher both in intracoronary and systemic blood. Circulating MPs, primarily of leukocyte origin, are considered a primary source for blood-borne tissue factor involved in thrombus propagation at the site of vascular injury.[30] In addition, human atherosclerotic plaques contain high levels of MPs expressing CD40L and bearing TF.[31] During plaque disruption and thrombus formation,

circulating TF-bearing MPs and pMPs might contribute to high levels of TF-activity at the thrombus triggering the formation of fibrin. Last but not least, cMPs generate and transport mCRP in MI patients.[32]

Although cMPs have emerged as potential biomarkers of cardiovascular disease,[22-24] cMP detection and quantification at large scale is still in its infancy. For instance, some groups are now focusing on small-size cMPs as independent predictors of cardiovascular disease.[33] However, conventional flow cytometry, the state-of-the-art technique for cMP characterization, can not distinguish this particular size-type of microvesicles and it is not know whether these are the most pathogenic ones. Moreover, other groups provide their measurements as protein content or PS^+-captured MPs.[34] Thus, under these inconsistencies and despite recent advances in this regard,[35-37] major improvements are urgently needed for standardization of current available techniques in terms of size, phenotype and quantity among laboratories. Until now, the majority of studies evaluate cMPs as biomarkers measuring just their levels; but MP assessment in terms of levels has been proved to be complex and susceptible to imprecision when used on larger cohorts for clinical purpose. A novel strategy of our study is to further characterize the particular cell and activation phenotype of annexin V-positive cMPs.

A limitation of this study is that cMPs from cardiomyocytes and smooth muscles cells were not measured due to lack of reliable and specific cell surface markers. However, they might also be contributors to the pathophysiology of STEMI patients.

No previous studies, to the best of our knowledge, have investigated the clustering of cell-type and activation status of cMPs in the intracoronary blood of STEMI patients undergoing primary PPCI nor comparison to peripheral blood levels. In conclusion, our results gave a profile signature of cMPs at both systemic and coronary levels in STEMI patients undergoing PPCI, which is associated to duration of pain-to-PPCI

ischemic time. Taken together, these data support a role for MPs in thrombus formation and propagation and indicates that MPs are sensitive to disease severity, reflecting the temporal evolution of disease, which can be used to improve the prognosis of STEMI patients.

Funding sources

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Table 1. Baseline clinical characteristics of STEMI-patients.

	Ischemia time ≤ 3 h (n=24)	Ischemia time > 3 h (n=16)	*P*-value
Age (years, mean±SE)	67.0±2.3	60.1±3.8	*P*=0.1104
Risk factors (n, %)			
Smoking	16 (66.7%)	6 (37.5%)	*P*=0.1064
Dyslipidemia	13 (54.2%)	11 (68.8%)	*P*=0.5121
Diabetes mellitus	4 (16.7%)	7 (43.7%)	*P*=0.0800
Systemic hypertension	17 (70.8%)	9 (56.2%)	*P*=0.5001
Obesity	4 (16.7%)	5 (31.2%)	*P*=0.4414
Medical History (n, %)			
Pre-AMI	3 (12.5%)	1 (6.2%)	*P*=0.6376
Pre-PCI	2 (8.3%)	3 (18.7%)	*P*=0.3725
Pre-CABG	1 (4.2%)	0 (0%)	*P*=0.4083
Pre-Peripheral vasculopathy	0 (0%)	1 (6.2%)	*P*=0.4000
STEMI-clinics			
Killip on admission (I / II-IV)	21 / 3	16 / 0	*P*=0.2615
Creatinine kinase at peak, U/L	3560.8±617.5	3275.4±508.3	*P*=0.7431
ACEF score, units	1.5±0.1	1.1±0.1	*P*=0.0885
LVEF, %	49±2.1	51±2.5	*P*=0.6118
Culprit vessel (n, %)			
LAD	14 (58.3%)	7 (43.7%)	*P*=0.3243
Cx	6 (25%)	4 (25%)	*P*=0.9999
RCA	18 (75%)	13 (81.2%)	*P*=0.7171
Localization (AL / IP)	10/14	4/12	*P*=0.3295
Diseased vessels (n, %)			
1	18 (75%)	11 (68.8%)	*P*=0.7275
2	4 (16.7%)	4 (25%)	*P*=0.6857
3	2 (8.3%)	1 (6.2%)	*P*=0.8064
Graft (n, %)	2 (8.3%)	0 (0%)	*P*=0.5077
IABP (n, %)	2 (8.3%)	1 (6.2%)	*P*=0.8064
Thrombectomy (n, %)	19 (79.2%)	15 (93.7%)	*P*=0.4159
Direct stenting (n, %)	11 (45.8%)	9 (56.2%)	*P*=0.5145
Stenting (n, %)			
Bare-metal stenting	20 (45.8%)	14 (87.5%)	*P*=0.7177
Drug-eluting stenting	4 (83.3%)	2 (12.5%)	*P*=0.7177
Total length of stent (mm)	35.0±4.3	31.7±5.3	*P*=0.6315
TIMI pre-PCI (n, %)			
0	19 (79.2%)	13 (81.2%)	*P*=0.8718
1	1 (4.2%)	0 (0%)	*P*=0.4083
2	3 (12.5%)	1 (6.2%)	*P*=0.6376
3	1 (4.2%)	2 (12.5%)	*P*=0.5530
TIMI post-PCI (n, %)			
0	0 (0%)	0 (0%)	*P*=0.9999
1	1 (4.2%)	0 (0%)	*P*=0.4083
2	2 (8.3%)	1 (6.2%)	*P*=0.8064
3	21 (87.5%)	15 (93.7%)	*P*=0.6376
MACE (n, %)	12 (50%)	1 (6.2%)	*P*=0.0053
Treatment (n, %)			
GPIIb/IIIa antagonists			
Abciximab	20 (8.3%)	14 (87.5%)	*P*=0.7177
Tirofiban	1 (4.2%)	0 (0%)	*P*=0.4083
Acetylsalicylic Acid	18 (75%)	11 (68.7%)	*P*=0.9424
Clopidogrel	7 (29.2%)	4 (25%)	*P*=0.7725
Heparin	24 (100%)	16 (100%)	*P*=0.9999

Table 1. Baseline clinical characteristics of STEMI-patients. ACEF, age, creatinine, and ejection fraction; AL, anterolateral; AMI, acute myocardial infarction; CABG, Coronary Artery Bypass Grafting; Cx, circumflex coronary artery; GP, glycoprotein; IABP, intra-aortic balloon pump; IP, inferoposterior; LAD, left anterior descending coronary artery; MACE, major adverse cardiac events; PPCI, primary percutaneous coronary intervention; RCA, right coronary artery; SE, standard error; STEMI, ST-segment elevation myocardial infarction; TIMI, thrombolysis in myocardial infarction.

Figure Legends

Figure 1. Cell origin distribution of circulating microparticles in STEMI-patients. Box and whisker plots of AV^+-cMPs from **(A)** leukocytes, lymphocytes, and monocytes, **(B)** endothelial cells , and **(C)** platelets in healthy controls (n=20), STEMI-patients (n=40) and post-MI patients (n=20). Data are expressed as median (IQR). **P*<0.05 vs controls and †*P*<0.05 vs STEMI (Non-parametric U-Mann Whitney test). **P*<0.05 (Kruskal Wallis test). **(D)** ROC curve analysis used to evaluate combination of cMPs to predict STEMI with AUC indicated along its 95%CI (*P*<0.0001).

Figure 2. Circulating microparticles from activated cells in STEMI-patients. Box and whisker plots of AV^+-cMPs from **(A)** activated leukocytes, **(B)** activated endothelial cells, **(C)** activated platelets, **(D)** both platelets and endothelial cells, and **(E)** tissue factor-positive cells in healthy controls (n=10) and STEMI-patients (n=40). Data are expressed as median (IQR). **P*<0.05 (Non-parametric U-Mann Whitney test). **(F)** ROC curve analysis used to evaluate combination of cMPs to predict STEMI with AUC indicated along its 95%CI (*P*<0.0001).

Figure 3. Cell origin distribution of circulating microparticles in peripheral and intracoronary blood of STEMI-patients. Box and whisker plots of AV^+-cMPs from **(A)** leukocytes, **(B)** endothelial cells, **(C)** platelets, and **(D)** erythrocytes in peripheral and intracoronary blood of STEMI-patients (n=40). Data are expressed as median (IQR). **P*<0.05 (Non-parametric Wilcoxon-Signed Rank test).

Figure 4. Circulating microparticles from activated cells in peripheral and intracoronary blood of STEMI-patients. Box and whisker plots of AV^+-cMPs from **(A)** activated monocytes, **(B)** activated platelets, **(C)**

activated endothelial cells, and **(D)** tissue factor-positive total cells and endothelial cells in peripheral and intracoronary blood of STEMI-patients (n=40). Data are expressed as median (IQR). *$P<0.05$ (Non-parametric Wilcoxon-Signed Rank test).

Figure 5. Intracoronary circulating microparticles in association to ischemic time of STEMI-patients. Box and whisker plots of AV^+-cMPs from **(A)** monocytes and activated monocytes, **(B)** endothelial cells, and **(C)** tissue factor-positive monocytes and platelets in intracoronary blood of STEMI-patients (n=40). Data are expressed as median (IQR). *$P<0.05$ (Non-parametric U-Mann Whitney test).

Figure 6. Circulating microparticles in peripheral and intracoronary blood related to ischemic time of STEMI-patients. Box and whisker plots of AV^+-cMPs from **(A)** activated monocytes and activated monocytes, **(B)** endothelial cells, **(C)** activated endothelial cells and tissue factor-positive endothelial cells, and **(D)** activated platelets in peripheral and intracoronary blood of STEMI-patients (n=40) classified in two groups: ischemic time ≤ 3h (n=24) and > 3h (n=16). Data are expressed as median (IQR). *$P<0.05$ (Non-parametric Wilcoxon-Signed Rank test).

Figure 7. Circulating microparticles of STEMI-patients related to diseased vessels and type of stent. Box and whisker plots of AV^+-cMPs in association with **(A)** number of diseased vessels (one *versus* two or more treated vessels) and **(B)** type of stent (drug-eluting stent [DES] *versus* bare metal stent [BMS]). AV^+-cMPs from endothelial cells **(A-I/II)** and lymphocytes **(B-I)** and granulocytes **(B-II)** in the peripheral blood of STEMI-patients (n=40). Data are expressed as median (IQR). * $P < 0.05$ (Non-parametric U-Mann Whitney test).

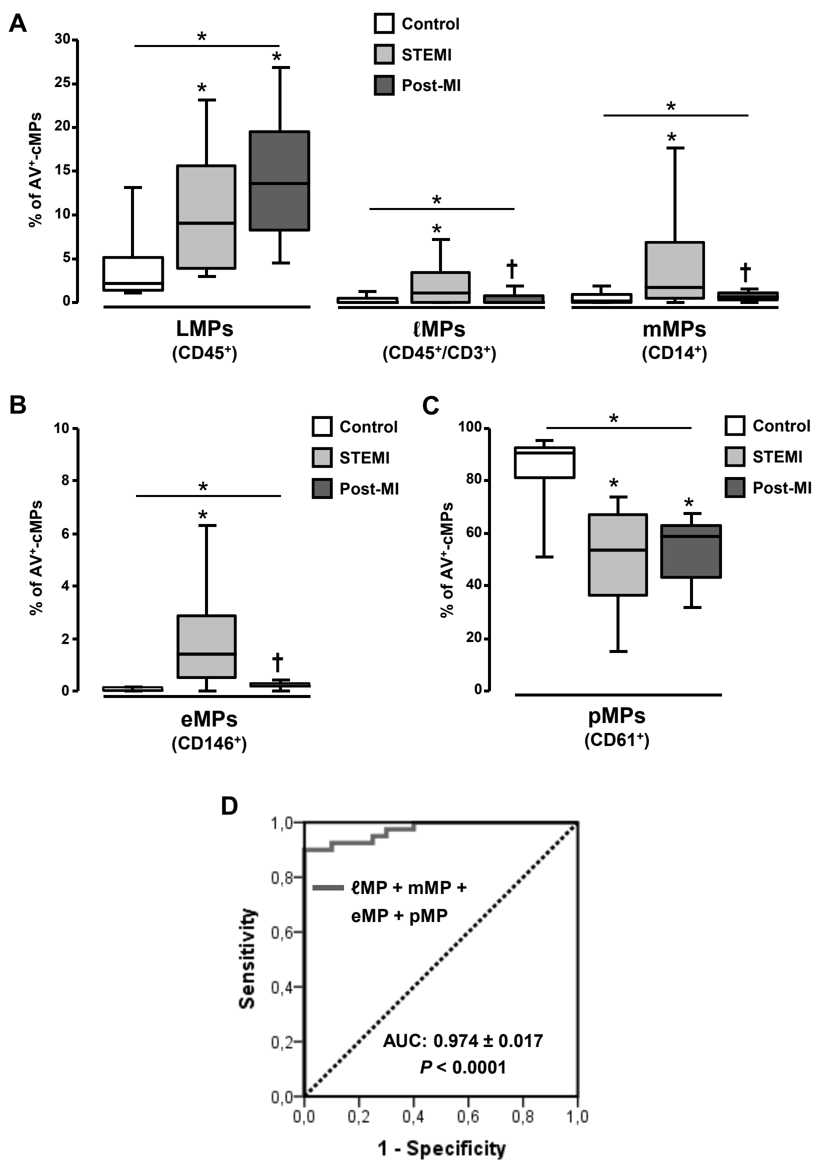


Figure 1

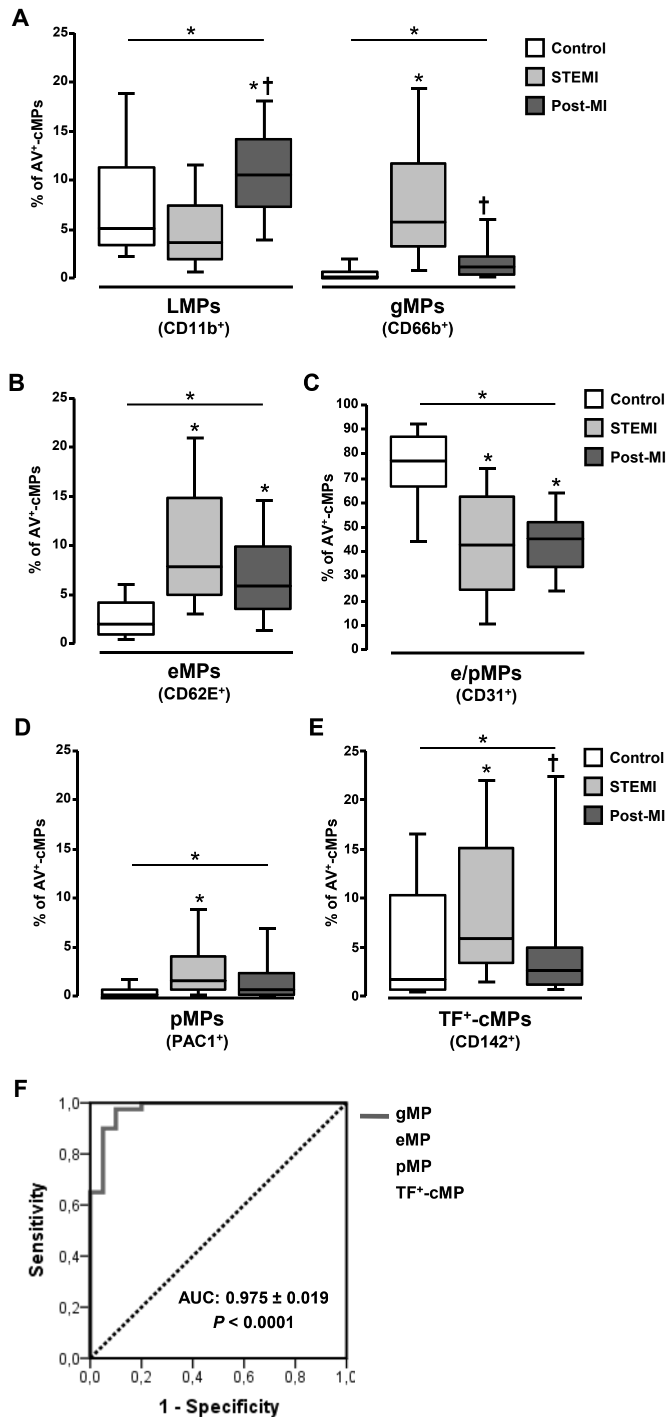


Figure 2

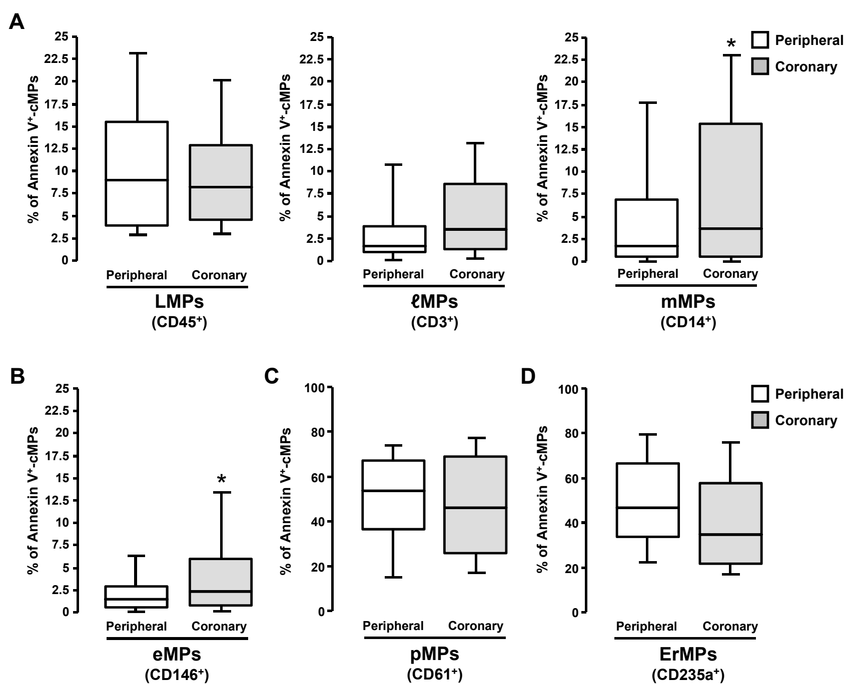


Figure 3

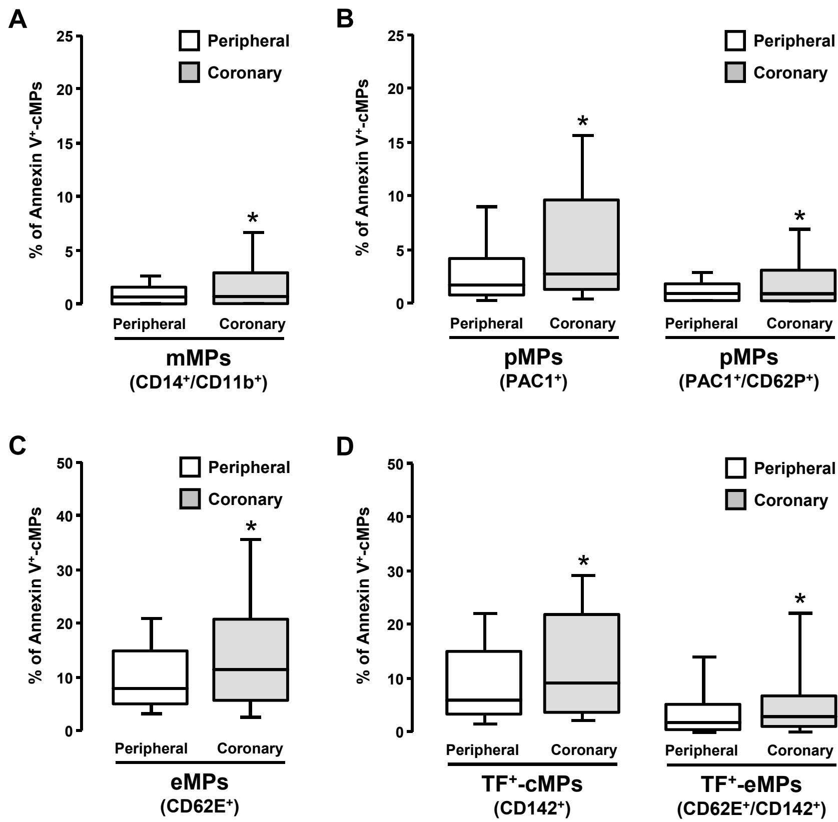


Figure 4

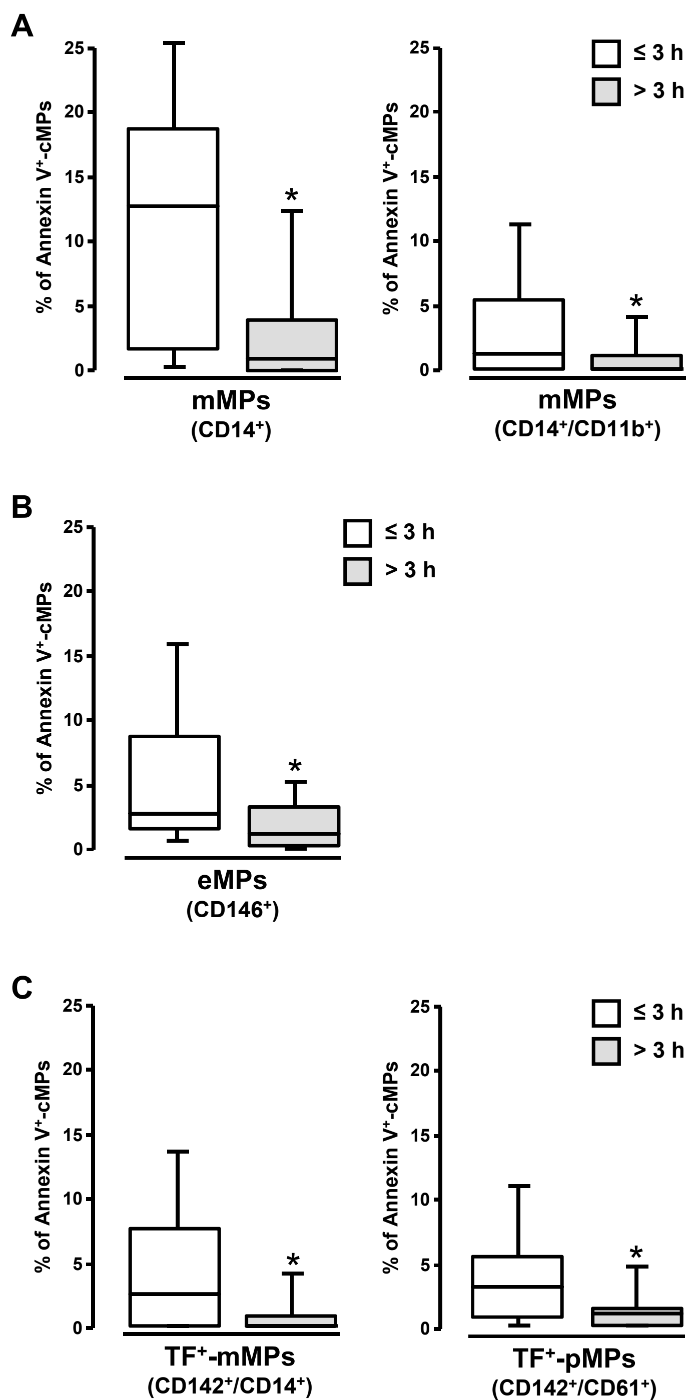


Figure 5

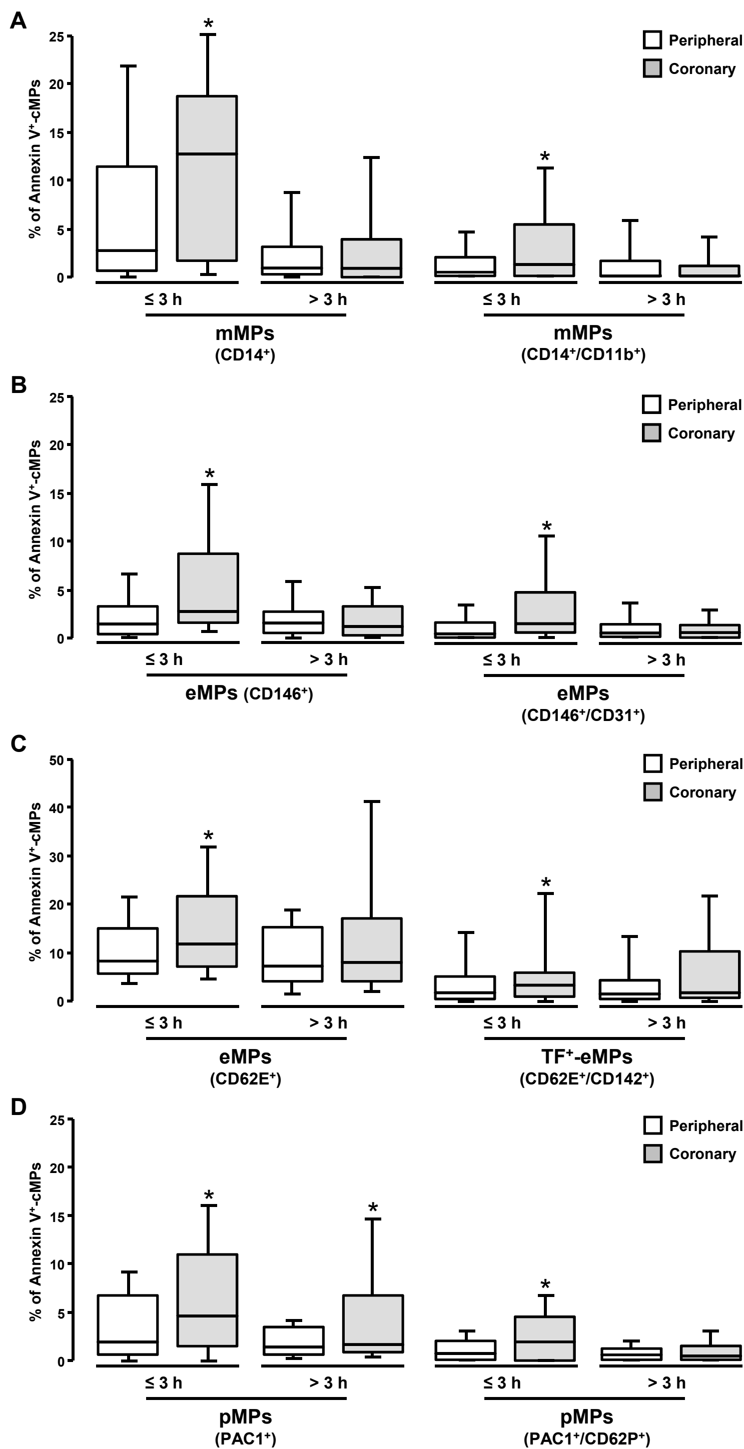


Figure 6

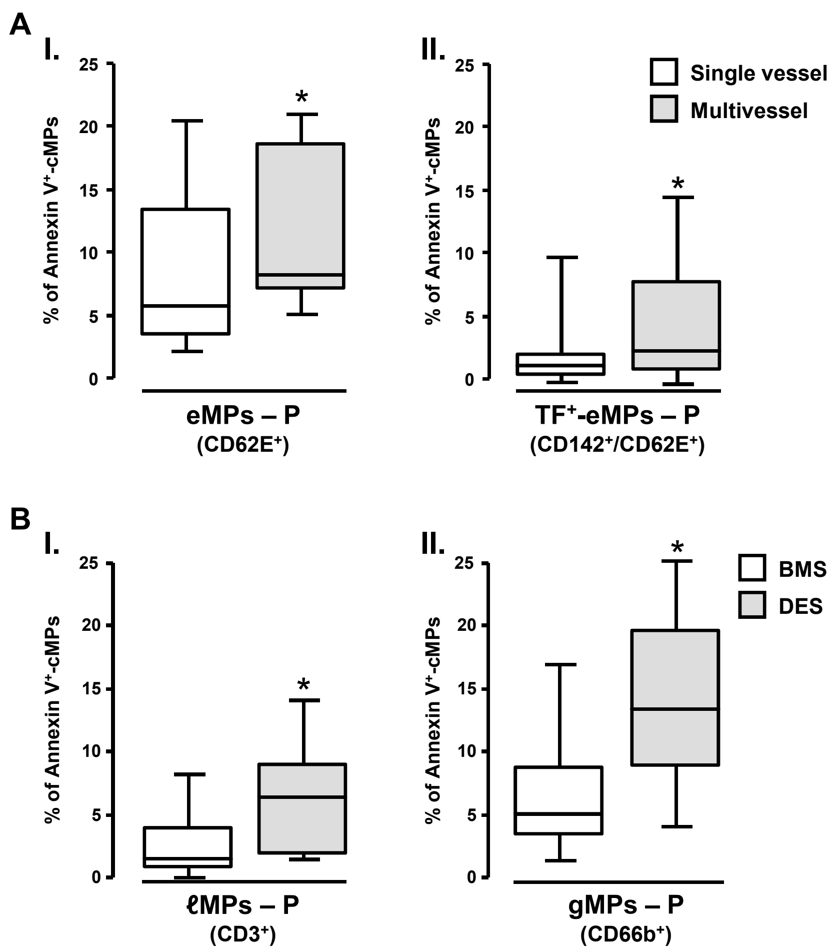


Figure 7

Supplemental Material

Supplemental Table I. Baseline clinical characteristics of study population.

	STEMI (n=40)	Post-MI 72h (n=20)	Controls (n=20)	Statistics (*P*-value) Controls *vs* STEMI	Controls *vs* post-MI	STEMI *vs* post-MI
Age (years, mean±SE)	64.2±2.11	63.6±3.0	58.4±1.9	*P*=0.0891	*P*=0.1807	*P*=0.8718
Risk factors (n, %)						
Smoking	22 (55%)	9 (45%)	7 (35%)	*P*=0.1439	*P*=0.5186	*P*=0.4650
Dyslipidemia	24 (60%)	13 (55%)	12 (60%)	*P*=0.9999	*P*=0.7440	*P*=0.7073
Diabetes mellitus	11 (27%)	5 (25%)	5 (25%)	*P*=0.8365	*P*=0.9999	*P*=0.8365
Systemic hypertension	26 (65%)	11 (55%)	11 (55%)	*P*=0.4526	*P*=0.9999	*P*=0.4526
Obesity	9 (22%)	3 (15%)	5 (25%)	*P*=0.8291	*P*=0.4292	*P*=0.4936
Drugs of abuse	3 (7.5%)	0 (0%)	0 (0%)	*P*=0.2089	*P*=0.9999	*P*=0.2089

MI, myocardial infarction; SE, standard error; STEMI, ST-segment elevation myocardial infarction.

Supplemental Table II. Patient treatment at time of blood collection.

	STEMI (n=40)	Post-MI 72h (n=20)	Statistics (*P*-value)
STEMI-clinics (n, %)			
Antithrombotic therapy			
Clopidogrel	11 (27%)	18 (90%)	$P<0.0001$
Acetylsalicylic Acid	29 (72%)	20 (100%)	$P=0.0095$
Anti-glycoprotein IIb/IIIa	35 (87%)	6 (30%)	$P<0.0001$
Heparin	24 (65%)	20 (100%)	$P=0.0010$
TIMI flow grade 3			
Pre-PCI	3 (1%)	1 (5%)	$P=0.7144$
Post-PCI	34 (85%)	17 (85%)	$P=0.9999$

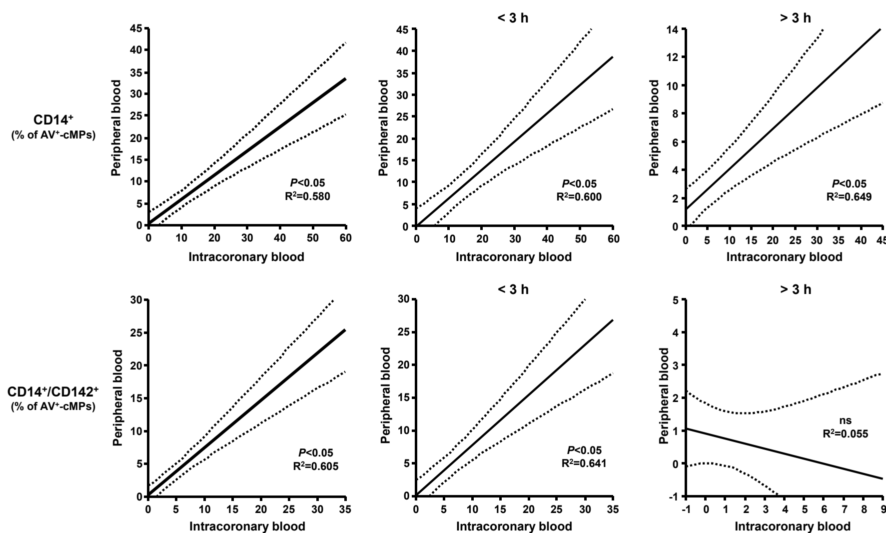
SE, standard error; STEMI, ST-segment elevation myocardial infarction; TIMI, thrombolysis in myocardial infarction.

Supplemental Table III. Cell surface molecules for circulating microparticle identification and characterization.

Marker	Alternative name	Expression	Conjugation	Clone	Company
Annexin V	PS-binding protein	Widely expressed	BD Horizon V450	-	BD Pharmingen
CD3	T3	Lymphocytes	FITC	HIT3a	BD Pharmingen
CD11b	MAC-1	Neutrophils, monocytes	FITC	VIM12	Molecular Probes
CD14	LPS-receptor	Macrophages, monocytes	PE	M5E2	BD Pharmingen
CD31	PECAM-1	Platelets, endothelial cells	PE	1F11	BD Pharmingen
CD45	LCA	Leukocytes	PE	Immu-19.2	Beckman Coulter
CD61	β_3-integrin	Platelets	PE	VI-PL2	BD Pharmingen
CD62E	E-selectin	Activated endothelial cells	PE	68-5H11	BD Pharmingen
CD62P	P-selectin	Activated platelets	PE	AK-4	BD Pharmingen
CD66b	CD67	Granulocytes	FITC	G10F5	BD Pharmingen
CD142	Tissue factor	Widely expressed	FITC	VD8	American Diagnostica
CD146	MUC18	Endothelial cells	FITC	P1H12	BD Pharmingen
CD235a	Glycophorin A	Erythrocytes	FITC	11E4B-7-6	Beckman Coulter
PAC1	$\alpha_{IIb}\beta_3$-integrin	Activated platelets	FITC	PAC1	BD Pharmingen
IgG1γ	-	-	FITC / PE	X40	BD Biosciences
IgG1κ	-	-	FITC / PE	MPOC21	BD Pharmingen

FITC indicates fluorescein isothiocyanate; PE, phycoerythrin; MAC-1, integrin alpha M; LPS, lipopolysaccharide; PECAM-1, platelet endothelial cell adhesion molecule-1; LCA, leukocyte common antigen; MUC18, melanoma-associated antigen.

Supplemental Figure



Supplemental Figure 1. Relationship between peripheral and intracoronary blood. Correlation of total monocyte-derived ($CD14^+$-mMPs) and tissue factor-rich mMPs ($CD14^+/CD142^+$) percentage between peripheral and intracoronary blood and categorized by time-to-PPCI (<3 and >3 hours) after symptom onset. Data expressed as percentage of annexin V-positive cMPs (AV^+-cMPs). Non-parametric Spearman correlation. *NS,* non significant.

Article 7

Microparticles from thrombin-induced platelets have a complex proteomic profile rich in prothrombotic components

Rosa Suades, Teresa Padró, Lina Badimon

Manuscript in preparation.

Objectives

The aim of this study was to investigate the potential thrombogenic content of microparticles derived from platelets activated with thrombin, the most common initial agonist in tissue factor-triggered coagulation, a process that commonly occurs in the onset of the clinical complications of atherothrombosis.

Highlights

- Thrombin-induced platelet activation triggers the release of microparticles with a complex proteomic profile modifying the expression pattern of some proteins related to thrombosis.
- This proteomic approach might help to elucidate basic molecular mechanisms of thrombin stimulation on platelets with relevant impact on atherothrombotic disease.

Microparticles from thrombin-induced platelets have a complex proteomic profile rich in prothrombotic components

Suades et al: Thrombin effect on platelet microparticle proteome

By:

Rosa Suades BS [1,2,3], **Teresa Padró** PhD [1,2], **Lina Badimon** PhD, FESC, FAHA [1,2,3,4]

From:

[1] Cardiovascular Research Center, CSIC-ICCC, Barcelona, Spain

[2] Biomedical Research Institute Sant-Pau (IIB-Sant Pau), Barcelona, Spain

[3] CIBEROBN-Pathophysiology of Obesity and Nutrition, Barcelona, Spain

[4] Cardiovascular Research Chair, UAB, Barcelona, Spain

Correspondence to:

Prof. Lina Badimon
Cardiovascular Research Center, c/Sant Antoni Mª Claret 167, 08025 Barcelona, Spain.
Phone: +34.935565880.
Fax: +34.935565559.
E-mail: lbadimon@csic-iccc.org

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Abstract

Introduction. Platelets play a fundamental role in pathological events underlying acute coronary syndromes (ACS). Microparticles (MPs) from different cellular origin are found elevated in the circulation of patients with thrombotic diseases and ACS. However, MP protein composition remains poorly defined. Platelet-derived microparticle (pMP) phenotype, rather than their quantity, may help to better understand atherothrombotic pathophysiologic processes.

Objective. The present study was performed to (1) characterize the pMP proteomic profile induced by thrombin activation, a process that commonly occurs in the onset of the clinical complications of atherothrombosis, and (2) to identify differential proteins related to platelet function and thrombogenicity.

Methods. MPs were prepared from a suspension of washed platelets obtained from healthy donors and activated *in vitro* with 0.5 uNIH/mL thrombin (3 min at 37ºC) by differential centrifugation and characterized by flow cytometry using Annexin V and CD41. Proteomic studies were performed by bidimensional electrophoresis and mass spectrometry (MALDI-ToF). Proteins were identified using Swiss-Prot database. Differences in the protein patterns were analyzed using specific data analysis software (PDQuest).

Results. Flow cytometric analysis revealed that pMP from non-activated platelets (C-MP) have binding capacity for annexin V, express high levels of CD41 and low levels of P-selectin and PAC1. After thrombin activation, P-selectin and PAC1 levels were significantly increased. The proteomic analysis showed a total number of 337 protein spots were found in both groups. By 2D-electrophoresis 382±9 different proteins features were detected in thrombin-activated pMPs (T-MP) and a total of 73 protein spots were differentially altered in T-MPs compared to C-MPs,

which were related to: a) cytoskeleton and involved in cell organization; b) signal transduction; c) metabolism and, d) vesicle-mediated transport.

Conclusion. Thrombin-induced platelet activation triggers the release of microparticles with a complex proteomic profile that may transfer proteins among blood and vascular cells facilitating thrombus formation. T-MPs carry proteins that may have a functional involvement in occlusive thrombus formation and the progression of atherosclerosis and will enable to investigate new therapeutic targets of atherothrombotic processes directed to the pMPs.

Key Words: atherothrombosis, microparticles, platelets, releasate, thrombin.

Introduction

Platelet-derived microparticles (pMPs) are a heterogeneous population of microvesicles, ranging from 100 nm to 1.0 µm, generated from the plasma membrane upon activation by various stimuli.[1-3] pMPs constitute the majority of the circulating microparticles in the bloodstream.[4-6] Although microparticles are present in the blood of healthy individuals,[7] elevation of platelet-derived microparticle levels occurs in cardiovascular disorders such as atherosclerosis and acute coronary syndromes.[8-10] pMPs are produced in different amounts and with differences in their proteome depending on the platelet stimulus.[11] pMPs contain a unique subset of proteins derived from the parent cell, and in recent years it has become clear that they have important biological functions, such as their participation in blood coagulation[12] and as carriers of bioactive messages.[13] Indeed, we have recently shown that platelet-derived microparticles enhance arterial thrombosis on atherosclerotic plaques.[14]

Platelets are major triggers of thrombosis on atherosclerotic plaques, the leading cause of cardiovascular disease.[15,16] Platelets are activated by a variety of agonists (thrombin, collagen, ADP) and mechanical stimuli (shear stress) and adhere and aggregate at sites of vascular injury.[17] In atherosclerotic plaque-triggered thrombosis the main initiator is the tissue factor present in the disrupted atherosclerotic plaque[18-20] that triggers thrombin formation a monolayer of fibrin and platelet activation to exponentially recruit further platelets and fibrin. Based on our recent findings of a significant effect of pMPs in promoting thrombus formation on injured vessel wall[14] we hypothesized that pMPs had to carry messengers to facilitate that prothrombotic effects and also modifiers of blood homeostasis. Henceforth our objective has been to indentify the proteins carried by pMPs when platelets are activated by thrombin. To fulfil this objective we have applied proteomics and systems biology approach.

Proteomics is a useful tool for the identification of proteins coordinately involved in platelet activation and vascular regulatory mechanisms.[21-23] Microparticle proteomics is of high importance as MPs are shed in biological fluids (blood flow, urine, saliva, and cerebrospinal fluid). Furthermore, focusing on a platelet subproteome such as MPs enables to reduce complexity as well as to improve assessment of low abundance proteins.

Several proteomic studies have been performed on plasma or platelet-derived microparticle proteomes within the last ten years. A defined platelet microparticle proteome obtained from activation of washed platelets by ADP was reported[24] and recently amplified.[25] Then, a comparative proteome of plasma-derived with platelet-derived microparticles was described.[6] Other studies have studied in-depth the plasma microparticle proteome from healthy donors[26,27] or from different pathologies.[28-32] The protein composition of plasma microparticles from healthy donors shows high variability;[33] however, a core set of plasma MP proteins found across population rather than in a subset of individuals has been reported.[34] To increase the complexity it has been shown that different size classes of pMPs contain different protein components[35] and that the different stimulation conditions (shear and shear plus thrombin) induce very different types of pMPs.[11] Releasates (soluble fraction or full secretome without removing MPs) from platelets activated by thrombin or TRAP was characterized,[36-38] although in these last studies microparticles were not specifically analyzed.

Because we found a significant effect of pMPs and cMPs in promoting thrombosis on damaged vascular wall,[14] here we have approached the proteomic study of pMPs concentrating in the prothrombotic proteome of microparticles derived from platelets activated with thrombin, the most common initial agonist in TF-triggered atherothrombosis.

Methods

Platelet activation and platelet-derived microparticle subfractionation

Human platelets from fresh healthy donor platelet concentrates were centrifuged (1200 ×g, 10 minutes, 20ºC), washed 3 times and resuspended in Ca^{2+} free - HEPES-Tyrode's buffer (HTB; containing 134mM NaCl, 0.34mM Na_2HPO_4, 2.9mM KCl, 12mM $NaHCO_3$, 1mM $MgCl_2$, 5mM $C_6H_{12}O_6$ and 20mM HEPES; pH 7.3). Platelet function was determined by optical aggregometry (*data not shown*). Washed platelets were counted and adjusted to a final concentration of $4.0x10^6$ platelets/μL (Medonic CA530-16 hematologic analyzer). The platelet suspension was activated with thrombin (human T, 0.5 uNIH/mL) or its buffer (control –C–) for 3 minutes at 37ºC with constant slow stirring. Immediately thereafter, platelets were pelleted by a centrifugation step (3220 ×g, 10 minutes, 20ºC) and stored at -80ºC for further studies. The supernatant was centrifuged once more (3220 ×g, 10 minutes, 20ºC) to assure the removal of platelets. The final supernatant contained only MPs (particles less than approximately 1.0 μm). One aliquot of MPs was taken for flow cytometry analysis for MP characterization. Platelet-derived microparticles (pMPs) were isolated from the supernatants of thrombin-stimulated platelets and their control by ultracentrifugation (150000 ×g, 90 minutes, 10ºC) with a 50.2Ti rotor (Beckman Coulter).[6] The final pMP pellet was resuspended in lysis buffer containing protease inhibitors (10mM Tris/HCl, 0.15M KCl, 0.1% Triton X-100, 2.9mM PMSF, 0.1mM DTT, 1μg/mL Leupeptin, 1μg/mL Aprotinin, pH 7.4). pMPs-aliquots were snap-frozen in liquid nitrogen and stored at -80ºC until proteomic studies were performed.

Flow cytometry characterization

pMPs were identified and size characterized by flow cytometry using size calibrated microspheres and fluorescent labelling for annexin V as surface marker. Samples (25 µl of each) were incubated 15 minutes in the dark with 5 µl of phycoerythrin (PE)-conjugated Annexin V (Molecular Probes). Next, the samples were diluted in Tyrode's Buffer supplemented with 5 mM $CaCl_2$ (the presence of low calcium concentrations is essential for annexin V binding) and analyzed in a Beckman Coulter Epics XL flow cytometer. The instrument was calibrated before analysis with Flow Check YG Size Range Calibration Kit (Polysciences). Light scatter and fluorescence channels were set at logarithmic gain. A platelet gate was created according to its light scatter. The fluorescence-positive particles were separated on another histogram on the basis of size (forward light scatter). Microparticle gate was defined as events smaller than 1-µm diameter (microspheres) and positively labelled with PE-Annexin V in FL/FSC fluorescence dot plot (size particle vs. fluorescence).

Proteomic analysis

Sample preparation: pMP-samples were subjected to IgG removal with protein G sepharose (GE Healthcare) in order to increase protein resolution and detection power. Then, the samples were cleaned by centrifugation using 3kDa centrifugal filter devices (Millipore) and sample buffer was exchange to a urea denaturing buffer (8mol/L urea, 2mol/L thiourea, 2% w/v CHAPS). Prior protein separation, samples were desalted and decontaminated (from ionic detergents, nucleic acids, lipids) by ReadyPrep 2D-CleanUp Kit (Bio-Rad), following supplier's manual. Protein concentration was determined in triplicate using Quant Kit protein assay reagents (GE Healthcare).

<u>Two-dimensional gel electrophoresis (2-DE)</u>: Extracts (120 µg for analytic gels or 300 µg for preparative gels) were diluted in rehydratation solution (7mol/L urea, 2mol/L thiourea, 2% w/v CHAPS, 100 mmol/L DTT, and 0.2% carrier ampholytes) and loaded by active rehydratation on 17-cm dry strips (pH 3-10 linear range, Bio-Rad) at 50 V during 16 hours. Strips were isoelectric focused (IEF) at 0.05 mA/strip for 70 kV/h at 20ºC using the Protean-IEF cell (BioRad). Once IEF was completed the strips were equilibrated with a reducing solution (50 mM Tris-HCl buffer, pH 8.8, containing 6 M urea, 2% SDS, 30% glycerol, and 2% DTT) and an alkylating solution (50 mM Tris-HCl buffer pH 8.8, 6 M urea, 2% SDS, 30% glycerol, and 2.5% iodoacetamide), for 15 minutes each. For the second dimension, strips were applied on top of 12% SDS-polyacrilamide separating gels and electrophoresis was performed using an Ettan Dalt Six system (GE Healthcare) at 17w/gel for analytical gels or a Protean system (Bio-Rad) at 40 mA/gel for preparative gels. For each independent experiment, 2-DE for protein extracts from the four treatment groups were processed in parallel. Gels were visualised by fluorescent staining (Flamingo Fluorescent Gel Stain, Bio Rad). Analysis for differences in protein patterns was performed with the PDQuest 8.0 (BioRad), using a single master that included all gels of each independent experiment. For image analysis, fluorescence-stained gels were scanned (Typhoon 9400, GE Healthcare) –all were scanned at 100µm resolution–, and analysed for differences in protein patterns between control and thrombin-stimulated samples with PDQuest 8.0 software (Bio-Rad). Each spot was assigned a relative value that corresponded to the single spot volume compared to the volume of all spots in the gel, following background extraction. Normalization between gels was based on local regression model (LOESS).

<u>Protein Identification by Maldi-ToF-MS Analysis</u>: Gel pieces containing protein spots of interest were excised from 2-DE gels, using

Spot Picker. After destaining (15 mmol/L potassium ferricyanide, 50 mmol/L thiosulfate solution), proteins spots were washed with 50 mmol/L ammonium bicarbonate, 50% methanol, dehydratated with 100% acetonitrile, and dried under vacuum before enzymatic digestion with sequence-grade modified porcine trypsin (Promega). Peptides from in-gel-trypsin digestion were applied to Prespotted AnchorChip plates surrounding the calibrants provided on the plates. Protein identification was performed by matrix-assisted laser desorption/ionization –time of flight (MALDI-TOF) mass spectrometry using an AutoFlex III SmartBeam MALDI-TOF/TOF (Bruker Daltonics). All spectra were acquired with flexControl on reflector mode (mass range 850–4000 m/z, reflector 1: 21.06 kV; reflector 2: 9.77 kV; ion source 1 voltage: 19 kV; ion source 2: 16.5 kV; detection gain 2.373) with an average of 3500 added shots at a frequency of 200 Hz. Each sample was processed with flexAnalysis (version 3.0, Bruker Daltonics) considering a signal-to-noise ratio over 3, applying statistical calibration, and eliminating background peaks. For protein identification, peaks between 850 and 1000 m/z were not considered because in general only matrix peaks are visible on this mass range. After processing, spectra were sent to the interface BioTools (version 3.2; Bruker Daltonics) and MASCOT search on Swiss-Prot 57.15 database was done (taxonomy: homo sapiens, mass tolerance 50–100, up to 2 trypsin miss cleavages, global modification: carbamidomethyl [C], variable modification: oxidation [M]). Identification was accepted with a score greater than 56 by peptide mass fingerprint and confirmed by tandem mass spectrometry.

Western blotting

Western blot analysis was carried out on nitrocellulose membranes using the following rabbit anti-flotillin monoclonal antibody. Membranes were exposed to horseradish peroxidise-labelled goat anti-rabbit antibody

(Millipore), processed using an enhanced-chemiluminiscence system and quantified by densitometry (BioRad).

Data mining

GO Slim / MGI (Mouse Genome Informatics) software was used for gene ontology (GO) assignments to identify proteins and determine significantly under-and-over-represented functional GO categories: on the basis of cellular component, molecular function and biological process categories. The annotations and analyses were made using the default MGI human database and the GO cell component, GO molecular function and GO biological process ontology. Signalling pathways were investigated using the Kyoto Encyclopedia of Genes and Genome (KEGG) database and with Phanter software.
Group of pMP identified proteins was also compared to Vesiclepedia and ExoCarta databases in order to better define our microparticle fraction.

Ingenuity pathways analysis

Ingenuity Pathways Analysis (IPA) software (Ingenuity Systems, CA) was used to investigate possible interactions among all the identified differentially expressed proteins. Interactive pathways were generated to observe potential direct and indirect relations among the differentially expressed proteins.

Statistical analysis

2-DE and WB analysis data are expressed as median and interquartile range [IQR]. N indicates the number of experiments. Statistical analysis was performed with StatView 5.0.1 software. Parametric student's T-test and non-parametric Mann-Whitney test were used for comparison between the two groups. A p-value <0.05 was considered significant.

Results

Platelet-derived microparticle characterization

Isolated microparticles were identified by flow cytometry based on their light scattering properties (forward scatter –FSC– and size scatter –SSC–) as events smaller than 1 μm. Flow cytometric gating of isolated pMPs using their FSC and SSC signals is shown in Figure 1A. The centrifugation conditions used for their isolation provide an optimal yield of microparticles, while cell and platelet contamination is minimal (less than 0.1%).

Flow cytometry against microparticle-associated marker annexin V was similar among all pMP samples. The presence of this well-established marker confirms that pMP-sample conform a properly-isolated homogenous and pure fraction. The data showed that platelets shed high amounts of microparticles upon thrombin-stimulation, with significantly higher percentage of platelet activation markers. Figure 1B shows that microparticles derived from thrombin-stimulated platelets (T-pMPs) had significantly increased $PAC1^+$ ($\alpha_{IIb}\beta_3$-integrin) and $CD62P^+$ (P-selectin) markers in their surface compared to microparticles derived from non-activated platelets (C-pMPs) (5.43%±1.67% *vs* 35.30%±1.10% and 2.54%±0.88% vs 10.13%±1.53%, respectively).

Comparison of pMPs and pMP-free platelet releasate fractions

SDS-PAGE 1D analysis shows a distinct band pattern (Figure 2) between pMP and pMP-free platelet *releasate* extract indicating that regulated secretion of MPs is more likely than their release following cellular breakage. Remarkably, MPs were especially rich in high molecular weight proteins (bigger than 160 kDa) which may correspond to post-translationally modified proteins and integral membrane proteins.

MPs were also analyzed by Western blotting using an antibody recognizing the known raft-marker Flotillin, a protein associated with membrane lipid rafts and caveolae (Figure 2). Lipid rafts seem to be also released in microparticles.

Platelet-derived microparticle proteome profiling

The proteome analysis was based on high-resolution 2D gels (17 x 21 cm). We focused on the pI 3-10 range in order to cover and study a broad spectrum of proteins. A total of 175 different proteins were identified. Overall protein composition of pMPs demonstrated that platelets release pMPs with a highly complex and characteristic proteomic profile (Figure 3A).

Proteins identified in pMPs were compared to the proteins found in (a) Vesiclepedia, a manually curated compendium of molecular data (lipid, RNA and protein) identified in extracellular vesicles and then also to (b) ExoCarta 3.2, a database with most comprehensive protein/mRNA listing of published information on the proteomics and RNA content of exosomes. When comparing our data to those in both databases, 65% of pMPs proteins were found in Vesiclepedia and 57% in ExoCarta. However, 61 proteins (over 35%) of our MS-identified proteins were not yet listed in Vesiclepedia and may be novel entries into the database. Moreover, 15 proteins found in Vesiclepedia were not present in Exocarta, whereas one protein found in Exocarta was not found in Vesiclepedia. The presence in pMPs fraction of the top 25 proteins more often identified in exosomes was also investigated and only 11 proteins were found in pMPs.

Identified proteins were classified based on Gene Ontology Slim (GO Slim) annotations for biological process, molecular function and cellular localization, specifically defined for MGI (Mouse Genome Informatics), an international database resource providing integrated genomic and biological data to facilitate the study of human health and

disease. GO annotations provide several processes, functions and locations for a single protein. Specifically, in our pMP data set, from the 175 proteins analyzed, a total of 1618 GO entries were retrieved: 1165 for GO Slim biological processes, 239 for molecular functions and 214 for cellular components. Number of proteins significantly enriched in all obtained GO Slim annotations for pMPs are shown in Figure 3B. The five top annotations for biological processes correspond to cell organization and biogenesis, protein metabolism, developmental processes, signal transduction and transport. In accordance, signal transduction and cytoskeletal activity were found to be the major molecular function categories. The proteins identified are associated with most of the major cellular organelles and subcellular/extracellular localizations: 64 proteins (19.8%) were annotated as cell plasma membrane proteins and 45 (13.9%) as extracellular (secreted or plasma-related). Interestingly, a high percentage of proteins were annotated to the category of cytosol (41 proteins, 12.7%) and cytoskeleton (40 proteins, 12.4%). As a caveat, it must be stated that numerous proteins we identified have multiple subcellular localizations, for instance, the endoplasmic reticulum chaperone glucose-regulated protein 78 may translocate to the cytoplasm, and even the cell surface. Thus, proteins found in pMPs are mainly derived from platelet plasma membrane or intracellularly associated to a variety of organelles.

Pathway annotations were found using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database, an information resource that contain wiring diagrams for molecular interactions and reaction networks. Table 1 shows the KEGG pathways to which 10 or more proteins in pMPs were annotated. As it could be expected from the high number of cytosolic and cytoskeletal-associated proteins, many proteins in pMPs were annotated to the metabolic and regulation of actin cytoskeleton pathways. Furthermore, several proteins were annotated to

the focal adhesion pathway. Interestingly, the annotation pathway in complement and coagulation that plays a critical role in thrombosis is also found in Table 1. Finally, 5 proteins were annotated to the KEGG pathway endocytosis (*not shown in Table 1*). We determined likewise the over-represented pathway terms in pMPs using the binomial statistical tool Panther as shown in Table 2. Results, which in this case are ordered by expected p-value, are similarly comparable to KEGG pathways and also include pathways related to disease such as blood coagulation, related to metabolism like glycolysis and related to cytoskeleton as integrin signalling pathway and cytoskeletal regulation by Rho GTPase.

Thrombin-induced differential proteome in pMPs

Within each group (control and thrombin-stimulated) five experiments were run. A total of 10 gels, two from each experiment, were analyzed. Microparticles from thrombin-activated platelets (T-pMPs) express different properties compared with microparticles from normal cells (C-pMPs). A mean of 395.2±9.7 protein features were found on pI 3-10 gels corresponding to C-pMPs, whereas a mean of 382.2.2±8.7 protein features were found on T-MPs (Figure 4A). A total of 337 protein spots were found in both groups of the five experiments thus showing the reproducibility of the method.

We focused on the identification of disappearing and appearing spots, as well as up- and down-regulation of spot intensities where the fold change was at least 1.5 (with $p<0.05$) and also special significance was given to proteins being regulated in at least four / five out of the five experiments performed. By applying these criteria, 73 protein features were detected as differentially regulated when comparing the proteome of MP obtained by thrombin stimulation of human platelets (Figure 4B). Of all the identified proteins that fit the criterion mentioned above, differentially regulated proteins were involved mainly in (1) cytoskeleton

and cell organization, (2) signal transduction, (3) metabolism, and (4) vesicle-mediated transport although there were other protein-associated functions fairly represented such as coagulation and cell adhesion (Figure 4C).

Microparticles carry proteins involved in thrombosis

Thrombin-induced changes in pMP protein content were mainly related to signal transduction, vesicle-mediated transport and cell adhesion as well as cytoskeleton and cell organization, metabolic and plasmatic proteins.

Proteins that displayed thrombin-induced differential expression on microparticles from control platelets are listed in Table 3. Among these, some representative examples of each functional group are shown in Figure 5. A close-up view of spots corresponding to cytoskeleton, motility and cell organization proteins are shown in Figure 5A. Coronin 1A (CORO1A), which functions in the invaginations and protrusions of the plasma membrane, is increased upon thrombin-stimulation, while myosin light chain 3 (MYL3) and myosin regulatory light polypeptide 9 (MYL9), the regulatory light chain of myosin and the myosin regulatory subunit, respectively, are decreased in T-pMPs. cAMP-dependent protein kinase type I-alpha regulatory subunit (PRKAR1A) protein (Figure 5B) involved in platelet signalling as a cAMP-dependent protein kinase regulator activity and kallikrein-1 (KLK1) protein (Figure 5F), a plasma protein related to proteolysis and blood coagulation, are up-regulated with thrombin. In contrast, the metabolic protein long-chain-fatty-acid-CoA ligase 3 (ACSL3; Figure C), the coagulation factor fibrinogen gamma (FGG; Figure E) and protein bicaudal D homolog 1 (BICD1) and annexin A4 (ANXA4) protein, both related to vesicle-mediated transport, are down-regulated with thrombin stimulation (Figure 5D).

Additional to these proteins, others proteins differential regulated by PAR receptors in microparticles from control platelets refer to proteins of cell adhesion, signalling proteins and cytoskeleton, and include proteins such as membrane glycoprotein gp140 (CDCP1), fermitin family homolog 3 (FERMT3), protocadherin α4 (PCDHA4), guanine nucleotide-binding protein G(1) / G(S) / G(T) subunit beta-1 (GNB1), kringle-containing transmembrane protein 1 isoform 1 (KREMEN1), and catenin alpha-2 (CTNNA2), which may play a fundamental role in platelet activation by thrombin.

Functional groups identified

Thrombin-induced differential proteins of pMPs from control platelets were grouped into associated functions, canonical pathways and disease networks using Integrated Pathway Analysis software (Table 4).

Figure 6 shows the interactomes of the top connecting network and functional associations for differentially regulated pMP proteins by thrombin from control platelets corresponding to the *Cellular Assembly and Organization, Cellular Function and Maintenance, Developmental Disorder* network. Figure 6 demonstrates the extraordinary connectivity of the coagulation protein system with that of the plasma membrane and cytoskeleton-related systems, and signalling complexes. These linkages from extracellular and cell surface localizations to intracellular signalling and cytoskeletal compounds possibly are manifesting in platelet activation and adhesion.

Discussion

Upon stimulation by thrombin as well as other agonists or mechanical flow factors, platelets become activated and secrete proteins as soluble molecules or as membrane vesicle-bound, forming the secretome with a major role in thrombosis and haemostasis. Therefore, pMP proteome represents a high platelet-specific subproteome. Due to their accessibility and specificity, pMP proteins represent good candidates for drug targets and disease biomarkers.

In the present study we have studied proteins transported by microparticles upon platelet activation. Using a proteomic approach, we have identified key components of the procoagulant platelet microparticle proteome directly involved in the pathophysiology of atherothrombotic disease. And with the application of analytical software in a systems biology approach, we have generated logical and functional classifications of the proteins detected. Most of identified proteins were represented by cytoskeleton and cytoskeleton-binding proteins (actin, cofilin, myosin), membrane-associated proteins involved in intracellular transport and signalling (annexins), protein folding (isomerases, chaperons) and in cell-cell interaction processes (membrane glycoprotein 140, fermitin and protocadherin).

The majority of ADP-induced pMP proteins belonged to the class of metabolism, energy pathways, signal transduction and communication.[24] In our study, due to platelet thrombin activation, we found more cytoskeleton-related proteins involved in cell assembly and platelet morphology. In fact, given the important role played by the cytoskeleton in cellular exocytosis, thrombin signalling may rely upon a cytoskeletal remodelling in order to induce MP generation. Some of the identified proteins are known to translocate from the soluble cytosol to the cytoskeleton associated to the actin scaffold in activated platelets, such as

vinculin, alpha actinin, filamin, the alpha, beta and gamma fibrinogen chains, the Arp2/3 complex and the coronin protein. This association might enable platelets to change their morphology, secrete granules and membrane blebs as well as to amplify the signals in order to adhere and aggregate.

We have recently reported that pMPs facilitate thrombus growth on thromboactive substrates.[14] Therefore, we have focused our attention on identifying proteins that could be involved in thrombosis. Interestingly, we have identified proteins involved in cell adhesion like protocadherin alpha-4, membrane glycoprotein 140 (gp140) and fermitin family homolog 3 (FERMT3). Gp140 is a glycoprotein CUB domain-containing protein 1, involved in cancer, which activates β1-integrin and induces motility signalling as well as regulates adhesion by forming complex with SRC-family kinases.[39] In the same line, FERMT3 plays a central role in cell adhesion in hematopoietic cells, by activating β1-β3 integrin and is required for platelet and leukocyte adhesion to endothelial cells.[40] Other proteins that may be crucial for pMP functionality in the pathogenesis are those of blood coagulation. For instance, fibrinogen gamma and beta as well as antithrombin III are clearly reduced on pMPs following thrombin stimulation, which indicates that are functionally active and implicated in the process. Furthermore, we have identified differential proteins of the cytoskeleton such as the myosin regulatory light chain (MYL9), a calcium ion binding, that plays and important role in the regulation of cytokinesis, receptor capping and cell locomotion, and of the vesicle-mediated transport, like Protein bicaudal D (BICD1) that regulates coat complex coatomer protein I (COPI)-independent Golgi-endoplasmic reticulum transport by recruiting the dynein-dynactin motor complex and annexin A4 (ANXA4) that promotes membrane fusion and is involved in exocytosis. We also found proteins involved in energy metabolism (long chain fatty acid-CoA ligase 3, ACSL3). It is also worth to stress the

signalling proteins. Thrombin activation triggers inside-out signalling and induces increase in protein kinase activity. Of interest it is the detection and modified expression of phosphatidylinositol-4-kinase alpha, the regulatory subunit of cAMP dependent protein kinase, annexin V, guanine nucleotide-binding protein G(1) / G(S) / G(T) subunit beta-1 and kringle-containing transmembrane protein 1 isoform 1 (KREMEN1). The latter one, KREMEN1 is involved in the Wnt-beta catenin signalling,[41-43] which recently has been identified on platelets and may enhance the amplification of platelet activation.[44,45]

A relevant example is the endoplasmic reticulum resident and cell surface chaperone protein disulfide isomerase (PDI, P4HB), considered as a critical mediator of wound healing and as a chaperone that inhibits aggregation of misfolded proteins, which was found to be reduced in microparticles released upon platelet activation by thrombin. Another oxireductase that has recently been shown to participate in thrombus formation is protein disulfide isomerise A3 (PDIA3, ERp57, GRP58).[46,47] Indeed, in a previous study of our group, Vilahur et al showed that PDIA3 increases in the secretome of thrombin-activated platelets and NO donor could modulate its release.[48] Now, in the present study, PDIA3 is found significantly increased in thrombin-induced pMPs, indicating that PDIA3 is secreted as not only soluble form but also as MP membrane-bound, in agreement with previous findings.[49-51] The fact that PDI levels are reduced and PDIA3 levels are increased upon platelet activation suggests that platelet surface may undergo a redox remodelling state which facilitates the different binding of thiol isomerases to mediate the disulphide rearrangements and activation of proteins such as $\alpha_{IIb}\beta_3$-integrin.[52]

Differentially regulated proteins were further investigated by IPA software. The highest score networks resulting from this analysis confirmed the involvement of membrane and cytoskeleton proteins in platelet function. In fact, we have identified several proteins implicated in

regulation of actin-based motility by Rho as well as signalling proteins, conforming satellite hub proteins of the network. Small GTPases have been shown earlier to control actin reorganization necessary for MP shedding by platelets.[53] The presence of elements of this pathway may reflect mechanism by which microparticles were formed.

It is important to note that our pMP fraction is composed of vesicular fragments of the plasma membrane (microparticles) and alpha granules (exosomes). The comparison of our group of procoagulant pMP proteins to Vesiclepedia and ExoCarta databases demonstrated that pMP fraction is highly enriched in proteins from both platelet shed microparticles and proteins derived from exosome release, covering the whole membrane-bound secretome. Little et al. identified a core group of plasma MP proteins expected to be found across most of the population.[34] Among this group, our pMP fraction shares 46% of these proteins, thus indicating that proteins found in this study are susceptible to be potential biomarkers or therapeutic targets.

The differential analysis of thrombin-induced platelet microparticle subproteome allowed us to investigate the effects of platelet activation on microparticle shedding during platelet activation since a potent agonist such as thrombin could modify the expression pattern of some proteins related to thrombosis. Our study reveals potential therapeutic targets implicated in the pathophysiology of atherothrombosis that are susceptible of anti-thrombotic strategies and further studies deserve their attention.

In summary, our results evidence that the proteomic approach used here may help to elucidate some of the molecular mechanisms of thrombin stimulation on platelets with relevant impact on atherothrombotic disease. These results will not only apply for atherosclerosis and cardiovascular disease, but also for cancer.

Figure legends

Figure 1. Flow cytometric characterization of platelet-derived microparticles. (A) Determination of forward scatter and side-scatter characteristics of platelet and pMPs in suspension. The microparticle gate was established based on light scattering properties and size, using calibration microspheres and defining pMPs as events both smaller than 1 μm and smaller than unstimulated platelets. **(B)** Diagram plots of size-selected events with expression of phosphatidylserine (annexin V^+) and platelet activation markers P-selectin ($CD62P^+$) and $\alpha_{IIb}\beta_3$-integrin ($PAC1^+$) on pMP surface from non-activated and activated platelets in the fluorochrome-conjugated gate. Data are expressed by labelling percentage of total population.

Figure 2. Comparison of platelet-derived subfractions. (A) Flamingo staining pattern of protein extracts of microparticles and MP-free *releasate* from control **(C)** and thrombin-stimulated **(T)** human platelets. Note that the pattern of bands present in both samples is different. **(B)** Representative image of western blot against flotillin-1 on pMP samples.

Figure 3. (A) Representative 2D proteome map of proteins in pMPs. Proteins were separated in a pH range 3-10 on 12% SDS-PAGE. **(B)** Classification of identified proteins by gene ontology (GO) classification –biological process, subcellular localization, and molecular function–. Number of annotated proteins.

Figure 4. Thrombin-induced effects on pMPs derived from control platelets and thrombin-induced platelets. (A) Venn diagrams depicting overlap in microvesicular protein in the different studied groups. **(B)** PDQuest differential analysis of pMP protein spots. **(C)** Distribution of thrombin-induced differential proteins among identified ones by functional categories.

Figure 5. Thrombin-induced effects on pMPs derived from control platelets and thrombin-stimulated platelets. Selection of proteins differentially regulated between control and thrombin-induced platelets and, specifically, enlargement of representative 2-DE images corresponding to spots for **(A)** cytoskeleton, motility and cell organization, **(B)** signal transduction, **(C)** metabolism, **(D)** vesicle-associated transport, **(E)** coagulation factors and, **(F)** plasma – related proteins. Each panel shows bar graphs for each spot showing variations in mean spot intensity ± SE in the different studied groups (C, control and T, thrombin-stimulated). Data are expressed in arbitrary units (AU). Differences were analyzed by Student T-test (n=5).

Figure 6. Analysis of differentially regulated proteins by Ingenuity Pathways Analysis Core Analysis. Potential interactions are shown in the following network: *Cellular Assembly and Organization, Cellular Function and Maintenance, Developmental Disorder*. Proteins identified by differential analysis are shown as shaded nodes with their gene names. Solid lines represent direct interactions, dotted represent indirect interactions. Arrows from one node to another indicate that this node acts upon the other. Lines without arrows represent binding. Node shapes are: double circle = complex or group; notched triangle = kinase; wavy shape = enzyme; circle = other. Proteins known to be involved in platelet activation by thrombin are indicated by a black solid arrow.

Table 1. KEGG pathways

KEGG ID	KEGG pathway	Organism	Nº of proteins
hsa01100	Metabolic pathways	Homo sapiens	21
hsa04810	Regulation of actin cytoskeleton	Homo sapiens	16
hsa04510	Focal adhesion	Homo sapiens	14
hsa04670	Leukocyte transendothelial migration	Homo sapiens	11
hsa04610	Complement and coagulation cascades	Homo sapiens	10

Table 2. Panther pathways

Panther ID	Panther pathway	Organism	Nº of proteins	P value
P00011	Blood coagulation	Homo sapiens	8	1.11E-06
P00034	Integrin signalling pathway	Homo sapiens	11	9.92E-05
P00049	Parkinson disease	Homo sapiens	7	2.47E-03
P00024	Glycolysis	Homo sapiens	4	7.81E-03
P00016	Cytoskeletal regulation by Rho GTPase	Homo sapiens	6	2.51E-02

Table 4. IPA analysis.

	T vs C	P-value / Score
Top canonical pathways	Intrinsic prothrombin activation pathway	$3.2\cdot10^{-7}$
	Coagulation system	$8.1\cdot10^{-7}$
	Extrinsic prothrombin activation pathway	$4.8\cdot10^{-6}$
	Regulation of actin-based motility by Rho	$8.5\cdot10^{-6}$
	RhoA signalling	$2.8\cdot10^{-5}$
Molecular and cellular top biofunctions	Cellular assembly and organization	0.05 - 0.0001
	Cellular function and maintenance	0.05 - 0.0001
	Cell-to-cell signalling and interaction	0.05 - 0.0005
	Cell morphology	0.05 - 0.001
	Cellular movement	0.05 - 0.001
Top networks	Cellular Assembly and Organization, Cellular Function and Maintenance, Developmental Disorder	55
	Gene Expression, Cell-To-Cell Signaling and Interaction, Hematological System Development and Function	18
	Cellular Function and Maintenance, Cardiovascular Disease, Cardiovascular System Development and Function	11

The scores (-log [p-values]) reflect the probabilities of such associations occurring by chance, with the threshold value for significance set as 1.25; as evident the scores are highly significant.

Table 3. Thrombin-induced differential proteins in microparticles derived from thrombin-stimulated platelets.

Function	Protein name	Swissprot number	Score	Fold change
Cytoskeleton, motility and cell organization	Coronin 1A	P31146	59	+ 1.52
	Filamin-A	P21333	87	+ 1.67
	Catenin alpha-2	P26232		+ 3.03
	Myosin light chain 3	P08590	95	− 2.94
	MRLC, polypeptide 9 isoform b	P24844	37	− 2.63
	Actin beta	P60709	144	− 2.11
Cell adhesion	Membrane glycoprotein gp140	Q9H5V8		+ 2.42
	Fermitin family homolog 3	Q86UX7	57	+ 1.48
	Protocadherin alpha 4	Q9UN74	72	− 7.69
Metabolism	ATP synthase subunit alpha	P06576	101	+ 3.97
	Protein disulfide isomerase A3	P30101	226	+ 1.46
	Purine nucleoside phosphorylase	P00491	80	− 1.27
	Protein disulfide isomerase	Q96C96		− 1.96
	Long-chain-fatty-acid-CoA ligase 3	O95573	60	− 1.72
Signal transduction	PRKAR1A	P10644	75	+ 1.49
	Phosphatidylinositol-4-kinase alpha	P42356	74	+ 1.55
	Annexin A5	P08758	30	+ 2.29
	Transducin beta chain 1*	P62873	56	− 1.89
	Ras suppressor protein 1	Q15404	84	− 1.61
	KREMEN 1	Q96MU8		− 1.89
Vesicle-mediated transport	Alpha-soluble NSF attachment protein	P54920	58	+ 1.92
	Protein bicaudal D homolog 1	Q96G01	60	D
	Vesicle transport protein	Q9NZ43	88	− 3.70
	SSX2-interacting protein	Q96QF0	80	− 2.50
	Annexin A4	P09525	52.6	− 1.25
Coagulation factors	Fibrin(ogen) gamma	P02679	92	D
	Anti-thrombin III	P01008	65	− 4.76
	Fibrin(ogen) beta	P02675	129	− 2.56
Plasma	C4b-binding protein alpha chain	P04003	79	+ 1.62
	Antitrypsin alpha 1	P01009	110	+ 1.36
	Kallikrein	P06870		+ 1.62
	Organic solute transport protein 1	Q8WVF1	61	− 1.59
Others	Ashwin	Q9BVC5	62	− 1.25
	HERV-K Sq13.3 provirus Rec protein	P61576	56	− 2.13
	F-box/LRR-repeat protein 20	Q96IG2	59	− 1.82

PRKAR1A, regulatory subunit of cAMP dependent protein kinase alpha type 1; KREMEN1, kringle-containing transmembrane protein 1.

* Guanine nucleotide-binding protein G(1) / G(S) / G(T) subunit beta-1.

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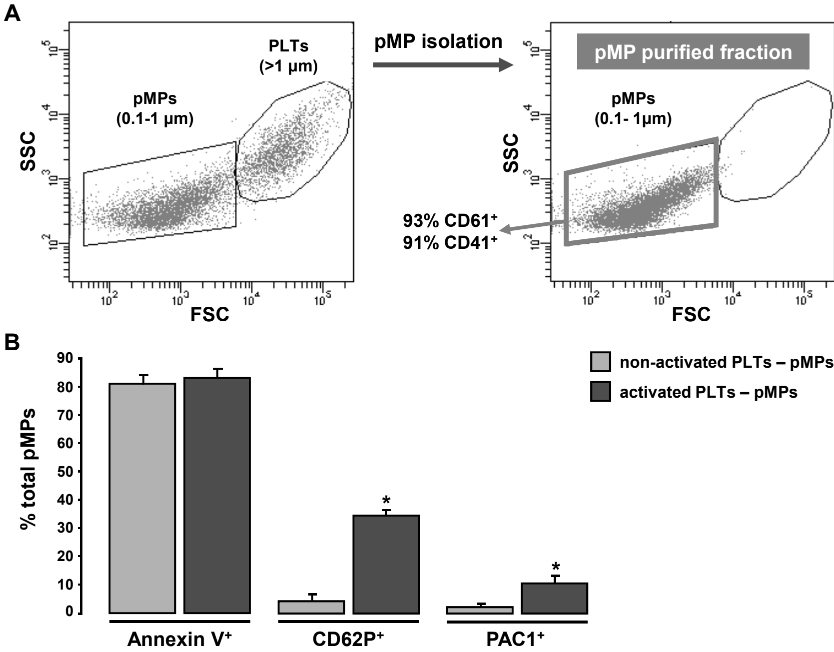
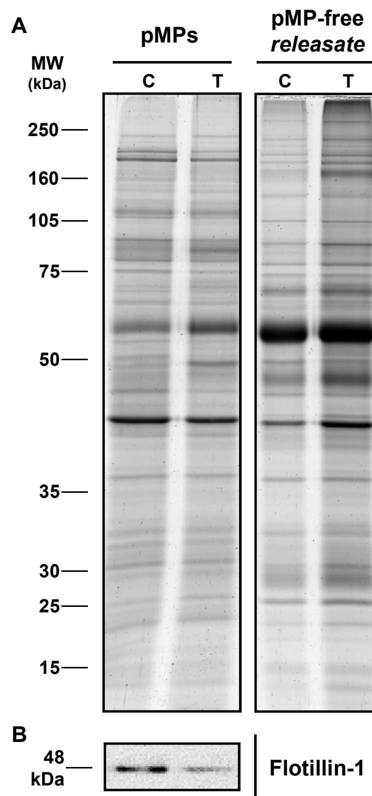


Figure 1



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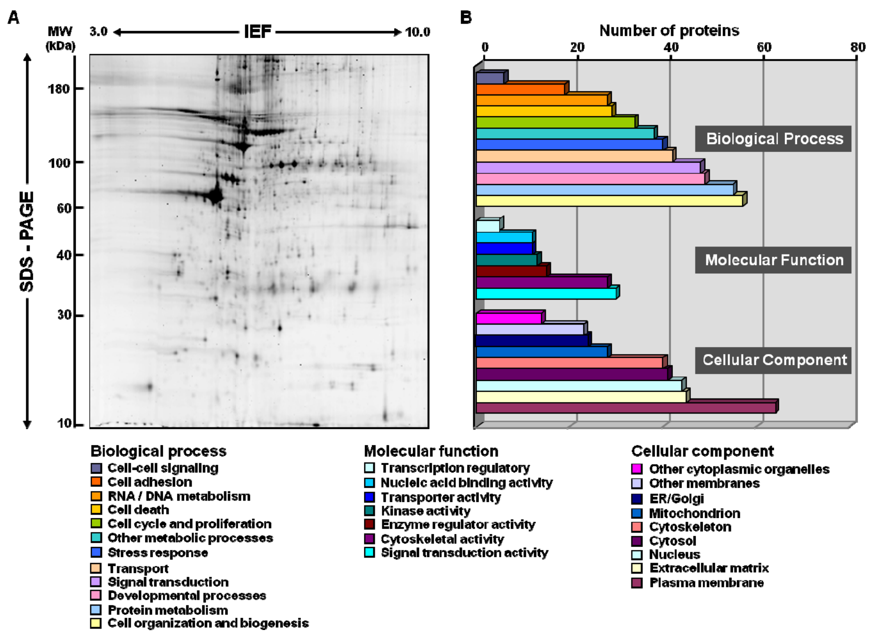

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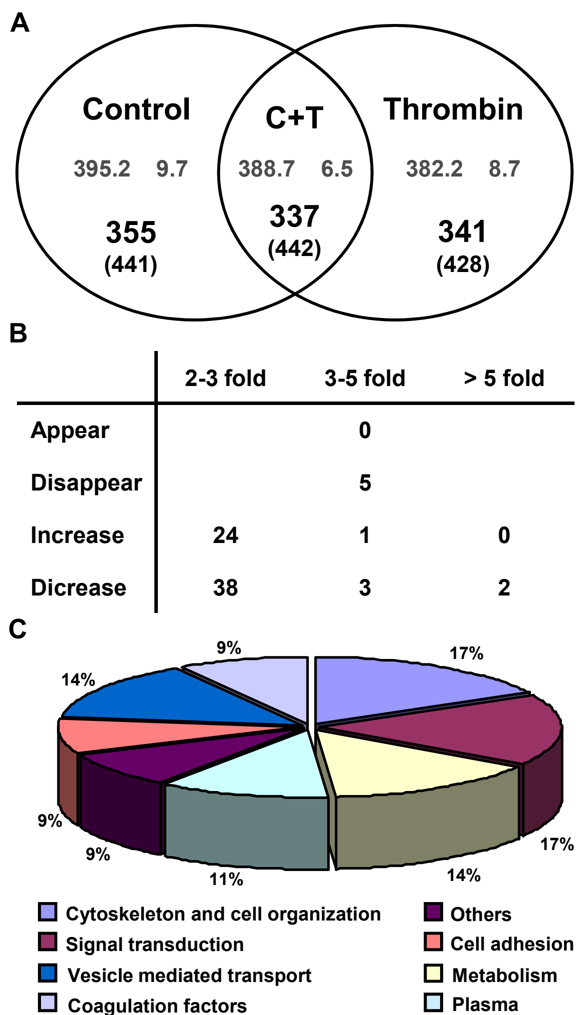

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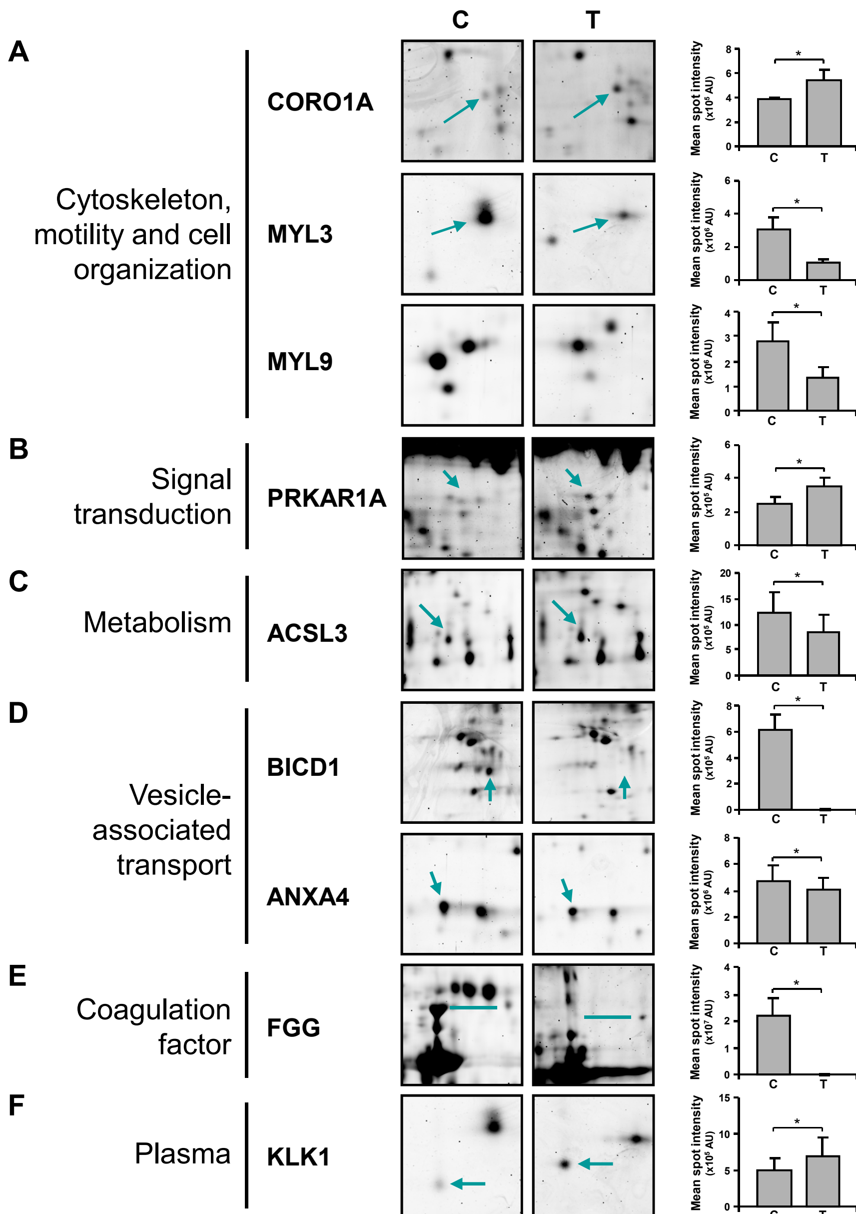

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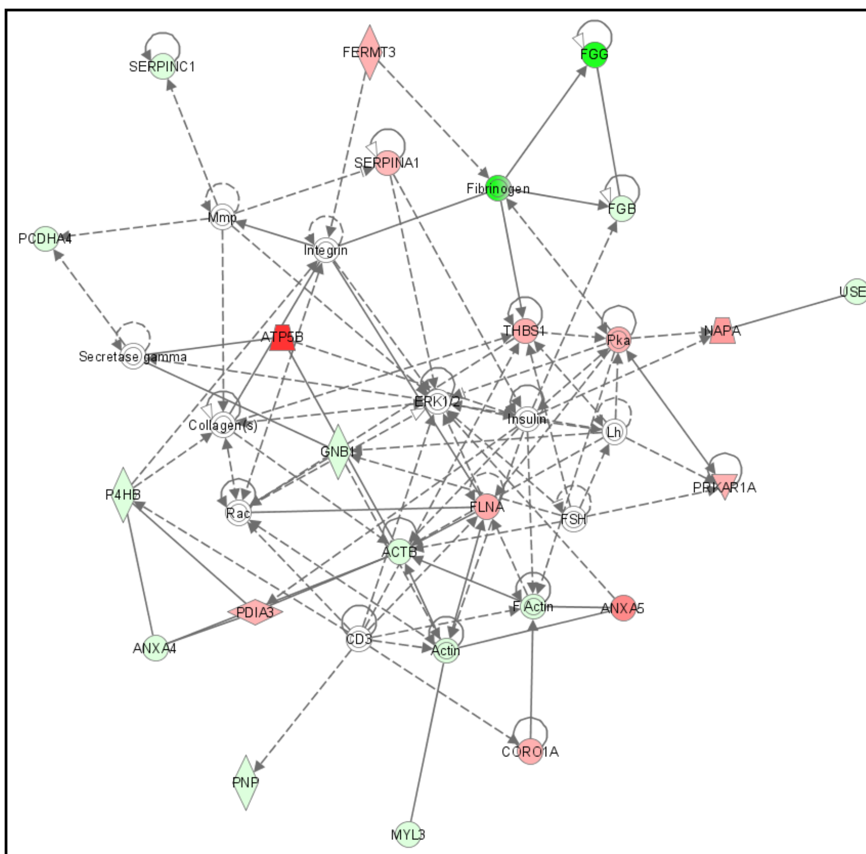

Figure 6

Article 8

Exosomal microRNA signature predicts future ischemic events in hypercholesterolemic patients

Rosa Suades, Teresa Padró, Rodrigo Alonso, Sandra Camino, Pedro Mata, Lina Badimon

Manuscript in preparation.

Objectives

The aim of this study was to determine microRNAs, non-coding RNAs involved in post-transcriptional regulation of gene expression, in circulating exosomes as prognostic markers of future cardiovascular events (CVE).

Highlights

- 21 exosomal microRNAs are differentially expressed in CVE patients compared to nCVE patients.
- A five-microRNA signature (miR-130b, miR-142-3p, miR-200c, miR-660, miR-744) is increased in CVE patients.
- Exosomal miRNA signature could be used as a predictor of ischemic event presentation in hypercholesterolemic patients.

Exosomal microRNA signature predicts future ischemic events in hypercholesterolemic patients

Suades et al: Circulating microRNAs and ischemic events

By:

Rosa Suades[1], Teresa Padró[1], Sandra Camino[1], Rodrigo Alonso[2], Pedro Mata[2], Lina Badimon[1,4]

From:

[1] Cardiovascular Research Center, CSIC-ICCC, IIB-Sant Pau, Barcelona, Spain

[2] Jimenez Diaz Foundation, Madrid, Spain

[3] Cardiovascular Research Chair, UAB, Barcelona, Spain

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Correspondence to:

Prof. Lina Badimon
Cardiovascular Research Center,
c/Sant Antoni Mª Claret 167, 08025 Barcelona, Spain.
Phone: +34.935565880.
Fax: +34.935565559.
E-mail: lbadimon@csic-iccc.org

Abstract

Background. High LDL-cholesterol plasma levels constitute an independent risk factor for the pathogenesis of atherothrombotic cardiovascular disease. We aimed to study miRNAs, non-coding RNAs involved in post-transcriptional regulation of gene expression, in circulating exosomes as prognostic markers of future cardiovascular events in heterozygous familial hypercholesterolemia (FH).

Methods. Exosomes were isolated from platelet-free plasma (PFP) obtained from patients that suffered an ischemic event (N=42) within 3.0 ± 0.4 years post-sampling (CVE) and from age/treatment-matched patients (N=30) that did not have an event within the same time-frame (nCVE). Fully clinically characterized patients were from the Spanish hypercholesterolemia SAFEHEART cohort. RNA from exosomes was obtained with the Exo-MiR extraction kit. miRNA profiling was performed using Megaplex pool A microRNA arrays. Differentially expressed miRNAs were validated by RT-qPCR that was measured with Taqman miR Custom Array Cards.

Results. microRNA profiling revealed that 21 exosomal miRNAs were differentially expressed in CVE patients compared to nCVE patients. RT-qPCR validation confirmed that nine of these miRNAs, including (miR-130b, miR-133a, miR-142-3p, miR-200c, miR-324-5p, miR-339-3p, miR-660, miR-744) are significantly increased in CVE patients while miR-122 is decreased. A ROC curve analyses of the predicted probabilities of a five-miRNA signature (including the best five discriminators) was calculated and an AUC of 0.795±0.069 [95%CI: 0.660-0.930] (P<0.001) for ischemic event presentation was obtained.

Conclusion. Ten miRNAs involved in the prognosis of acute CVE have been indentified. Exosomal miRNA signature could be used as a predictor of ischemic event presentation in high-risk hypercholesterolemic patients.

Key words: atherothrombosis, exosomes, hypercholesterolemia, miRNAs

Introduction

Despite traditional risk factor models and lipid-lowering strategies, there are still a percentage of patients that suffer cardiovascular events without having any sign or symptom, and even any major cardiovascular risk factor.[1] Therefore, one of the main challenges of cardiovascular research is the identification of subjects at risk of developing cardiovascular disease (CVD) and its clinical atherothrombotic manifestation such as acute coronary syndromes.[2] In this context, the search for novel specific biomarkers aiming at the identification and stratification of high-risk patients is of huge importance. Dyslipidemia has been recognized as an independent risk factor for the pathogenesis and progression of atherosclerotic CVD. Familial hypercholesterolemia (FH) patients represent a useful clinical model to study prognostic markers of future cardiovascular events. FH patients, due to their high LDL-cholesterol level, have an early development of atherosclerotic plaques and, ultimately, atherothrombotic CVD presentation.[3-7] Up to now, however, the use of lipid-related markers or their combinations in conventional risk estimation algorithms has led to only slight improvement in CVD prediction.

MicroRNAs (miRNAs), non-coding RNAs involved in post-transcriptional regulation of gene expression have been implicated in CVD pathophysiology.[8-10] Recently, miRNAs have been found in stable circulating form in blood and other body fluids mostly packed into microvesicles such as exosomes.[11,12] Exosomes are small vesicles ranging from 20 to 200 nm, constantly secreted by all healthy and pathological cells and are present abundantly in all body fluids such as plasma, saliva, and breast milk. Exosomes formed by the inward budding of cellular compartments are defined as internal vesicles or endosomes.[13] Once formed, the exosomes are secreted by merging with the cell membrane.

These vesicles, loaded with unique RNA and protein cargo, have a wide range of biological functions, inducing cell-to-cell communication and signalling transferring phenotypic traits from the parent cell.[14] The biogenesis, release, and uptake of exosomes are tightly regulated processes governed by diverse signalling mechanisms, which can be altered in pathologies such as cardiovascular disease.[15]

An advantage of exosomes as microRNA delivery vehicles is that they are protected from the environment by their lipid bilayer and are more likely to reach their targets cells.[16,17] Recently, circulating microRNAs have emerged as prognostic and predictive biomarkers and their utility in personalized medicine represents a promising biomedical tool. They could lead to the development of minimally invasive diagnostics and next generation therapies within the next few years.

It is our main objective to determine whether there is a specific miRNA expression signature associated to microvesicles that could predict cardiovascular events. To this aim, we have characterized the microRNAs derived from exosomes as surrogate markers of ischemic events in high-risk FH patients who had an ischemic event within five years after their inclusion in comparison with healthy controls and non-FH hypercholesterolemic patients without any cardiovascular episode at follow-up.

Methods

Clinical population

The study design included an initial profiling phase of circulating exosomal miRNAs in patients with familial hypercholesterolemia who suffered an ischemic cardiovascular event (CVE) compared with a healthy control group. All healthy subjects and patients used in this study were selected from the SAFEHEART cohort, as previously described.[18] For the validation phase, circulating exosomal miRNA signature was also studied in plasma samples of FH patients, who suffered an ischemic CVE within approximately 3 years post-sampling after entering the cohort (N=42), compared to non-FH hypercholesterolemic patients who did not suffer any ischemic CVE (N=30) within the same time-frame. Ischemic events included sudden death, fatal and non-fatal myocardial infarction, unstable angina, and cerebrovascular disease. Table 1 shows the main clinical characteristics of the selected population. All FH patients fulfilled the WHO criteria and were receiving lipid-lowering therapy (LLT) according to clinical guidelines.[19-21] Maximum statin dose were: simvastatin 40 mg/day, pravastatin 40mg/day, lovastatin 80 mg/day, fluvastatin 80 mg/day, atorvastatin 80 mg/day, rosuvastatin 20-40 mg/day.[22]

Blood sampling for analysis

Venous blood was withdrawn from the cubital vein without tourniquet using a 20-gauge needle after 10-14 hours of fasting EDTA-anticoagulated tubes for microRNA analysis. Blood cells were removed by double low-speed centrifugation to obtain the platelet-free plasma (PFP), which was snap-frozen in liquid nitrogen and stored at -80ºC until miRNA studies.

Microvesicle isolation and RNA extraction

miRNAs were measured from total RNA fraction contained in exosomes using the Exo-MiR extraction kit (Bioo Scientific), specifically designed to isolate RNA from the microvesicle fractions (microparticles and exosomes) of the sample, according to the manufacturer. Briefly, exosomes were obtained by size exclusion filtration from EDTA-plasma samples thawed in melting ice. Briefly, a clarifying pre-spin was previously performed in order to ensure cell and lipoprotein depletion. Then, plasma samples were diluted and pushed through two different sized filters provided in the kit: the first MP-specific sized filter (>200 nm) retains the larger microvesicles, while the second filter captures the exosomes sized between 20-200 nm. Total RNA was isolated and extracted from the filter-trapped MVs by eluting the sample off the filters with the lysis solution. RNA was obtained with an organic extraction with chloroform and isopropanol precipitation and an ethanol-based purification steps and the final pellet was resuspended in RNAse-free water. Finally, total RNA was quantified with Nanodrop and used for further analysis. All samples were spiked-in with 25 fmol/μL of Caenorhabditis elegans miR-39 prior to RNA extraction for normalization in downstream analyses.

miRNA profiling

For microRNA profile analysis, total RNA from plasma exosomes was analysed using the low-density TaqMan® Array Human MicroRNA A Card v2.0 according to the manufacturer's protocol. This array card set enables assaying 377 most relevant specific human microRNAs, aligned with Sanger miRBase v20 database. Seven control miRNAs were also included. Briefly, miRNA screening was performed by reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis

from total RNA samples (input of 100 ng) that were reversely transcribed to cDNA with MegaPlex RT primers pool A followed by a pre-amplification step using MegaPlex PreAmp Primer Pool Set v2.0. Real-time PCR amplification of miRNAs using low-density TaqMan Arrays was performed on an Applied Biosystem 7900HT system using SDS software v2.4. Assays were median normalised.

miRNA validation

For detection and quantification of selected miRNAs and cel-miR-39 spike-in, Custom TaqMan Array MicroRNA Cards were used. Single-stranded cDNA was synthesized using the multi-scribe reverse transcriptase kit and custom RT primer pool specific to the miRNAs being assessed. cDNA was preamplified with TaqMan PreAmp MasterMix, and then specific miRNAs were measured by quantitative PCR using either specific human TaqMan miRNA Assays or Custom TaqMan Array MicroRNA cards (Life Technologies) on an Applied Biosystem 7900HT system using SDS software v2.4.

miRNA data analysis

A RQ Study was set up to review the amplification plots, analyze comparative cycle thresholds (Ct), and to adjust when necessary the baseline and the threshold settings using RQ Manager software v1.2.1. Ct values were median normalized (global normalization) for miRNA profiling. For miRNA validation, Ct values were normalized to cel-miR-39 by the formula 2–(Ct [miRNA] – Ct[cel-miR-39]) with DataAssist software v3.01. Of note, undetectable miRNA levels (Ct values ≥ 35) were considered as undetermined and were not considered.

Target gene prediction and integrated analysis by IPA

miRNAs found to be significantly associated with FH status were further analyzed by bioinformatics analysis (Ingenuity Pathway Analysis, IPA) to identify miRNA biological functions and/or diseases.[23] Right-tailed Fisher's exact test was used to calculate a significant *P*-value for each functional category and each *P*-value was further adjusted using the Benjamini–Hochberg correction. We focused our analysis with an adjusted *P*-value (P<0.05) directly related to cellular functions and diseases. A list of predicted mRNA targets for the 5 selected differential miRNAs was generated using IPA Target prediction.[23] The list, restricted to the common mRNAs, was then used to run a canonical pathway analysis in order to identify potential functional implications of the altered miRNA expression. The significance of the associations was measured by Fisher's exact test and a P<0.05 was selected as a threshold.

Statistical analysis

All data are presented as either median (interquartile range), except when indicated. An initial descriptive analysis was provided using number of cases and percentages for qualitative variables and mean ± SE for quantitative variables. Frequencies of qualitative variables (such as risk factors) were compared between groups by using the Chi-square analysis. Mean values of quantitative variables were compared with two-sided parametric unpaired Student T-test. Median values were compared with two-sided non-parametric tests. The statistical significances for differences between two groups were determined with U-Mann Whitney and multiple comparisons by Kruskal Wallis. When significant, Bonferroni post-hoc analysis was used to assess intergroup differences. StatView (5.0.1, Abacus Concepts) was used for all statistical tests and a P<0.05 was considered statistically significant.

To evaluate the prognostic value provided by exosomal miRNAs, associated receiver operating characteristic (ROC) curve analyses for each miRNA were generated and the corresponding area under the curve (AUC) along with its 95% CI were calculated. To evaluate combination of prognostic markers (miRNA signatures), a binary logistic regression model with miRNA levels was carried out to estimate the likelihood of an ischemic event by creating predicted probabilities before ROC curve analyses. SPSS Statistics Version 21.0.0 (21.0.0, SPSS, Chicago) was used for c-statistics analyses and a $P<0.05$ was considered statistically significant.

Results

Clinical characteristics

Table 1 provides baseline characteristics of the entire study populations from the SAFEHEART cohort. In the profiling study, CVE-patients and healthy controls were age- and gender-matched. Groups did not show significant differences in clinical and biochemical characteristics, except in some cardiovascular risk factors and HDL-cholesterol. In the validation phase, age- and treatment-matched CVE- and nCVE-patients were included. There were no differences in clinical characteristics, but more CVE-patients presented arterial hypertension. Although most of the patients of both groups were hypercholesterolemic and were treated with LLT, there were significant differences in lipid profile (total cholesterol, LDL-cholesterol, HDL-cholesterol, and triglycerides) likely due to FH condition.

Exosomal microRNA profiling phase

To determine miRNAs involved in ischemic events, we compared levels of 377 circulating exosome-associated miRNAs in plasma samples of FH patients with CVE and healthy controls. As shown in Table 2, miRNA profiling analysis performed in hypercholesterolemic patients revealed that 21 miRNAs were differentially expressed (*fold change* >1.5) in circulating exosomes of CVE- compared to healthy subjects (N=5/group).
To identify biological functions and/or diseases that were most significant to our data set of 21 miRNAs, a functional annotation analysis with IPA software was performed. Figure 1 shows the most significant functional categories reported by IPA, related to cardiovascular disease, which included: inflammatory response, immunological and inflammatory disease, cellular development, growth and proliferation, cell death and

survival, metabolic disease, cell morphology, function and maintenance, and cardiovascular system development.

Validation of differential exosomal microRNAs

Validation studies were performed for the 21 miRNAs selected as differential during the profiling phase based on their direct association with ischemic events in FH patients. miRNA levels in the circulating exosomal fraction were determined by RT-qPCR in FH-patients presenting and ischemic event (CVE) compared to non-FH hypercholesterolemic patients (nCVE), matched by LLT. Ten miRNAs showed significantly differential levels in CVE- compared to nCVE-patients (Figure 2). Nine of these exosome-associated miRNAs were higher in CVE- than nCVE-patients, while one was lower. These miRNAs included miR-130b, miR-133a, miR-142-3p, miR-200c, miR-324-5p, miR-339-3p, miR-425-5p, miR-660, and miR-744 accounting for increased miRNA levels in exosomes of CVE-patients and miR-122 for decreased exosome-miRNA level in nCVE-patients (Figure 2).

To further evaluate the potential use of circulating exosomal miRNAs as prognostic biomarkers of ischemic events in high cardiovascular risk patients, ROC curves analyses were performed. First, AUCs were calculated for the ten miRNAs individually (Figure 3). Of all miRNAs, miR-660 showed the highest AUC (0.706 ± 0.086; 95%CI:0.538-0.874; Figure 3A). Then, the panel of all nine increased miRNAs showed the highest AUC compared with that of miR-660 alone (0.850 ± 0.058; 95%CI:0.736-0.963) (Figure 4A). However, due to sample size and drawbacks of excessive multi-panels in clinical use, a signature of five miRNAs including the ones with highest AUCs (miR-130b, miR-133a, miR-200c, miR-660 and miR-744) was also tested. By applying this 5-

miRNA combination, an AUC of 0.795 ± 0.069 [95%CI:0.660-0.930] was achieved (Figure 4B).

To further investigate the global association between the five miRNAs and their target mRNAs in FH, we used IPA pathway analysis software to perform target predication and functional analysis of the 5 exosomal miRNA signature. From a large number of potential target genes predicted for the screened miRNAs (N= 639), we performed an integrated analysis of common mRNAs regulated by at least two of these five miRNAs, which resulted in a target list consisting of 303 potential genes from all the predicted targets. Forty-two different target genes were found to be regulated by the selected miRNAs cooperatively in the functional disorder of cardiovascular disease (prediction *P*-value: 2.30E-04 – 3.25E-02). In addition, as shown in Table 3, the target genes were related with gene expression, cell death and survival, cellular growth assembly and organization, and cell movement. Moreover, the five differentially expressed exosomal miRNAs were found to be involved in relevant canonical pathways acting at various critical points of the signalling network. Specifically, the highest scored canonical pathways showed that 10 targets genes were associated to PPAR activation signalling (Figure 5), 9 targets genes to RhoA signalling (Figure 6) and 11 targets genes to tight junction signalling (Figure 7).

Discussion

miRNAs have been recently implicated in the pathogenesis of cardiovascular disease.[9,10] In the present study, circulating exosomal miRNAs were profiled as possible novel biomarkers of cardiovascular ischemic events. First, our results identified 21 differentially expressed miRNAs in hypercholesterolemic patients associated to ischemic events and demonstrated that nine of these miRNAs, including miR-130b, miR-133a, miR-142-3p, miR-200c,miR-324-5p, miR-339-3p, miR-425-5p, miR-660, and miR-744, are significantly increased in CVE-patients while miR-122 is significantly decreased compared to nCVE. Besides, the specific signature of five of these miRNAs (miR-130b, miR-142-3p, miR-200c, miR-660, and miR-744) demonstrated a discriminatory power for ischemic events. Thus, exosomal miRNA signature could be potentially used as predictor of ischemic event presentation in high-risk hypercholesterolemic patients.

Changes in exosomal miRNA cargo were studied in a high-risk group of patients such as FH-patients. Why subjects with similar genetic background show different prevalence of adverse coronary artery disease[5] is a question that remains unresolved. Here we described for first time that the level of 10 circulating miRNAs embedded in exosomes differed profoundly and showed significant changes in patients that presented an ischemic event within five years after inclusion, giving a clinical relevance to the role of circulating microvesicles and their microRNA content in the cellular crosstalk of atherothrombotic disease precipitation. Indeed, we have demonstrated that circulating microparticles enhance blood thrombogenic potential on human atherosclerotic plaques and, therefore, have functional effects on atherothrombosis beyond being mere biomarkers of cell activation.[24] Besides, functional analysis revealed that these deregulated miRNAs in FH patients were related to cardiovascular

disease, immunological, inflammatory and metabolic diseases, as well as many cell functions all of which were involved in atherosclerosis development and cardiovascular atherothrombotic disease.

Nine of the 10 miRNAs differentially expressed were significantly increased in the CVD groups. Among them, miR-339-3p has been recently related to platelet activation[25] and miR-425-5p to atrial natriuretic peptide regulation in arterial hypertension.[26] In addition, the myomiR-133a (cardiac-enriched miRNA) has recently been related to myocardial infarction and coronary artery disease diagnosis[27,28] and miR-324-5p has also been involved in heart failure diagnosis.[29] From our study, we also identified that levels of miR-122 were significantly decreased in CVE patients. miR-122 is a liver-specific miRNA that has a role in lipid metabolism and, as a consequence, has been related to atherosclerosis and has been found downregulated in aortic stenosis.[30,31] Due to the relevance of the lipid metabolism in FH, changes in miR-122 associated to CV events in FH patients deserve further studies on its potential implication in the pathophysiology.

Circulating miRNAs have been recently pointed out as potential biomarkers due to their characteristics: stable, easily accessible, sensible and associated to pathology, among others.[32] As circulating miRNAs in plasma have multiple origins and locations, a subfraction such exosome-associated miRNAs in FH was studied because it confers a high degree of specificity and conforms a promising strategy for their prognostic potential.[33] The multi-miRNA approach provided a panel of five miRNAs (miR-130b, miR-142-3p, miR-200c, miR-660, and miR-744) as discriminators of cardiovascular ischemic events in FH patients, with an AUC of 0.795 ± 0.069 [95% CI:0.660-0.930], suggesting their potentially clinical usefulness in terms of improving risk stratification and early diagnosis in the management of CVD. Hence predicted target genes of

this miRNAs were associated to cardiovascular system, and molecular and cellular functions described in atherothrombosis.

Signal transduction pathways associated with target genes of the differentially expressed miRNAs were also investigated *in silico* suggesting PPAR activation, and RhoA and tight junction signalling as the most relevant mechanisms regulated by the five-miRNA profile. This observations are in line with the fact that PPAR-γ exacerbate the tendency to atherosclerotic plaque formation.[34] Additionally, PPARs are involved in lipid metabolism through regulating specific genes as well as they are implicated in the regulation of endothelial function, proliferation and migration of vascular smooth muscle cells, and activation of macrophages.[35] Interestingly, miR-130b is directly involved in the PPARγ regulation.[36] It targets directly the 3'-UTR of PPAR-γ and thereby suppresses its expression. miR-130b also regulates physiological vascular function and reflects the degree of obesity, why it is considered a marker of hypertriacylglycerolemia and metabolic syndrome.[37] Indeed, microvesicle-shuttled miR-130b has been shown to reduce adipogenesis and lipogenesis, and fat deposition in adypocytes.[36] Of particular importance is that the other best-rated predicted pathways were RhoA and tight junction signalling, which regulate processes like cytoskeletal regulation, shape change, endocytosis, vesicle transport, and membrane and vesicle trafficking. In its turn, these processes are responsible of inducing a wide range of fundamental cell functions such as contraction, motility, proliferation, and apoptosis characteristic of major cardiovascular disorders (atherosclerosis, restenosis, hypertension, and cardiac hypertrophy).[38]

Several limitations of this study deserve consideration. First, in the profiling phase, the relatively small sample size might hamper the detection of some miRNAs also relevant for CVE and some detected differentially miRNAs might represent the prevalence of FH alone rather

than CVE. However, in the validation phase, nCVE-patients included secondary hypercholesterolemia cases. Thus, prospective studies expanding these results in a larger samples size for the prognostic value of miRNA signature are warranted. Finally, methodology for exosome-associated miRNA isolation and RT-qPCR evaluation needs to be further developed in order to be used in an easily fashion in the clinical setting.

In summary, miRNAs that circulate in the bloodstream can be taken up by distant cells and exert cell-to-cell communication; therefore, they have the potential of regulating gene expression simultaneously in different tissues and cells. From the clinical context, a specific biomarker-based prognosis of atherothrombotic events is of high clinical interest in order to improve CVD prevention. In this scenario, we provide preliminary evidence that an exosomal miRNA signature can lead to the prognosis of ischemic events in a high-risk population such as familial hypercholesterolemia patients even in the absence of symptoms.

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Figure Legends

Figure 1. Functional analysis identified by IPA associated with differential expressed miRNAs in the profiling phase. Inferred enriched microRNAs functional categories on 21 differentially expressed exosomal miRNAs (N=10) obtained from IPA analysis. *P*-values (log-transformed) for each category are represented in the x-axis. Arrows highlight the most interesting categories for our population of study.

Figure 2. Exosomal microRNA validation phase. Box and whisker plots showing levels (per µL of PFP) of specific miRNAs: miR-122, miR-130b, miR-133a, miR-142-3p, miR-200c, miR-324-5p, miR-339-3p, miR-425-5p, miR-660, and miR-744 in FH patients who had an ischemic event 3 years after their inclusion (CVE, N=42) compared with non-FH hypercholesterolemic patients without any cardiovascular episode at follow-up (nCVE, N=30). Data are expressed as median (interquartile range). * $P < 0.05$ using U-Mann Whitney non-parametric test.

Figure 3. Discriminatory power of miRNAs for the prediction of ischemic CV events. Associated receiver operator characteristic (ROC) curves along with their area under the curve (AUC) are given for all validated differential miRNAs in predicting an ischemic event (CVE) in high-risk hypercholesterolemic patients (N=72) in comparison to a Reference Line (AUC=0.500).

Figure 4. Discriminatory power of the nine- and five-miRNA signatures for the prediction of ischemic cardiovascular events. Associated receiver operator characteristic (ROC) curve along with their area under the curve (AUC) are given for the nine-miRNA panel **(A)** and the five-miRNA panel **(B)** in predicting an ischemic event (CVE) in high-

risk hypercholesterolemic patients (N=72) in comparison to a Reference Line (AUC=0.500). **(A)** The nine-miRNA signature includes the miRNAs: miR-130b, miR-133a, miR-142-3p, miR-200c, miR-324-5p, miR-339-3p, miR-425-5p, miR-660, and miR-744, and **(B)** the five-miRNA signature includes the miRNAs: miR-130b, miR-142-3p, miR-200c, miR-660, and miR-744.

Figure 5. PPAR activation signalling. PPAR activation signal transduction pathway associated with common target genes of the differential 5-miRNA signature obtained as the first scored canonical pathway analysis by IPA.

Figure 6. RhoA signalling. RhoA signal transduction pathway associated with common target genes of the differential 5-miRNA signature obtained as the second scored canonical pathway analysis by IPA.

Figure 7. Tight junction signalling. Tight junction signal transduction pathway associated with common target genes of the differential 5-miRNA signature obtained as the second scored canonical pathway analysis by IPA.

Table 1. Characteristics of the the subjects selected for miRNA profiling as well as the overall study population, CVE-FH- and nCVE-patients.

	miRNA profiling			miRNA validation		
	CVE (N=6)	HC (N=6)	*P*-value	CVE (N=42)	nCVE (N=30)	*P*-value
Age (years)	57 ± 2.7	52.7 ± 3.9	*P* = 0.43	60.7 ± 2.2	51.0 ± 2.7	*P* = 0.01
BMI (Kg/m^2)	30.3 ± 1.6	27.6 ± 1.6	*P* = 0.24	28.9 ± 0.6	27.1 ± 0.9	*P* = 0.08
Diabetes mellitus (%)	3 (50%)	0 (0%)	*P* = 0.04	6 (14.3%)	1 (100%)	*P* = 0.12
Hypertension (%)	3 (50%)	0 (0%)	*P* = 0.04	19 (45.2%)	3 (10%)	*P* < 0.01
Smoking (%)	1 (16.7%)	1 (16.7%)	*P* = 0.99	16 (38.1%)	10 (33.3%)	*P* = 0.68
Obesity (%)	4 (66.7%)	0 (0%)	*P* = 0.01	11 (26.2%)	4 (13.3%)	*P* = 0.18
Hyperlipidemia (n, %)	6 (100%)	1 (16.7%)	*P* < 0.01	41 (97.6%)	28 (93.3%)	*P* = 0.37
TC (mg/dL)	215 ± 15.6	223 ± 16.2	*P* = 0.75	266 ± 12.3	204.3 ± 6.4	*P* < 0.01
LDL-c (mg/dL)	148 ± 11.3	146 ± 15.7	*P* = 0.93	195 ± 11.5	130.1 ± 6.1	*P* < 0.01
HDL-c (mg/dL)	39.5 ± 2.8	51.8 ± 3.7	*P* = 0.02	42.1 ± 1.8	55.4 ± 2.5	*P* < 0.01
Triglyceride (mg/dL)	139 ± 41.3	123 ± 22.9	*P* = 0.73	141 ± 12.9	94.2 ± 7.4	*P* < 0.01
LLT with statins (%)	5 (100%)	0 (0%)	*P* < 0.01	38 (90.5%)	28 (93.3%)	*P* = 0.66
hs-CRP (mg/L)	2.0 ± 0.6	2.1 ± 0.9	*P* = 0.78	3.0 ± 0.4	2.7 ± 0.8	*P* = 0.71
Ischemic CVE						
- Sudden death	1	-	-	9	-	-
- ACV	2	-	-	7	-	-
- MI	2	-	-	20	-	-
- UA	1	-	-	6	-	-
Sampling-to-CVE / analysis time (years)	1,64 ± 0.25	3.33 ± 0.21	*P* < 0.01	3.06 ± 0.37	3.20 ± 0.07	*P* = 0.75

Comparison of circulating miRNAs in plasma samples of CVE-patients and nCVE-controls. BMI, body mass index; hs-CRP, high-sensitive C-reactive protein; CVE, cardiovascular event; HC, healthy controls; LLT, lipid-lowering treatment;

Table 2. Exosomal microRNA profiling phase.

Differential miRNA	Change	Ratio	Fold change (increase / decrease)
hsa-let-7e	↑	1.98	+ 1.98
hsa-miR-122	↓	0.50	– 2.00
hsa-miR-130b	↑	2.33	+ 2.33
hsa-miR-132	↓	0.38	– 2.63
hsa-miR-133a	↑	2.14	+ 2.14
hsa-miR-138	↓	0.46	– 2.17
hsa-miR-142-3p	↑	1.80	+ 1.80
hsa-miR-143	↓	0.65	– 1.54
hsa-miR-155	↑	1.60	+ 1.60
hsa-miR-19a	↓	0.43	– 2.33
hsa-miR-199-5p	↑	1.90	+ 1.90
hsa-miR-200c	↑	2.68	+ 2.68
hsa-miR-28-5p	↑	1.82	+ 1.82
hsa-miR-324-5p	↑	1.77	+ 1.77
hsa-miR-339-3p	↑	1.60	+ 1.60
hsa-miR-340	↑	1.55	+ 1.55
hsa-miR-425	↓	0.62	– 1.61
hsa-miR-494	↓	0.46	– 2.17
hsa-miR-660	↑	1.60	+ 1.60
hsa-miR-744	↑	2.40	+ 2.40
hsa-miR-885-5p	↓	0.45	– 2.22

Results of the profiling phase of circulating miRNAs in patients with FH and CVE (N=5) with healthy controls (N=5). ↓ and – indicates decrease; ↑ and +, increase.

Table 3. Top biological functions obtained from IPA associated to common targets genes regulated by 5-miRNA signature.

Name	*P*-value	Molecules
Gene expression	2.17E-09 – 3.25E-02	68
Cell death and survival	2.13E-07 – 3.25E-02	77
Cellular growth and proliferation	2.59E-07 – 3.25E-02	86
Cellular assembly and organization	5.03E-07 – 3.25E-02	49
Cellular movement	3.31E-06 – 3.25E-02	51

Top 5 molecular and cellular functions with their respective *P*-value and the number of molecules involved obtained from IPA analysis of common target genes regulated by miRNAs from the studied 5-miRNA signature.

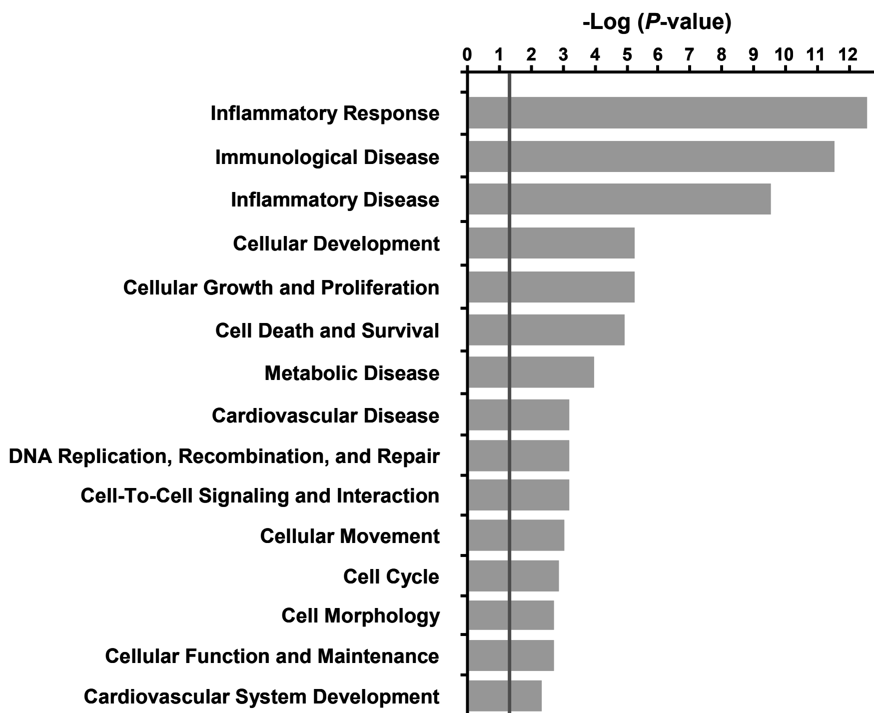


Figure 1

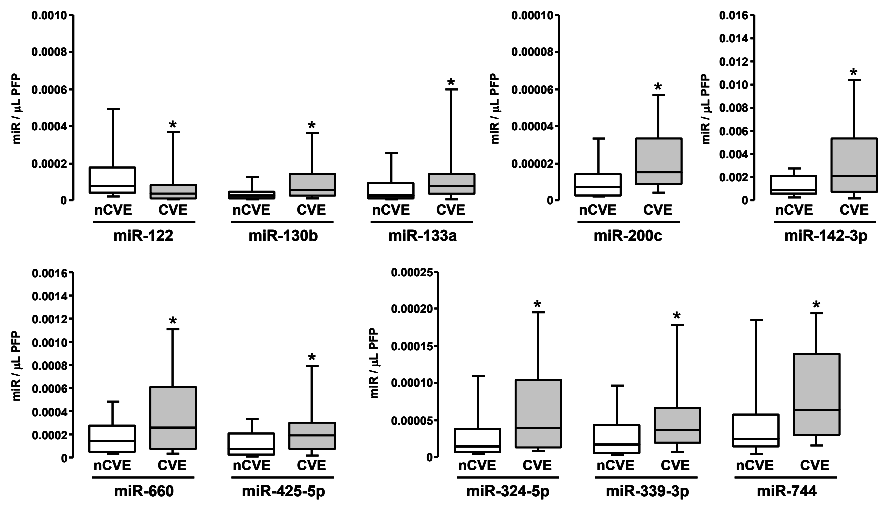
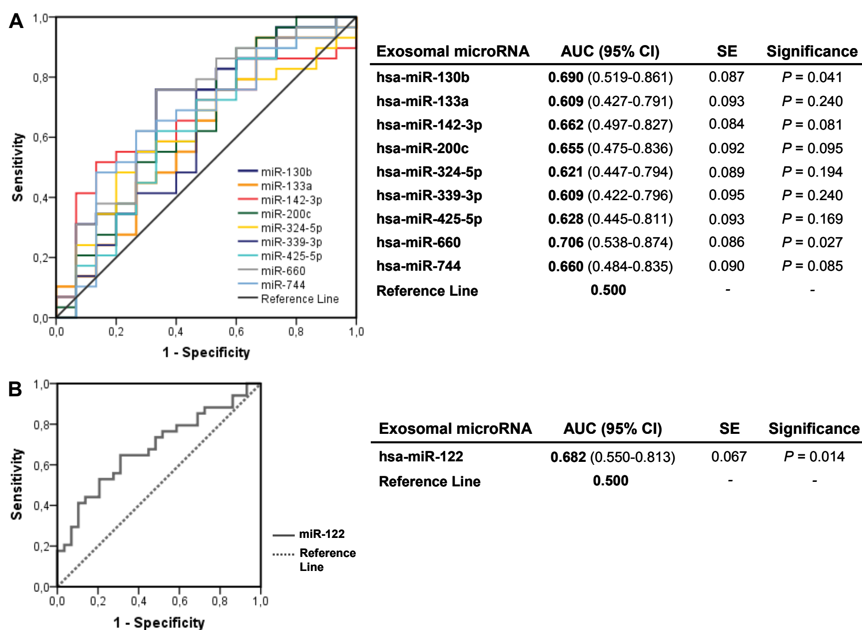


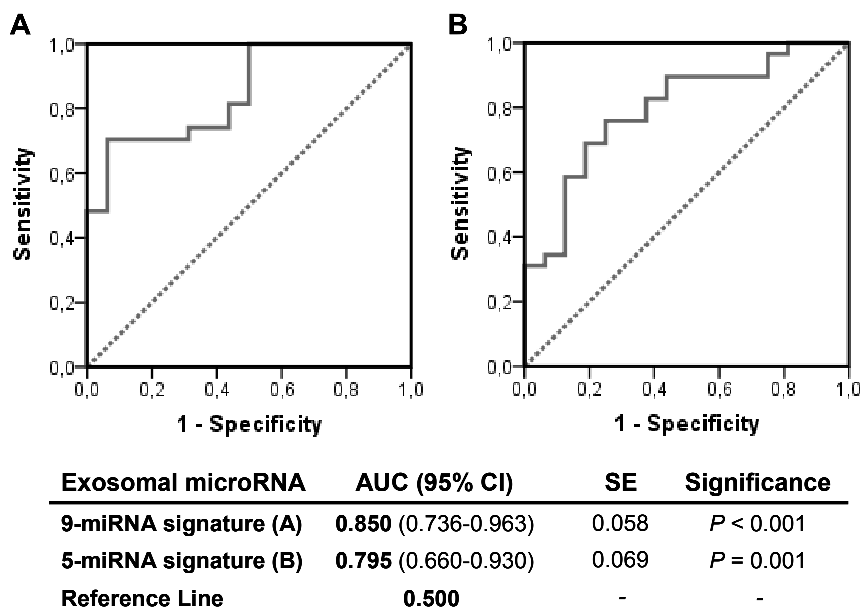
Figure 2



Exosomal microRNA	AUC (95% CI)	SE	Significance
hsa-miR-130b	**0.690** (0.519-0.861)	0.087	*P* = 0.041
hsa-miR-133a	**0.609** (0.427-0.791)	0.093	*P* = 0.240
hsa-miR-142-3p	**0.662** (0.497-0.827)	0.084	*P* = 0.081
hsa-miR-200c	**0.655** (0.475-0.836)	0.092	*P* = 0.095
hsa-miR-324-5p	**0.621** (0.447-0.794)	0.089	*P* = 0.194
hsa-miR-339-3p	**0.609** (0.422-0.796)	0.095	*P* = 0.240
hsa-miR-425-5p	**0.628** (0.445-0.811)	0.093	*P* = 0.169
hsa-miR-660	**0.706** (0.538-0.874)	0.086	*P* = 0.027
hsa-miR-744	**0.660** (0.484-0.835)	0.090	*P* = 0.085
Reference Line	**0.500**	-	-

Exosomal microRNA	AUC (95% CI)	SE	Significance
hsa-miR-122	**0.682** (0.550-0.813)	0.067	*P* = 0.014
Reference Line	**0.500**	-	-

Figure 3



Exosomal microRNA	AUC (95% CI)	SE	Significance
9-miRNA signature (A)	**0.850** (0.736-0.963)	0.058	*P* < 0.001
5-miRNA signature (B)	**0.795** (0.660-0.930)	0.069	*P* = 0.001
Reference Line	**0.500**	-	-

Figure 4

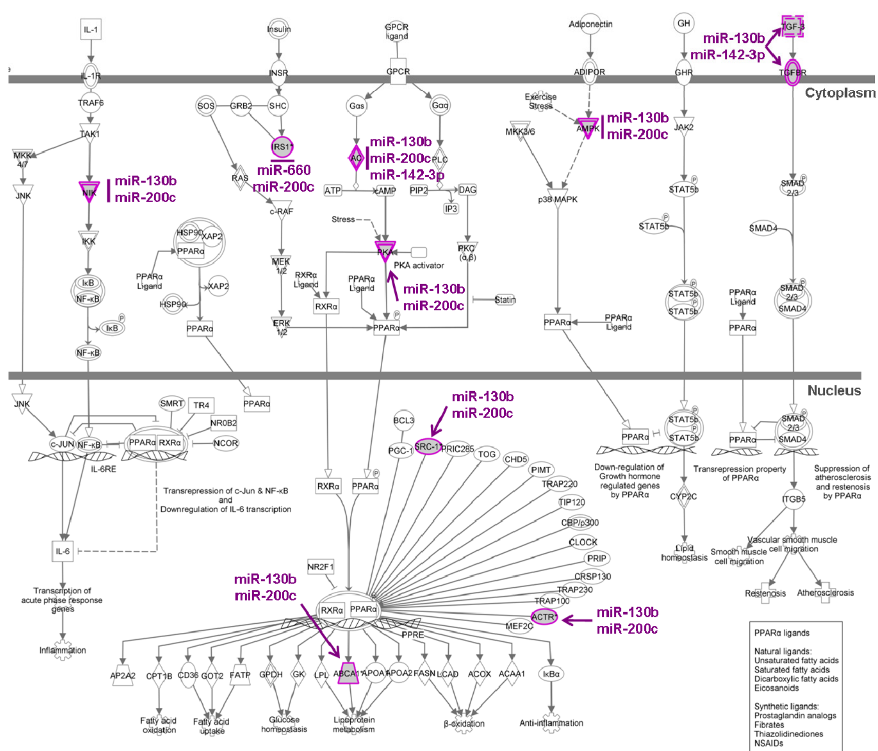


Figure 5

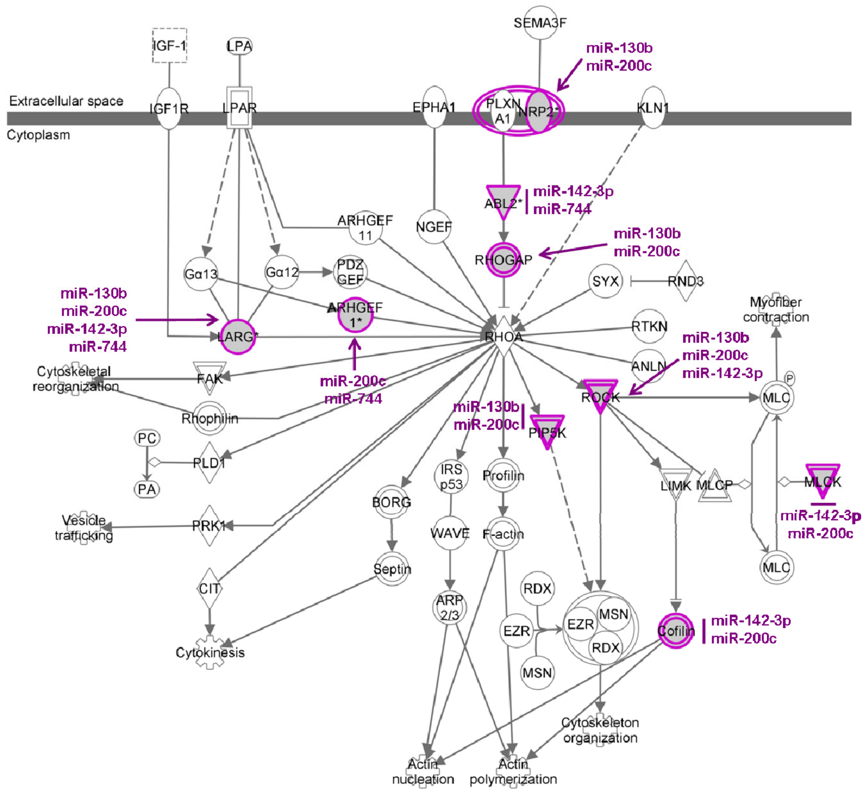


Figure 6

Figure 7

V. DISCUSSION

Atherosclerosis is a chronic inflammatory disease that is caused by high LDL plasma levels. Indeed, familial hypercholesterolemia, a major risk factor for atherosclerosis, is associated with early development of CVD and hence constitutes a suitable model to study lipid-dependent atherosclerosis. Despite accumulating progress has been made in the scenario of atherosclerosis, it typically remains undetected until a rupture or a reduction in blood flow results in a cardiovascular event, such myocardial infarction or stroke. The risk of developing CVD can be partly assessed by calculating a risk score that takes into account traditional risk factors including hypertension, high blood LDL, low HDL, and smoking. However, most cardiovascular events (CVE) occur in subjects with a low or average risk score.[170,613] Moreover, many mediators of inflammation, such as interleukins and hsCRP, are notoriously difficult to interpret since levels can be elevated as a result of other independent co-morbidities or may only be elevated in the acute phase, when it is likely too late for intervention. Therefore, there is an urgent need to find novel biomarkers in order to identify earlier vulnerable patients and refine cardiovascular risk prediction.

For this reason, a major objective of the present thesis has been to evaluate cMPs, small membranous vesicles released by activated blood cells, as potential new markers of atherosclerotic plaque burden. MPs depict markers of parental cells and contain anionic phospholipids on their surface. Activated cells generate phosphatidylserine-positive cMPs, but also PS-negative cMPs can be found in the circulation.[165] Surface exposure of PS on MPs appears to confer them procoagulant activity and capacity to regulate both their internalization and clearance by phagocytosis, and to be mediators of their own formation.[614] Recent studies have correlated AV-negative cMPs to clinical parameters in systemic diseases,[615] which emphasize the importance of including characterization of AV binding in cMPs. Despite both types of cMPs were detected, the present thesis have focused mainly on AV-positive cMPs and on total cMPs, because of their relevant association to atherothrombotic disease.[49]

Previous studies have shown elevated cMP levels in patients with clustering of cardiovascular risk factors[51,162,544] and many pathological states[10,50,164] such as CAD.[50] Expanding this information, the present study has found that asymptomatic FH patients receiving long-term LLT depicted a relevant change in their cMP phenotype related to cell origin and have significant higher number of overall cMPs, in particular of those derived from endothelial cells, monocytes and lymphocytes than non-FH hypercholesterolemic patients, demonstrating that endothelial dysfunction and vascular inflammation are present in FH patients despite being treated as per guideline (*Paper 3*).

Higher levels of endothelial-derived ($CD146^+$) MPs were detected in FH than in non-FH patients (*Paper 3*). High plasma levels of eMPs are an indicator of endothelial cell activation and dysfunction.[616] Indeed, high plasma cholesterol levels directly impair flow-mediated dilatation and induce aortic stiffness, both associated with the onset and progression of atherosclerosis.[617] eMPs have been proposed as potential prognostic markers for future cardiovascular events in high CV risk patients.[181] Pirro et al showed high levels of leuko-eMPs in newly-diagnosed never-LLT-treated hypercholesterolemic subjects.[618] Besides, Huber et al found that those microvesicles containing oxidized phospholipids, typically in lipid-related diseases, could induce monocyte-endothelial interactions,[619] contributing to atherogenesis.[620] The findings of this thesis are in agreement with a recent study that points out eMPs as biomarkers to monitor endothelial function in hypertension with hyperlipidemia.[621] Interestingly, in a large community-based sample, eMP levels have been associated with the presence of cardiometabolic risk factors, particularly metabolic profiles associated to high cardiovascular risk (HCVR): dyslipidemia, TG levels, hypertension, hypertriglyceridemic waist, and metabolic syndrome.[622]

While the number of total non-activated leukocyte-derived MPs was found similar in FH and in non-FH patients, a significantly higher number of monocyte-derived MPs was detected in the FH patients (*Paper 3*), suggesting a link between cMPs, endothelial dysfunction and inflammation, which might be supported by the fact that monocyte MPs might induce atherogenic effects[623] and amplify inflammation by activation of endothelium.[494] It was also described that atherosclerotic plaques contain large amounts of LMPs[56] and that plaque-MPs promote plaque progression by promoting inflammatory cell adhesion to ECs.[500] Furthermore, FH patients had significantly higher number of cMPs bearing leukocyte activation marker MAC-1 (α_M-integrin, $CD11b^+$) even though the aggressive LLT in these patients. MAC-1 is conjugated to $\alpha_M\beta_2$-integrin and its activation indicates a proinflammatory/prothrombotic state due to ongoing platelet interaction to leukocytes.[619] Previously, lymphocyte-derived cMPs (ℓMPs) were shown to promote production of TNFα and IL-1β by monocytes[70] and were proposed as markers of reduced vasculoprotective properties of ECs in patients at HCVR.[276] Accordingly, increased levels of ℓMPs ($CD45^+/CD3^+$) were found in FH patients, being even higher in those patients with lipid-rich plaques.

In a previous study, our group reported a high degree of atherosclerotic plaque burden using aortic-MRI in a group of FH patients also investigated in this thesis.[106] Indeed, it has been found that aortic atherosclerosis is a marker for coronary atherosclerosis.[624] Lipid-rich plaques, characterized by the presence of a thin fibrous cap and inflammatory cells, are associated with the highest clinical risk because they are prone to rupture.[18] Interestingly, lipidic plaques were found to correlate positively and significantly with

higher levels of total circulating microparticles and cMPs carrying markers of activation of their mother-inflammatory cells, even in the presence of LLT, as compared to fibrous/mixed plaques. Specifically, within the FH group, ℓMPs were significantly higher in those patients with lipid-rich plaques. Thus, these data suggest that circulating ℓMP levels are a robust predictor of presence of lipidic plaques in FH, in accordance with data from patients with high-grade carotid stenosis and leukocyte-derived MPs.[625] Here, a cut-off value of 20,000 ℓMPs/mL in plasma was obtained by using ROC curve analysis (P=0.008) as a discriminator of lipid-rich atherosclerotic plaques in FH patients (*Paper 3*), which deserves further proof-of-principle studies in other patients with CAD. This remarkable positive correlation found with atherosclerotic plaque type by aortic-MRI and the specific cMP profile in statin-treated FH patients expands the mechanistic insights generated by MP characterization. The fact that $CD45^+/CD3^+$-ℓMPs map aortic lipid-rich plaques highlights the role of lymphocytes in atherosclerosis. Indeed, activated lymphocytes are considered effectors of atherogenesis by promoting lesion formation and exacerbating atherosclerotic disease.[626] In contrast to the previous observation, lower levels of cMPs were found in FH patients with calcified atherosclerotic plaques and a large mean aortic wall area, which were characteristic of non-lipidic low risk plaques.[106] Accordingly, a recent study has highlighted that MP procoagulant activity was higher in moderate calcified plaque group compared to non-calcified and totally calcified groups.[627] Although total calcium (measured by different scores) is indicative of atherosclerotic burden, calcified plaques in itself are not markers of vulnerability. Indeed, the PROSPECT study showed that calcification *per se* may not cause coronary events[36] and other clinical studies have shown that, in fact, calcification might confer stability to the established atherosclerotic plaques.[43]

Atherosclerosis, induced by high cholesterol levels, is characterized in the early phase by endothelial dysfunction and vessel inflammation, and evolves to advanced plaques with vessel wall remodelling. When the remodelling becomes inward and reduces the lumen diameter reducing flow, plaques may rupture and trigger thrombosis. Thrombosis and vessel spasm, with reduction or abrogation of blood, leads to the presentation of the acute coronary syndromes. Because FH patients are at high CV risk, a 100mg/dL cut-off point for LDL-c was considered as the therapeutic target according to clinical guidelines.[585,586,628] Different studies indicate that only 13% of patients attained LDL-c goal on their initial lipid-lowering treatment (REALITY study[629]) and only 3% of high coronary risk patients achieve lipid therapeutic targets recommended by European guidelines (DARIOS study[630]). Similarly, only a low percentage of HCVR-FH patients achieved the therapeutic LDL target level; however, the shedding of cMPs was independent of LDL levels in these patients with chronic exposure to high plasma levels of LDL. These results did not overrule the effects of

cholesterol on MP shedding as seen in *in vitro* studies where enrichment of cholesterol in monocytes[364] and VMSC[631] trigger the release of procoagulant MPs. HCVR-FH patients are chronically exposed to high LDL during their lifetime with effects on all membranes. The impact of cumulative risk exposure on susceptible tissues and vessels and the ensuing clinically silent vascular activation in asymptomatic FH-HCVR patients seems to confer them a higher susceptibility to premature atherosclerosis and CAD. Although statins seem to exert effects on MP budding (*Paper 2*), the differences in cMP shedding detected between FH and non-FH patients may reflect the lifetime exposure to LDL-c that is not fully corrected by guideline-driven LLT. The findings of this thesis suggest that longer time and further LDL-lowering strategies are needed to protect HCVR-FH patients. The data presented here indicates that cMP analysis may be helpful to improve risk assessment and define the optimal use of treatment in asymptomatic patients. The fact that the gold standard inflammatory marker, hsCRP, did not correlate with cMPs in well-controlled patients indicates that although systemic inflammatory markers can be regulated by statin therapy, as seen in the Jupiter trial,[632] vascular cell activation in HCVR-FH patients evidenced by measuring cMP release is not completely abolished.

Lipid-rich plaques accumulate different inflammatory cells what may explain the changes observed in the activated cMP pattern. Additionally, lipid infiltration in the vessel wall has been described to induce tissue factor release from vascular resident cells.[35] TF activation is one of the main triggers of thrombosis on ruptured atherosclerotic plaques[633] and it is present within the lipid-rich core components.[603] In addition, activated platelets contribute to thrombogenicity triggering the coagulation cascade and the subsequent formation of fibrin.[78] Thus, a critical component of atherosclerotic progression leading to thrombotic events occurrence is the thrombogenic potential of blood. It was hypothesized that the state of activation of the cells in the blood-vascular interface would amplify the shedding of MPs being markers of the high atherothrombotic risk in these patients. Therefore, this thesis has investigated the prothrombotic state in high CV risk patients, associated to preclinical atherosclerosis (*Paper 4*). HCVR-FH patients had a significantly higher number of cMPs carrying epitopes of cell activation and TF than non-FH control patients. Indeed, HCVR-FH patients with MRI-detected atherosclerotic plaque burden have cMPs rich in TF that is biologically active and capable of triggering procoagulant activity. Furthermore, significantly elevated activated platelet-derived cMPs and TF-rich cMPs were found in the circulation of well-treated HCVR-patients with lipid-rich plaques, indicating a chronic burden of vascular cell activation, subclinical atherosclerosis and premature CVD.

TF^{+}-cMPs are likely to have different functional significance depending on their cellular source. TF-positive microparticles derived from both

monocytes and platelets were detected in higher numbers in HCVR-patients than in the control group. The presence of TF in platelets has been the subject of a long-standing controversy. While some groups have failed to detect TF on platelets,[634] today is generally accepted that platelets carry TF[635] and that platelets possess mechanisms to internalize TF-rich MPs[532] suggesting that TF in platelets could be the result of plasma transfer, expression or even be acquired by internalization of mMPs.[294,636] In contrast, there is agreement on monocytes as the most important source of TF^{+}-cMPs.[637] cMP-associated TF activity had been mainly attributed to mMPs.[638] The results of this thesis points that HCVR-patients have increased levels of monocyte- but also platelet-derived TF^{+}-MPs, which are functional in terms of PCA and correlate with atherosclerotic plaque burden, indicating that TF^{+}-cMPs might contribute to atherothrombosis. Following this hypothesis, the biological significance of cell-released-TF^{+}cMPs, further to their biomarker value, may be the amplification of TF-driven effects, including TF procoagulant activity and thrombosis, by accumulation in atherosclerotic lesions and propagation to distant areas and vascular territories. Recently, increased plasma TF^{+}-MPs were found in hyperlipidemic mice and monkeys as well as in severe FH-patients who required LDL-aphaeresis.[639] Hence, the presence of high levels of TF-rich cMPs appears to be a key step in the propagation of distal thrombosis, especially once it is locally initiated by vascular damage, contributing to local hypercoagulation.

In addition, HCVR-patients showed enhanced platelet activation as evidenced by significantly high numbers of pMPs carrying activated platelet markers, which also directly correlated with lipid-rich plaque burden. There is increasing evidence of the importance of platelets, not only in the acute phase of myocardial infarction but also in the early stages of vascular injury, leading to atherosclerosis.[78] In FH, platelets might become activated because of the chronic hypercholesterolemia and the ensuing inflammatory processes.[640] Thus, pMPs might contribute to the development and progression of atherosclerosis and premature CAD in HCVR-patients by several mechanisms. Indeed, previous studies have reported that pMPs can enhance the expression of adhesion molecules on monocytes and endothelial cells,[93] induce PCA (*Paper 1*), promote thrombin generation[638] and interact with leukocytes.[22]

As previously stated, the continuous exposure to high LDL levels in HCVR-FH patients confers them a very high susceptibility to premature CAD that is poorly detected with the existing risk scores. In this study, it is shown that MP assessment can help in the risk prediction. Indeed, the level of TF^{+}-pMPs ($CD142^{+}/TSP^{+}$) showed to be a useful predictor of lipid-rich atherosclerotic plaques by the area under the ROC curve. Interestingly, when c-statistics was applied combining predicted probabilities for TF-rich MPs and activated platelet-derived MPs together, the prediction of lipidic plaques

gave an AUC of 0.931 [95%CI: 0.821-1.000]. Furthermore, when these prothrombotic cMPs were added to a risk factor model (AUC of 0.716 [95%CI: 0.526-0.906]), the discrimination capacity significantly increased to 0.955 [95%CI: 0.883-1.000]. Thus, MPs arise as promising predictors of subclinical atherosclerosis that may add incremental value to currently used risk prediction models (*Paper 4*).

Taken together, the present results (*Paper 3 and 4*) show for the first time a relation of the type of plaque, lipid-rich or calcified, with cMP number, phenotype and activation status of the cell source in the FH-HCVR patients. Of special interest are the highly significant increases in lymphocyte-derived $CD45^+/CD3^+$-MPs in one hand and TF^+-MPs and activated pMPs in the other hand found in patients with lipid-rich atherosclerotic plaques. The prediction of lipid-rich atherosclerotic plaque burden and, hence, high cardiovascular risk by specific cMPs, demonstrating an incremental prognostic value, even in a moderate number of patients, should be expanded to prospective clinical studies. Nevertheless, prior to large-scale studies, it would be desirable that cMP analysis should be internationally standardized. Until now, the majority of studies evaluating cMPs as biomarkers have been proved to be complex and susceptible to variability when performed on larger cohorts for clinical purpose. Thus, under these drawbacks and despite recent advances in this regard,[582,598,641] major improvements are warranted for standardization of both pre-analytical conditions and current available techniques in terms of size, phenotype and quantity among laboratories in order to implement and translate advances in research into the clinical practice.

The identified increased circulating ℓMPs in patients with atherosclerotic plaque burden highlights not only their potential use as biomarkers of subclinical atherosclerosis but also their role in the early phases of atherogenesis. Furthermore, the burden in prothrombotic cMPs could also signal for a state of vulnerability of the vessel wall to trigger arterial thrombosis and hence clinical CVD manifestation upon sudden plaque structural changes and rupture. Thus, all together these proof-of-principle studies with patients from a large cohort study sustained the concept of cMPs as effectors beyond markers of vascular disease.

Interestingly, the present thesis has investigated cMPs as novel biomarkers at different stages of CVD progression, not only in the early phases of atherosclerosis and as predictors of disease occurrence, but also in the clinical setting of MI. Most cases of persistent ST-segment elevation MI are caused by atherosclerotic plaque rupture and thrombosis, which ultimately leads to occlusion of a major coronary artery.[31] For that reason, proof-of-concept studies were firstly devoted to investigate the characteristic cMP profile released into the perfusing blood while the thrombus is formed on

substrates with different thrombogenic potential (*Paper 5*). Next, translational studies in patients with acute coronary thrombosis were aimed to determine whether occlusive coronary thrombi causing STEMI may release cMPs of a determined phenotype in order to disseminate the prothrombotic message and affect the distal vasculature and the microcirculation (*Papers 5 and 6*).

Paper 5 depicts the shift in blood cMP profile as a consequence of thrombus formation induced under controlled conditions of shear stress and exposure of atherosclerotic substrates in an *in vitro* model using blood from healthy donors. It was shown that total cMP shedding is increased after thrombosis elicited by high shear and thrombogenic lesions, conditions that mimic stenotic coronary blood flow on damaged vascular wall. Under these conditions, there was an increase in circulating red blood cell-MPs, whereas pMPs bearing epitopes involved in adhesion were reduced after perfusion in both chambers and pMPs carrying activation markers were found mainly decreased after blood perfusion on collagen surfaces, which only support platelet adhesion, but not on vascular wall that anchor growing thrombi.

As aforementioned, platelet-derived microparticles are of high importance in the pathogenesis of CVD.[468] pMPs provide the membrane surfaces necessary for assembly of the tenase and prothrombinase complexes. A number of studies have highlighted the fact that shear stress enhance their formation with a variety of stimulus and conditions.[11,84,184,189,307,642-647] Indeed, in a substudy of this thesis, detailed below, it was found that cMPs and, in particular pMPs, enhance the deposition of platelets and fibrin to atherosclerotic vessel wall (*Paper 1*). To this respect, P-selectin-containing MPs enhance leukocyte aggregation and accumulation on selectin-expressing substrates under high shear stress.[22] In this study, a blunt decrease of pMPs with surface markers of adhesion and activation in the post-thrombus blood was detected, likely indicating retention into the growing thrombus, as demonstrated in *Paper 1*.

The significant elevation of erythrocyte-MPs after exposing human blood to damaged arterial substrates and collagen, under high shear rate, highlights the importance of red blood cells in arterial thrombosis, an aspect largely overlooked so far. Indeed, a recent study has revealed a previously unrecognised ability of RBCs to participate in thrombosis by mediating platelet adhesion to an intact endothelial surface, in a $FeCl_3$-mediated thrombosis model.[648] ErMPs have also recently emerged as potential mediators of transfusion-related morbidity. However, the pathophysiological role of RBC-MPs has not been fully elucidated. High levels of ErMPs have been detected in haematological disorders[649,650] such as sickle cell disease (SCD).[651,652] ErMPs have recently been shown to amplify systemic inflammation by thrombin-dependent activation of complement.[653] Due to their haemoglobin content and PS exposure, ErMPs may account for NO

scavenging and PCA enhancement.[654] In fact, ErMPs are able to support coagulation by decreasing prothrombin time[655] and, together with pMPs, have demonstrated to trigger thrombin generation in a FXII-dependent/TF-independent manner,[638] being their coagulation activation involvement corroborated in the setting of SCD.[656,657] Recently, ErMPs have shown to induce endothelial injury and facilitate vaso-occlusive events in a murine model of SCD, connecting sickle cell anaemia to vascular disease.[658] Therefore, elevated levels of ErMPs, triggered by incipient thrombus formation, may also play a pathogenic role in the thrombotic profile of patients with cardiovascular diseases. Indeed, STEMI patients, undergoing primary percutaneous coronary intervention (pPCI), showed a high percentage of AV^{+}-$CD235a^{+}$-cMPs both at systemic levels and at the coronary culprit site, suggesting circulating ErMPs as a marker for an ongoing thrombotic event on the coronary artery. As expected, ErMPs were reduced in systemic blood of patients at 72 hours post-ischemia compared to the acute phase, suggesting the activation trigger for ErMPs (occlusive forming thrombi) had disappeared, but they were still higher than controls probably due to a not yet completed thrombus resolution and clearance. Thus, ErMPs could be considered as a haemorheological index to characterize arterial thrombosis. Taken together, blood perfusing thrombogenic damaged atherosclerotic vessel wall at a high shear stress triggers platelet deposition and thrombus formation that induce a rapid cMP release into the circulation. Present data on ErMP release at sites of arterial thrombosis suggests that ErMP analysis could be a novel method of detection of ongoing thrombosis.

Other cell-derived cMPs and their activation status during the temporal evolution of STEMI were further analyzed by a multi-panel procoagulant annexin V^{+}-cMP approach (*Paper 6*). High levels of cMPs in STEMI patients have been well-described[49,155,659-661] and associated to the area at risk during MI.[662,663] Expanding the previous findings, *paper 6* demonstrates a significant shift in the profile of cMPs in acute STEMI patients undergoing pPCI compared to healthy controls and 72 h post-STEMI patients after pPCI. Higher percentages of proinflammatory-, endothelium-, activated platelet-, and RBC-derived as well as TF-rich cMPs were found in the acute phase of STEMI. pPCI represents the reperfusion strategy of choice in patients with STEMI, provided it is delivered in a timely fashion and being most beneficial when performed within the first 2 h after symptom onset.[664] In accordance with the high rate of successful revascularization of the culprit artery, a global decrease in cMPs was found at 72 h post-STEMI after pPCI. However, post-STEMI patients maintained similar levels of activated pMPs, eMPs, and TF^{+}-cMPs. Surprisingly, pan-LMPs increased even after acute-MI, likely due to the inflammatory burst occurred at STEMI onset. Interestingly, cMPs from endothelium, monocytes, activated platelets and cMPs rich in TF were in a significantly higher proportion in blood samples

collected from the culprit coronary artery than those obtained from the peripheral artery of the same patients, suggesting a local shedding of activated cells in the leading edge of growing thrombus. Thus, changes in prothrombotic, proinflammatory and endothelial dysfunction can be found by measuring cMPs both at systemic and intracoronary level, reflecting the sensitivity of cMPs as markers of ongoing thrombus formation. Likewise, when the procoagulant cMP phenotype was investigated as a function of ischemic time, intracoronary cMPs were directly related to the duration of pain-to-pPCI ischemic time, suggesting their local release and contribution to intracoronary thrombus. In addition, peripheral cMPs also associated to the number of diseased vessels. In the present study, changes in cMP signature in the culprit coronary artery of STEMI-patients reveal their sensitivity to detect thrombo-occlusive vascular process developing in the coronary arteries of STEMI-patients and its impact at the systemic level, reflecting the temporal evolution of disease, which could be used to improve the prognosis of STEMI patients.

Taken together, MPs that changed in STEMI patients undergoing pPCI (*Papers 5 and 6*) were derived from vascular and circulating cells known to play a direct effect on the clinical context of atherothrombosis, helping to establish the atherosclerotic milieu. In particular, eMPs have a role in endothelial dysfunction[50] and $CD31^+/AV^+$-cMPs are an independent predictor of CV events in stable CAD patients.[665] The release of ErMPs has been shown to be also an integral part of the thrombotic process. Erythrocytes by mechanical fluid dynamic forces push platelets to circulate in the boundary liquid layer over the surface of the injured vessel and platelets recruited at the site of thrombosis are activated and able to shed MPs which can disseminate a procoagulant state and provide a trigger for further thrombogenicity. It is interesting to note that STEMI patients with exacerbated platelet activation had higher numbers of total pMPs and activated pMPs. In a deleterious vascular environment, the generation of cMPs may further accelerate disease progression by cross-talk with other blood cells, inducing their activation and amplifying arterial thrombus formation. cMPs, primarily of leukocyte origin, are considered a primary source for blood-borne TF involved in thrombus propagation at the site of vascular injury.[517] In addition, human atherosclerotic plaques contain high levels of MPs expressing CD40L and bearing TF.[495] During plaque disruption and thrombus formation, circulating TF-rich MPs and pMPs might contribute to high levels of TF-activity at the thrombus triggering the formation of fibrin. Last but not least, cMPs generate and transport mCRP in MI patients.[666] Thus, the specific cell-derived MPs shed could represent distinct biological vectors contributing to vascular disease.

Increasing evidence supports the concept that cMPs are not merely markers of cell activation in the circulation but also could be causal inducers of

atherosclerosis and atherothrombosis. An unresolved question in the presentation of the acute coronary syndromes is that a similar type of underlying atherosclerotic lesion can trigger different types of ACS, either STEMI, NSTEMI or even UA. The type and size of thrombotic mass developing during ACS, either mural or fully occlusive, has important clinical implications with differences in morbidity and mortality.[80] Thus, the mechanisms driving ACS outcome need to be investigated. Up to now the contributing factors beyond the underlying triggering atherosclerotic plaque are still not fully identified and systemic factors have been mainly centred in characterizing the mass/size of thrombus, platelets and fibrin growing on top of the plaque, and the role of inflammatory mediators in the circulation; however, the determinants of the different clinical outcomes remain unknown. During atherosclerosis, blood cells and platelets become activated and release MPs and their numbers are increased in patients with CAD.[49,667,668] Thus, cMPs may be one of the factors contributing to thrombosis. Whether circulating MPs had a role on the growth of the thrombotic mass triggered on top of atherosclerotic plaques had not been demonstrated. In this context, *Paper 1* showed for the first time that increased numbers of blood-cMPs significantly enhance platelet adhesion and thrombus formation either on atherosclerotic lesions or on vessel wall with vascular injury. The study was based on blood-derived cMPs purified from healthy donors and spontaneously generated pMPs in blood bank platelet concentrates. Full characterization by flow cytometry depicted high binding capacity for annexin V (PS) and markers for $\alpha_{IIb}\beta_3$-integrin, CD36, and PECAM-1. CD36 associates to MPs in diabetics,[669] and PECAM-1 increases in cMPs of patients with different cardiovascular outcomes.[665] pMPs also carry thrombospondin-1 and CD63, an antigen found to be increased in pMP subpopulations of peripheral arterial disease and myocardial infarction patients,[155] as well as low levels of activation markers like P-selectin, activated $\alpha_{IIb}\beta_3$-integrin and tissue factor.

The pattern of thrombus formation on human atherosclerotic lesions is directly regulated by local rheological conditions and vessel wall composition.[599,600] The present study, using two well-characterized flow perfusion devices to rheologically model stenotic coronary blood conditions,[599,600,602,670] demonstrated that cMPs have a direct functional role in enhancing thrombus formation (*Paper 1*). When adjusted to an identical concentration, pMPs induced a stronger stimulation of platelet deposition than cMPs, suggesting a reduced contribution of other cell-derived MPs present in the circulation to the thrombogenic stimulus. High pMP numbers enhance the rate, extent and height of platelet and fibrin deposition on human atherosclerotic substrates under conditions mimicking coronary blood flow. Previous studies had shown that pMPs could adhere to subendothelial matrix proteins as collagen type-I,[89] as well as to fibrinogen, von Willebrand factor and surface immobilized platelets.[570] Here, using

fluorescence-tagged pMPs, it was shown that under high shear rate conditions pMPs also localize within the growing platelet thrombi on exposed collagen. In particular, pMPs were found to be able to bind to activated and adhered platelets, under high shear rate conditions, which might stimulate further platelet deposition and thrombus growth. Therefore, these data reinforce *Paper 5* where using the flat flow perfusion chamber it was shown that activated pMPs were reduced in stressed effluent blood as a result of this interaction. Altogether these data indicate that the presence of a high concentration of pMPs in blood (6000 MPs/μL) promote platelet adhesion due to a high tendency to adhere, supporting their clear implication in the atherothrombotic process.

pMPs not only promote thrombus formation under flow conditions but also stimulate platelet activation as shown by PFA-100 analysis. Closure time, measured using an epinephrine/collagen cartridge, was shortened in the presence of increased pMPs, in agreement with Kim et al.[671] In addition, this thesis shows for the first time pMP dose-dependent and incubation time-dependent effects on closure time shortening. This assay was also used to fix the pMP levels for the *in vitro* studies, in conjunction with the literature, specifically, in high CVD risk patients.[155] Interestingly, pMPs also shortened the clotting time of whole blood when analyzed by thromboelastography and showed a proaggregatory effect on LTA when platelets were challenged with low ADP concentrations.

In summary, data here presented indicates that when a plaque ruptures or the vascular lumen is damaged and considerably reduced by stenosis, blood MPs might contribute to platelet deposition and thrombus formation. In this proof of concept study cMPs, and specifically pMPs, have shown an enhancing effect on platelet aggregation, coagulation, and thrombosis on atherosclerotic and damaged vessel wall.

Because of the aforementioned association between MPs and atherosclerotic burden and the impact of MPs in promoting thrombus formation on injured atherosclerotic vessel wall (*Paper 1,3 and 4*), it was hypothesized that MPs might carry different messengers in order to facilitate such proatherothrombotic effects and also modifiers of blood homeostasis. To this respect, it is worth to mention that not only the quantity but also the quality of the circulating MPs may be essential to their effects. Thus, the use of proteomic approaches may provide key information to better understand MPs composition and their role as relevant interplayers of cellular crosstalk and interactions. During the atherothrombotic process, upon stimulation by thrombin as well as other agonists or mechanical flow factors, platelets become activated and secrete proteins, as soluble molecules or as membrane vesicle-bound, with a major role in thrombosis and haemostasis. Therefore, pMP proteome represents a high platelet-specific subproteome. Due to their

accessibility and specificity, pMP proteins are potential candidates for drug targets and disease biomarkers. Henceforth, proteins carried by pMPs derived from activated platelets were identified (*Paper 7*).

Proteins transported by pMPs upon platelet activation by thrombin were studied using a quantitative proteomic assessment by which key components of the procoagulant pMP proteome directly involved in the pathophysiology of atherothrombotic disease were identified. A functional classification of these proteins was generated with the application of analytical software in a systems biology approach. Most of identified proteins were represented by cytoskeleton and cytoskeleton-binding proteins (actin, cofilin, myosin, and myosin regulatory light chain), membrane-associated proteins involved in intracellular transport and signalling (annexins), protein folding (isomerases) and cell interaction processes (membrane glycoprotein 140 [gp140], fermitin and protocadherin).

The majority of ADP-induced pMP proteins have been described to belong to the class of metabolism, energy pathways, signal transduction and communication.[388,470] Here, platelets were activated by thrombin; since it is the most abundant protein of the coagulation cascade and a potent mediator of platelet function. Accordingly, more cytoskeleton-related proteins involved in cell assembly and platelet morphology were found. In fact, given the important role played by the cytoskeleton in cellular exocytosis, thrombin signalling may rely upon a cytoskeletal remodelling in order to induce MP generation. Some of the identified proteins are known to translocate from the soluble cytosol to the cytoskeleton associated to the actin scaffold in activated platelets, such as vinculin, alpha actinin, filamin, the alpha, beta and gamma fibrinogen chains, the Arp2/3 complex and the coronin protein. This association might enable platelets to change their morphology, secrete granules and membrane blebs as well as to amplify the signals in order to adhere and aggregate.

Interestingly, proteins involved in cell adhesion like protocadherin alpha-4, gp140 and fermitin family homolog 3 (FERMT3) were also identified. Gp140 is a glycoprotein CUB domain-containing protein 1, which activates β1-integrin and induces motility signalling as well as regulates adhesion by forming complex with SRC-family kinases.[672] In the same line, FERMT3 plays a central role in cell adhesion in hematopoietic cells, by activating β1-β3 integrin and is required for platelet and leukocyte adhesion to ECs.[673] Proteins of blood coagulation may also be crucial for pMP function in the thrombotic process. For instance, fibrinogen gamma and beta as well as antithrombin III were clearly reduced on pMPs following thrombin stimulation, which indicates that are functionally active and implicated in the pathogenesis. Furthermore, other differential proteins were identified related to: (a) vesicle-mediated transport, like Protein bicaudal D that regulates coat

complex coatomer protein I-independent Golgi-endoplasmic reticulum transport by recruiting the dynein-dynactin motor complex and, annexin A4 that promotes membrane fusion and is involved in exocytosis; and (b) energy metabolism (long chain fatty acid-CoA ligase 3). It is also worth to stress signalling proteins since thrombin activation triggers inside-out signalling and induces an increase in protein kinase activity. Of interest it is the detection and change in the levels of phosphatidylinositol-4-kinase alpha, the regulatory subunit of cAMP dependent protein kinase, annexin V, guanine nucleotide-binding protein G(1) / G(S) / G(T) subunit beta-1 and kringle-containing transmembrane protein 1 isoform 1, which is involved in the Wnt-beta catenin signalling[674-676] that recently has been identified on platelets and might enhance the amplification of platelet activation.[677,678]

The oxireductase protein disulfide isomerise A3 (PDIA3, ERp57, GRP58) has recently been shown to participate in thrombus formation.[679,680] A previous study of our group showed that PDIA3 increases in the secretome of thrombin-activated platelets and NO donor could modulate its release.[681] In the present study, PDIA3 is found significantly increased in thrombin-induced pMPs, indicating that PDIA3 is secreted almost in part as a MP membrane-bound protein. Besides, protein disulfide isomerase (PDI, P4HB), considered as a critical mediator of wound healing and as a chaperone that inhibits aggregation of misfolded proteins, was found to be reduced in pMPs released upon platelet thrombin-activation. The fact that PDI levels are reduced and PDIA3 levels are increased upon platelet activation suggests that platelet surface may undergo a redox remodelling state which facilitates the different binding of thiol isomerases to mediate the disulphide rearrangements and activation of proteins like $\alpha_{IIb}\beta_3$-integrin.[682]

In summary, thrombin-activation of platelets results in shedding of MPs with a differential expression pattern of several proteins related to thrombosis. Accordingly, *paper 7* data reveals potential therapeutic targets that are susceptible of anti-thrombotic strategies. pMPs due to their protein content and characteristics might be proatherogenic, procoagulant and proinflammatory factors. Nevertheless, many mechanisms still need to be disentangled and further studies deserve their attention. The proteomic study in this thesis evidences a novel approach that may help to elucidate some of the molecular mechanisms of thrombin stimulation on platelets by pMPs with relevant impact on atherothrombotic disease.

Besides to proteins, microvesicles have shown to contain codifying and non-codifying nucleic acids such as microRNAs with relevant functions in regulation of protein translation. MiRNAs achieved their stability in plasma partly by their specific package into cMPs, hence favouring intercellular transport and influencing pathophysiological processes as atherothrombosis. Since extracellular vesicle-associated miRNAs are an active partaker in the

complex cell-to-cell communication network, their screening in plasma can potentially serve to provide novel non-invasive biomarkers for disease-specific diagnosis. In the last studies performed in this thesis, the prognostic value, for future cardiovascular events, of miRNAs in circulating microvesicles was investigated. In this regard, microRNA profiling of *paper 8* revealed that 21 exosomal miRNAs were differentially expressed in patients that suffered an ischemic event post-sampling (CVE) compared to nCVE patients in the same time-frame. RT-qPCR validation confirmed that five of these miRNAs, including miR-130b, miR-142-3p, miR-200c, miR-660, and miR-744 were significantly increased in CVE patients. A ROC curve analysis of the predicted probabilities of this miRNA signature was calculated and an AUC of 0.795 for ischemic event presentation was obtained. Further validation studies of this exosomal miRNA signature as a predictor of ischemic event presentation are needed.

miRNA-143 had been reported to induce the differentiation phenotype of vascular smooth muscle cells contributing to detain atherosclerosis.[683] Besides, miRNA-143 has been linked to atheroprotective shear stress-mediated communication between ECs and VSMCs through a microvesicle-mediated mechanism.[439] Interestingly, lower levels of cMP-associated miR-143 were detected in HCVR-FH patients compared to non-FH controls and, importantly, cMP-miR-143 correlated negatively with lipid-rich plaque burden, suggesting that the selective secretion of cMP-packed miRNA-143 is reduced in patients with high atherothrombotic risk. Likewise, the inclusion of MP-associated miRNA-143 together with prothrombotic cMPs to a risk factor model increased the AUC of the ROC curve for the prediction of lipid-rich atherosclerotic plaques to 0.972 [95%CI: 0.919-1.000]. Thus, detection of low levels of miR-143-cMPs could be useful as prognostic circulating biomarker for CVD (*Paper 4*).

Despite methodological biases in extracellular miRNA analysis, circulating miRNAs appear as promising novel *micromaps* of atherothrombosis disease in hypercholesterolemic patients. Indeed, a robust relationship between cMP-specific microRNA and *in vivo* measured atherosclerotic burden was identified. Lipidic plaques were found to correlate positively and significantly with lower levels of cMP-miR-143 in the circulation of well-treated HCVR-patients, indicating miRNA involvement in subclinical atherosclerosis and premature CVD.

In view of main results presented in this thesis, from the increased levels of cMPs in hyperlipidemia to the effects of cMPs on thrombus formation and their potential prothrombotic components, the inhibition of cMP release may have significant implications both in plaque identification and inhibition of plaque progression. High plasma cholesterol levels are a causal factor for atherothrombosis and CVD. Effective treatment of hyperlipidemia is of

great importance in the overall management of vascular risk and prevention of cardiovascular disease. Large well-controlled clinical trials have demonstrated that statins are effective in primary and secondary prevention of CVD.[147] In primary prevention results of clinical trials have shown less clear beneficial effects but yet reductions in CAD are evident (WOSCOP,[684] AFCAP/TexCAP,[685] ASCOT_LLA[686]) and even mortality was reduced in the JUPITER trial.[632] However, the benefit on all-cause mortality has not been proved in a recent meta-analysis.[687] For most hypercholesterolemic patients unable to achieve recommended lipid level goals with therapeutic lifestyle changes, statins are considered first option for treatment. Statins are hypolipidemic drugs, which not only decrease plasma cholesterol levels but also exert beneficial effects in CVD prevention due to their pleiotropic effects. However, there is very limited information about the effect of LLT with statins on cMPs. This thesis demonstrates that the blood of patients treated with lipid-lowering therapy with statins have lower microparticle numbers, especially of platelet, leukocyte and endothelial cell-derived cMPs, than the blood from untreated patients with the same plasma lipid levels (*Paper 2*). This study was designed to evidence effects of statin use in a population of primary prevention patients with a median of LDL-cholesterol in target levels (<130 mg/dL). Interestingly, blood from statin-treated patients had cMPs with reduced markers of cell activation. Markers from activated-platelets, inflammatory cells and endothelial cells were lower than in untreated patient's blood. These results indicate a direct effect of statin in cell activation and membrane homeostasis.

As already discussed above, circulating MPs and, specifically pMPs, play an important role in mural thrombosis and also in coagulation (*Paper 1*). Thus, it is conceivable that the effects detected may have implications in the protection against atherosclerosis exerted by statins. It has been described that statins could improve plasma adiponectin levels, a circulating adipokine that suppresses the attachment of monocytes to ECs and stimulates NO production in vascular ECs improving endothelial function.[534,688] Interestingly, low numbers of cell-activation markers, such as $\alpha_{IIb}\beta_3$-integrin, P-selectin, α_M-integrin and tissue factor were detected in cMPs of the statin-treated patients suggesting that statins acting on various multiple cellular targets may exhibit anti-inflammatory and anti-thrombotic actions. These results are in accordance to a recent report highlighting the broader benefit of statins decreasing inflammation and preventing MP release, an effect not observed with ezetimibe alone.[689]

Hypercholesterolemic patients were treated with four commonly used statins at low dose (*Paper 2*), which were shown to be equally effective in the modulation of cMP shedding in the asymptomatic hypercholesterolemic patients. Contrarily, patients treated with statins had a better cMP profile depending on the time on-treatment, suggesting that chronic use of statins

helps to reduce the vascular dysfunction burden in hyperlipidemia. Therefore, the duration of the treatment more than the type of statin seems to influence these effects. Within asymptomatic hypercholesterolemic patients, cMP levels were significantly correlated with their cardiovascular risk. Some specific cMPs were related to Framingham CV risk score while the majority of cMPs correlated with REGICOR (Registre Gironí del Cor) risk score, which is used to indentify HCVR in the Spanish population.

Overall these results showed that statins significantly reduce the shedding of blood cells and vascular cell MPs. The specific reduction of cMPs derived from activated parental cells suggests how statins can affect evolution of disease. The lower cMP shedding may ameliorate the vascular and inflammatory effects associated to the progression of atherothrombotic disease in asymptomatic patients contributing to statin protective effects. Benefits of statins operating at this level may explain their proved beneficial effects seen in patients with low cholesterol levels. In summary, effective lipid-lowering treatment with statins may prevent the development of premature CVD by reducing vascular and inflammatory cell activation as detected by a reduced cMP shedding of the cell membranes. Further studies measuring changes in MP number and phenotype before and after LLT will help to complete our understanding of statin effects on the vessel wall.

This and other studies of the present thesis (*Papers 2*-6) have some common issues that need to be commented. First, the number of patients included in these studies may be considered a limitation but it is suitable for this type of analysis (discovery and proof-of-principle studies) on cMPs. These are cross-sectional studies from an observational (non-randomized) prospective clinical study and some bias related to the indication of treatment may be taken into account. However, in the case of FH patients all cases receiving LLT have been treated at least one year before the inclusion in the study with the same LLT. Besides, these patients are studied following a full genetic characterization.

The present thesis demonstrated that cMPs actively contribute to atherosclerosis progression and complication by enhancing thrombus formation and propagation. Although existing cardiovascular risk algorithms such as Framingham risk score provide reliable information of atherothrombotic risk, identification of novel valuable biomarkers signalling for subclinical atherosclerosis may facilitate the identification of patients at high cardiovascular risk. In summary, the results of the present thesis outlined that cMPs not only support a contributing role for MPs in thrombosis but also support their potential use as proinflammatory and prothrombotic biomarkers of cardiovascular disease.

VI. CONCLUSIONS

The main conclusions of the work presented in this thesis are:

1) **Circulating microparticle (cMP) release from different vascular resident, inflammatory and prothrombotic cells is directly associated to the increased atherosclerosis burden in patients with high cardiovascular risk and familial hypercholesterolemia (FH).**
   - FH patients have higher number of overall cMPs and of those derived from endothelial cells, monocytes and lymphocytes than non-FH hypercholesterolemic patients.
   - Circulating $CD45^{+}/CD3^{+}$-lymphocyte microparticles are biomarkers of asymptomatic subclinical lipid-rich atherosclerotic plaques in FH.
   - High cardiovascular risk FH patients have higher numbers of cMPs derived from activated platelets as well as tissue factor-rich MPs than controls.
   - Prothrombotic cMPs (tissue factor-positive and platelet-derived) correlate with atherosclerotic plaque burden, adding prognostic value for the prediction of lipid-rich atherosclerotic plaques.

2) **Microparticles derived from vascular and circulating cells depict changes in their profile under characterized flow and substrate conditions, contributing to the pathophysiology of ST elevation myocardial infarction (STEMI). Thus, circulating MPs may be sensitive systemic markers of the thrombo-occlusive vascular process developing in the coronary arteries of STEMI-patients.**

   - High shear rates and thrombogenic substrates (vascular wall and isolated collagen) increase the number of released cMPs and modulate their specific phenotype.
   - Conditions that mimic stenotic coronary blood flow on damaged vascular wall increase circulating erythrocyte-derived microparticles (ErMPs), while activated pMPs are retained into the growing thrombi. Plasma levels of ErMPs are increased after an acute coronary syndrome, indicating that circulating ErMPs could be markers of ongoing mural thrombosis.
   - cMP cell origin and activation status display a significantly different profile in STEMI-patients compared to controls within the first 72 hours after the ischemic event.
   - cMPs from endothelium, monocytes, activated platelets and carrying tissue factor are in higher proportion in the culprit coronary artery blood than in peripheral blood of STEMI-patients, especially in the shorter thrombotic occlusion times, revealing their sensitivity to detect the thrombotic occlusion homeostatic effect.

3) **Blood microparticles, even in blood from healthy donors, induce the thrombogenic potential and have functional effects on cardiovascular atherothrombotic disease beyond being mere biomarkers of cell activation.**
   - Increased numbers of cMPs in human blood, and more specifically, platelet-derived microparticles (pMPs) enhance platelet adhesion and thrombus formation on vessel wall with vascular injury.
   - High human pMPs content in blood enhance platelet deposition and thrombus formation on human complex atherosclerotic lesions.
   - pMPs show an enhancing affect on platelet function and aggregation as well as coagulation.

4) **Microvesicle composition, in terms of both protein and microRNA, reflects cell activation and enables MPs to serve as messengers and cellular crosstalk effectors in the systemic circulation.**
   - Thrombin-activated platelets release MPs with a highly complex procoagulant proteome directly involved in atherothrombosis.
   - MP-associated miRNA-143 content is decreased in hypercholesterolemic patients with atherosclerotic plaque burden.
   - Circulating exosomal microRNA signature predicts ischemic event presentation in high-risk hypercholesterolemic patients.

5) **Beyond cholesterol lowering, statins protect against vascular cell activation with direct effects on reducing activated cell membrane shedding of cMPs.**
   - At equal LDL-cholesterol levels, patients treated with statins have less overall cMPs and less cMPs carrying markers of parental cell activation, specifically of platelet, inflammatory (lymphocyte and monocyte), and endothelial cell origin.
   - The effect of statins on cMP shedding is increased with years of treatment.

The main contribution of this thesis can be summarized as follows:

Blood contained microparticles, released from blood and vascular cells, are a rich source of information of the cardiovascular compartment. First, they show a role as active functional messengers of cell-cell activation; second, they are potential prognostic biomarkers of silent atherothrombotic disease measuring the cellular temporal-spatial activation; third, they may be instrumental in helping to improve prediction of cardiovascular risk in intermediate-risk patients; and fourth, MP can be considered vehicles with a possible use for biological target interference.

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