ESTUDIOS
DE LA FUNDACIÓN
SERIE TESIS

ROLE OF MICROPARTICLES IN ATHEROTHROMBOSIS

Rosa Suades Soler

funcas

ESTUDIOS
DE LA FUNDACIÓN

SERIE TESIS

ESTUDIOS
DE LA FUNDACIÓN

SERIE TESIS

ROLE OF MICROPARTICLES IN ATHEROTHROMBOSIS

Rosa Suades Soler

funcas

Impreso en España
Edita: Funcas
Caballero de Gracia, 28, 28013 - Madrid

ISBN: 978-84-15722-57-1
ISBN: 978-84-15722-58-8
Depósito legal: M-39873-2016
Maquetación: Funcas
Imprime: Cecabank

Esta tesis doctoral ha sido distinguida con el
PREMIO ENRIQUE FUENTES QUINTANA A TESIS DOCTORALES,
CATEGORÍA CIENCIAS DE LA SALUD,
en la convocatoria 2014-2015

Tesis doctoral presentada en la
Universitat Pompeu Fabra
Departamento de Ciencias Experimentales y de la Salud

Directora de la tesis:
Lina Badimon Maestro

Codirectora de la tesis:
Teresa Padró Capmany

Tutor de la tesis:
Josep-Eladi Baños Díez

A la memòria de l'avi Candi

A la meva família

I ara que tot s'acaba i tot comença,
fem del passat i del present el repte
per un futur esperançat i càlid.
Que cap mirall fal•laç no ens enlluerni.
Tindrem només allò que cada dia
conquerim amb esforç, i tanmateix,
aquest àmbit de llum ha de bastar-nos
per creure cada cop més en nosaltres
si no oblidem que amb el batec del viure
tot s'acaba i, alhora, tot comença.

Miquel Martí i Pol

ACKNOWLEDGMENTS

I love what I do in order to improve people's health and quality of life. Passion is what will change the world and enable us to live longer and better. It is not an easy job but magical moments, moments that are priceless and make sense, allow me to love and believe in the advancement of science. I feel very lucky to have found people who have accompanied and helped me in this journey, I therefore dedicate you a few words of sincere gratitude.

To Prof. Lina Badimon, an exceptional, brilliant and, at the same time, close director, the opportunity of being trained by you. It has been a pleasure to learn from someone with a huge scientific and creative talent, visionary, innovative, with an extraordinary intelligence and tenacity and a great sensitivity. Thank you Lina for being my mentor, for your constant guide, to encourage me to learn and improve every day, communicate and advance, to tackle together setbacks and to help me to grow and mature both personally and professionally.

To Dr. Teresa Padró, because you have always known how to give me optimism and enthusiasm during this journey. With you I have learnt to deal with complicated situations, find options to address specific problems and to be persistent. Thank you Teresa for helping me to look for talent, being willing to help me, working as a team and, ultimately, for your encouragement, will, smile and joy.

Dr. Josep-Eladi Baños, my supervisor at University Pompeu Fabra (UPF), to be an inspiring source, to transmit your enthusiasm for medicinal plants, to show us novel methods of learning and teaching, to give me wise advice and help me with professionalism, naturalness and simplicity.

To all the colleagues from ICCC since living together every day is sharing many small moments that ultimately are the ones that remain.

To Dr. Gemma Vilahur, an inspiring figure with a great intellectual capacity and mental endurance. Thanks for being a scientific model, for your support during these years, for teaching me to laugh at oneself and the value of respect, and for being always ready to cooperate.

To Nia, to whom I acquired knowledge of platelets and proteomics, as well as the basis of laboratory work. You have always been a guide, especially in the beginning. Thank you for teaching me a lot, for your priceless advice, for giving me energy and for being so special.

Thank you very much to the 004 group because next to you I have grown, matured and learned to live the experience to the fullest, being constantly aware of how lucky I was living a real dream. Ilaria, your example has taught me to express my best, to not demand anything to anyone and to surround myself with the right people. Thanks for the Italian lessons and sharing stories of your annual travel. Judit, it has been a pleasure to be with you. From the beginning you have kept an eye on me and I appreciate it. With you I have learned above all life lessons. Thanks to your concern and constant monitoring, I discovered myself and I felt strong, important and valuable. Among many experiences, I remember the shared moments of innocence, laughter, enthusiasm and generosity. I really appreciate you, you're unique, I give you encouragement and forth with everything! Lolita, despite being a year older than me, you will always be younger. Thank you for livening up the laboratory, sharing laughter and pranks, being the 'boss' of the lab, asking for presents every time we travel, and specially because together we are unstoppable! Do not be swayed by anyone and be yourself. Carmen many thanks for your personality and coherence, to pass child spirit on the group and keep us constantly updated on last news.

To Ma. José and Silvia, for their key help in abstracts, manuscripts, meetings and endless procedures that you dominate like nobody else does and you solve them with an innate determination. At the same time, you have always been there so thoughtful, with tips, smiles and what was required. Joaquim and Pepi, thanks for your valuable help in management that has also been essential. I would also appreciate the professionalism and sincere help of the entire administration team: Núria, Meritxell, Maria and Montse. Thank you for your flawless and perfect work that makes everyday life easier. Finally, the positive attitude of Vicenç not only in informatics matters but also in life. Thanks for sharing with me your progression as a pilot and your flight moments!

I would like to thank Anna and Diego, and to my neighbours Esther Gregoris and Dr. Bayés from the Foundation. Doctor thanks for the annual calendar from Pilarín (a childhood idol!), for your smile every morning and, in short, for the valuable example: an eminence who every day show us that the best preparation for tomorrow is doing your best today, you have to exert always yourself, and the importance of keeping an active mind and a constant enthusiasm for what you do.

At ICCC I could share moments with extraordinary people. In particular, I would like to emphasize the scientific integrity of Esther P. and Gemma A., the common sense and determination of Maribel, the dynamism of Maria B., the authenticity of Rosa A., the truth and commitment of Laura C., the serenity and easy going of Rachel F., the vision, tenacity and enthusiasm of Sandra (thank you for your willingness to help), the dose of humour of Javi (required to face the challenge of MPs, thank you!) and the understanding and confidence of Oriol. Thanks Oriol for your lessons of histopathology, to help me in morphometric evaluation (four months of full-time microscope cannot remain you indifferent!), for shared advice and conversations. Thanks Maria A., Lourdes and Gemma Ch. for providing fresh air to the centre and to have the opportunity to learn and upgrade. To Maisa and Anna N.

for the musical environment in the laboratory and for their 'MS', always cooperating with those proteomics aspects you control.

I have been fortunate to share moments inside and outside the center with Carolina, Maite, Rafael, Raquel T. and Sònia E. Thanks Carolina for your serenity, Maite for your diplomacy and curiosity, Rafael for your unconditional help with miRNA software and for all prolific dissertations about miRNAs, Raquel to always make us laugh, Sònia for your simplicity and being so nice and to all for being a bright thesis colleagues!

The support in laboratory skills at specific moments has become key and vital. Thank you Ma. Àngels for both the immersion to histology techniques that was very rewarding and your attentive and well-mannered behavior. Thank you very much Montse G. and Olaya for your excellent help. Thanks Ona for getting involved in perfusion cameras work although being brief and Mònica P. for your help in blood sample extraction. Thanks Pablo and Mari as it is always a relief to know that you are animal facility. Likewise also thank to the other fellows of animal facility because you have always support me: Angel, Fani (for your kindness), Javier (for your talks about trips), Josep (for your willingness, you worth much), Pablo G., Sergi L. and Sergi F.

Many thanks to all the members of other research groups as it has been a real pleasure to share conversations, events and knowledge with Leif, Cristina E., Adela, Pepe, Cristina R., Judith, Mar, Ingrid, Beatriz, Saray, María G., Vicenta, Alberto, David, and Elena.

To the rest of colleagues who have offered me the essential environment: Sonia H. thanks for the moments at the FHF trip (a long walk to reach the Tower of Hercules!); Esther Gerbolés for your vitality and good mood; Laura N. and Silvia A. for your expertise; Monica A. for your smile, closeness, and solidarity; Quim G. for your common sense; Laura B. for your early greetings and perfect material control; Filo, Carmen and Maria for accompanying each other during the afternoon, and Mònica B. and Roger for transmitting calm.

I am also grateful to those who are not longer at ICCC but left me a mark: Montse Saurí for your smile and expression; Roberta for your time and help; Sonia V. for your sympathy and spontaneity; Chema good luck in your new stage; Blanca O. for the master, the first years of the thesis and for Nice!; Rodrigo for the scientific talks about TF; Javier F. for involving me in your own fight against HPLC and for the jokes with the tips; Marta V .; Norma for your discretion; Ricardo for being so nice; Roi C.; Marta S.; Patricia A.; Álvaro; Blanca M.; John Mc Gregor; Mark S.; Emil; Rosa; Pilar; Àngel; Jaume; Patricia; Anna C.; Maurizio; Lluís; Judith S.; Marta T.; Carlota; José; Anna G.; Xevi; Anna L.; Joaquín L.; David I.; María G.; María C.; Pablo F.; Olivier; Paula; Valentina; Elena Mendoza; Elena Martínez; Juanjo; Raul; Marga; Mònica T.; Eli; Juli; Jerónimo; Erick, among others. Sergi M. thank you for teaching me humility. Collaborating with you allowed me to meet and open my mind to new perspectives. I hope everything goes on well in Boston. To Albert M., Maria L.,

Jaione, Marta G., Laura S. and Nan X. for the opportunity not only for surprising me but also to do diagrams, transmit, supervise and guide. I have greatly enriched with you and your youth and innocence reminded me the hope of first years.

To all the medical doctors to whom we have had the pleasure of working together, especially Dr. Pedro Mata and Dr. Rodrigo Alonso, and also to all patients, nurses and FHF members for their collaboration. To the members of the Clinical Chemistry Lab of Amsterdam Medical Centre, an amazing group of smart, efficient and friendly people. To Dr. Rienk Nieuwland, Chi M Hau, and Anita Boing for your help and your inputs. The project could never have gone ahead without your support. To Sergi and to the rest of Blood and Tissue Bank from Hospital de la Santa Creu i Sant Pau (HSCSP) for the provided help, kindness and facilities. To Teresa P. and Ignasi G. for statistical enquiries, to Esther A. and Gemma C. from BD Applications for their help with BD FACS Canto II, to Elisabet L. from Palex and Montse B. from HSCSP for their help in the use of automated DXS system, to Natalia R., Miriam C., Aitor C. and Ramon M. from UPF for all the resolved questions and doubts. To the CIBERObn and PFIS-ISCIII fellowships as well as Jesus Serra foundation and ICCC for the economical support.

To the whole research group in Thrombosis and Hemostasis from Hospital La Fe (Valencia): Mª Teresa, Juana, Amparo, Mª Ángeles, Paco, Toni, Aitana, Virtudes, Mercedes, Pilar, Elena, Laura, Silvia, Josep, Sara, etc. because it is always a great pleasure to meet you, for making me feel another member of the group in the MLTD congress and for your complicity, support and trust.

I would also like to thank those who have helped with my previous training. To my degree supervisor Núria C., for your exceptional guidance and advice. To the team of Pharmacology at Almirall with whom I began in cardiovascular research during the final internship: Amadeu G. Jesús Ll., Jordi G., Marisa V., Josep LL., José M., Luisa, Clara A., among others. To Dr. Mike Polkey thank you for giving me the opportunity to join your research group at the Royal Brompton Hospital as an undergraduate and introducing me to respiratory medicine research. Also thanks to Amanda S. N., Ed C. and Alastair M. for your support. To Dr. Rafael M. and Dr. Olga V. who gave me the opportunity to do an internship at the Neuropharmacology Laboratory and learn from Andrés O. and Ester A. Also thanks to Fina F., Glòria G., Glòria B. and Ma. Dolors E. from Althaia laboratories in Manresa, and to Kataryzna S. from University of Bydgosczc. To the EDN teachers who provide me a complete learning with a critical point of view and interest in science. To Carme B. from Berga through to whom I enjoy and learn English.

Moreover, doctoral studies period has also been marked by other curiosities, though not devote too much time, helped me a lot. Thanks to Mercè S. for awaking me the knowledge and awareness through yoga. To Jordi V. from Social Work of HSCSP and Carme L. , Ferran P. and all the volunteers from ALICIA because I feel very lucky to participate in their initiative.

I had the good fortune that family and friends gave me strength during this journey and without them, I would never get where I am. The fact of having persons who really loves you makes it easier to fight for truth and find your own way.

To my close friends together with all the group from Navàs (David P., David M., Gemma Sabata, Gemma Vilalta, Gisela, Helen, Jeroen, Jordi, Jordina, Laia R., Lluís, Pep, Quar, Mònica, Nere i Silvia i els petitons Aina, Climent i Marc) because we have lived many things together and you are very important to me. Gemma S. we have always been together since we were a child and you have been at my side, Gemma V. despite living in Belgium you are always present, small Silvia thank you for your trust and contagious vitality that gives strength, David M. we still have more dreams to fulfill, Mònica I am lucky to have you, talk about everything, you bring positivism and you are worth (as you usually say: laugh and live and never give up!), Laia thanks for your support and constant peace, Nere we have shared many things, (Roxette, boys scouts, courses, including a doctorate), thanks to understand me as nobody else and do not let the eagle of Patum stop turning! Gis, thank you for being so great as a person and a friend.

To the group from Vic: Àdam, Albert P., Aloma, Bego, Eli, Enric, Fatsi, Idoia, Joan B., Jordi 'Alsi', Jornet, Maria, Marin, Mimi, Núria, Plade, Pol, Quim, Roger T., Sabèlia, Sanji, Sara, Uri, Txell, Vila, Vivó i Vilella for the new years' eve, 'Castanyades', summer solstices and Saint John parties, FC Barcelona football matches, vacations, family dinners in Alps, Puigmal and several meetings, which are always a dose of power that have helped to disconnect and oxygenate the mind. To Albert A., Jaume A., Joan V., Laia Ll., Magda, Marta, Mireia i Roger A. for carnivals, 'calçotades', Fussimanya and Stromboli volcano, without you it would not be the same! To the degree and master mates, namely Ma . Teresa, Helena A., Anna L., and Susanna M. for the years of world discovering.

Thank you all for joining me, you are a very important support, thanks for the lived moments, endless conversations after meals, for your understanding during all this process and to impregnate me with adventurous spirit and desire to achieve my goals.

Finally, to my whole family for supporting me unconditionally in all the aspects of my life and give me the soundest possible platform for my personal growth. In particular, to my uncles and cousins for your constant interest and helping me to be natural and authentic. Thanks Miquel, Carme, Maria and Jordi C. for giving me the opportunity of knowing you better, for taking care of me and for giving me the courage and strength to pursue my dreams.

To my aunt Ignasieta for great summers in Berga I will always remember, to give me every day her hope despite the circumstances, for being like an older sister to my mother and, especially, for laughs and fantastic moments that we usually share when we are all together.

Also a memory for the godfathers who left too early and for my grandfather Candi, who taught me everything and more. You left because your heart suddenly

stopped, and since that time this work unconsciously began to take shape. To my grandmother Ines for always keeping me present, missing me and loving and wishing me the best.

To Joan for behaving as both an older brother and best friend and being an example to me, for protecting, hearing, helping and advising me over the years, and for being always at my side.

To my parents for being as you are, for the strength and courage you radiate, for taking care and understanding me, and for all the education, help and love you offered me. Heartfelt thanks for teaching me to be authentic and honest. And thanks also for conveying the language of enthusiasm, of things made with love, kindness and desire, looking for what I want or I believe, and for always encouraging me to live and choose what makes me happy.

I cannot finish without expressing my gratitude and deepest appreciation to my lovely sweet partner and best friend, dearest Jordi, for your constant and selflessly support, encouragement, calm and love and for always striving me to be a better person. I keenly look forward to all adventures that await us together.

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.

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.

LIST OF FIGURES

LIST OF TABLES

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 A2
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

1 INTRODUCTION

1.1. CARDIOVASCULAR DISEASE

1.1.1.Background

Cardiovascular disease (CVD) is the leading cause of death worldwide and, consequently, a global health problem (Alwan, 2010). 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 (Mathers and Loncar, 2006) with a significant negative impact on socioeconomics and quality of life in our society (Folsom *et al.,* 2011) Coronary artery disease (CAD) and stroke constitute the two leading causes of death in the world (Lozano *et al.,* 2011). In Spain, CVD is also the main cause of death accounting for 29% of all-cause mortality, being atherosclerosis its major cause (Regidor and Gutiérrez-Fisac, 2013).

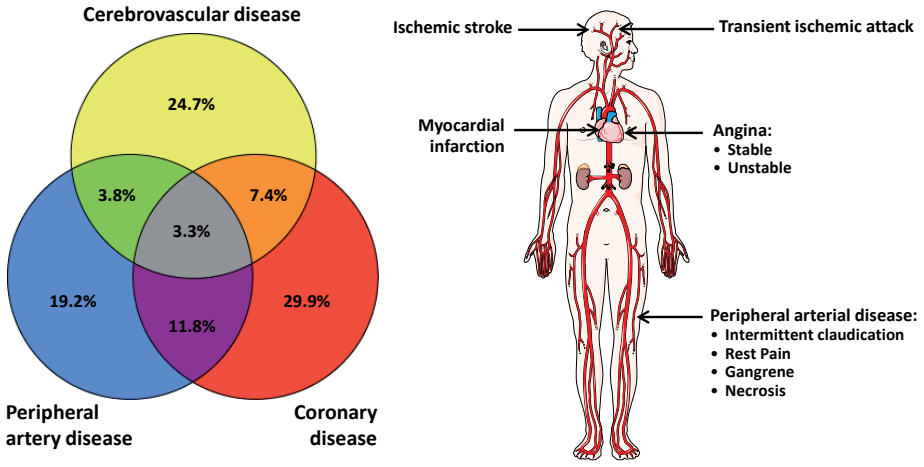
CVD, a generalised and diffused pathology, refers to all the diseases of the heart and the circulatory system. Depending on the affected area, CVD will have distinct clinical manifestations, including: coronary heart disease (acute coronary syndromes [ACS] or aortic stenosis), cerebrovascular disease (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 (Santos-Gallego, Picatoste and Badimon, 2014). Atherothrombosis underlies the majority of CV events independently of the specific vascular bed in which they occur (Chapman, 2007). 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) (Coccheri, 1998).

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) (Muller *et al.,* 2003). Indeed, high concentrations of circulating cMPs have been reported in patients with atherosclerosis, acute vascular syndromes and diabetes mellitus (Bernal-Mizrachi *et al.,* 2004; Nomura, Suzuki, *et al.,* 1995; Nomura, Imamura *et al.,* 2000), 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.

Figure 1

CARDIOVASCULAR DISEASE PRESENTATION



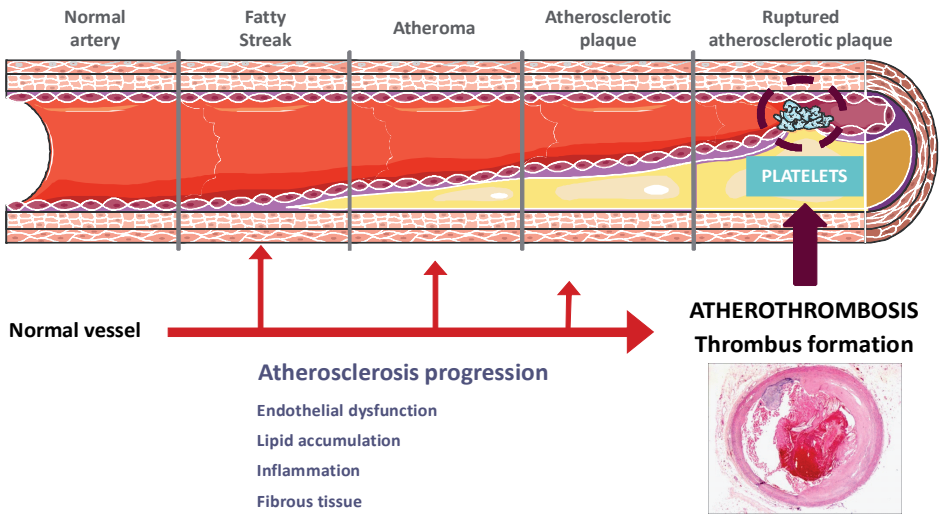
Note: Percentage of atherothrombotic events depending on the vascular territories affected.
Source: Data obtained from CAPRIE trial (Coccheri, 1998).

1.1.2. 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



Note: Atherothrombosis chronically develop from early fatty streak to atheromatous plaque formation that by unpredictable disruption leads to platelet activation and thrombus formation.
Sources: Pepine (1998); Ross (1999); Libby, Ridker and Maseri (2002).

1.1.2.1. 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):

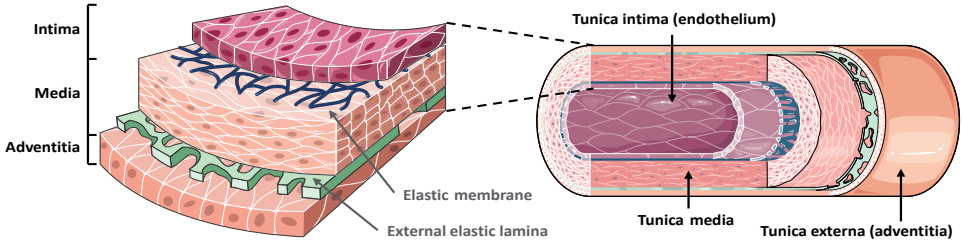
- *Intima,* the innermost layer, is in contact with the flowing blood. The intima layer is composed by a monolayer of endothelial cells (EC), a very thin basal lamina and a subendothelial layer formed by collagen and elastic fibrils.

- *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



Note: The artery wall consists of three concentric layers, called tunica intima, media and adventitia, separated by elastic membranes.
Source: Own elaboration.

1.1.2.2. 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 (Berenson, 1998). 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).

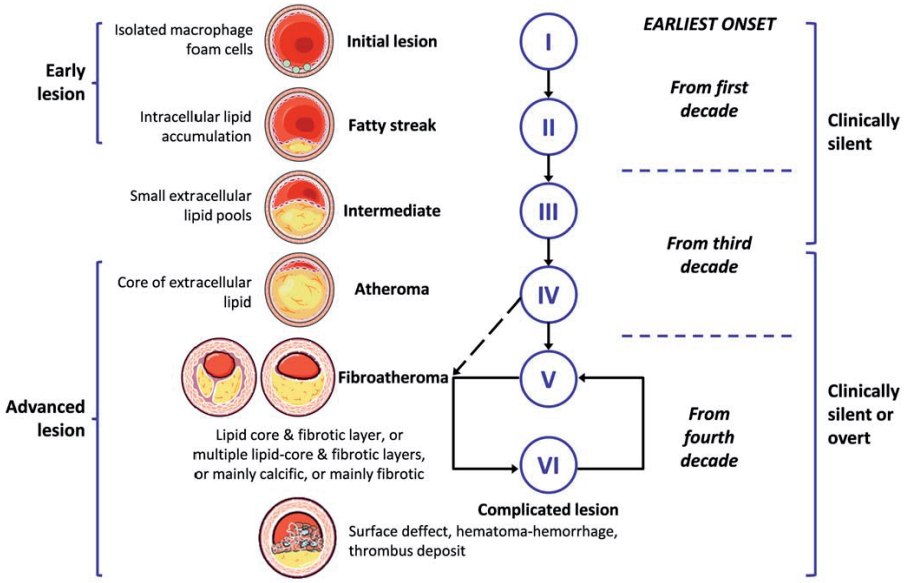
The complex interaction between disease processes (explained below) in the development of atherothrombosis has been a matter of debate. A well-established histological classification of atherosclerotic lesions was provided by the American Heart Association (AHA), which relates morphologic characteristics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

A simple modification of this classification providing a link to clinical findings has emerged (Virmani, 2000) 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.

Figure 4

AHA ATHEROSCLEROTIC LESION CLASSIFICATION



Source: Adapted from Stary *et al.* (1995).

- 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.

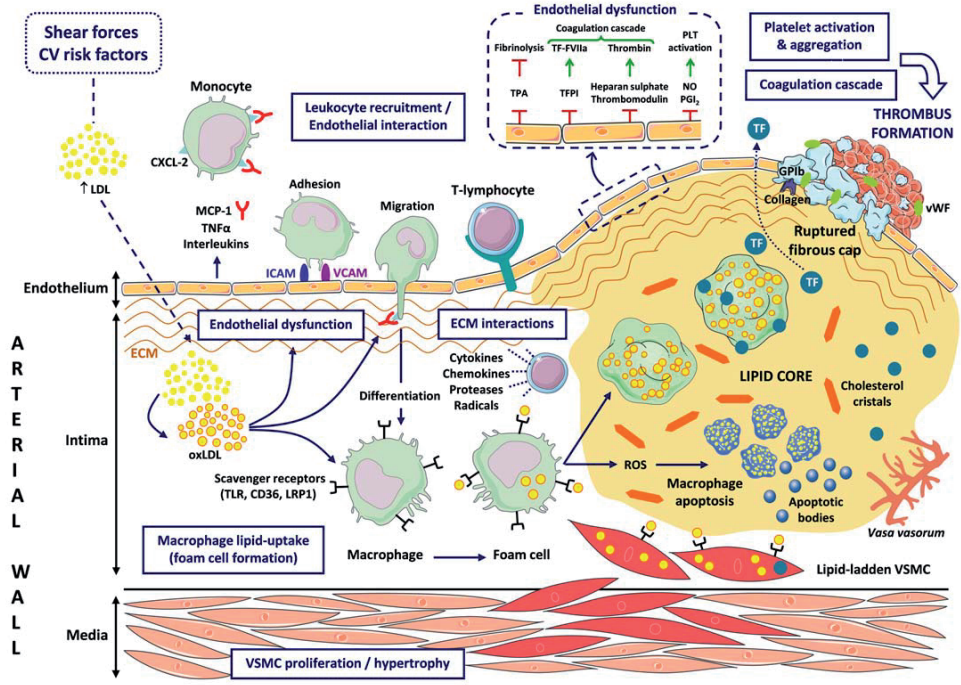
Advanced lesion types (fibroatheromas and fibrocalcific plaques) may evolve simultaneously and interrelated and their distinction is difficult. Nevertheless, 'complicated' lesions may lead to the formation of either a mural thrombus causing angina or an occlusive thrombus causing an ACS (unstable angina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

1.1.2.2.1. 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

Figure 5

PATHOPHYSIOLOGY OF ATHEROTHROMBOSIS



Notes: 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.
Source: Own elaboration.

prostacyclin to the proaggregant and vasoconstrictor thromboxane), with three major consequences (Figure 5):

- 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.
- 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.
- 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 (Tabas, Williams and Boren, 2007), 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 (Mause *et al.,* 2005).

1.1.2.2.2. 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 (Ong, 2011).

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 (Santos-Gallego, Picatoste and Badimon, 2014). Indeed, pMPs have shown to induce leukocyte aggregation and recruitment via P-selectin/PSGL-1-dependent interactions (Forlow, McEver and Nollert, 2000). 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 (Cristell *et al.,* 2011; Ferrante *et al.,* 2010) cMPs also possess procoagulant properties that lead to thrombin generation (Morel, *et al.,* 2006). 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 (Sambola *et al.,* 2003) 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 (Lopez-Vilchez *et al.,* 2012). Increased TF-positive procoagulant MPs are present in the circulating blood of patients under pathophysiologic conditions (Hugel *et al.,* 1999); 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 (Aras, 2004).

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 (Virmani *et al.,* 2000 and 2005). 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 (Falk *et al.,* 2005) and appears to be a common cause of asymptomatic progression to severe stenosis (Burke *et al.,* 2001). Among ruptured plaques, those exposing high contents of collagen with small amounts of TF, provide the most procoagulant and, thus, occlusive combination (Lopez-Vilchez, Tonda *et al.,* 2009).

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 (Farb *et al.,* 1996). 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 (Sugiyama *et al.,* 2004).

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 (Virmani *et al.,* 2000). 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 (Xu *et al.,* 2012).

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 (Madhavan *et al.,* 2014). Quantification of coronary artery calcification (CAC) has been widely proposed as a marker of CAD (Peters *et al.,* 2012; Polonsky *et al.,* 2010). Despite this, its utility is controversial since evidence suggests that it may be protective against development of ACS (Nicoll and Henein, 2013). 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 (Motoyama *et al.,* 2007; Ehara *et al.,* 2004). Indeed, heavily calcified plaque is significantly less likely to develop a thrombus than

uncalcified or mixed plaque (Beckman *et al.,* 2001). 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 (Mauriello *et al.,* 2013).

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.* (2013) 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 (Chung and Lip, 2003) (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.

1.1.2.3. Main participant cells

1.1.2.3.1. 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 (Bombeli, Schwartz and Harlan, 1998), which further induce atherosclerosis development.

cMPs derived from ECs (eMPs) have been related to vascular proinflammatory activity and thromboembolic complications (Diehl *et al.,* 2011). Interestingly, eMPs

are found increased in ACS (Mallat *et al.,* 2000) CAD (Werner *et al.,* 2006), severe hypertension (Preston *et al.,* 2003) end-stage renal failure (Amabile *et al.,* 2005), and pulmonary hypertension (Amabile *et al.,* 2008; Esposito *et al.,* 2006) eMPs seem to be markers of inflammation (Werner *et al.,* 2006), endothelial injury (Amabile *et al.,* 2005) and endothelial function (Esposito *et al.,* 2006).

1.1.2.3.2. 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 (Ardoin, Shanahan and Pisetsky, 2007). Indeed, atherosclerotic plaques contain LMPs (Leroyer *et al.,* 2007; Mallat *et al.,* 1999). As such LMPs have been associated to cytokine release (Mesri, Altieri, 1998 and 1999), expression of EC adhesion molecules and functional TF (Mesri, Altieri, 1999), PS exposure, and monocyte adhesiveness (Barry *et al.,* 1997) leading to proinflammatory and procoagulant activity.

– *Innate immunity* –

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 (Rajavashisth *et al.,* 1998). Moreover, monocytes are proinflammatory by releasing myeloperoxidase (Gordon and Taylor, 2005) and also expressing other proinflammatory molecules (tumour necrosis factor [TNF] α, matrix metalloproteinases [MMPs], transforming growth factor [TGF] β, interleukins [ILs], cathepsins) (Ferrante *et al.,* 2010). 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 (Rogacev *et al.,* 2012).

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 (Purushothaman *et al.,* 2012).

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 (Drechsler *et al.,* 2010; Sun *et al.,* 2007; Ionita *et al.,* 2010). Neutrophil-derived MPs have recently been shown to induce myeloperoxidase-mediated damage of vascular ECs (Pitanga *et al.,* 2014).

– 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 (Zhou *et al.,* 2000). Similarly, lymphocyte-derived MPs (ℓMPs) were shown to increase the production of TNFα and IL-1β (Scanu *et al.,* 2008).

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 (Caligiuri *et al.,* 2002).

1.1.2.3.3. Platelets

Platelets are key players in the pathogenesis of atherothrombotic processes (Fuster *et al.,* 1998). 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 (Huo and Ley, 2004) 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 (Huo and Ley, 2004). 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 (Huo and Ley, 2004).

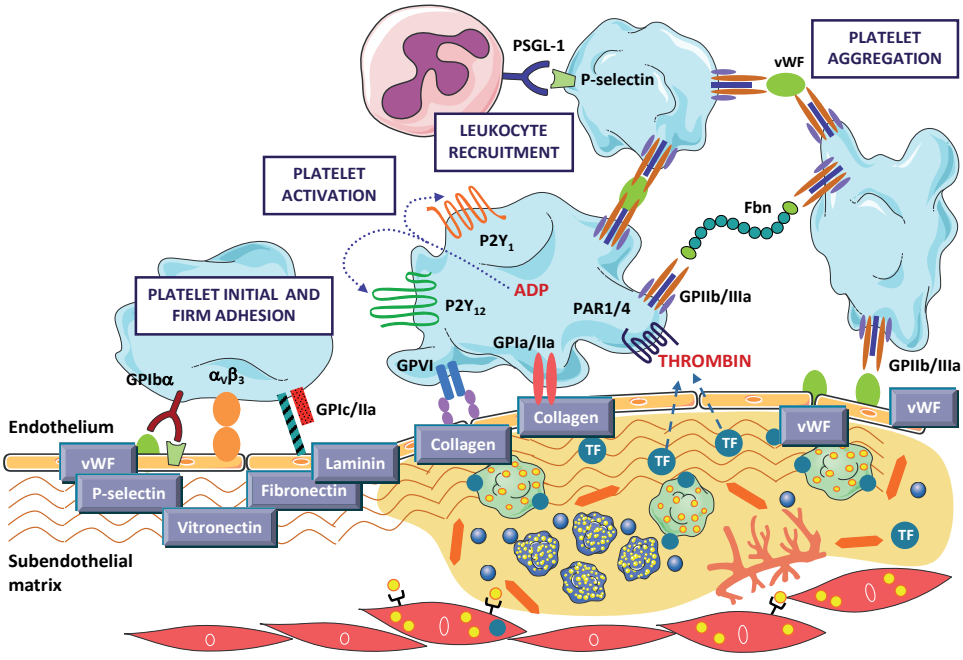
In the early stages of atherosclerosis, platelet *rolling* in the activated endothelium is primarily mediated by P-selectin and followed by firm adhesion through integrin binding (Massberg *et al.,* 2004). Platelet adhesion to intact but dysfunctional or activated EC layer may also be initiated by interaction of GPIbα and $\alpha_{IIb}\beta_3$-integrin with endothelial P-selectin and vWF (Massberg *et al.,* 2004). 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 (Jennings, 2009). In fact, platelet-related secretory effectors mediate the leukocyte-endothelium interactions (Libby, 2010). 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,

Figure 6

PLATELET-VESSEL WALL ADHESIVE INTERACTIONS AT SITES OF VASCULAR INJURY

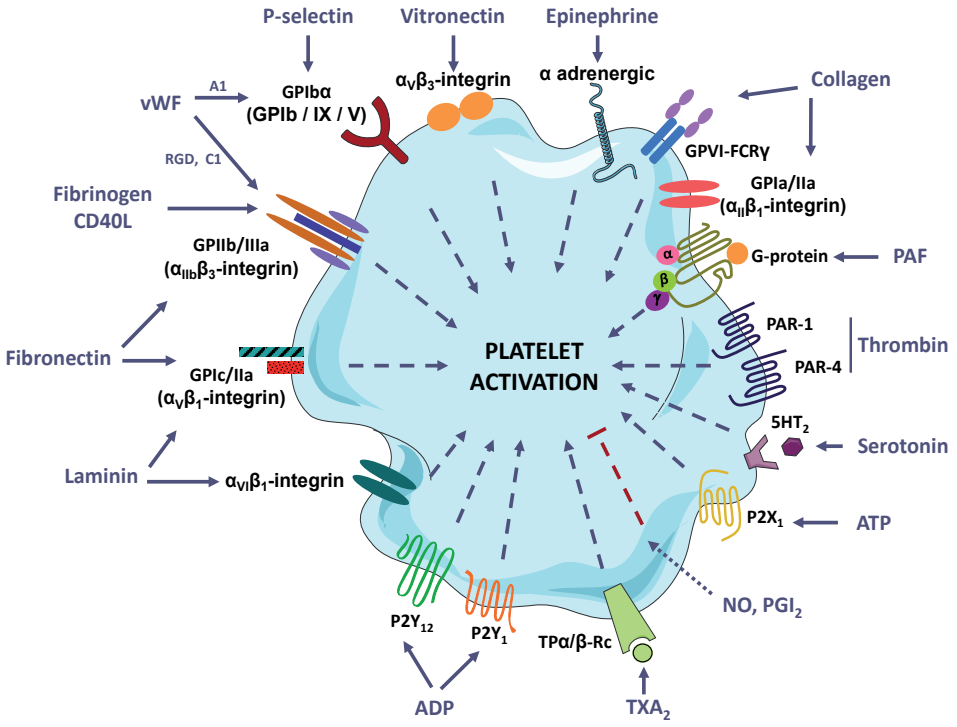


Note: Key platelet-vessel mechanisms indispensable for platelets to adhere, activate, form stable aggregates with other activated platelets, and promote thrombus formation.
Source: Adapted from (Badimon, Storey and Vilahur, 2011).

leads to the stabilisation of the platelet aggregates (Figure 6) (Badimon and Badimon, 1989). In advanced disrupted plaques, platelet adhesion varies according to the shear rate (Badimon, Storey and Vilahur, 2011). 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

Figure 7

MAJOR AGONISTS AND RECEPTORS INVOLVED IN PLATELET FUNCTION



Notes: 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.
Source: Own elaboration.

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 (Badimon, Storey and Vilahur, 2011). 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 (Stenberg *et al.,* 1985).

Besides collagen, circulating agonists such as epinephrine, serotonin, ADP and thrombin also activate platelets via specific surface receptors (Figure 7) (Badimon *et. al.,* 2002). For instance, serotonin enhances interaction of platelets with TF^+-MPs, increasing platelet activation and their overall procoagulant activity (Lopez-Vilchez, Diaz-Ricart *et. al.,* 2009).

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) (Parise, 1999). 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 (Gilbert *et al.,* 1991; Miyazaki *et al.,* 1996; Sims *et al.,* 1989) 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 (Healey *et al.,* 2006), which bind to the monocytes (Vogl *et al.,* 2007) and also induce EC apoptosis (Viemann *et al.,* 2007). Platelets also generate lipid mediators like TXA_2 that activate different signalling cascades depending on the trigger.

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 (Merten, Pakala and Thiagarajan, 1999) at the sites of endothelial injury or onto the forming fibrin (Pakala, 2004; Siljander, Carpen and Lassila, 1996) enable adhesive functions and thrombin generation, which further stimulates aggregation (Berckmans *et al.,* 2001;. Nomura *et al.,* 2001; Raturi *et al.,* 2008)

1.1.2.3.4. 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 (Valles *et al.,* 1998) Thus, erythrocytes can actively contribute as signalling cells to platelet-driven thrombogenesis and microvascular occlusion (Valles *et al.,* 2002). 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 (Jy *et al.,* 2005).

1.1.3. 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

Table 1

CARDIOVASCULAR RISK FACTORS CLASSIFIED AS MODIFIABLE AND NON-MODIFIABLE

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.

Table 1 (continued)

CARDIOVASCULAR RISK FACTORS CLASSIFIED AS MODIFIABLE AND NON-MODIFIABLE

CVD risk factors		
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.*

Notes: * Major cardiovascular risk factors. CHD indicates chronic heart disease; CVD, cardiovascular disease; LDL-c, low-density lipoprotein cholesterol; LV, left ventricular.
Source: Own elaboration.

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.

1.1.3.1. Major modifiable risk factors

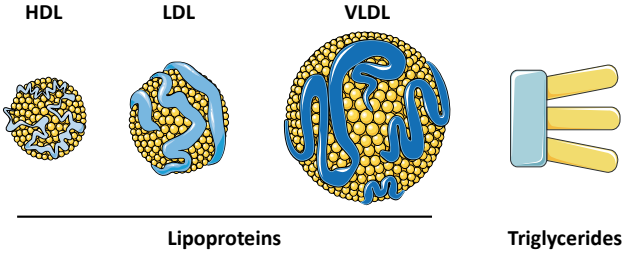
In the developed countries, at least one-third of all CVD is attributable to five major modifiable risk factors, named also classical cardiovascular risk factors: dyslipidemia, diabetes, hypertension, obesity, and smoking. Other modifiable risk factors exist (Table 1), and all of them increase the risk of cardiovascular disease. Together, known modifiable risk factors explain >90% of the occurrence of MI (Yusuf *et al.,* 2004).

1.1.3.1.1. Dyslipidemia

High blood cholesterol has been considered as one of the most important modifiable risk factors associated with CHD (Castelli *et al.,* 1992). 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-

Figure 8

MAIN TYPES OF LIPIDS IN THE CIRCULATION



Notes: Major classes of lipoproteins and triglycerides. HDL indicates high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very low-density lipoprotein.
Source: Own elaboration.

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

Table 2

CLINICAL LIPID MANAGEMENT GUIDELINES BASED ON LIPID TARGETS

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

Notes: 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.
Source: Own elaboration.

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.

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 (Grundy, 1998). 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 (Stone *et al.,* 2013).

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 (Gill, Harnden and Karpe, 2012). 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 (Hansson, 2005; Neefjes *et al.,* 2011). 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 (Sjouke *et al.,* 2011) as seen in FH patients with presence of aortic plaque burden detected by magnetic resonance imaging (MRI) (Caballero *et al.,* 2012). 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 (Yuan, Wang and Hegele, 2006).

1.1.3.1.2. 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 (Sambola *et al.,* 2003). Platelets from diabetic patients have increased reactivity and hyper-aggregability and expose a variety of activation-dependent adhesion proteins(Osende *et al.,* 2001), leading to increased accumulation of platelet on the altered vessel wall (Rauch, Crandall *et al.,* 2000). 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 (Lim *et al.,* 2012) 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 (Badimon *et al.,* 1999) and increasing fibrinogen levels (Miller, 1992).

1.1.4. 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.

1.1.4.1. 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 [Ridker *et al.,* 2000]), IL-18 ([Blankenberg *et al.,* 2002; Badimonm, 2012]) and IL-10 ([Chalikias *et al.,* 2000]) and *C-reactive protein* (CRP), which is used as a marker of worst outcomes and mortality in ACS (Lindahl *et al.,* 2000). 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* (Oei *et al.,* 2005) and *myeloperoxidase* that may have implications for early atherosclerosis and risk assessment (Brennan *et al.,* 2003). Additionally, plaque-derived biomarkers may be useful for predicting the risk of new vascular complications such as *osteopontin* (De Kleijn *et al.,* 2010) and *matrix metalloproteinases* (MMP-9, MMP-11) (Sangiorgi *et al.,* 2006), but until now they have only been proved useful for aortic dissection. Other inflammatory biomarkers include *oxidized LDL* that highly correlated with CV events (Witztum, 1991) and acute-phase proteins *serum amyloid A, pentraxin 3* and *growth differentiation factor 15* (Daniels *et al.,* 2011).

1.1.4.2. 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* (Heeschen *et al.,* 2004), *adhesion molecules* (Rallidis *et al.,* 2003), *soluble CD40 ligand* (Henn *et al.,* 1998) *von Willebrand Factor* (Ray *et al.,* 2005), *d-dimer* (Hazui *et al.,* 2005) and *fibrinogen* (Danesh *et al.,* 1998) and platelet markers, such as *platelet count* (Nikolsky *et al.,* 2007), *mean platelet volume* (Chu *et al.,* 2010), and *reticulated platelet fraction* (Grove, Hvas and Kristensen, 2009).

1.1.4.3. 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 (Cubedo *et al.,* 2011).

1.1.4.4. Biomarkers of neuroendocrine activation and left ventricular function

Brain natriuretic peptide (Januzzi *et al.,* 2006) and *mid regional-proadenomedullin* (Hinson *et al.,* 2000), 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 (Thygesen *et al.,* 2010) Their detection indicates myocardial cell necrosis (Thygesen *et al.,* 2007), being superior compared other biomarkers of myocardial necrosis, such as *creative kinase muscle and brain, creative phosphokinase, aspartate transaminase* and *myoglobin* (Hochholzer *et al.,* 2008). Unfortunately and despite high sensitivity cTn assays (Weber *et al.,* 2013), cTns are not specific markers of ACS (Collinson and Troponin, 1998) and they cannot detect MI in the early hours after symptom onset, due to the delayed increase of circulating levels (Collinson, Wiggins and Gaze, 2001). Finally, *copeptin* or *C-terminal pro-vasopressin* is a potential diagnostic and prognostic marker for heart failure (Nickel, Bingisser and Morgenthaler, 2012) and for ACS (Morgenthaler *et al.,* 2006) due to its stability and pattern of rapid secretion; however, it has failed to distinguish patients with UA from non-ischemic patients (Reichlin *et al.,* 2009). Since accurate ACS diagnosis is not achieved by current strategies, useful prognostic markers are needed.

1.1.4.5. 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 (Salic and, De Windt, 2012) 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.

Table 3

MAIN KNOWN AND NOVEL CVD BIOMARKERS

CVD BIOMARKERS	Associated disease
Endothelial dysfunction	
Adhesion molecules (ICAM-1, VCAM-1) (Rallidis *et al.,* 2003)	CAD
E-selectin (Rallidis *et al.,* 2003)	ACS
Atherogenesis	
Matrix metalloproteinases (MMP-3, MMP-11) (Sangiorgi *et al.,* 2006)	ACS
Pregnancy-associated plasma protein A (PAPP-A)	ACS
Cathepsin S or K	CAD
Free-fatty acids (FFA) / Pentraxin 3 (PTX3) (Daniels *et al.,* 2011)	CAD
Growth factors (Heeschen *et al.,* 2004)	ACS, CAD
Lipoproteins (TC, LDL, HDL, VLDL, Lp (a)) (Witztum, 1991)	CAD
Apoliproteins (ApoAI, ApoB, ApoE, ApoJ)	ACS, CAD, MI
Paraoxonase-1 / Follistatin / Osteopontin (De Kleijn *et al.,* 2010)	ACS
Plasminogen activator inhibitor 1 (PAI-1)	ACS
Inflammation	
High sensitivity C-reactive protein (hs-CRP) (Lindahl *et al.,* 2000)	ACS
Chemotactic molecules	CAD, ACS
Homocysteine / Cystatin C	ACS
Myeloperoxidase (MPO) (Brennan *et al.,* 2003) / Neopterin	ACS, CAD, CHF
Interleukins (IL-1β, IL-1Ra, IL-6, IL-10; IL-18) (Ridker *et al.,* 2000; Blankenberg *et al.,* 2002; Badimon, 2012; Chalikias *et al.,* 2007)	ACS, CAD, CHF
Myeloid-related proteins (MRP-8, MRP-14)	ACS
Growth differentiation factor 15 (GDF-15) (Daniels *et al.,* 2011)	ACS, CAD
Lp-associated phospholipase A_2 (Lp-PLA_2) (Oei *et al.,* 2005)	ACS, CAD
Galectin-3 / Serum amyloid A (Daniels *et al.,* 2011)	CHF
Osteoprogenterin (OPG) / Fetuin-A	CAD
Thrombosis	
Von Willebrand Factor (vWF) (Ray *et al.,* 2005)	ACS, CAD
Fibrinogen (Fbn) (Danesh *et al.,* 1998) / Soluble CD40 ligand (sCD40L) (Henn *et al.,* 1998)	ACS
Prothrombin fragment 1.2 / Fibrinopeptide A	ACS, CAD
D-dimer (Hazui *et al.,* 2005)	ACS, PE

Table 3 (continued)

MAIN KNOWN AND NOVEL CVD BIOMARKERS

CVD BIOMARKERS	Associated disease
Neurohormonal activation	
Prohormone brain natriuretic peptide (proBNP) (Januzzi *et al.,* 2005)	CHF
Mid regional proadenomedullin (MR-proADM) (Hinson, Kapas and Smith, 2000)	CHF, MI
Copeptin (Morgenthaler *et al.,* 2006)	ACS, CHF
Ischemia / Necrosis	
Ischemia-modified albumin (IMA)	CAD
Heart type-fatty acid binding protein (H-FABP)	ACS
Cardiac troponins (cTnT, cTnI) (Thygesen *et al.,* 2010)	CAD, CHF, MI
Myoglobin (Hochholzer *et al.,* 2008)	ACS

Notes: 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.
Source: Own elaboration.

1.1.5. Prevention and treatment

1.1.5.1. 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 (Estruch *et al.,* 2013). 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.

1.1.5.2. Pharmacological interventions

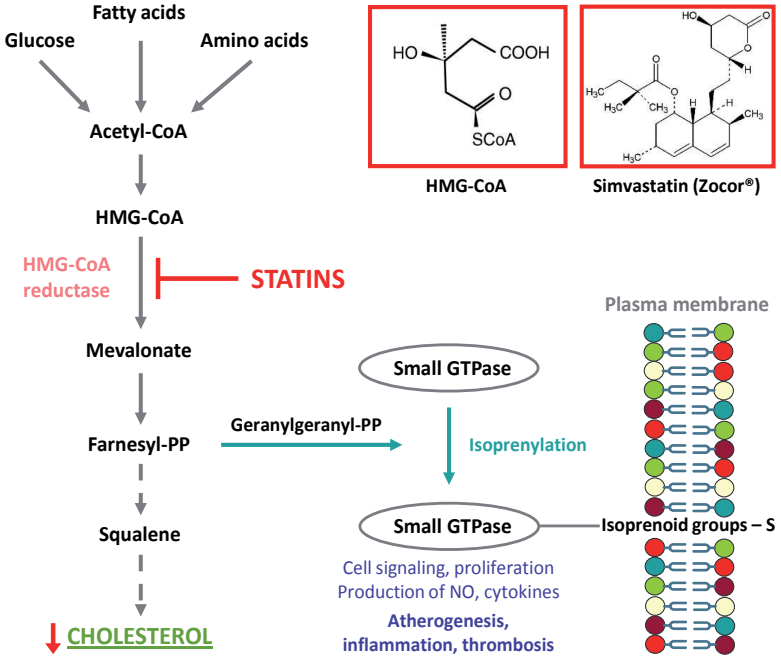
Since hypercholesterolemia is considered the primary risk for CVD, among pharmacological prevention of CVD, effective lipid management stands out as a key strategy to control vascular risk and reduce morbidity and mortality among patients with cardiovascular risk. Thus, *lipid-lowering therapy (LLT)* 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 (Baigent *et al.,* 2005). 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 (Kozai

Figure 9

MECHANISM OF ACTION OF STATIN-MEDIATED REDUCTION OF CHOLESTEROL AND PLEIOTROPIC EFFECTS



Note: 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.
Source: Own elaboration.

et al., 2005; Undas *et al.,* 2005). 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.

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) (Michelson, 2010). 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).

1.2. CELL-DERIVED MICROPARTICLES

1.2.1. Background

1.2.1.1. 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 (Jy, Horstman *et al.,* 2004). 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 (Doeuvre *et al.,* 2009). Indeed, MP levels show gender-specific differences (Robert *et al.,* 2009; Toth *et al.,* 2007) and changes are observed with age (Van der Zee *et al.,* 2006), during pregnancy (Bretelle *et al.,* 2003), after exercise (Chaar *et al.,* 2011; Maruyama, Kadono and Morishita, 2011) and after a high-fat meal (Tushuizen *et al.,* 2006). Levels of circulating microparticles have been shown to correlate with disease progression and severity (Burnier *et al.,* 2009). 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 (Burnier *et al.,* 2009), 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.

1.2.1.1.1. Microparticles in cardiovascular pathophysiology

Although cMPs are released in health under basal conditions (Berckmans *et al.,* 2001), 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 (Preston *et al.,* 2003; Koga *et al.,* 2005). Elevated microparticle levels have been correlated with a higher calculated 10-year Framingham CAD risk (Ueba *et al.,* 2010). Increases in microparticle levels have also been correlated to the degree of CAD –myocardial infarction>unstable angina>stable angina– (Bernal-Mizrachi *et al.,* 2003 and 2004) and to clinical parameters, such as coronary artery endothelial function in CAD patients (Werner *et al.,* 2006). 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 (Perez-Pujol, Marker and Key, 2007). 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.

Table 4

CLINICAL CONDITIONS ASSOCIATED TO CIRCULATING MICROPARTICLES

Clinical condition	MP subtype change	References
CARDIOVASCULAR DISEASE		
Cardiovascular risk factors		
Type-2 diabetes mellitus	↑ eMP, ↑ pMP, ↑ LMP	Bernard *et al.,* 2009; Diamant *et al.,* 2002; Koga *et al.,* 2006
Severe hypertension	↑ pMP, ↑ eMP, ↑ LMP	Preston *et al.,* 2003; Nomura *et al.,* 2002
Metabolic syndrome	↑ eMP, ↑ ErMP, ↑ pMP, ↑ LMP	Agouni *et al.,* 2008; Helal *et al.,* 2011; Arteaga *et al.,* 2006; Goichot *et al.,* 2006
Obesity	↑ pMP	Goichot *et al.,* 2006; Murakami *et al.,* 2007
Hypertriglyceridemia	↑ eMP	Ferreira *et al.,* 2005
Smoking	↑ eMP	Goichot *et al.,* 2006; Heiss *et al.,* 2008

Table 4 (continued)

CLINICAL CONDITIONS ASSOCIATED TO CIRCULATING MICROPARTICLES

Clinical condition	MP subtype change	References
CARDIOVASCULAR DISEASE		
Endothelial dysfunction (ED)		
Acute endothelial dysfunction	↑ eMPs	Heiss *et al.,* 2008
Chronic renal failure with ED	↑ eMP	Amabile *et al.,* 2005
Atherosclerosis		
Subclinical atherosclerosis	↑ LMP	Chironi *et al.,* 2006
Coronary calcification	↑ eMP, ↑ pMP	Jayachandran *et al.*, 2008
Acute coronary syndrome	↑ eMP, ↑ pMP	Bernal-Mizrachi *et al.,* 2004; Mallat *et al.,* 2000; Bernal-Mizrachi *et al.,* 2003; Nomura *et al.,* 2003; Li and Cong H, 2009
Stable coronary artery disease	↑ eMPs	Bernal-Mizrachi *et al.,* 2003
Coronary heart disease	↑ pMP	Ueba *et al.,* 2010; Nozaki *et al.,* 2009
Peripheral artery disease	↑ pMP	Zeiger *et al.,* 2000; Van der Zee *et al.,* 2006
Heart failure & vascular disease		
Congestive heart failure	↑ eMP	Nozaki *et al.,* 2006; Rossig *et al.,* 2000
Cyanotic congenital heart disease	↑ pMP	Horigome *et al.,* 2002
Cardiopulmonary bypass	↑ PS^+-MP	Nieuwland *et al.,* 1997
Coronary stents	↑ pMP	Inoue *et al.,* 2008
Heart transplantation	↑ eMP	García *et al.,* 2005
Heart transplant rejection	↑ eMP	Morel *et al.,* 2008
Severe aortic stenosis	↑ eMP, ↑ LMP	Diehl *et al.,* 2008
Pulmonary hypertension	↑ eMP, ↑ pMP, ↑ LMP	Diehl *et al.,* 2011
Acute pulmonary embolism	↑ pMP	Bal *et al.,* 2010
Valvular atrial fibrillation	↑ pMP	Azzam and Zagloul, 2009
Deep vein thrombosis and venous thromboembolism	↑ eMP, ↑ pMP, ↑ LMP	Rectenwald *et al.,* 2005; Chirinos *et al.,* 2005
Buerger's disease	↑ pMP	Darnige *et al.,* 2010
CEREBROVASCULAR DISEASE		
Acute ischemic stroke	↑ pMP, ↑ eMPs	Lee *et al.,* 1993; Lukasik *et al.,* 2010; Simak *et al.,* 2006
Cerebral vasospasm	↑ eMP, ↑ pMP, ↑ LMP, ↑ ErMP	Lackner *et al.,* 2010
Lacunar infarcts	↑ pMP	Lee *et al.,* 1993
Multi-infarct dementia	↑ pMP	Lee *et al.,* 1993
Carotid atherosclerosis	↑ pMP	Michelsen *et al.,* 2009
Cerebrovascular atherosclerosis	↑ eMPs	Jung *et al.,* 2009

Table 4 (continued)

CLINICAL CONDITIONS ASSOCIATED TO CIRCULATING MICROPARTICLES

Clinical condition	MP subtype change	References
HEMATOLOGIC DISEASES		
Immune thrombocytopenia purpura	↑ pMP	Tantawy *et al.,* 2010
Thrombotic thrombocytopenia purpura	↑ eMP, ↑ pMP	Galli *et al.,* 1996; Jimenez *et al.,* 2003
Paroxysmal nocturnal hemoglobinuria	↑ eMP, ↑ pMP	Hugel *et al.,* 1999; Simak *et al.,* 2004
Heparin-induced thrombocytopenia	↑ pMP	Warkentin *et al.,* 1994; Hughes *et al.,* 2000
Castaman syndrome	↑ pMP	Castaman *et al.,* 1996 and 1997
INFECTIOUS DISEASES		
Sepsis	↑ eMP, ↑ gMP, ↑ pMP	Joop *et al.,* 2001; Mostefai, Meziani *et al.,* 2008; Soriano *et al.,* 2005; Nieuwland *et al.,* 2009
Prion disease	↑ pMP	Robertson *et al.,* 2006
HIV-1 infection	↑ ℓMP	Aupeix *et al.,* 1997
AUTOIMMUNE DISEASES		
Systemic lupus erythematosus	↑ eMP, ↑ pMP	Pereira *et al.,* 2006; Sellam *et al.,* 2009
Antiphospholipid syndrome	↑ eMP, ↑ pMP	Jy *et al.,* 2009; Dignat-George *et al.,* 2004
Systemic sclerosis	↑ eMP, ↑ pMP, ↑ LMP	Guiducci *et al.,* 2008; Nomura *et al.,* 2008
Rheumatoid arthritis	↑ pMP	Sellam *et al.,* 2009; Boilard *et al.,* 2010; Knijff-Dutmer *et al.,* 2002
Acute and systemic vasculitis	↑ eMP, ↑ pMP, ↑ LMP	Daniel *et al.,* 2006; Brogan and Dillon, 2004; Erdbruegger *et al.,* 2008
Type-1 diabetes mellitus	↑ eMP, ↑ pMP	Sabatier *et al.,* 2002
Multiple sclerosis	↑ eMP, ↑ pMP	Sheremata *et al.,* 2008; Minagar *et al.,* 2001
Sjögren syndrome	↑ pMP	Sellam *et al.,* 2009
CANCER		
Gastric cancer	↑ pMP	Kim *et al.,* 2003
Lung cancer	↑ PS^+-MP	Janowska-Wieczorek *et al.,* 2005
Breast cancer	↑ pMP	Janowska-Wieczorek *et al.,* 2006; Toth *et al.,* 2008
Prostate cancer	↑ PS^+-MP	Dashevsky *et al.,* 2009; Helley *et al.,* 2009

Table 4 (continued)

CLINICAL CONDITIONS ASSOCIATED TO CIRCULATING MICROPARTICLES

OTHER		
Obstructive sleep apnea	↑ eMP, ↑ pMP, ↑ LMP	Ayers *et al.,* 2009; Yun *et al.,* 2010
Renal disease	↑ eMP, ↑ pMP	Faure *et al.,* 2006; Dursun *et al.,* 2009
Decompression sickness	↑ pMP	Faille *et al.,* 2009
Preeclampsia	↑ pMP, ↑ ℓMP, ↑ LMP, ↑ eMP	Lok *et al.,* 2007 and 2008; VanWijk *et al.,* 2002; González-Quintero *et al.,* 2003
Spontaneous abortion	↑ pMP	Kaptan *et al.,* 2008
Alzheimer disease	↑ pMP	Matsubara *et al.,* 2002
Uremia	↑ pMP	Ando *et al.,* 2002
Chron's disease	↑ PS^+-MP	Chamouard *et al.,* 2002

Notes: Main changes in MPs among clinical pathologies. ↑ indicates increase,–, no change.
Source: Own elaboration.

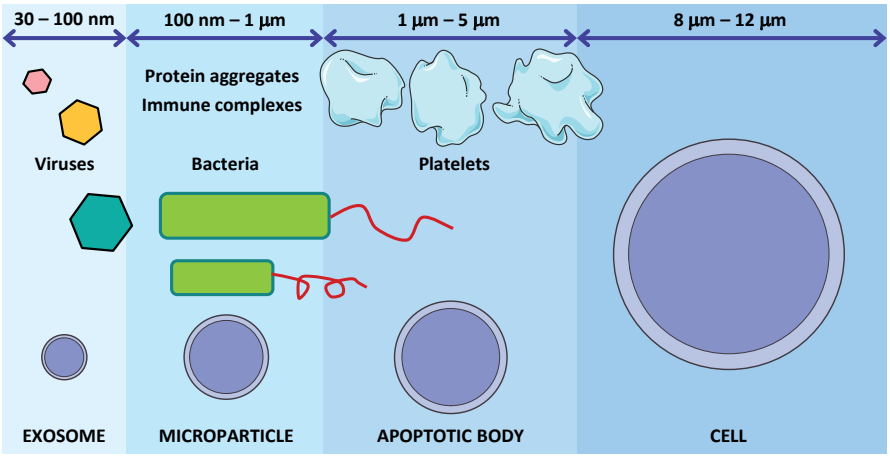
1.2.1.2. Definition and nomenclature

Microparticles are defined as phospholipid microvesicles (MVs) containing certain membrane receptors as well as other protein and molecular components inherent to their parental cells (Morel *et al.,* 2005). 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 (Heijnen *et al.,* 1999). 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 (Simak and Gelderman, 2006). 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 (Perez-Pujol, Marker and Key, 2007). In contrast, *exosomes* (20 to 100 nm) are cup-shaped vesicles released from multivesicular bodies and exocytosis of endocytic bodies (Johnstone, 2006), with a density of 1.10-1.18 g/mL.

Figure 10

SIZE RANGES OF EXTRACELLULAR-RELEASED MEMBRANE VESICLES



Notes: 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.
Sources: Adapted from Williams *et al.* (2011) and Gyorgy *et al.* (2011).

Exosomes were first described in platelets (Wickman *et al.,* 2013) and in general form a more homogenous population than MPs, both by size and molecular content (Heijnen *et al.,* 1999). *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 (Hristov *et al.,* 2004; Jiménez *et al.,* 2003). 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 (Wickman *et al.,* 2013). Figure 10 depicts the size ranges of the different shedding vesicles.

1.2.2. Modulation

1.2.2.1. Microparticle release

It is now widely accepted that MPs (and probably exosomes) are generated by all eukaryotic cells, including cells in the vasculature (Aatonen, Gronholm and Siljander, 2012) Therefore, MPs can originate from platelets, endothelial cells, leukocytes, monocytes, lymphocytes, granulocytes, erythrocytes (red blood cells) and other

cell types (smooth muscle cells, cardiomyocytes) (Jiménez *et al.,* 2003). Besides such vascular cellular source, epithelial cells and tumour cells are also capable of producing MPs (Abrahams *et al.,* 2003). Moreover, all extracellular body fluids contain microvesicles: plasma (Lasser, Alikhani *et al.,* 2011), saliva (Berckmans *et al.,* 2011), urine (Raj *et al.,* 2012), breast milk (Lasser, Alikhani *et al.,* 2011), bile (Masyuk *et al.,* 2010), tears, semen (Poliakov *et al.,* 2009), nasal mucus or secretions(Lasser, O'Neil *et al.,* 2011), amniotic fluid (Liu *et al.,* 2014), cerebrospinal fluid (Street *et al.,* 2012), bronchoalveolar lavage (Admyre *et al.,* 2003), synovial fluid (Berckmans *et al.,* 2002), ascytes (Dai *et al.,* 2008), ocular effluent and aqueous humour (Perkumas *et al.,* 2007), and pleural effusions (Bard *et al.,* 2004).

Microparticle release is an active and highly controlled energy-dependent process (Simak J, Gelderman, 2006). Therefore, cells release MPs in response to a variety of stimuli, via (a) *cell activation* through multiple agonist-dependent signalling pathways (Burnier et al., 2009), (b) *apoptotic cell blebbing* or *senescence* (Doeuvre *et al.,* 2009; Schoenwaelder *et al.,* 2009) or, finally, (c) *mechanic forces* due to certain blood flow conditions such as high shear stress (Miyazaki *et al.,* 1996). 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 (Doeuvre *et al.,* 2009; Freyssinet *et al.,* 2003; Kolowos *et al.,* 2005; Martin *et al.,* 2004). 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 (Simak and Gelderman, 2006; Connor *et al.,* 2010). 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 (Morel *et al.,* 2011; Pasquet *et al.,* 1996). 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 (Schoenwaelder *et al.,* 2009).

1.2.2.2. 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 (Flaumenhaft, 2006), or even less than 10 minutes

in rabbits (Rand, 2006). Recently, apheresis-derived pMPs were shown to circulate for more than 5 hours (Rank, 2011). Discrepancy in the MP turnover may account for distinct 'eat-me' signals due to secretion process (Dasgupta *et al.,* 2009; Abdel-Monem, 2010).

MPs could be cleared from the circulating blood by phagocytosis, due to: (1) direct mechanisms such as PS exposure (Schlegel and Williamson, 2001), or (2) indirect mechanisms such as opsonization by proteins like growth arrest-specific gene 6 product, protein S, thrombospondin, complement and IgM (Wu, Tibrewal and Birge, 2006; Pittoni and Valesini, 2002; Ravichandran, 2003; Willekens *et al.,* 2005; Litvack, Post and Palaniyar, 2011) through, for instance, discriminating glycosilation patterns (Bilyy *et al.,* 2012). PS exposure is potentially important as interacts with several receptors on macrophages, resulting in phagocytosis of T-cell MPs (Distler *et al.,* 2005) or removal of apoptotic cells (Ravichandran and Lorenz, 2007). Therefore, PS-negative MPs may evade this fate, and could serve as a distinct long-lasting 'surveillance reservoir' of MPs (Del Conde *et al.,* 2005; Falati *et al.,* 2002;. Rauch *et al.,* 2000). Endogenous lactadherin may also be an important 'tag', binding to PS on MPs and marking them for clearance (Dasgupta *et al.,* 2009).

Table 5

MAIN STIMULI FOR MP FORMATION DEPENDING ON CELL TYPE

Cell type		Stimulus	Reference
Platelet	Proinflammatory	LPS, shiga toxin	Stahl, Sartz and Karpman (2011)
		Soluble CD40 ligand	Prasad *et al.* (2003)
		Cytokines	Nomura, Nakamura *et al.* (2000)
	Procoagulant	Adenosine diphosphate	Combes *et al.* (1997)
		Thrombin	Terrisse *et al.* (2010)
		Collagen	Takano *et al.* (2004)
		PAR agonists	Chung *et al.* (2004)
		TRAP	Tschuor *et al.* (2008)
	Other	Shear stress	Reininger *et al.* (2006)
		Epinephrine/noradrenaline	Tschuor *et al.* (2008)
		Calcium ionophore	Nomura *et al.* (2000)
		Complement C5b-9	Wiedmer *et al.* (1990)
		Prolyl gallate	Xiao *et al.* (2002)
		Storage	Owens (1994)
		Dibucaine	Fox *et al.* (1990)

Table 5 (continued)

MAIN STIMULI FOR MP FORMATION DEPENDING ON CELL TYPE

Cell type		Stimulus	Reference
Endothelial cell	Proinflammatory	Tumour Necrosis Factor α	Brown *et al.* (2011) and Peterson *et al.* (2008)
		LPS	Del Turco *et al.* (2007)
		Interleukin-1α	Abid Hussein *et al.* (2007)
		C-reactive protein	Devaraj, Kumaresan and Jialal (2011) and Wang *et al.* (2007)
	Procoagulant	Thrombin	Abid Hussein *et al.* (2007); Simoncini *et al*. (2009)
		PAI1	Peterson *et al.* (2008) and Brodsky *et al.* (2002)
		Activated protein C	Perez-Casal *et al.* (2011)
	Uraemic toxins	p-cresyl sulphate	Meijers *et al.* (2009)
		Indoxyl sulphate	Faure *et al.* (2006)
		Homocysteine	Sekula *et al.* (2011)
	Other	High glucose	Tschuor *et al.* (2008)
		Angiotensin II	Burger *et al.* (2011)
		Calcium ionophore	Hamilton *et al.* (1990)
		Complement	Combes *et al.* (1999)
		Camptothecin	Simak, Holada and Vostal (2002)
		Growth factor deprivation	Jiménez *et al.* (2003)
		Reactive oxygen species	Szotowski *et al*. (2007)
		Shear stress	Boulanger *et al.* (2007)
Leukocyte	Proinflammatory	Tumour Necrosis Factor α	Scanu *et al.* (2008) and Eyre *et al.* (2011)
		TLR-3 and TLR-4	Gauley and Pisetsky (2010)
		LPS, bacterial infection	Ben-Hadj-Khalifa-Kechiche *et al.* (2010) and Satta *et al.* (1994)
	Other	Phytohemagglutinin	Ullal and Pisetsky (2010)
		Fas ligand	Shefler *et al.* (2010) and González-Cano *et al.* (2010)
		Etoposide	Terrisse *et al.,* 2010
		Staurosporin	Martin *et al.* (2004)
		Actinomycin D	Ullal *et al.* (2010) and Mastronardi *et al.* (2011)
		Calcium ionophore	Cerri *et al.* (2006)
		Endotoxin	Watanabe *et al.* (2003)

Table 5 (continued)

MAIN STIMULI FOR MP FORMATION DEPENDING ON CELL TYPE

Cell type		Stimulus	Reference
Erythrocyte	Other	Calcium ionophore	Hugel *et al.* (1999)
		Acid pH (5.4)	Hugel *et al.* (1999)
		Reactive oxygen species	Freikman *et al.* (2008)
		Diamide	Wagner *et al.* (1986)

Notes: LPS indicates lipopolysaccharide; PAR, protease activated receptor; PAI-1, plasminogen activator inhibitor; TRAP, thrombin receptor activating peptide; TLR, toll-like receptor.
Source: Own elaboration.

Alternatively, MPs may be cleared by the activity of circulating phospholipases (Fourcade *et al.,* 1995). 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 (Dasgupta *et al.,* 2012), functioning as a bridging molecule between integrins and PS.

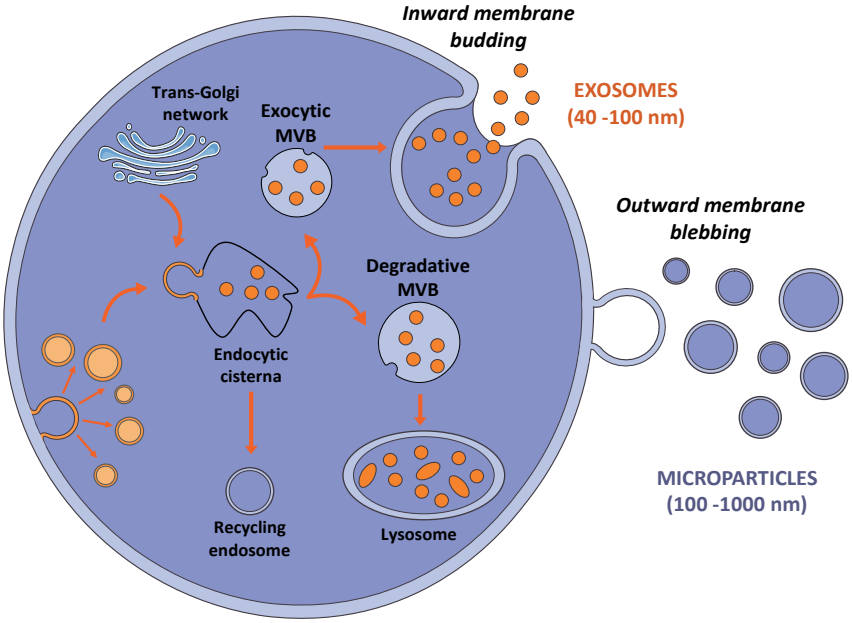
1.2.3. Microvesicle formation

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 (Van Nispen *et al.,* 2010) 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 (Van der Zee *et al.,* 2006; Biró *et al.,* 2005) and *vice versa,* many common pMP proteins are detected on subsets of platelet exosomes (Heijnen *et al.,* 1999). 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 (Elmore and Apoptosis, 2007).

Figure 11

MICROVESICLE BIOGENESIS



Note: 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.
Sources: Adapted from Cocucci *et al.* (2009) and Raposo *et al.*(2013).

1.2.3.1. Microparticle formation

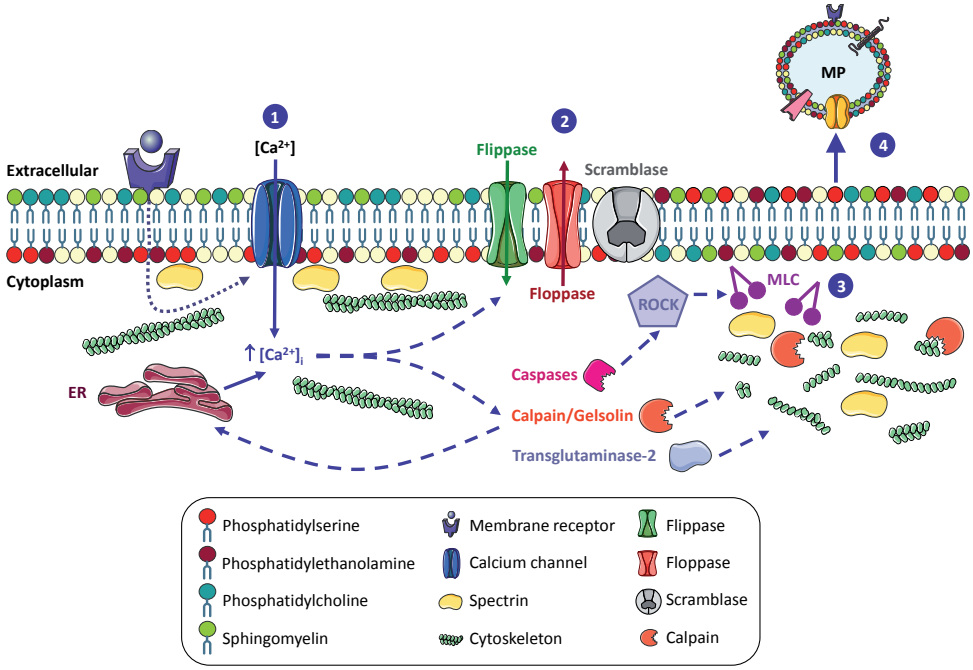
Microparticles emanate from cells through the outward blebbing of their plasma membranes *(ectocytosis)* by successive mechanisms of a complex nature that implicate various membrane (lipid transporters, receptors, and calcium channels) and cytoplasmatic (cytoskeleton, calpains) actors (Figure 12) (Zwaal, Comfurius and Bevers, 2005).

- *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 (Daleke, 2003). In addition, calcium may interact with and activate specific proteins involved in MP formation, such as calpain (Dachary-Prigent *et al.,* 1995) and caspases (Sebbagh *et al.,* 2001).

■ *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 (Zwaal and Schroit, 1997). 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 (Williamson *et al.,* 1995). *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

Figure 12

MICROPARTICLE FORMATION



Notes: 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.
Sources: Own elaboration.

are involved in plasma membrane remodelling, such as protein disulfide isomerase, which modulates *flippase* and *floppase* activities (Popescu and Lupu, 2010) and, acid sphingomyelinase, necessary for MP formation in glial cells (Bianco *et al.,* 2009).

- *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 (Yano *et al.,* 1994).
- *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 (Cocucci, Racchetti and Meldolesi, 2009).

1.2.3.1.1. 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 (Balasubramanian and Schroit, 2003). The release of MP is observed some hours after apoptosis induction (Aupeix *et al.,* 1997), whereas a swift MP release occur a few minutes after stimulation in platelets (Dachary-Prigent *et al.,* 1993) as they have the highest scrambling rate in contrast to other vascular cell types (Bevers and Williamson, 2010). In some cases, the processes of PS translocation and MP generation can be separated (Siljander *et al.,* 2001). 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 (Pasquet, Dachary-Prigent and Nurden, 1998). On the other hand, a portion of the MPs released from blood cells does not expose accessible PS on their surface (Simak *et al.,* 2004; Shet *et al.,* 2003). 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 (Kolowos *et al.,* 2005).

Furthermore, cellular plasmatic membranes also display lipid microdomains, named rafts, which are responsible of lateral mobility of proteins and recruitment of signalling molecules (Anderson and Jacobson, 2002). Recent data indicate that the lipid rafts, rich in cholesterol and sphingolipids, are essential for PS transmembrane redistribution (López, Del Conde and Shrimpton, 2005). Indeed, PS exposure was colocalized with membrane lipid rafts regions (Fischer *et al.,* 2006). In addition, perturbation of lipid domains was associated with alterations in MP formation (Liu *et al.,* 2007) and, similarly, disruption of lipid-rich domains impairs MP formation (Burger *et al.,* 2011). Interestingly, TF-rich microparticles have been found to arise from lipid rafts (Del Conde *et al.,* 2005). 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 (Jy *et al.,* 2002) and (b) several proteins which localize to lipid rafts have been also identified in MPs (CD39, flotillin-2, caveolin-1) (Banz *et al.,* 2008). However, the exact mechanism involving lipid rafts remains unknown.

1.2.3.1.2. 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 (Manno, Takakuwa and Mohandas, 2002). 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, *spectrin* 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, *caspases* 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 (Sebbagh *et al.,* 2001) and *caspase-2* (Sapet *et al.,* 2006). *Caspase 1–like enzymes* also contribute to the externalization of membrane PS residues by hydrolysis of cytoskeletal proteins (moesin, filamin A) (Vanags *et al.,* 1996). 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 (Fox *et al.,* 1991). Additionally, *gelsolin* is involved in the cleavage of the actin capping proteins (McLaughlin *et al.,* 1993). Rearrangements of cytoskeleton proteins and protein cleavage activate various receptors and proenzymes (Zwaal and Schroit 1997), such as *RhoA,* a member of the Rho family, also involved in MP biogenesis (Li *et al.,* 2012).

In addition, other potential calcium-independent mechanisms involving numerous signalling transduction pathways are involved in MP formation such as integrin $\alpha_{IIb}\beta_3$ (Cauwenberghs *et al.,* 2006) calmodulin activation (Wiedmer and Sims, 1991) p21-activated kinases (Crespin *et al.,* 2009) p38-mitogen activated protein kinase (Curtis *et al.,* 2009), TRAIL/Apo2L complex (Simoncini *et al.,* 2009), toll-like receptor 4 (Hashimoto *et al.,* 2009) and others. Although participation of kinases (Siljander *et al.,* 2001) and phosphatase inhibition (Wiedmer and Sims, 1991) 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 (Van den Akker et al., 2012).

1.2.3.2. 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) (Williams and Urbe, 2007). 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 (Pilzer *et al.,* 2005); (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 (Stoorvogel *et al.,* 2002; Trajkovic *et al.,* 2008). 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 (Hsu *et al.,* 2010; Ostrowski *et al.,* 2010). A part from the canonical biogenesis, other mechanisms for exosome release have been proposed: (a) a role for the tetraspanin protein family (Trajkovic *et al.,* 2008); (b) a lipid self-assembly process, consisting in a spontaneous secondary microvesiculation triggered by enrichment of larger artificial structures in ceramide (Bianco *et al.,* 2009); and (c) an ARF6-regulated endosomal pathway (Muralidharan-Chari *et al.,* 2009).

1.2.4. Structure and composition

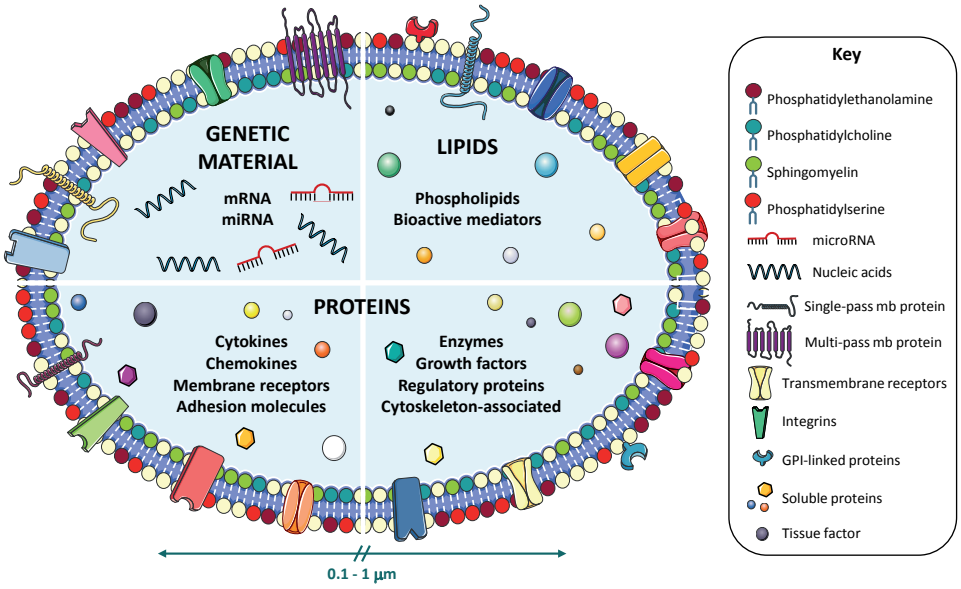
MPs are not a random sample of the parental cellular content, but rather MPs are assembled through a highly selective process (Valadi *et al.,* 2007), the nature of which remains unclear.

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 (Shai *et al.,* 2012; García *et al.,* 2005; Senzel, Gnatenko and Bahou, 2009). 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 (Sims *et al.,* 1989).

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 (Jiménez *et al.,* 2003), as shown in Figure 13. MPs not only

Figure 13

MICROPARTICLE STRUCTURE AND COMPOSITION



Note: Schematic representation of the repertoire of MP components.
Source: Adapted from Norling *et al.* (2013).

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.

1.2.4.1. 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 (Shai *et al.,* 2012). To increase the complexity it has been shown that different size classes of pMPs contain different protein components (Dean *et al.,* 2009). 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 (Robertson *et al.,* 2006; Perini *et al.,* 1996) or contractile proteins like thrombosthenin (Crawford, 1971).

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 (Abid Hussein *et al.,* 2003). Table 6 shows the most common markers depending on cell type.

1.2.4.2. 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 (Nolte-'t Hoen *et al.,* 2012). 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 (Nolte-'t Hoen *et al.,* 2012; Huang *et al.,* 2013).

Table 6

CELL-DERIVED MARKERS FOR MICROPARTICLE PHENOTYPING

Cellular origin	Markers	Alternative name
Red blood cell	CD235a	Glycophorin-A
Leukocyte	CD11b	Integrin alpha M
	CD45	Leukocyte common antigen
	CD62L	L-selectin
	CD64a	Immunoglobulin γ Fc Receptor I
	CD162	P-selectin glycoprotein ligand 1
Granulocyte	CD15	Lewis X
	CD16b	Immunoglobulin γ Fc Receptor IIIB
	CD66b	Glycosylphosphatidylinositol-linked protein
Monocyte	CD14	Lipopolysaccharide-receptor
	Interleukin-1β	Proinflammatory cytokine
	Anti-oxLDL antibody	
Lymphocyte	CD3	T3
	CD4	T4
	CD8	T8
	CD19	B4
	CD20	B1
	CD80	B7
	CD83	HB-15
	CD154	CD40 ligand
Platelet	CD41	αIIb-integrin (glycoprotein IIb)
	CD42a	Glycoprotein IX
	CD42b	Glycoprotein Ibα
	CD61	β3-integrin (glycoprotein IIIa)
	CD62P	P-selectin
	CD63	Lysosomal-associated membrane protein 3
	TSP1	Thrombospondin-1
	PAF	Platelet-activating factor
	β-amyloid precursor	
	Anticoagulant protein C/S	
	Complement C5b-9	

Table 6 (continued)

CELL-DERIVED MARKERS FOR MICROPARTICLE PHENOTYPING

Cellular origin	Markers	Alternative name
Endothelial cell	CD11a	Integrin αL
	CD34	Hematopoietic progenitor cell antigen 1
	CD54	Intercellular adhesion molecule-1
	CD62E	E-selectin
	CD51	α_V-integrin
	CD309	KDR or vascular endothelial growth factor 2
	CD105	Endoglin
	CD106	Vascular cell adhesion molecule-1
	CD133	Prominin 1 (also marker of stem cells)
	CD144	Cadherin-5
	CD146	Melanoma-associated antigen MUC-18
Inflammatory & others markers	CD31	Platelet endothelial cell adhesion molecule 1
	CD36	Scavenger receptor class B member 3
	CD71	Transferrin
	CD142	Tissue factor
	FasL	Fas ligand

Source: Own elaboration.

1.2.4.2.1. 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 (Chen *et al.,* 2008; Lawrie *et al.,* 2008; Mitchell *et al.,* 2008), which means that circulating miRNAs are selectively exported and packaged in order to avoid RNAses and prevent their degradation (Wang *et al.,* 2010). Indeed, miRNAs are protected against degradation not only (1) by their inclusion in protein and lipid microvesicles (exosomes and microparticles) (Valadi *et al.,* 2007; Camussi *et al.,* 2011) as well as in apoptotic bodies (Zernecke *et al.,* 2009), but also leaked into (2) RNA binding proteins (Argonaute 2 [Ago2]) (Arroyo *et al.,* 2011), or associated with (3) lipoproteins (high density lipoproteins) (Vickers *et al.,* 2011; Wagner *et al.,* 2011; Norata *et al.,* 2013), 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 (Pigati *et al.,* 2010), 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 (Kosaka *et al.,* 2010).

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 (Fabian, Sonenberg and Filipowicz, 2010). This regulation is dynamic; temporary, when miRNA release the mRNA or, permanent, causing mRNA strand degradation (Fabian, Sonenberg and Filipowicz, 2008). Thus, miRNAs have the ability to regulate a wide range of biological processes (Flowers and Aouizerat, 2013). Indeed, bioinformatic predictions indicate that mammalian miRNAs can regulate ≈30% of all protein-coding genes (Lewis *et al.,* 2005). 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 (Kozomara and Griffiths-Jones, 2011). Importantly, stable regulatory non-coding RNA was detected not only in plasma or serum (Chen *et al.,* 2008; Lawrie *et al.,* 2008; Mitchell *et al.,* 2008; Hunter *et al.,* 2008; Skog *et al.,* 2008) but also, similarly to MPs, in other body fluids including blood plasma, urine, tears, breast milk, amniotic fluid, cerebrospinal fluid, saliva, and semen (Cortez *et al.,* 2011; Hanke *et al.,* 2010; Park *et al.,* 2009; Weber *et al.,* 2009), varying their concentration in each one. miRNAs are likely found in these compartments because they are in the process of degradation, reabsorption, or excretion (Turchinovich *et al.,* 2011). However, their abundance and stability also implies a possible role in cell-cell communication (Turchinovich *et al.,* 2011; Lotvall and Valadi, 2007; Montecalvo *et al.,* 2012).

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 (Zhang *et al.,* 2012) or other means (fungal or bacterial origins) (Wang *et al.,* 2012). Epigenetic events are the sum of genetic risk factors and sustained environmental exposures that drive an organism towards adaptive responses to maintain homeostasis (Wahid *et al.,* 2010). 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 (Richardson *et al.,* 2013), among others (Wang *et al.,* 2012).

a. microRNA biogenesis

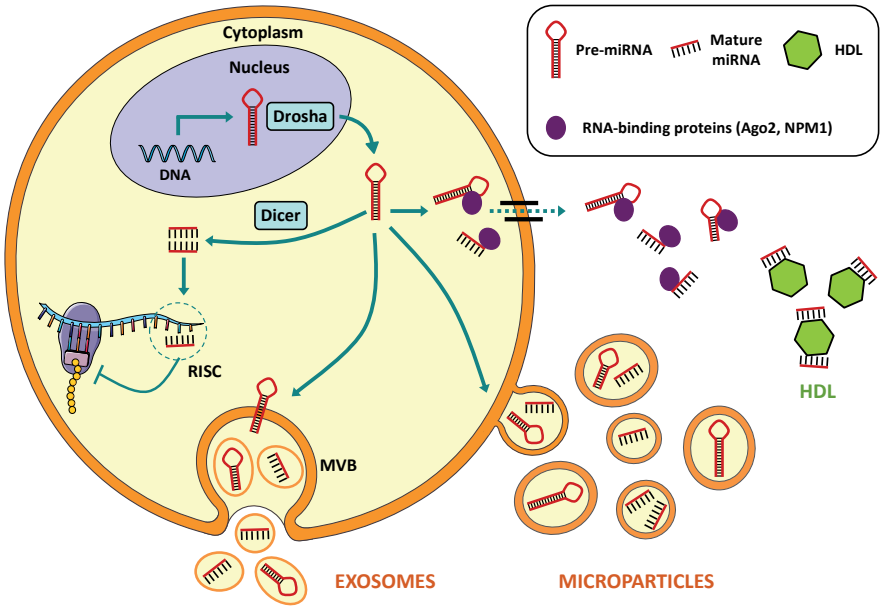
All miRNAs are originally transcribed from DNA by RNA polymerase II in the cell nucleus as primary miRNAs (pri-miRNAs) transcripts (Starega-Roslan *et al.,* 2011), which are cleaved and spliced into ≈70-100 nucleotide (nt) precursor miRNA (pre-miRNA) by Drosha complex (Lee *et al.,* 2002). Pre-miRNAs 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 (Erson and Petty, 2008). The maturity of miRNA depends on the separation of dsRNA by various helicases (Katahira and Yoneda, 2011), leaving one of the strands associated with an Ago2 protein, forming then the RNA-induced silence complex (RISC) (Braun, Huntzinger and Izaurralde, 2013). 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

Figure 14

EXTRACELLULAR MICRORNA BIOGENESIS



*Notes:*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).
Source: Own elaboration.

remains unknown. During RISC disassembling in the cytoplasm, miRNAs can be sorted into MVBs which by fusion with the plasma membrane form exosomes (Simons and Raposo, 2009) or directly packed into microparticles (Cocucci, Racchetti and Meldolesi, 2009; Muralidharan-Chari *et al.,* 2009). 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.

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 (Valadi *et al.,* 2007;. Skog *et al.,* 2008; Pegtel *et al.,* 2010). Indeed, the fact that cMV-miRNAs can be efficiently transferred to recipient cells and be functional has been demonstrated in endothelial cells (Zernecke *et al.,* 2009; Zhang *et al.,* 2010), fibroblasts (Morello *et al.,* 2013; Yuan *et al.,* 2010), peripheral blood mononuclear cells (Janowska-Wieczorek *et al.,* 2005), smooth muscle cells (Hergenreider *et al.,* 2012), antigen-presenting cells (Mittelbrunn *et al.,* 2011) and cardiomyocytes (Halkein *et al.,* 2013), 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 (Care *et al.,* 2007; Janssen *et al.,* 2013; Lee *et al.,* 2012; Selvamani *et al.,* 2012; Suckau *et al.,* 2009). To this respect, different studies have reported on the association of miRNAs with CV risk factors (Karolina *et al.,* 2012) and clinical conditions, such as myocardial infarction (D'Alessandra *et al.,* 2010; Wang *et al.,* 2010; Kuwabara *et al.,* 2011; Ai *et al.,* 2010; Adachi *et al.,* 2010; Cheng *et al.,* 2010; Corsten *et al.,* 2010; Devaux *et al.,* 2010; Meder *et al.,* 2011; Wang *et al.,* 2011) heart failure (Fukushima *et al.,* 2011; Tijsen *et al.,* 2010) and atherosclerosis (Fichtlscherer *et al.,* 2010; Taurino *et al.,* 2010; Hoekstra *et al.,* 2010), 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

Table 7

CIRCULATING MICRORNA IN CARDIOVASCULAR DISEASE

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

Notes: HCMV indicates human cytomegalovirus; PMN, polymorphonuclear leukocytes; UA, unstable angina.
Source: Own elaboration.

tool to monitor therapeutic treatments is under discussion (Flowers *et al.,* 2013). 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.

1.2.4.3. 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 (Weerheim *et al.,* 2002; Sadallah *et al.,*

2011). Notwithstanding lipid composition depends on the vascular milieu and/or inflammatory environment (Fourcade *et al.,* 1995) as well as the oxidation status of lipids (Huber *et al.,* 2002).

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 (Morel *et al.,* 2006; Sims *et al.,* 1988; Tan and Lip, 2005). Additionally, PS enhances the procoagulant activity of TF, one of the main cellular promoters of blood coagulation (Key, 2010). 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 (Sinauridze *et al.,* 2007). The interaction of membrane-PS with coagulation factors is inhibited by its affinity ligand annexin V (AV) in presence of calcium (Gidon-Jeangirard *et al.,* 1999). 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 (Jiménez *et al.,* 2003). 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 (Connor *et al.,* 2010; Beleznay *et al.,* 1993). Nevertheless, annexin V-negative MPs may be biologically distinct, as previously stated in the MP formation section.

1.2.4.4. Exosomes

Exosomes differed markedly from MPs in that contain different types of molecular cargo and less exposed PS (Bianco *et al.,* 2009; Thery *et al.,* 2001). 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 (Thery *et al.,* 1999; Van Niel *et al.,* 2006). 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.

1.2.5. 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).

1.2.5.1. 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.

1.2.5.2. 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 (Johnstone, 2006).

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 (Morel *et al.,* 2010).

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 (Owens and Mackman, 2011).

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 (Broze, 1995; Steppich *et al.,* 2005); thrombomodulin, endothelial protein C receptor, and activated protein C (Pérez-Casal *et al.,* 2005 and 2009; Tans *et al.,* 1991). 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 (Satta, Freyssinet and Toti, 1997). 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 (Dejouvencel *et al.,* 2010; Lacroix and Dignat-George, 2013; Lacroix *et al.,* 2007).

1.2.5.3. MPs in atherosclerosis

1.2.5.3.1. 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 (Amabile *et al.,* 2005; Brodsky *et al.,* 2004) and increase endothelial permeability (Densmore *et al.,* 2006). Besides, lymphocyte MPs from T cells increase ROS production and decrease NO synthesis in ECs (Mostefai, Agouni et al., 2008). Moreover, MPs isolated from metabolic syndrome (Agouni *et al.,* 2008) and CAD patients (Boulanger *et al.,* 2001) reduce in vitro NO production and increase superoxide dismutase expression in ECs, impairing endothelium-dependent relaxation.

1.2.5.3.2. 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 (Jy, Minagar *et al.,* 2004) and enhance macrophage adhesion (Burger *et al.,* 2011). In addition, pMPs were also found to enhance monocyte adhesion to the vascular endothelium (Barry *et al.,* 1998). 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 (Mause *et al.,* 2005; Vasina *et al.,* 2011; Wang *et al.,* 2011). pMPs also could favour leukocyte-leukocyte interactions through

P-selectin expression under flow conditions (Forlow, McEver and Nollert (2000). Furthermore, MPs from macrophages expressing CD40L may play essential roles in inflammation during plaque progression (Leroyer *et al.,* 2008). Lymphocyte-derived MPs can stimulate monocytes with cytokine production (Scanu *et al.,* 2008) and induce smooth muscle cell inflammation through nuclear factor kappa light chain (NF-κB) and ciclooxigenase-2 (COX-2) upregulation (Scanu *et al.,* 2008; Tesse *et al.,* 2005) Leukocyte-derived MPs might also stimulate cytokine release by endothelial cells, leading to increased proinflammatory activity (Mesri and Altieri, 1998 and 1999; Altieri, 1999) leukocyte recruitment and monocyte chemotaxis (Barry *et al.,* 1997).

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 (Priou *et al.,* 2010) and in mouse aorta (Meziani *et al.,* 2006). 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 (Barry *et al.,* 1998). Thus, these MPs may be implicated in the recruitment of inflammatory cells and in the promotion of atherosclerotic plaque progression (Rautou *et al.,* 2011).

1.2.5.3.3. 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 (Distler *et al.,* 2005; Huber *et al.,* 2007). Other studies revealed the presence of caspases in MPs. MPs from endothelial cells and platelets may also contain active executive caspase-3 (Abid *et al.,* 2007; Abid Hussein *et al.,* 2005; Boing *et al.,* 2008). And mMPs induce death of target cells by delivering caspase-1 (Sarkar *et al.,* 2009). Interestingly, MPs isolated from hypertensive patients induce H_2O_2 production, cellular senescence and apoptosis (Huang *et al.,* 2010).

1.2.5.3.4. Angiogenesis

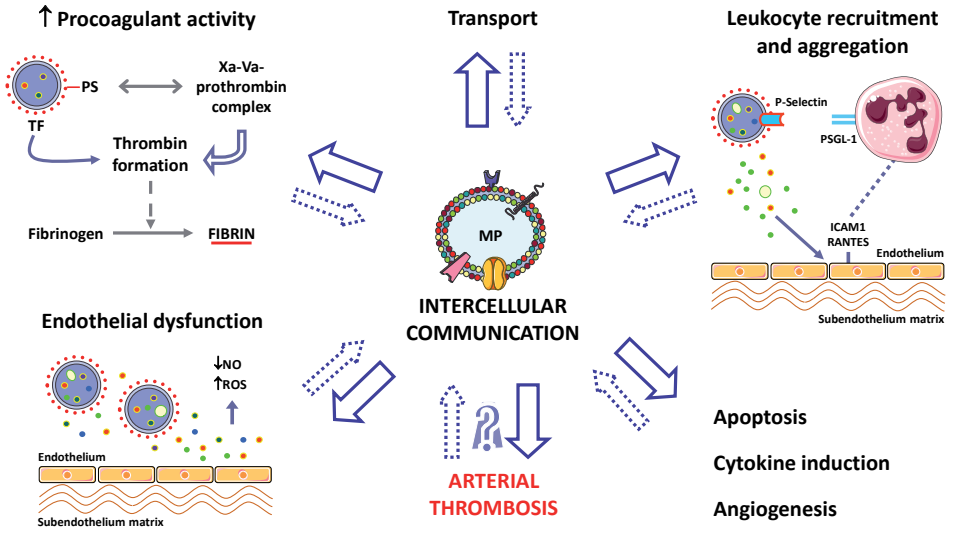
Microparticles could regulate endothelial proliferation and capillary tube formation. Platelet-derived microparticles have been shown to promote angiogenesis in a number of studies (Brill *et al.,* 2005; Kim *et al.,* 2004; Mause *et al.,* 2010). MPs from other cells may also influence angiogenesis. MPs from activated lymphocytes harbour Sonic hedgehog and induce angiogenesis (Benameur *et al.,* 2010; Soleti *et al.,* 2009; Soleti and Martinez, 2009). MPs isolated from atherosclerotic plaques might be involved in neovessel formation and the progression of plaques to a vulnerable state prone to rupture (Leroyer *et al.,* 2008). Nevertheless, contradictory results exists (Yang *et al.,* 2008; Ou *et al.,* 2011) and it has been suggested a balance between pro- and anti-angiogenic activities of MPs of different cell origin present in human plasma (Leroyer *et al.,* 2007).

1.2.5.4. 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 (Berckmans *et al.,* 2001), enhances platelet adhesion to the endothelium, and is important for tissue factor function (Del Conde *et al.,* 2005) and thrombin generation and clot formation (Morel *et al.,* 2006). TF-enriched MPs are taken up by platelets and induce platelet aggregation in the presence of factor VII (Lopez-Vilchez *et al.,* 2007). Besides, the interaction between MP PSGL-1 and platelet P-selectin that favour fibrin formation (Falati *et al.,* 2003) 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 (Falati *et al.,* 2002; Gross *et al.,* 2005). MPs bearing active TF, may accumulate and allow for unregulated thrombus formation and fibrin generation (Owens and Mackman, 2011) and thrombus propagation (Zwicker *et al.,* 2011) predisposing to thrombotic complications.

Figure 15

BIOLOGICAL FUNCTIONS OF MICROPARTICLES



Note: Due to their phenotypic 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.
Source: Own elaboration.

1.2.6. 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 8 (Donadee *et al.,* 2011;. Touyz *et al.,* 2011).
- Effects on the extracellular matrix by proteolytic enzymes of MPs, which are able to cleave signalling molecules (Canault *et al.,* 2007; Lozito and Tuan 2012).
- 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 (Terrisse *et al.,* 2010) as demonstrated by platelet (Lo *et al.,* 2006), endothelial (Terrisse *et al.,* 2010), smooth muscle (Essayagh *et al.,* 2005) and neutrophil-derived MPs (Pluskota *et al.,* 2008).

- 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* (Joly and Hudrisier, 2003) or internalized, through *endocytosis, phagocytosis* or *macropinocytosis* (Faille *et al.,* 2012), in a dose-dependent manner by macrophages (Rozmyslowicz *et al.,* 2003), endothelial cells (Tang *et al.,* 2010), and other cell types (Bebawy *et al.,* 2009). The endocytic uptake is differentially regulated in various cell types and mainly depends on the actin cytoskeleton, PI3K activity, and dynamin-2 function (Tian *et al.,* 2010; Barres *et al.,* 2010). As a featured example of the internalization process, platelets possess mechanisms to capture and incorporate TF-rich vesicles (Escolar *et al.,* 2008).

MP fusion and internalization can lead to:

- Transfer of their contents, including RNA (Diehl *et al.,* 2012), bioactive lipids and proteins (Rautou *et al.,* 2011) into the recipient cell. For instance, MPs can transfer fully operational surface receptors onto the recipient cells, such as CXC4R and CD41 of pMPs (Rozmyslowicz *et al.,* 2003).

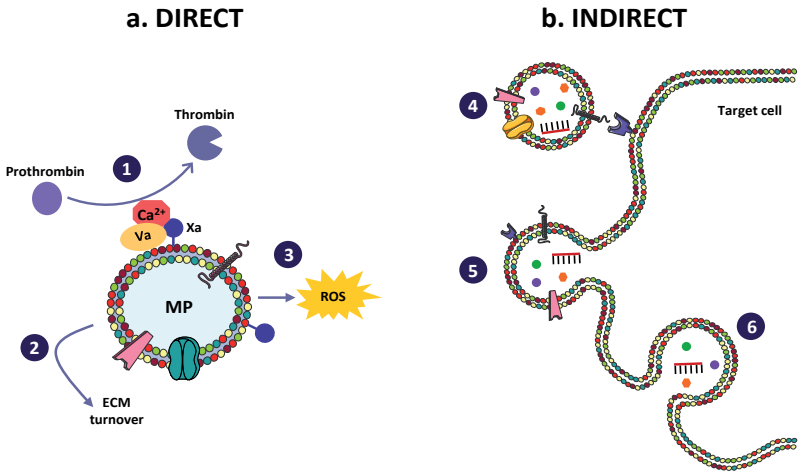
Interestingly, recent studies suggest a scenario, whereby microvesicular transfer would encompass multiple effectors at once (Skog *et al.,* 2008).

- 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



Notes: (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.
Source: Own elaboration.

1.2.7. 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 (Nomura *et al.,* 1998 and 2011) or their composition (Mobarrez *et al.,* 2011), the lowering of cMP load in the circulation may prove to be a novel therapeutic strategy for treatment (Murakami *et al.,* 2007). 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 (Essayagh *et al.,* 2005; Goto *et al.,* 2005; Morel *et al.,* 2004) acetylsalicylic acid (Bulut, Becker and Mugge, 2011), clopidogrel (Judge *et al.,* 2010), and ticlopidine (Nomura, Takahashi *et al.,* 2004; Shouzu *et al.,* 2004) have shown to reduce MP levels. Similarly, anti-hypertensive drugs like angiotensin II receptor antagonists (Labios *et al.,* 2010) and calcium channel blockers (Nomura *et al.,* 2005 and 2007), anti-oxidants (Morel *et al.,* 2003), and peroxisome proliferator-activator receptor (PPAR) activators (Esposito, Ciotola and Giugliano 2006; Kagawa *et al.,* 2001) 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 (Diamant *et al.,* 2008; Mobarrez *et al.,* 2012; Nomura *et al.,* 2008) others found that statin treatment promote MP inhibition (Nomura, Shouzu *et al.,* 2004; Sommeijer *et al.,* 2005; Tramontano *et al.,* 2004). Besides, there are currently under study a variety of inhibitors of MP release such as calpain inhibitors (Croce *et al.,* 1999; Fox *et al.,* 1990; Rendu *et al.,* 2001), ROCK inhibitors (Antonyak, Wilson and Cerione, 2012), TNFα inhibitor (Wassmer *et al.,* 2005), and pantethine and cystamine (Penet *et al.,* 2008). 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 (Van Dommelen *et al.,* 2011). 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 (Getts *et al.,* 2014). Nevertheless, further characterization of the biological effects of these MPs is warranted.

1.2.8. 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.

1.2.8.1. Preanalytical stages for microparticle isolation

Major causes of MP variability in analysis of blood samples include (Jy, Horstman *et al.,* 2004; Delobel *et al.,* 2010; Mullier *et al.,* 2013; Yuana, Bertina and Osanto, 2011):

- *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 (Lacroix *et al.,* 2012).
- *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 (Jayachandran *et al.,* 2012). 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 (George *et al.,* 1982). 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.

1.2.8.2. 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.

Table 8

CENTRIFUGATION CONDITIONS FOR MP OBTENTION

Centrifugation protocol			
Platelet free plasma	Microparticles	Technique	References
1500 xg 15′ 13000 xg 1′	100000 g x 90′ 10ºC	FC, EM	Combes *et al.* (1999)
1500 xg 20′ 20ºC	17500 g x 20′ 20ºC	FC	Nieuwland *et al.* (2000)
1500 xg 20′ 20ºC	17570 g x 30′ 20ºC	FC	Joop *et al.* (2001)
1500 xg 20′ 20ºC	17570 g x 30′ 20ºC	FC	Diamant *et al.* (2002)
1500 xg 10′ 13000 xg 2′ 20ºC	-	FC	Sabatier *et al.* (2002)
1500 xg 10′ 20ºC 1300 xg 20′ 20ºC	100000 g x 60′ 2 times 20ºC	FC, ELISA, PCA	Shet *et al.* (2003)
1500 xg 10′	-	FC	Preston *et al.* (2003)
160 xg 10′ 1000 xg 6′	-	FC	Bernal-Mizrachi *et al.* (2003)
1550 xg 20′ 20ºC	18000 g x 30′ 20ºC	FC	Biró *et al.* (2003)
2700 xg 2 times	19800 g x 10′ 20ºC	FC	Simak *et al.* (2004)

Table 8 (continued)

CENTRIFUGATION CONDITIONS FOR MP OBTENTION

Centrifugation protocol			
Platelet free plasma	Microparticles	Technique	References
710 xg 15′	150000 g x 90′ 4ºC	FC, Proteomics	García *et al.* (2005)
3200 xg 30′	250000 g x 1h	FC, Proteomics	Jin *et al.* (2005)
160 xg 10′ 1000 xg 8′	-	FC	Chirinos *et al.* (2005)
1550 xg 20′ 20ºC	-	FC	Tushuizen *et al.* (2006)
13000 xg 2′ 20ºC	50000 g x 45′ 4ºC	FC, Functional assays	Keuren *et al.* (2007)
1500 xg 20′ 20ºC	-	ELISA	Goichot *et al.* (2006)
13000 xg 2′	-	FC	Werner *et al.* (2006)
1500 xg 20′ 20ºC	17570 g x 30′ 20ºC	FC	Van der Zee et al. (2006)
786 xg 15′ 20ºC 13000 xg 2′ 20ºC	18000 g x 45′	FC	Magdeleyns *et al.* (2007)
710 xg 15′ 20ºC	150000 g x 90′ 10ºC	FC, Proteomics	Smalley *et al.* (2007)
500 xg 15′ 9500 xg 5′	-	FC	Heiss *et al.* (2008)
1500 xg 20′ 20ºC	-	FC	Bernard *et al.* (2009)

Notes: 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.
Source: Own elaboration.

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.

1.2.8.3. 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.

1.2.8.3.1. Standard methods

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

- *Flow cytometry* (FC) (Nieuwland *et al.,* 2000; Yun *et al.,* 2010; Tantawy *et al.,* 2013) 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 (Perez-Pujol, Marker and Key, 2007). 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 (Keuren *et al.,* 2007). 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) (Simak J, Gelderman *et al.,* 2006; Mrvar-Brecko *et al.,* 2010) 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* (Combes *et al.,* 1999) 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* (Rubin *et al.,* 2010). 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* (Huang *et al.,* 2007) and *high performance liquid chromatography* (Valadi *et al.,* 2007) that is the method of choice for lipid content.

1.2.8.3.2. 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) (Headland *et al.,* 2014).

- *Dynamic light scattering* (DLS) (Lawrie *et al.,* 2009; Maurer-Spurej *et al.,* 2009; Xu, Nakane and Maurer-Spurej, 2011). 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) (Yuana, Bertina and Osanto, 2011; Leong *et al.,* 2011) 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.

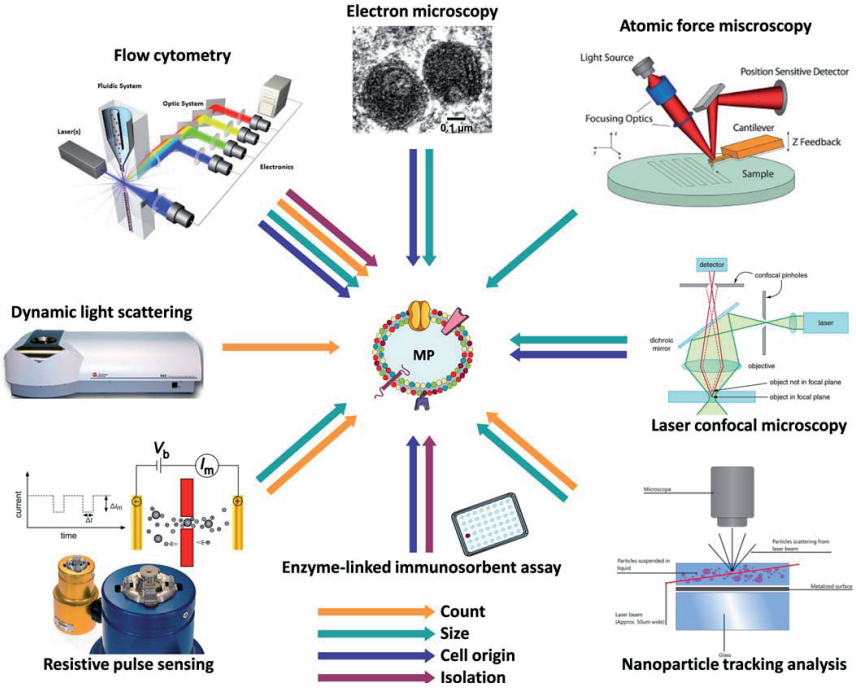
- *Atomic force microscopy* (AFM) (Yuana, Bertina and Osanto, 2011; Dragovic *et al.,* 2011) allows performing a nanoscale measurement of individual MPs. In AFM, a mechanical cantilever is passed over a surface, with deflections indicating the presence of surface structures. With the possibility of

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) (Van der Pol *et al.,* 2013), 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.

Figure 17

MICROPARTICLE DETECTION METHODS



Notes: 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
Source: Own elaboration.

- *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 (Van der Pol *et al.,* 2013).

1.3. 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.

REFERENCES

Aatonen, M.; Gronholm, M., and Siljander, P. R. (2012), "Platelet-derived microvesicles: multitalented participants in intercellular communication," *Semin Thromb Hemost,* 38(1): 102-113.

Abdel-Monem, H. *et al.* (2010), "Phagocytosis of platelet microvesicles and beta2- glycoprotein I," *Thromb Haemost,* 104(2): 335-341.

Abid Hussein, M. N. *et al.* (2003), "Antigenic characterization of endothelial cell-derived microparticles and their detection ex vivo," *J Thromb Haemost,* 1(11): 2434-2443.

Abid Hussein, M. N. *et al.* (2005), "Cell-derived microparticles contain caspase 3 in vitro and in vivo," *J Thromb Haemost,* 3(5): 888-896.

Abid Hussein, M. N. *et al.* (2007), "Inhibition of microparticle release triggers endothelial cell apoptosis and detachment," *Thromb Haemost,* 98(5).

Abrahams, V. M. *et al.* (2003), "Epithelial ovarian cancer cells secrete functional Fas ligand," *Cancer Res,* 63(17): 5573-5581.

Adachi, T. *et al.* (2010), "Plasma microRNA 499 as a biomarker of acute myocardial infarction," *Clin Chem,* 56(7): 1183-1185.

Admyre, C. *et al.* (2003), "Exosomes with major histocompatibility complex class II and co-stimulatory molecules are present in human BAL fluid," *Eur Respir J,* 22(4): 578-583.

Agouni, A. *et al.* (2008), "Endothelial dysfunction caused by circulating microparticles from patients with metabolic syndrome," *Am J Pathol,* 173(4): 1210-1219.

Ai, J. *et al.* (2010), "Circulating microRNA-1 as a potential novel biomarker for acute myocardial infarction," *Biochem Biophys Res Commun,* 391(1): 73-77.

Altieri, D. C. (1999), "Regulation of leukocyte-endothelium interaction by fibrinogen," *Thromb Haemost,* 82(2): 781-786.

Alwan, A. (2010), "Global status report on noncommunicable disaeses," *Noncommunicable diseases and mental health,* Geneva, World Health Organization: 176.

Amabile, N. *et al.* (2005), "Circulating endothelial microparticles are associated with vascular dysfunction in patients with end-stage renal failure," *J Am Soc Nephrol,* 16(11): 3381-3388.

Amabile, N. *et al.* (2008), "Circulating endothelial microparticle levels predict hemodynamic severity of pulmonary hypertension," *Am J Respir Crit Care Med,* 177(11): 1268-1275.

Anderson, R. G., and Jacobson, K. (2002), "A role for lipid shells in targeting proteins to caveolae, rafts, and other lipid domains," *Science,* 296(5574): 1821-1825.

Ando, M. *et al.* (2002), "Circulating platelet-derived microparticles with procoagulant activity may be a potential cause of thrombosis in uremic patients," *Kidney Int,* 62(5): 1757-1763.

Antonyak, M. A.; Wilson, K. F., and Cerione, R. A. (2012), "R(h)oads to microvesicles," *Small GTPases,* 3(4): 219-224.

Aras, O. et al. (2004), "Induction of microparticle- and cell-associated intravascular tissue factor in human endotoxemia," *Blood,* 103(12): 4545-4553.

Ardoin, S. P.; Shanahan, J. C., and Pisetsky, D. S. (2007), "The role of microparticles in inflammation and thrombosis," *Scand J Immunol,* 66(2-3): 159-165.

Arroyo, J. D. *et al.* (2011), "Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma," *Proc Natl Acad Sci U S A,* 108(12): 5003-5008.

Arteaga, R. B. *et al.* (2006), "Endothelial microparticles and platelet and leukocyte activation in patients with the metabolic syndrome," *Am J Cardiol,* 98(1): 70-74.

Aupeix, K. *et al.* (1997), "The significance of shed membrane particles during programmed cell death in vitro, and in vivo, in HIV-1 infection," *J Clin Invest,* 99(7): 1546-1554.

Ayers, L. *et al.* (2009), "Circulating cell-derived microparticles in patients with minimally symptomatic obstructive sleep apnoea," *Eur Respir J,* 33(3): 574-580.

Azzam, H., and Zagloul, M. (2009), "Elevated platelet microparticle levels in valvular atrial fibrillation," *Hematology,* 14(6): 357-360.

Badimon, L. (2012), "Interleukin-18: a potent pro-inflammatory cytokine in atherosclerosis," *Cardiovasc Res,* 96(2): 172-175; discussion 176-180.

Badimon, L., and Badimon, J. J. (1989), "Mechanisms of arterial thrombosis in nonparallel streamlines: platelet thrombi grow on the apex of stenotic severely injured vessel wall. Experimental study in the pig model," *J Clin Invest,* 84(4): 1134-1144.

Badimon, L. *et al.* (1999), "A sudden increase in plasma epinephrine levels transiently enhances platelet deposition on severely damaged arterial wall--studies in a porcine model," *Thromb Haemost,* 82(6): 1736-1742.

Badimon, L. *et al.* (2002), "Pathogenesis of the acute coronary syndromes and therapeutic implications," *Pathophysiol Haemost Thromb,* 32(5-6): 225-231.

Badimon, L.; Storey, R. F., and Vilahur, G. (2011), "Update on lipids, inflammation and atherothrombosis," *Thromb Haemost,* 105 Suppl 1: S34-42.

Baigent, C. *et al.* (2005), "Efficacy and safety of cholesterol-lowering treatment: prospective meta-analysis of data from 90,056 participants in 14 randomised trials of statins," *Lancet,* 366(9493): 1267-1278.

Bal, L. *et al.* (2010), "Factors influencing the level of circulating procoagulant microparticles in acute pulmonary embolism," *Arch Cardiovasc Dis,* 103(6-7): 394-403.

Balasubramanian, K., and Schroit, A. J. (2003), "Aminophospholipid asymmetry: A matter of life and death," *Annu Rev Physiol,* 65: 701-734.

Banz, Y. *et al.* (2008), "CD39 is incorporated into plasma microparticles where it maintains functional properties and impacts endothelial activation," *Br J Haematol,* 142(4): 627-637.

Bard, M. P. *et al.* (2004), "Proteomic analysis of exosomes isolated from human malignant pleural effusions," *Am J Respir Cell Mol Biol,* 31(1): 114-121.

Barres, C. *et al.* (2010), "Galectin-5 is bound onto the surface of rat reticulocyte exosomes and modulates vesicle uptake by macrophages," *Blood,* 115(3): 696-705.

Barry, O. P. *et al.* (1997), "Transcellular activation of platelets and endothelial cells by bioactive lipids in platelet microparticles," J Clin Invest, 99(9): 2118-2127.

Barry, O. P. *et al.* (1998), "Modulation of monocyte-endothelial cell interactions by platelet microparticles," *J Clin Invest,* 102(1): 136-144.

Bebawy, M. *et al.* (2009), "Membrane microparticles mediate transfer of P-glycoprotein to drug sensitive cancer cells," *Leukemia,* 23(9): 1643-1649.

Beckman, J. A. *et al.* (2001), "Relationship of clinical presentation and calcification of culprit coronary artery stenoses," *Arterioscler Thromb Vasc Biol,* 21(10): 1618-1622.

Beleznay, Z. *et al.* (1993), "Inhibition of aminophospholipid translocation in red cell vesicles," *Biochem Soc Trans,* 21(2): 76S.

Benameur, T. *et al.* (2010), "Microparticles carrying Sonic hedgehog favor neovascularization through the activation of nitric oxide pathway in mice," *PLoS One,* 5(9): e12688.

Ben-Hadj-Khalifa-Kechiche, S. *et al.* (2010), "Differential inhibitory effect of fondaparinux on the procoagulant potential of intact monocytes and monocyte-derived microparticles," *J Thromb Thrombolysis,* 30(4): 412-418.

Berckmans, R. J. *et al.* (2001), "Cell-derived microparticles circulate in healthy humans and support low grade thrombin generation," *Thromb Haemost,* 85(4): 639-646.

Berckmans, R. J. *et al.* (2002), "Cell-derived microparticles in synovial fluid from inflamed arthritic joints support coagulation exclusively via a factor VII-dependent mechanism," *Arthritis Rheum,* 46(11): 2857-2866.

Berckmans, R. J. *et al.* (2011), "Cell-derived vesicles exposing coagulant tissue factor in saliva," *Blood,* 117(11): 3172-3180.

Berenson, G. S. *et al.* (1998), "Association between multiple cardiovascular risk factors and atherosclerosis in children and young adults. The Bogalusa Heart Study," *N Engl J Med,* 338(23): 1650-1656.

Bernal-Mizrachi, L. *et al.* (2003), "High levels of circulating endothelial microparticles in patients with acute coronary syndromes," *Am Heart J,* 145(6): 962-970.

Bernal-Mizrachi, L. *et al.* (2004), "Endothelial microparticles correlate with high-risk angiographic lesions in acute coronary syndromes," *Int J Cardiol,* 97(3): 439-446.

Bernard, S. *et al.* (2009), "Increased levels of endothelial microparticles CD144 (VE-Cadherin) positives in type 2 diabetic patients with coronary noncalcified plaques evaluated by multidetector computed tomography (MDCT)," *Atherosclerosis,* 203(2): 429-435.

Bevers, E. M., and Williamson, P. L. (2010), "Phospholipid scramblase: an update," *FEBS Lett,* 584(13): 2724-2730.

Bianco, F. *et al.* (2009), "Acid sphingomyelinase activity triggers microparticle release from glial cells," *Embo J,* 28(8): 1043-1054.

Bilyy, R. O. *et al.* (2012), "Macrophages discriminate glycosylation patterns of apoptotic cell-derived microparticles," *J Biol Chem,* 287(1): 496-503.

Biró, E. *et al.* (2003), "Human cell-derived microparticles promote thrombus formation in vivo in a tissue factor-dependent manner," *J Thromb Haemost,* 1(12): 2561-2568.

Biró, E. *et al.* (2005), "The phospholipid composition and cholesterol content of platelet-derived microparticles: a comparison with platelet membrane fractions," *J Thromb Haemost,* 3(12): 2754-2763.

Blankenberg, S. *et al.* (2002), "Interleukin-18 is a strong predictor of cardiovascular death in stable and unstable angina," *Circulation,* 106(1): 24-30.

Boilard, E. *et al.* (2010), "Platelets amplify inflammation in arthritis via collagen-dependent microparticle production," *Science,* 327(5965): 580-583.

Boing, A. N. *et al.* (2008), "Platelet microparticles contain active caspase 3," Platelets, 19(2): 96-103.

Bombeli, T.; Schwartz, B. R., and Harlan, J. M. (1998), "Adhesion of activated platelets to endothelial cells: evidence for a GPIIbIIIa-dependent bridging mechanism and novel roles for endothelial intercellular adhesion molecule 1 (ICAM-1), alphavbeta3 integrin, and GPIbalpha," *J Exp Med,* 187(3): 329-339.

Boulanger, C. M. *et al.* (2001), "Circulating microparticles from patients with myocardial infarction cause endothelial dysfunction," *Circulation,* 104(22): 2649-2652.

Boulanger, C. M. *et al.* (2007), "In vivo shear stress determines circulating levels of endothelial microparticles in end-stage renal disease," *Hypertension,* 49(4): 902-908.

Braun, J. E.; Huntzinger, E., and Izaurralde, E. (2013), "The role of GW182 proteins in miRNA-mediated gene silencing," *Adv Exp Med Biol,* 768: 147-163.

Brennan, M. L. *et al.* (2003), "Prognostic value of myeloperoxidase in patients with chest pain," *N Engl J Med,* 349(17): 1595-1604.

Bretelle, F. *et al.* (2003), "Circulating microparticles: a marker of procoagulant state in normal pregnancy and pregnancy complicated by preeclampsia or intrauterine growth restriction," *Thromb Haemost,* 89(3): 486-492.

Brill, A. *et al.* (2005), "Platelet-derived microparticles induce angiogenesis and stimulate post-ischemic revascularization," *Cardiovasc Res,* 67(1): 30-38.

Brodsky, S. V. *et al.* (2002), "Plasminogen activator inhibitor-1 promotes formation of endothelial microparticles with procoagulant potential," *Circulation,* 106(18): 2372-2378.

Brodsky, S. V. *et al.* (2004), "Endothelium-derived microparticles impair endothelial function in vitro," Am *J Physiol Heart Circ Physiol,* 286(5): H1910-1915.

Brogan, P. A., and Dillon, M. J. (2004), "Endothelial microparticles and the diagnosis of the vasculitides," *Intern Med,* 43(12): 1115-1119.

Brown, M. D. *et al.* (2011), "Racial differences in tumor necrosis factor-alpha-induced endothelial microparticles and interleukin-6 production," *Vasc Health Risk Manag,* 7: 541-550.

Broze, G. J., Jr. (1995), "Tissue factor pathway inhibitor," *Thromb Haemost,* 74(1): 90-93.

Bulut, D.; Becker, V., and Mugge, A. (2011), "Acetylsalicylate reduces endothelial and platelet-derived microparticles in patients with coronary artery disease," *Can J Physiol Pharmacol,* 89(4): 239-244.

Burger, D. *et al.* (2011), "Endothelial microparticle formation by angiotensin II is mediated via Ang II receptor type I/NADPH oxidase/ Rho kinase pathways targeted to lipid rafts," *Arterioscler Thromb Vasc Biol,* 31(8): 1898-1907.

Burke, A. P. *et al.* (2001), "Healed plaque ruptures and sudden coronary death: evidence that subclinical rupture has a role in plaque progression," *Circulation,* 103(7): 934-940.

Burnier, L. *et al.* (2009), "Cell-derived microparticles in haemostasis and vascular medicine," *Thromb Haemost,* 101(3): 439-451.

Caballero, P. *et al.* (2012), "Detection of subclinical atherosclerosis in familial hypercholesterolemia using non-invasive imaging modalities," *Atherosclerosis,* 222(2): 468-472.

Caligiuri, G. *et al.* (2002), "Protective immunity against atherosclerosis carried by B cells of hypercholesterolemic mice," *J Clin Invest,* 109(6): 745-753.

Camussi, G. *et al.* (2011), "Exosome/microvesicle-mediated epigenetic reprogramming of cells," *Am J Cancer Res,* 1(1): 98-110.

Canault, M. *et al.* (2007), "Microparticles of human atherosclerotic plaques enhance the shedding of the tumor necrosis factor-alpha converting enzyme/ADAM17 substrates, tumor necrosis factor and tumor necrosis factor receptor-1," *Am J Pathol,* 171(5): 1713-1723.

Care, A. *et al.* (2007), "MicroRNA-133 controls cardiac hypertrophy," *Nat Med,* 13(5): 613-618.

Castaman, G. *et al.* (1997), "Characterization of a novel bleeding disorder with isolated prolonged bleeding time and deficiency of platelet microvesicle generation," *Br J Haematol,* 96(3): 458-463.

Castaman, G.; Yu-Feng, L., and Rodeghiero, F. (1996), "A bleeding disorder characterised by isolated deficiency of platelet microvesicle generation," *Lancet,* 347(9002): 700-701.

Castelli, W. P. *et al.* (1992), "Lipids and risk of coronary heart disease. The Framingham Study," *Ann Epidemiol,* 2(1-2): 23-28.

Cauwenberghs, S. *et al.* (2006), "Shedding of procoagulant microparticles from unstimulated platelets by integrin-mediated destabilization of actin cytoskeleton," *FEBS Lett,* 580(22): 5313-5320.

Cerri, C. *et al.* (2006), "Monocyte/macrophage-derived microparticles up-regulate inflammatory mediator synthesis by human airway epithelial cells," *J Immunol,* 177(3): 1975-1980.

Chaar, V. *et al.* (2011), "Effect of strenuous physical exercise on circulating cell-derived microparticles," *Clin Hemorheol Microcirc,* 47(1): 15-25.

Chalikias, G. K. *et al.*(2007), "Interleukin-18/interleukin-10 ratio is an independent predictor of recurrent coronary events during a 1-year follow-up in patients with acute coronary syndrome," *Int J Cardiol,* 117(3): 333-339.

Chamouard, P. *et al.* (2005), "Circulating cell-derived microparticles in Crohn's disease," *Dig Dis Sci,* 50(3): 574-580.

Chapman, M. J. (2007), "From pathophysiology to targeted therapy for atherothrombosis: a role for the combination of statin and aspirin in secondary prevention," *Pharmacol Ther,* 113(1): 184-196.

Chen, X. *et al.* (2008), "Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases," *Cell Res,* 18(10): 997-1006.

Cheng, Y. *et al.* (2010), "A translational study of circulating cell-free microRNA-1 in acute myocardial infarction," *Clin Sci (Lond),* 119(2): 87-95.

Chirinos, J. A. *et al.* (2005), "Elevation of endothelial microparticles, platelets, and leukocyte activation in patients with venous thromboembolism," *J Am Coll Cardiol,* 45(9): 1467-1471.

Chironi, G. *et al.* (2006), "Circulating leukocyte-derived microparticles predict subclinical atherosclerosis burden in asymptomatic subjects," *Arterioscler Thromb Vasc Biol,* 26(12): 2775-2780.

Chu, S. G. *et al.* (2010), "Mean platelet volume as a predictor of cardiovascular risk: a systematic review and meta-analysis," *J Thromb Haemost,* 8(1): 148-156.

Chung, A. W. *et al.* (2004), "Platelet-leukocyte aggregation induced by PAR agonists: regulation by nitric oxide and matrix metalloproteinases," *Br J Pharmacol,* 143(7): 845-855.

Chung, I., and Lip, G. Y. (2003), "Virchow's triad revisited: blood constituents," *Pathophysiol Haemost Thromb,* 33(5-6): 449-454.

Coccheri, S. (1998), "Distribution of symptomatic atherothrombosis and influence of atherosclerotic disease on risk of secondary ischaemic events: Results from CAPRIE," *Eur Heart J, 19(Suppl)*: 227.

Cocucci, E.; Racchetti, G., and Meldolesi, J. (2009), "Shedding microvesicles: artefacts no more," *Trends Cell Biol,* 19(2): 43-51.

Collinson, P. O. (1998), "Troponin T or troponin I or CK-MB (or none?)," *Eur Heart J,* 19 Suppl N: N16-24.

Collinson, P. O.; Wiggins, N., and Gaze, D. C. (2001), "Clinical evaluation of the ACS:180 cardiac troponin I assay," *Ann Clin Biochem,* 38(Pt 5): 509-519.

Combes, V. *et al.* (1997), "A new flow cytometry method of platelet-derived microvesicle quantitation in plasma," *Thromb Haemost,* 77(1): 220.

Combes, V. *et al.* (1999), "In vitro generation of endothelial microparticles and possible prothrombotic activity in patients with lupus anticoagulant," *J Clin Invest,* 104(1): 93-102.

Connor, D. E. *et al.* (2010), "The majority of circulating platelet-derived microparticles fail to bind annexin V, lack phospholipid-dependent procoagulant activity and demonstrate greater expression of glycoprotein Ib," *Combes,* 103(5): 1044-1052.

Corsten, M. F. *et al.* (2010), "Circulating MicroRNA-208b and MicroRNA-499 reflect myocardial damage in cardiovascular disease," *Circ Cardiovasc Genet,* 3(6): 499-506.

Cortez, M. A. *et al.* (2011), "MicroRNAs in body fluids--the mix of hormones and biomarkers," *Nat Rev Clin Oncol,* 8(8): 467-477.

Crawford, N. (1971), "The presence of contractile proteins in platelet microparticles isolated from human and animal platelet-free plasma," *Br J Haematol,* 21(1): 53-69.

Crea, F., and Liuzzo, G. (2013), "Pathogenesis of acute coronary syndromes," *J Am Coll Cardiol,* 61(1): 1-11.

Crespin, M. *et al.* (2009), "Activation of PAK1/2 during the shedding of platelet microvesicles," *Blood Coagul Fibrinolysis,* 20(1): 63-70.

Cristell, N. *et al.* (2011), "High-sensitivity C-reactive protein is within normal levels at the very onset of first ST-segment elevation acute myocardial infarction in 41% of cases: a multiethnic case-control study," *J Am Coll Cardiol,* 58(25): 2654-2661.

Croce, K. *et al.* (1999), "Inhibition of calpain blocks platelet secretion, aggregation, and spreading," *J Biol Chem,* 274(51): 36321-36327.

Cubedo, J. *et al.* (2012), "Differential proteomic distribution of TTR (pre-albumin) forms in serum and HDL of patients with high cardiovascular risk," *Atherosclerosis,* 222(1): 263-269.

Curtis, A. M. *et al.* (2009), "p38 mitogen-activated protein kinase targets the production of proinflammatory endothelial microparticles," *J Thromb Haemost,* 7(4): 701-709.

Dachary-Prigent, J. *et al.* (1993), "Annexin V as a probe of aminophospholipid exposure and platelet membrane vesiculation: a flow cytometry study showing a role for free sulfhydryl groups," *Blood,* 81(10): 2554-2565.

Dachary-Prigent, J. *et al.* (1995), "Calcium involvement in aminophospholipid exposure and microparticle formation during platelet activation: a study using Ca2+-ATPase inhibitors," *Biochemistry,* 34(36): 11625-11634.

Dai, S. *et al.* (2008), "Phase I clinical trial of autologous ascites-derived exosomes combined with GM-CSF for colorectal cancer," *Mol Ther,* 16(4): 782-790.

Daleke, D. L. (2003), "Regulation of transbilayer plasma membrane phospholipid asymmetry," *J Lipid Res,* 44(2): 233-242.

D'Alessandra, Y. *et al.* (2010), "Circulating microRNAs are new and sensitive biomarkers of myocardial infarction," *Eur Heart J,* 31(22): 2765-2773.

Danesh, J. *et al.* (1998), "Association of fibrinogen, C-reactive protein, albumin, or leukocyte count with coronary heart disease: meta-analyses of prospective studies," *Jama,* 279(18): 1477-1482.

Daniel, L. *et al.* (2006), "Increase of circulating neutrophil and platelet microparticles during acute vasculitis and hemodialysis," *Kidney Int,* 69(8): 1416-1423.

Daniels, L. B. *et al.* (2011), "Growth-differentiation factor-15 is a robust, independent predictor of 11-year mortality risk in community-dwelling older adults: the Rancho Bernardo Study," *Circulation*, 123(19): 2101-2110.

Darnige, L. *et al.* (2010), "Platelet microparticle levels: a biomarker of thromboangiitis obliterans (Buerger's disease) exacerbation," *J Cell Mol Med,* 14(1-2): 449-451.

Dasgupta, S. K. *et al.* (2009), "Lactadherin and clearance of platelet-derived microvesicles," *Blood,* 113(6): 1332-1339.

Dasgupta, S. K. *et al.* (2012), "Developmental endothelial locus-1 (Del-1) mediates clearance of platelet microparticles by the endothelium," *Circulation,* 125(13): 1664-1672.

Dashevsky, O.; Varon, D., and Brill, A. (2009), "Platelet-derived microparticles promote invasiveness of prostate cancer cells via upregulation of MMP-2 production," *Int J Cancer,* 124(8): 1773-1777.

De Kleijn, D. P. *et al.* (2010), "Local atherosclerotic plaques are a source of prognostic biomarkers for adverse cardiovascular events," *Arterioscler Thromb Vasc Biol,* 30(3): 612-619.

Dean, W. L. *et al.* (2009), "Proteomic and functional characterisation of platelet microparticle size classes," *Thromb Haemost,* 102(4): 711-718.

Dejouvencel, T. *et al.* (2010), "Fibrinolytic cross-talk: a new mechanism for plasmin formation," *Blood,* 115(10): 2048-2056.

Del Conde, I. *et al.* (2005), "Tissue-factor-bearing microvesicles arise from lipid rafts and fuse with activated platelets to initiate coagulation," *Blood,* 106(5): 1604-1611.

Del Turco, S. *et al.* (2007), "Parallel decrease of tissue factor surface exposure and increase of tissue factor microparticle release by the n-3 fatty acid docosahexaenoate in endothelial cells," *Thromb Haemost,* 98(1): 210-219.

Delobel, J. *et al.* (2010), "Biomarker analysis of stored blood products: emphasis on pre-analytical issues," *Int J Mol Sci,* 11(11): 4601-4617.

Densmore, J. C. *et al.* (2006), "Endothelium-derived microparticles induce endothelial dysfunction and acute lung injury," *Shock,* 26(5): 464-471.

Devaraj, S.; Kumaresan, P. R., and Jialal, I. (2011), "C-reactive protein induces release of both endothelial microparticles and circulating endothelial cells in vitro and in vivo: further evidence of endothelial dysfunction," *Clin Chem,* 57(12): 1757-1761.

Devaux, Y. *et al.* (2012), "Use of circulating microRNAs to diagnose acute myocardial infarction," *Clin Chem,* 58(3): 559-567.

Diamant, M. *et al.* (2002), "Elevated numbers of tissue-factor exposing microparticles correlate with components of the metabolic syndrome in uncomplicated type 2 diabetes mellitus," *Circulation,* 106(19): 2442-2447.

Diamant, M. *et al.* (2008), "Simvastatin-induced endothelial cell detachment and microparticle release are prenylation dependent," *Thromb Haemost,* 100(3): 489-497.

Diehl, P. *et al.* (2008), "Increased levels of circulating microparticles in patients with severe aortic valve stenosis," *Thromb Haemost,* 99(4): 711-719.

Diehl, P. *et al.* (2011), "Increased platelet, leukocyte and endothelial microparticles predict enhanced coagulation and vascular inflammation in pulmonary hypertension," *J Thromb Thrombolysis,* 31(2): 173-179.

Diehl, P. *et al.* (2012), "Microparticles: major transport vehicles for distinct microRNAs in circulation," *Cardiovasc Res,* 93(4): 633-644.

Dignat-George, F. *et al.* (2004), "Endothelial microparticles: a potential contribution to the thrombotic complications of the antiphospholipid syndrome," *Thromb Haemost,* 91(4): 667-673.

Distler, J. H. *et al.* (2005), "The release of microparticles by apoptotic cells and their effects on macrophages," *Apoptosis,* 10(4): 731-741.

Doeuvre, L. *et al.* (2009), "Cell-derived microparticles: a new challenge in neuroscience," *J Neurochem,* 110(2): 457-468.

Donadee, C. *et al.* (2011), "Nitric oxide scavenging by red blood cell microparticles and cell-free hemoglobin as a mechanism for the red cell storage lesion," *Circulation,* 124(4): 465-476.

Dragovic, R. A. *et al.* (2011), "Sizing and phenotyping of cellular vesicles using Nanoparticle Tracking Analysis," *Nanomedicine,* 7(6): 780-788.

Drechsler, M. *et al.* (2010), "Hyperlipidemia-triggered neutrophilia promotes early atherosclerosis," *Circulation,* 122(18): 1837-1845.

Dursun, I. *et al.* (2009), "The relationship between circulating endothelial microparticles and arterial stiffness and atherosclerosis in children with chronic kidney disease," *Nephrol Dial Transplant,* 24(8): 2511-2518.

Ehara, S. *et al.* (2004), "Spotty calcification typifies the culprit plaque in patients with acute myocardial infarction: an intravascular ultrasound study," *Circulation,* 110(22): 3424-3429.

Elmore, S. (2007), "Apoptosis: a review of programmed cell death," *Toxicol Pathol,* 35(4): 495-516.

Erdbruegger, U. *et al.* (2008), "Diagnostic role of endothelial microparticles in vasculitis," *Rheumatology (Oxford),* 47(12): 1820-1825.

Erson, A. E., and Petty, E. M. (2008), "MicroRNAs in development and disease," *Clin Genet,* 74(4): 296-306.

Escolar, G. *et al.* (2008), "Internalization of tissue factor by platelets," *Thromb Res,* 122 Suppl 1: S37-41.

Esposito, K.; Ciotola, M., and Giugliano, D. (2006), "Pioglitazone reduces endothelial microparticles in the metabolic syndrome," *Arterioscler Thromb Vasc Biol,* 26(8): 1926.

Essayagh, S. *et al.* (2005), "Microparticles from apoptotic vascular smooth muscle cells induce endothelial dysfunction, a phenomenon prevented by beta3-integrin antagonists," *Thromb Haemost,* 94(4): 853-858.

Estruch, R. *et al.* (2013), "Primary prevention of cardiovascular disease with a Mediterranean diet," *N Engl J Med,* 368(14): 1279-1290.

Eyre, J. *et al.* (2011), "Monocyte- and Endothelial-Derived Microparticles Induce an Inflammatory Phenotype in Human Podocytes," *Nephron Exp Nephrol,* 119(3): e58-e66.

Fabian, M. R.; Sonenberg, N., and Filipowicz, W. (2010), "Regulation of mRNA translation and stability by microRNAs," *Annu Rev Biochem,* 79: 351-379.

Faille, D. *et al.* (2009), "Platelet microparticles: a new player in malaria parasite cytoadherence to human brain endothelium," *FASEB J.*

Faille, D. *et al.* (2012), "Endocytosis and intracellular processing of platelet microparticles by brain endothelial cells," *J Cell Mol Med,* 16(8): 1731-1738.

Faille, S. *et al.* (2002), "Real-time in vivo imaging of platelets, tissue factor and fibrin during arterial thrombus formation in the mouse," *Nat Med,* 8(10): 1175-1181.

Falati, S. *et al.* (2003), "Accumulation of tissue factor into developing thrombi in vivo is dependent upon microparticle P-selectin glycoprotein ligand 1 and platelet P-selectin," *J Exp Med,* 197(11): 1585-1598.

Falk, E. *et al.* (2013), "Update on acute coronary syndromes: the pathologists' view," *Eur Heart J,* 34(10): 719-728.

Farb, A. *et al.* (1996), "Coronary plaque erosion without rupture into a lipid core. A frequent cause of coronary thrombosis in sudden coronary death," *Circulation,* 93(7): 1354-1363.

Faure, V. *et al.* (2006), "Elevation of circulating endothelial microparticles in patients with chronic renal failure," *J Thromb Haemost,* 4(3): 566-573.

Ferrante, G. *et al.* (2010), "High levels of systemic myeloperoxidase are associated with coronary plaque erosion in patients with acute coronary syndromes: a clinicopathological study," *Circulation,* 122(24): 2505-2513.

Ferreira, A. C. *et al.* (2004), "Postprandial hypertriglyceridemia increases circulating levels of endothelial cell microparticles," *Circulation,* 110(23): 3599-3603.

Fichtlscherer, S. *et al.* (2010), "Circulating microRNAs in patients with coronary artery disease," *Circ Res,* 107(5): 677-684.

Filipowicz, W.; Bhattacharyya, S. N., and Sonenberg, N. (2008), "Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight?," *Nat Rev Genet,* 9(2): 102-114.

Fischer, K. *et al.* (2006), "Antigen recognition induces phosphatidylserine exposure on the cell surface of human CD8+ T cells," *Blood,* 108(13): 4094-4101.

Flaumenhaft, R. (2006), "Formation and fate of platelet microparticles," *Blood Cells Mol Dis,* 36(2): 182-187.

Flowers, E., and Aouizerat, B. E. (2013), "MicroRNA associated with dyslipidemia and coronary disease in humans," *Physiol Genomics,* 45(24): 1199-1205.

Flowers, E. *et al.* (2013), "MicroRNA associated with atherogenic dyslipidemia in South Asian men," *Int J Cardiol,* 168(5): 4884-4885.

Folsom, A. R. *et al.* (2011), "Community prevalence of ideal cardiovascular health, by the American Heart Association definition, and relationship with cardiovascular disease incidence," *J Am Coll Cardiol,* 57(16): 1690-1696.

Forlow, S. B.; Mcever, R. P., and Nollert, M. U. (2000), "Leukocyte-leukocyte interactions mediated by platelet microparticles under flow," *Blood,* 95(4): 1317-1323.

Fourcade, O. *et al.* (1995), "Secretory phospholipase A2 generates the novel lipid mediator lysophosphatidic acid in membrane microvesicles shed from activated cells," *Cell,* 80(6): 919-927.

Fox, J. E.; Reynolds, C. C., and Austin, C. D. (1990), "The role of calpain in stimulus-response coupling: evidence that calpain mediates agonist-induced expression of procoagulant activity in platelets," *Blood,* 76(12): 2510-2519.

Fox, J. E. *et al.* (1990), "Role of the membrane skeleton in preventing the shedding of procoagulant-rich microvesicles from the platelet plasma membrane," *J Cell Biol,* 111(2): 483-493.

Fox, J. E. *et al.* (1991), "Evidence that agonist-induced activation of calpain causes the shedding of procoagulant-containing microvesicles from the membrane of aggregating platelets," *J Biol Chem,* 266(20): 13289-13295.

Freikman, I. *et al.* (2008), "Oxidative stress causes membrane phospholipid rearrangement and shedding from RBC membranes-an NMR study," *Biochim Biophys Acta,* 1778(10): 2388-2394.

Freyssinet, J. M. (2003), "Cellular microparticles: what are they bad or good for?," *J Thromb Haemost,* 1(7): 1655-1662.

Freyssinet, J. M. *et al.* (1999), "Apoptosis in vascular disease," *Thromb Haemost,* 82(2): 727-735.

Fukushima, Y. *et al.* (2011), "Assessment of plasma miRNAs in congestive heart failure," *Circ J,* 75(2): 336-340.

Fuster, V. *et al.* (1988), "Insights into the pathogenesis of acute ischemic syndromes," *Circulation,* 77(6): 1213-1220.

Galli, M.; Grassi, A., and Barbui, T. (1996), "Platelet-derived microvesicles in thrombotic thrombocytopenic purpura and hemolytic uremic syndrome," *Thromb Haemost,* 75(3): 427-431.

García, B. A. *et al.* (2005), "The platelet microparticle proteome," *J Proteome Res,* 4(5): 1516-1521.

García, S. *et al.* (2005), "Phenotypic assessment of endothelial microparticles in patients with heart failure and after heart transplantation: switch from cell activation to apoptosis," *J Heart Lung Transplant,* 24(12): 2184-2189.

Gauley, J., and Pisetsky, D. S. (2010), "The release of microparticles by RAW 264.7 macrophage cells stimulated with TLR ligands," *J Leukoc Biol,* 87(6): 1115-1123.

George, J. N. *et al.* (1982), "Isolation of human platelet membrane microparticles from plasma and serum," *Blood,* 60(4): 834-840.

Getts, D. R. *et al.* (2014), "Therapeutic inflammatory monocyte modulation using immune-modifying microparticles," *Sci Transl Med,* 6(219): 219ra217.

Gidon-Jeangirard, C. *et al.* (1999), "Annexin V delays apoptosis while exerting an external constraint preventing the release of CD4+ and PrPc+ membrane particles in a human T lymphocyte model," *J Immunol,* 162(10): 5712-5718.

Gilbert, G. E. *et al.* (1991), "Platelet-derived microparticles express high affinity receptors for factor VIII," *J Biol Chem,* 266(26): 17261-17268.

Gill, P. J.; Harnden, A., and Karpe, F. (2012), "Familial hypercholesterolaemia," Bmj, 344: e3228.

GOICHOT, B. *et al.* (2006), "Circulating procoagulant microparticles in obesity," *Diabetes Metab,* 32(1): 82-85.

González-Cano, P. *et al.* (2010), "Mycobacterium tuberculosis H37Rv induces ectosome release in human polymorphonuclear neutrophils," *Tuberculosis (Edinb),* 90(2): 125-134.

González-Quintero, V. H. *et al.* (2003), "Elevated plasma endothelial microparticles in preeclampsia," *Am J Obstet Gynecol,* 189(2): 589-593.

Gordon, S., and Taylor, P. R. (2005), "Monocyte and macrophage heterogeneity," *Nat Rev Immunol,* 5(12): 953-964.

Goto, S. *et al.* (2003), "Different effects of various anti-GPIIb-IIIa agents on shear-induced platelet activation and expression of procoagulant activity," *J Thromb Haemost,* 1(9): 2022-2030.

Gross, P. L. *et al.* (2005), "Leukocyte-versus microparticle-mediated tissue factor transfer during arteriolar thrombus development," *J Leukoc Biol,* 78(6): 1318-1326.

Grove, E. L.; Hvas, A. M., and Kristensen, S. D. (2009), "Immature platelets in patients with acute coronary syndromes," *Thromb Haemost,* 101(1): 151-156.

Grundy, S. M. (1998), "Statin trials and goals of cholesterol-lowering therapy," *Circulation,* 97(15): 1436-1439.

Guiducci, S. *et al.* (2008), "The relationship between plasma microparticles and disease manifestations in patients with systemic sclerosis," *Arthritis Rheum,* 58(9): 2845-2853.

Gyorgy, B. *et al.* (2011), "Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles," *Cell Mol Life Sci,* 68(16): 2667-2688.

Halkein, J. *et al.* (2013), "MicroRNA-146a is a therapeutic target and biomarker for peripartum cardiomyopathy," *J Clin Invest,* 123(5): 2143-2154.

Hamilton, K. K. *et al.* (1990), "Complement proteins C5b-9 induce vesiculation of the endothelial plasma membrane and expose catalytic surface for assembly of the prothrombinase enzyme complex," *J Biol Chem,* 265(7): 3809-3814.

Hanke, M. *et al.* (2010), "A robust methodology to study urine microRNA as tumor marker: microRNA-126 and microRNA-182 are related to urinary bladder cancer," *Urol Oncol,* 28(6): 655-661.

Hansson, G. K. (2005), "Inflammation, atherosclerosis, and coronary artery disease," *N Engl J Med,* 352(16): 1685-1695.

Hashimoto, K. *et al.* (2009), "Aggregation and microparticle production through toll-like receptor 4 activation in platelets from recently menopausal women," *J Cardiovasc Pharmacol,* 54(1): 57-62.

Hazui, H. *et al.* (2005), "Simple and useful tests for discriminating between acute aortic dissection of the ascending aorta and acute myocardial infarction in the emergency setting," Circ J, 69(6): 677-682.

HEADLAND, S. E. *et al.* (2014), "Cutting-Edge Analysis of Extracellular Microparticles using ImageStream(X) Imaging Flow Cytometry," *Sci Rep,* 4: 5237.

Healey, J. S. *et al.* (2006), "Cardiovascular outcomes with atrial-based pacing compared with ventricular pacing: meta-analysis of randomized trials, using individual patient data," *Circulation,* 114(1): 11-17.

Heeschen, C. *et al.* (2004), "Prognostic value of placental growth factor in patients with acute chest pain," *Jama,* 291(4): 435-441.

Heijnen, H. F. *et al.* (1999), "Activated platelets release two types of membrane vesicles: microvesicles by surface shedding and exosomes derived from exocytosis of multivesicular bodies and alpha-granules," *Blood,* 94(11): 3791-3799.

Heiss, C. *et al.* (2008), "Brief secondhand smoke exposure depresses endothelial progenitor cells activity and endothelial function: sustained vascular injury and blunted nitric oxide production," *J Am Coll Cardiol,* 51(18): 1760-1771.

Helal, O. *et al.* (2011), "Increased levels of microparticles originating from endothelial cells, platelets and erythrocytes in subjects with metabolic syndrome: relationship with oxidative stress," *Nutr Metab Cardiovasc Dis,* 21(9): 665-671.

Helley, D. *et al.* (2009), "Platelet microparticles: a potential predictive factor of survival in hormone-refractory prostate cancer patients treated with docetaxel-based chemotherapy," *Eur Urol,* 56(3): 479-484.

Henn, V. *et al.* (1998), "CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells," *Nature,* 391(6667): 591-594.

Hergenreider, E. *et al.* (2012), "Atheroprotective communication between endothelial cells and smooth muscle cells through miRNAs," *Nat Cell Biol,* 14(3): 249-256.

Hinson, J. P.; Kapas, S., and Smith, D. M. (2000), "Adrenomedullin, a multifunctional regulatory peptide," *Endocr Rev,* 21(2): 138-167.

Hochholzer, W. *et al.* (2008), "New definition of myocardial infarction: impact on long-term mortality," *Am J Med,* 121(5): 399-405.

Hoekstra, M. *et al.* (2010), "The peripheral blood mononuclear cell microRNA signature of coronary artery disease," *Biochem Biophys Res Commun,* 394(3): 792-797.

Horigome, H. *et al.* (2002), "Overproduction of platelet microparticles in cyanotic congenital heart disease with polycythemia," *J Am Coll Cardiol,* 39(6): 1072-1077.

Hristov, M. *et al.* (2004), "Apoptotic bodies from endothelial cells enhance the number and initiate the differentiation of human endothelial progenitor cells in vitro," *Blood,* 104(9): 2761-2766.

Hsu, C. *et al*. (2010), "Regulation of exosome secretion by Rab35 and its GTPase-activating proteins TBC1D10A-C," *J Cell Biol,* 189(2): 223-232.

Huang, P. H. *et al.* (2010), "Increased circulating CD31+/annexin V+ apoptotic microparticles and decreased circulating endothelial progenitor cell levels in hypertensive patients with microalbuminuria," *J Hypertens,* 28(8): 1655-1665.

Huang, Y. *et al.* (2013), "Molecular functions of small regulatory noncoding RNA," *Biochemistry (Mosc),* 78(3): 221-230.

Huber, J. *et al.* (2002), "Oxidized membrane vesicles and blebs from apoptotic cells contain biologically active oxidized phospholipids that induce monocyte-endothelial interactions," *Arterioscler Thromb Vasc Biol,* 22(1): 101-107.

Huber, L. C. *et al.* (2007), "The role of membrane lipids in the induction of macrophage apoptosis by microparticles," *Apoptosis,* 12(2): 363-374.

Hugel, B. *et al.* (1999), "Elevated levels of circulating procoagulant microparticles in patients with paroxysmal nocturnal hemoglobinuria and aplastic anemia," *Blood,* 93(10): 3451-3456.

Hughes, M. *et al.* (2000), "Morphological analysis of microparticle generation in heparin-induced thrombocytopenia," *Blood,* 96(1): 188-194.

Hunter, M. P. *et al.* (2008), "Detection of microRNA expression in human peripheral blood microvesicles," *PLoS One,* 3(11): e3694.

Huo, Y., and Ley, K. F. (2004), "Role of platelets in the development of atherosclerosis," *Trends Cardiovasc Med,* 14(1): 18-22.

Inoue, T. *et al.* (2008), "Increased circulating platelet-derived microparticles are associated with stent-induced vascular inflammation," *Atherosclerosis,* 196(1): 469-476.

Ionita, M. G. *et al.* (2010), "High neutrophil numbers in human carotid atherosclerotic plaques are associated with characteristics of rupture-prone lesions," *Arterioscler Thromb Vasc Biol,* 30(9): 1842-1848.

Janowska-Wieczorek, A. *et al.* (2005), "Microvesicles derived from activated platelets induce metastasis and angiogenesis in lung cancer," *Int J Cancer,* 113(5): 752-760.

Janowska-Wieczorek, A. *et al.* (2006), "Enhancing effect of platelet-derived microvesicles on the invasive potential of breast cancer cells," *Transfusion,* 46(7): 1199-1209.

Janssen, H. L. *et al.* (2013), "Treatment of HCV infection by targeting microRNA," *N Engl J Med,* 368(18): 1685-1694.

Januzzi, J. L. *et al.* (2006), "NT-proBNP testing for diagnosis and short-term prognosis in acute destabilized heart failure: an international pooled analysis of 1256 patients: the International Collaborative of NT-proBNP Study," *Eur Heart J,* 27(3): 330-337.

Jayachandran, M. *et al.* (2008), "Characterization of blood borne microparticles as markers of premature coronary calcification in newly menopausal women," *Am J Physiol Heart Circ Physiol,* 295(3): H931-H938.

Jayachandran, M. *et al.* (2012), "Methodology for isolation, identification and characterization of microvesicles in peripheral blood," *J Immunol Methods,* 375(1-2): 207-214.

Jennings, L. K. (2009), "Mechanisms of platelet activation: need for new strategies to protect against platelet-mediated atherothrombosis," *Thromb Haemost,* 102(2): 248-257.

Jiménez, J. J.; Ji, W.; Mauro, L. M.; Horstman, L. L. *et al.* (2003), "Endothelial microparticles released in thrombotic thrombocytopenic purpura express von Willebrand factor and markers of endothelial activation," *Br J Haematol,* 123(5): 896-902.

Jiménez, J. J.; Ji, W.; Mauro, L. M.; Soderland, C. *et al.* (2003), "Endothelial cells release phenotypically and quantitatively distinct microparticles in activation and apoptosis," *Thromb Res,* 109(4): 175-180.

Jin, M. *et al.* (2005), "Distinct proteome features of plasma microparticles," *Proteomics,* 5(7): 1940-1952.

Johnstone, R. (2006), "Exosomes biological significance: A concise review," *Blood Cells Mol Dis,* 36(2): 315-321.

Joly, E., and Hudrisier, D. (2003), "What is trogocytosis and what is its purpose?," *Nat Immunol,* 4(9): 815.

Joop, K. *et al.* (2001), "Microparticles from patients with multiple organ dysfunction syndrome and sepsis support coagulation through multiple mechanisms," *Thromb Haemost,* 85(5): 810-820.

Judge, H. M. *et al.* (2010), "Relationship between degree of P2Y12 receptor blockade and inhibition of P2Y12-mediated platelet function," *Thromb Haemost,* 103(6): 1210-1217.

Jung, K. H. *et al.* (2009), "Circulating endothelial microparticles as a marker of cerebrovascular disease," *Ann Neurol,* 66(2): 191-199.

Jy, W. *et al.* (2002), "Agonist-induced capping of adhesion proteins and microparticle shedding in cultures of human renal microvascular endothelial cells," *Endothelium,* 9(3): 179-189.

Jy, W.; Hortsman, L. L. *et al.* (2004), "Measuring circulating cell-derived microparticles," *J Thromb Haemost,* 2(10): 1842-1851.

Jy, W.; Minagar, A. *et al.* (2004), "Endothelial microparticles (EMP) bind and activate monocytes: elevated EMP-monocyte conjugates in multiple sclerosis," *Front Biosci,* 9: 3137-3144.

Jy, W. *et al.* (2005), "Circulating cell-derived microparticles in thrombotic and inflammatory disorders," *Current Clinical Neurology: Inflammatory Disorders of the Nervous System:Pathogenesis, Immunology, and Clinical Management,* A. M. a. J. S. Alexander. Totowa, NJ, Humana Press Inc.: 91-102.

Jy, W. *et al.* (2007), "Platelet activation rather than endothelial injury identifies risk of thrombosis in subjects positive for antiphospholipid antibodies," *Thromb Res,* 121(3): 319-325.

Kagawa, H. *et al.* (2001), "Effect of bezafibrate on soluble adhesion molecules and platelet activation markers in patients with connective tissue diseases and secondary hyperlipidemia," *Clin Appl Thromb Hemost,* 7(2): 153-157.

Kaptan, K. *et al.* (2008), "Platelet-derived microparticle levels in women with recurrent spontaneous abortion," *Int J Gynaecol Obstet,* 102(3): 271-274.

Karolina, D. S. *et al.* (2012), "Circulating miRNA profiles in patients with metabolic syndrome," *J Clin Endocrinol Metab,* 97(12): E2271-2276.

Katahira, J., and Yoneda, Y. (2011), "Nucleocytoplasmic transport of microRNAs and related small RNAs," *Traffic,* 12(11): 1468-1474.

Keuren, J. F. *et al.* (2007), "Microparticles adhere to collagen type I, fibrinogen, von Willebrand factor and surface immobilised platelets at physiological shear rates," *Br J Haematol,* 138(4): 527-533.

KEY, N. S. (2010), "Analysis of tissue factor positive microparticles," *Thromb Res,* 125(Suppl 1): S42-45.

KIM, H. K. *et al.* (2004), "Platelet microparticles induce angiogenesis in vitro," *Br J Haematol,* 124(3): 376-384.

KIM, H. K. *et al.* (2003), "Circulating numbers of endothelial progenitor cells in patients with gastric and breast cancer," *Cancer Lett,* 198(1): 83-88.

KNIJFF-DUTMER, E. *et al.* (2002), "Elevated levels of platelet microparticles are associated with disease activity in rheumatoid arthritis," *Arthritis Rheum,* 46(6): 1498-1503.

KOGA, H. *et al.* (2005), "Elevated levels of VE-cadherin-positive endothelial microparticles in patients with type 2 diabetes mellitus and coronary artery disease," *J Am Coll Cardiol,* 45(10): 1622-1630.

KOGA, H. *et al.* (2006), "Elevated levels of remnant lipoproteins are associated with plasma platelet microparticles in patients with type-2 diabetes mellitus without obstructive coronary artery disease," *Eur Heart J,* 27(7): 817-823.

KOLOWOS, W. *et al.* (2005), "Microparticles shed from different antigen-presenting cells display an individual pattern of surface molecules and a distinct potential of allogeneic T-cell activation," *Scand J Immunol,* 61(3): 226-233.

KOSAKA, N. *et al.* (2010), "Secretory mechanisms and intercellular transfer of microRNAs in living cells," *J Biol Chem,* 285(23): 17442-17452.

KOZAI, T. *et al.* (2005), "Statins prevent pulsatile stretch-induced proliferation of human saphenous vein smooth muscle cells via inhibition of Rho/Rho-kinase pathway," *Cardiovasc Res,* 68(3): 475-482.

KOZOMARA, A., and GRIFFITHS-JONES, S. (2011), "miRBase: integrating microRNA annotation and deep-sequencing data," *Nucleic Acids Res,* 39(Database issue): D152-157.

KUWABARA, Y. *et al.* (2011), "Increased microRNA-1 and microRNA-133a levels in serum of patients with cardiovascular disease indicate myocardial damage," *Circ Cardiovasc Genet,* 4(4): 446-454.

LABIOS, M. *et al.* (2004), "Effect of eprosartan on cytoplasmic free calcium mobilization, platelet activation, and microparticle formation in hypertension," *Am J Hypertens,* 17(9): 757-763.

LACKNER, P. *et al.* (2010), "Cellular microparticles as a marker for cerebral vasospasm in spontaneous subarachnoid hemorrhage," *Stroke,* 41(10): 2353-2357.

LACROIX, R., and DIGNAT-GEORGE, F. (2013), "Microparticles: new protagonists in pericellular and intravascular proteolysis," *Semin Thromb Hemost,* 39(1): 33-39.

LACROIX, R. *et al.* (2007), "Activation of plasminogen into plasmin at the surface of endothelial microparticles: a mechanism that modulates angiogenic properties of endothelial progenitor cells in vitro," *Blood,* 110(7): 2432-2439.

LACROIX, R. *et al.* (2012), "Impact of pre-analytical parameters on the measurement of circulating microparticles: towards standardization of protocol," *J Thromb Haemost,* 10(3): 437-446.

LASSER, C.; ALIKHANI, V. S. *et al.* (2011), "Human saliva, plasma and breast milk exosomes contain RNA: uptake by macrophages," *J Transl Med,* 9: 9.

LASSER, C.; O'NEIL, S. E. *et al.* (2011), "RNA-containing exosomes in human nasal secretions," *Am J Rhinol Allergy,* 25(2): 89-93.

LAUGHLIN, P. J. *et al.* (1993), "Structure of gelsolin segment 1-actin complex and the mechanism of filament severing," *Nature,* 364(6439): 685-692.

LAWRIE, A. S. *et al.* (2009), "Microparticle sizing by dynamic light scattering in fresh-frozen plasma," *Vox Sang,* 96(3): 206-212.

LAWRIE, C. H. *et al.* (2008), "Detection of elevated levels of tumour-associated microRNAs in serum of patients with diffuse large B-cell lymphoma," *Br J Haematol*, 141(5): 672-675.

Lee, S. T. *et al.* (2012), "miR-206 regulates brain-derived neurotrophic factor in Alzheimer disease model," *Ann Neurol,* 72(2): 269-277.

Lee, Y. J. *et al.* (1993), "Elevated platelet microparticles in transient ischemic attacks, lacunar infarcts, and multiinfarct dementias," *Thromb Res,* 72(4): 295-304.

Lee, Y. *et al.* (2002), "MicroRNA maturation: stepwise processing and subcellular localization," *Embo J,* 21(17): 4663-4670.

Leong, H. S. *et al.* (2011), "Validation of flow cytometric detection of platelet microparticles and liposomes by atomic force microscopy," *J Thromb Haemost,* 9(12): 2466-2476.

Leroyer, A. S. *et al.* (2007), "Cellular origins and thrombogenic activity of microparticles isolated from human atherosclerotic plaques," *J Am Coll Cardiol,* 49(7): 772-777.

Leroyer, A. S. *et al.* (2008), "CD40 ligand+ microparticles from human atherosclerotic plaques stimulate endothelial proliferation and angiogenesis a potential mechanism for intraplaque neovascularization," *J Am Coll Cardiol,* 52(16): 1302-1311.

Lewis, B. P.; Burge, C. B., and Bartel, D. P. (2005), "Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets," Cell, 120(1): 15-20.

Li, B. *et al.* (2012), "RhoA triggers a specific signaling pathway that generates transforming microvesicles in cancer cells," *Oncogene,* 31(45): 4740-4749.

Li, X., and Cong, H. (2009), "Platelet-derived microparticles and the potential of glycoprotein IIb/IIIa antagonists in treating acute coronary syndrome," *Tex Heart Inst J,* 36(2): 134-139.

Libby, P. *et al.* (2010), "Inflammation in atherosclerosis: transition from theory to practice," *Circ J,* 74(2): 213-220.

Libby, P.; Ridker, P. M., and Maseri, A. (2002), "Inflammation and atherosclerosis," *Circulation,* 105(9): 1135-1143.

Lim, S. S. *et al.* (2012), "A comparative risk assessment of burden of disease and injury attributable to 67 risk factors and risk factor clusters in 21 regions, 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010," *Lancet,* 380(9859): 2224-2260.

Lindahl, B. *et al.* (2000), "Markers of myocardial damage and inflammation in relation to long-term mortality in unstable coronary artery disease. FRISC Study Group. Fragmin during Instability in Coronary Artery Disease," *N Engl J Med,* 343(16): 1139-1147.

Litvack, M. L.; Post, M., and Palaniyar, N. (2011), "IgM promotes the clearance of small particles and apoptotic microparticles by macrophages," *PLoS One,* 6(3): e17223.

Liu, M. L. *et al.* (2007), "Cholesterol enrichment of human monocyte/macrophages induces surface exposure of phosphatidylserine and the release of biologically-active tissue factor-positive microvesicles," *Arterioscler Thromb Vasc Biol,* 27(2): 430-435.

Liu, S. *et al.* (2014), "Procoagulant activity and cellular origin of microparticles in human amniotic fluid," *Thromb Res.*

Lo, S. C. *et al.* (2006), "Involvement of platelet glycoprotein Ib in platelet microparticle mediated neutrophil activation," *J Biomed Sci,* 13(6): 787-796.

Lok, C. A. *et al.* (2007), "Microparticle-associated P-selectin reflects platelet activation in preeclampsia," *Platelets,* 18(1): 68-72.

Lok, C. A. *et al.* (2008), "Changes in microparticle numbers and cellular origin during pregnancy and preeclampsia," *Hypertens Pregnancy,* 27(4): 344-360.

López, J. A.; Del Conde, I., and Shrimpton, C. N. (2005), "Receptors, rafts, and microvesicles in thrombosis and inflammation," *J Thromb Haemost,* 3(8): 1737-1744.

López-Vilchez, I. *et al.* (2007), "Tissue factor-enriched vesicles are taken up by platelets and induce platelet aggregation in the presence of factor VIIa," *Thromb Haemost,* 97(2): 202-211.

López -vilchez, I.; Diaz-Ricart, M. *et al.* (2009), "Serotonin enhances platelet procoagulant properties and their activation induced during platelet tissue factor uptake," *Cardiovasc Res,* 84(2): 309-316.

López -vilchez, I.; tonda, R. *et al.* (2009), "Relative contributions of collagen and tissue factor to thrombus formation on damaged vascular vessels: in-vitro studies with circulating blood," *Coron Artery Dis,* 20(6): 392-399.

López -vilchez, I. *et al.* (2012), "Platelet-associated tissue factor enhances platelet reactivity and thrombin generation in experimental studies in vitro," *Thromb Res,* 130(6): e294-300.

Lotvall, J., and Valadi, H. (2007), "Cell to cell signalling via exosomes through esRNA," Cell Adh Migr, 1(3): 156-158.

Lozano, R. *et al.* (2011), "Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010," *Lancet,* 380(9859): 2095-2128.

Lozito, T. P., and Tuan, R. S. (2012), "Endothelial cell microparticles act as centers of matrix metalloproteinsase-2 (MMP-2) activation and vascular matrix remodeling," *J Cell Physiol,* 227(2): 534-549.

Lukasik, M. *et al.* (2010), "Platelet activation and reactivity in the convalescent phase of ischaemic stroke," *Thromb Haemost,* 103(3): 644-650.

Madhavan, M. V. *et al.* (2014), "Coronary artery calcification: pathogenesis and prognostic implications," *J Am Coll Cardiol,* 63(17): 1703-1714.

Magdeleyns, E. J.; Keuren, J. F., and Curvers, J. (2007), "Factor Va, bound to microparticles released during platelet storage, is resistant to inactivation by activated protein C," *Transfusion,* 47(10): 1880-1888.

Mallat, Z. *et al.* (1999), "Shed membrane microparticles with procoagulant potential in human atherosclerotic plaques: a role for apoptosis in plaque thrombogenicity," *Circulation,* 99(3): 348-353.

Mallat, Z. *et al.* (2000), "Elevated levels of shed membrane microparticles with procoagulant potential in the peripheral circulating blood of patients with acute coronary syndromes," *Circulation,* 101(8): 841-843.

Manno, S.; Takakuwa, Y., and Mohandas, N. (2002), "Identification of a functional role for lipid asymmetry in biological membranes: Phosphatidylserine-skeletal protein interactions modulate membrane stability," *Proc Natl Acad Sci U S A,* 99(4): 1943-1948.

Martin, S. *et al.* (2004), "Shed membrane particles from T lymphocytes impair endothelial function and regulate endothelial protein expression," *Circulation,* 109(13): 1653-1659.

Maruyama, K.; Kadono, T., and Morishita, E. (2012), "Plasma levels of platelet-derived microparticles are increased after anaerobic exercise in healthy subjects," *J Atheroscler Thromb,* 19(6): 585-587.

MAssberg, S. *et al.* (2004), "Enhanced in vivo platelet adhesion in vasodilator-stimulated phosphoprotein (VASP)-deficient mice," *Blood,* 103(1): 136-142.

Mastronardi, M. L. *et al.* (2011), "Microparticles from apoptotic monocytes enhance nitrosative stress in human endothelial cells," *Fundam Clin Pharmacol,* 25(6): 653-660.

Masyuk, A. I. *et al.* (2010), "Biliary exosomes influence cholangiocyte regulatory mechanisms and proliferation through interaction with primary cilia," *Am J Physiol Gastrointest Liver Physiol,* 299(4): G990-999.

Mathers, C. D., and Loncar, D. (2006), "Projections of global mortality and burden of disease from 2002 to 2030," *PLoS Med,* 3(11): e442.

Matsubara, E. *et al.* (2002), "Platelet microparticles as carriers of soluble Alzheimer's amyloid beta (sAbeta)," *Ann N Y Acad Sci,* 977: 340-348.

Maurer-Spurej, E. *et al.* (2009), "Platelet quality measured with dynamic light scattering correlates with transfusion outcome in hematologic malignancies," *Transfusion,* 49(11): 2276-2284.

Mauriello, A. *et al.* (2013), "Coronary calcification identifies the vulnerable patient rather than the vulnerable Plaque," *Atherosclerosis,* 229(1): 124-129.

Mause, S. F. *et al.* (2005), "Platelet microparticles: a transcellular delivery system for RANTES promoting monocyte recruitment on endothelium," *Arterioscler Thromb Vasc Biol,* 25(7): 1512-1518.

Mause, S. F. *et al.* (2010), "Platelet microparticles enhance the vasoregenerative potential of angiogenic early outgrowth cells after vascular injury," *Circulation,* 122(5): 495-506.

Meder, B. *et al.* (2011), "MicroRNA signatures in total peripheral blood as novel biomarkers for acute myocardial infarction," *Basic Res Cardiol,* 106(1): 13-23.

Meijers, B. K. *et al.* (2009), "The uremic retention solute p-cresyl sulfate and markers of endothelial damage," *Am J Kidney Dis,* 54(5): 891-901.

Merten, M. *et al.* (1999), "Platelet microparticles promote platelet interaction with subendothelial matrix in a glycoprotein IIb/IIIa-dependent mechanism," *Circulation,* 99(19): 2577-2582.

Mesri, M., and Altieri, D. C. (1999), "Leukocyte microparticles stimulate endothelial cell cytokine release and tissue factor induction in a JNK1 signaling pathway," *J Biol Chem,* 274(33): 23111-23118.

Meziani, F. *et al.* (2006), "Shed membrane particles from preeclamptic women generate vascular wall inflammation and blunt vascular contractility," *Am J Pathol,* 169(4): 1473-1483.

Michelsen, A. E. *et al.* (2009), "Elevated levels of platelet microparticles in carotid atherosclerosis and during the postprandial state," *Thromb Res,* 123(6): 881-886.

Michelson, A. D. (2010), "Antiplatelet therapies for the treatment of cardiovascular disease," *Nat Rev Drug Discov,* 9(2): 154-169.

Miller, G. J. (1992), "Hemostasis and cardiovascular risk. The British and European experience," *Arch Pathol Lab Med,* 116(12): 1318-1321.

Minagar, A. *et al.* (2001), "Elevated plasma endothelial microparticles in multiple sclerosis," *Neurology,* 56(10): 1319-1324.

Mitchell, P. S. *et al.* (2008), "Circulating microRNAs as stable blood-based markers for cancer detection," *Proc Natl Acad Sci U S A,* 105(30): 10513-10518.

Mittelbrunn, M. *et al.* (2011), "Unidirectional transfer of microRNA-loaded exosomes from T cells to antigen-presenting cells," *Nat Commun,* 2: 282.

Miyazaki, Y. *et al.* (1996), "High shear stress can initiate both platelet aggregation and shedding of procoagulant containing microparticles," *Blood,* 88(9): 3456-3464.

Mobarrez, F. *et al.* (2011), "Atorvastatin reduces thrombin generation and expression of tissue factor, P-selectin and GPIIIa on platelet-derived microparticles in patients with peripheral arterial occlusive disease," *Thromb Haemost,* 106(2): 344-352.

Mobarrez, F. *et al.* (2012), "Release of endothelial microparticles in vivo during atorvastatin treatment; a randomized double-blind placebo-controlled study," *Thromb Res,* 129(1): 95-97.

Montecalvo, A. *et al.* (2012), "Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes," *Blood,* 119(3): 756-766.

Morel, O. *et al.* (2003), "Protective effects of vitamin C on endothelium damage and platelet activation during myocardial infarction in patients with sustained generation of circulating microparticles," *J Thromb Haemost,* 1(1): 171-177.

Morel, O. *et al.* (2004), "Circulating procoagulant microparticles and soluble GPV in myocardial infarction treated by primary percutaneous transluminal coronary angioplasty. A possible role for GPIIb-IIIa antagonists," *J Thromb Haemost,* 2(7): 1118-1126.

Morel, O. *et al.* (2005), "[The significance of circulating microparticles in physiology, inflammatory and thrombotic diseases]," *Rev Med Interne,* 26(10): 791-801.

Morel, O. *et al.* (2006), "Procoagulant microparticles: disrupting the vascular homeostasis equation?," *Arterioscler Thromb Vasc Biol,* 26(12): 2594-2604.

Morel, O. *et al.* (2008), "Endothelial cell activation contributes to the release of procoagulant microparticles during acute cardiac allograft rejection," *J Heart Lung Transplant,* 27(1): 38-45.

Morel, O. *et al.* (2010), "Mechanisms of microparticle generation: on the trail of the mitochondrion!," *Semin Thromb Hemost,* 36(8): 833-844.

Morel, O. *et al.* (2011), "Cellular mechanisms underlying the formation of circulating microparticles," *Arterioscler Thromb Vasc Biol,* 31(1): 15-26.

Morello, M. *et al.* (2013), "Large oncosomes mediate intercellular transfer of functional microRNA," *Cell Cycle,* 12(22): 3526-3536.

Morgenthaler, N. G. *et al.* (2006), "Assay for the measurement of copeptin, a stable peptide derived from the precursor of vasopressin," *Clin Chem,* 52(1): 112-119.

Mostefai, H. A.; Agouni, A. *et al.* (2008), "Phosphatidylinositol 3-kinase and xanthine oxidase regulate nitric oxide and reactive oxygen species productions by apoptotic lymphocyte microparticles in endothelial cells," *J Immunol,* 180(7): 5028-5035.

Mostefai, H. A.; Meziani, F. *et al.* (2008), "Circulating microparticles from patients with septic shock exert protective role in vascular function," *Am J Respir Crit Care Med,* 178(11): 1148-1155.

Motoyama, S. *et al.* (2007), "Multislice computed tomographic characteristics of coronary lesions in acute coronary syndromes," *J Am Coll Cardiol,* 50(4): 319-326.

Mrvar-Brecko, A. *et al.* (2010), "Isolated microvesicles from peripheral blood and body fluids as observed by scanning electron microscope," *Blood Cells Mol Dis,* 44(4): 307-312.

Muller, I. *et al.* (2003), "Intravascular tissue factor initiates coagulation via circulating microvesicles and platelets," *Faseb J,* 17(3): 476-478.

Mullier, F. *et al.* (2013), "Pre-analytical issues in the measurement of circulating microparticles: current recommendations and pending questions," *J Thromb Haemost,* 11(4): 693-696.

Murakami, T. *et al.* (2007), "Impact of weight reduction on production of platelet-derived microparticles and fibrinolytic parameters in obesity," *Thromb Res,* 119(1): 45-53.

Muralidharan-Chari, V. *et al.* (2009), "ADP-ribosylation factor 6 regulates tumorigenic and invasive properties in vivo," *Cancer Res,* 69(6): 2201-2209.

Neefjes, L. A. *et al.* (2011), "Accelerated subclinical coronary atherosclerosis in patients with familial hypercholesterolemia," *Atherosclerosis,* 219(2): 721-727.

Nickel, C. H.; Bingisser, R., and Morgenthaler, N. G. (2012), "The role of copeptin as a diagnostic and prognostic biomarker for risk stratification in the emergency department," *BMC Med,* 10: 7.

Nicoll, R., and Henein, M. Y. (2013), "Arterial calcification: friend or foe?," *Int J Cardiol,* 167(2): 322-327.

Nieuwland, R. *et al.* (1997), "Cell-derived microparticles generated in patients during cardiopulmonary bypass are highly procoagulant," *Circulation,* 96(10): 3534-3541.

Nieuwland, R. *et al.* (2000), "Cellular origin and procoagulant properties of microparticles in meningococcal sepsis," *Blood,* 95(3): 930-935.

Nikolsky, E. *et al.* (2007), "Impact of baseline platelet count in patients undergoing primary percutaneous coronary intervention in acute myocardial infarction (from the CADILLAC trial)," *Am J Cardiol,* 99(8): 1055-1061.

Nolte-'t Hoen, E. N. et al. (2012), "Deep sequencing of RNA from immune cell-derived vesicles uncovers the selective incorporation of small non-coding RNA biotypes with potential regulatory functions," *Nucleic Acids Res,* 40(18): 9272-9285.

Nomura, S. (2008), "Statin and endothelial cell-derived microparticles," *Thromb Haemost,* 100(3): 377-378.

Nomura, S. *et al.* (1995), "Platelet-derived microparticles may influence the development of atherosclerosis in diabetes mellitus," *Atherosclerosis,* 116(2): 235-240.

Nomura, S. *et al.* (1998), "Effect of cilostazol on soluble adhesion molecules and platelet-derived microparticles in patients with diabetes," *Thromb Haemost,* 80(3): 388-392.

Nomura, S. *et al.* (2001), "High-shear-stress-induced activation of platelets and microparticles enhances expression of cell adhesion molecules in THP-1 and endothelial cells," *Atherosclerosis,* 158(2): 277-287.

Nomura, S. *et al.* (2003), "Enzyme immunoassay detection of platelet-derived microparticles and RANTES in acute coronary syndrome," *Thromb Haemost,* 89(3): 506-512.

Nomura, S. *et al.* (2005), "Benidipine improves oxidized LDL-dependent monocyte and endothelial dysfunction in hypertensive patients with type 2 diabetes mellitus," *J Hum Hypertens,* 19(7): 551-557.

Nomura, S. *et al.* (2007), "Effect of nifedipine on adiponectin in hypertensive patients with type 2 diabetes mellitus," *J Hum Hypertens,* 21(1): 38-44.

Nomura, S. *et al.* (2008), "Significance of microparticles in progressive systemic sclerosis with interstitial pneumonia," *Platelets,* 19(3): 192-198.

Nomura, S. *et al.* (2011), "Effects of miglitol in platelet-derived microparticle, adiponectin, and selectin level in patients with type 2 diabetes mellitus," *Int J Gen Med,* 4: 539-545.

Nomura, S.; Imamura, A. *et al.* (2000), "Platelet-derived microparticles in patients with arteriosclerosis obliterans: enhancement of high shear-induced microparticle generation by cytokines," *Thromb Res,* 98(4): 257-268.

Nomura, S.; Kanazawa, S., and Fukuhara, S. (2002), "Effects of efonidipine on platelet and monocyte activation markers in hypertensive patients with and without type 2 diabetes mellitus," *J Hum Hypertens,* 16(8): 539-547.

Nomura, S.; Nakamura, T. *et al.* (2000), "Cytometric analysis of high shear-induced platelet microparticles and effect of cytokines on microparticle generation," *Cytometry,* 40(3): 173-181.

Nomura S.; Shouzu, A. *et al.* (2004), "Effects of losartan and simvastatin on monocyte-derived microparticles in hypertensive patients with and without type 2 diabetes mellitus," *Clin Appl Thromb Hemost,* 10(2): 133-141.

Nomura, S.; Takahashi, N. *et al.* (2004), "Probucol and ticlopidine: effect on platelet and monocyte activation markers in hyperlipidemic patients with and without type 2 diabetes," *Atherosclerosis,* 174(2): 329-335.

Norata, G. D. *et al.* (2013), "MicroRNAs and lipoproteins: a connection beyond atherosclerosis?," *Atherosclerosis,* 227(2): 209-215.

Norling, L. V., and Dalli, J. (2013), "Microparticles are novel effectors of immunity," *Curr Opin Pharmacol,* 13(4): 570-575.

Nozaki, T. *et al.* (2009), "Significance of a multiple biomarkers strategy including endothelial dysfunction to improve risk stratification for cardiovascular events in patients at high risk for coronary heart disease," *J Am Coll Cardiol,* 54(7): 601-608.

Oei, H. H. *et al.* (2005), "Lipoprotein-associated phospholipase A2 activity is associated with risk of coronary heart disease and ischemic stroke: the Rotterdam Study," *Circulation,* 111(5): 570-575.

Ong, P. *et al.* (2011), "3-year follow-up of patients with coronary artery spasm as cause of acute coronary syndrome: the CASPAR (coronary artery spasm in patients with acute coronary syndrome) study follow-up," *J Am Coll Cardiol,* 57(2): 147-152.

Osende, J. I. *et al.* (2001), "Blood thrombogenicity in type 2 diabetes mellitus patients is associated with glycemic control," *J Am Coll Cardiol,* 38(5): 1307-1312.

Ostrowski, M. *et al.* (2010), "Rab27a and Rab27b control different steps of the exosome secretion pathway," *Nat Cell Biol,* 12(1): 19-30; sup pp 11-13.

Ou, Z. J. *et al.* (2011), "Endothelium-derived microparticles inhibit angiogenesis in the heart and enhance the inhibitory effects of hypercholesterolemia on angiogenesis," *Am J Physiol Endocrinol Metab,* 300(4): E661-668.

Owens, A. P., and Mackman, N. (2011), "Microparticles in hemostasis and thrombosis," *Circ Res*, 108(10): 1284-1297.

Owens, M. R. (1994), "The role of platelet microparticles in hemostasis," *Transfus Med Rev,* 8(1): 37-44.

Pakala, R. (2004), "Serotonin and thromboxane A2 stimulate platelet-derived microparticle-induced smooth muscle cell proliferation," *Cardiovasc Radiat Med,* 5(1): 20-26.

Parise, L. V. (1999), "Integrin alpha(IIb)beta(3) signaling in platelet adhesion and aggregation," *Curr Opin Cell Biol,* 11(5): 597-601.

Park, N. J. *et al.* (2009), "Salivary microRNA: Discovery, characterization, and clinical utility for oral cancer detection," *Clin Cancer Res,* 15(17): 5473-5477.

Pasquet, J. M.; Dachary-Prigent, J., and Nurden, A. T. (1996), "Calcium influx is a determining factor of calpain activation and microparticle formation in platelets," *Eur J Biochem,* 239(3): 647-654.

— (1998), "Microvesicle release is associated with extensive protein tyrosine dephosphorylation in platelets stimulated by A23187 or a mixture of thrombin and collagen," *Biochem J,* 333(Pt 3): 591-599.

Pegtel, D. M. *et al.* (2010), "Functional delivery of viral miRNAs via exosomes," *Proc Natl Acad Sci U S A,* 107(14): 6328-6333.

Penet, M. F. *et al.* (2008), "Protection against cerebral malaria by the low-molecular-weight thiol pantethine," *Proc Natl Acad Sci U S A,* 105(4): 1321-1326.

Pepine, C. J. (1998), "The effects of angiotensin-converting enzyme inhibition on endothelial dysfunction: potential role in myocardial ischemia," *Am J Cardiol,* 82(10A): 23S-27S.

Pereira, J. *et al.* (2006), "Circulating platelet-derived microparticles in systemic lupus erythematosus. Association with increased thrombin generation and procoagulant state," *Thromb Haemost,* 95(1): 94-99.

Pérez-Casal, M. *et al.* (2005), "Activated protein C induces the release of microparticle-associated endothelial protein C receptor," *Blood,* 105(4): 1515-1522.

Pérez-Casal, M. *et al.* (2009), "Microparticle-associated endothelial protein C receptor and the induction of cytoprotective and anti-inflammatory effects," *Haematologica,* 94(3): 387-394.

Pérez-Casal, M. *et al.* (2011), "The clinical and functional relevance of microparticles induced by activated protein C treatment in sepsis," *Crit Care,* 15(4): R195.

Pérez-Pujol, S.; Marker, P. H., and Key, N. S. (2007), "Platelet microparticles are heterogeneous and highly dependent on the activation mechanism: studies using a new digital flow cytometer," *Cytometry A,* 71(1): 38-45.

Perini, F. *et al.* (1996), "PrP27-30 is a normal soluble prion protein fragment released by human platelets," *Biochem Biophys Res Commun,* 223(3): 572-577.

Perkumas, K. M. *et al.* (2007), "Myocilin-associated exosomes in human ocular samples," *Exp Eye Res,* 84(1): 209-212.

Peters, S. A. *et al.* (2012), "Added value of CAC in risk stratification for cardiovascular events: a systematic review," *Eur J Clin Invest,* 42(1): 110-116.

Peterson, D. B. *et al.* (2008), "Comparative proteomic analysis of PAI-1 and TNF-alpha-derived endothelial microparticles," *Proteomics,* 8(12): 2430-2446.

Pigati, L. *et al.* (2010), "Selective release of microRNA species from normal and malignant mammary epithelial cells," *PLoS One,* 5(10): e13515.

Pilzer, D. *et al.* (2005), "Emission of membrane vesicles: roles in complement resistance, immunity and cancer," *Springer Semin Immunopathol,* 27(3): 375-387.

Pitanga, T. N. *et al.* (2014), "Neutrophil-derived microparticles induce myeloperoxidase-mediated damage of vascular endothelial cells," *BMC Cell Biol,* 15: 21.

Pittoni, V., and *Valesini, G.* (2002), "The clearance of apoptotic cells: implications for autoimmunity," *Autoimmun Rev,* 1(3): 154-161.

Pluskota, E. *et al.* (2008), "Expression, activation, and function of integrin alphaMbeta2 (Mac-1) on neutrophil-derived microparticles," *Blood,* 112(6): 2327-2335.

Poliakov, A. *et al.* (2009), "Structural heterogeneity and protein composition of exosome-like vesicles (prostasomes) in human semen," *Prostate,* 69(2): 159-167.

Polonsky, T. S. *et al.* (2010), "Coronary artery calcium score and risk classification for coronary heart disease prediction," *Jama,* 303(16): 1610-1616.

Popescu, N. I.; Lupu, C., and Lupu, F. (2010), "Extracellular protein disulfide isomerase regulates coagulation on endothelial cells through modulation of phosphatidylserine exposure," *Blood,* 116(6): 993-1001.

Prasad, K. S. *et al.* (2003), "Soluble CD40 ligand induces beta3 integrin tyrosine phosphorylation and triggers platelet activation by outside-in signaling," *Proc Natl Acad Sci U S A,* 100(21): 12367-12371.

Preston, R. A. *et al.* (2003), "Effects of severe hypertension on endothelial and platelet microparticles," *Hypertension,* 41(2): 211-217.

Priou, P. *et al.* (2010), "Endothelial dysfunction and circulating microparticles from patients with obstructive sleep apnea," *Am J Pathol,* 177(2): 974-983.

Purushothaman, K. R. *et al.* (2012), "Increased expression of oxidation-specific epitopes and apoptosis are associated with haptoglobin genotype: possible implications for plaque progression in human atherosclerosis," *J Am Coll Cardiol,* 60(2): 112-119.

Raj, D. A. *et al.* (2012), "A multiplex quantitative proteomics strategy for protein biomarker studies in urinary exosomes," *Kidney Int,* 81(12): 1263-1272.

Rajavashisth, T. *et al.* (1998), "Heterozygous osteopetrotic (op) mutation reduces atherosclerosis in LDL receptor- deficient mice," *J Clin Invest,* 101(12): 2702-2710.

Rallidis, L. S. *et al.* (2003), "Usefulness of elevated levels of soluble vascular cell adhesion molecule-1 in predicting in-hospital prognosis in patients with unstable angina pectoris," *Am J Cardiol,* 92(10): 1195-1197.

Rand, M. L. *et al.* (2006), "Rapid clearance of procoagulant platelet-derived microparticles from the circulation of rabbits," *J Thromb Haemost,* 4(7): 1621-1623.

Rank, A. *et al.* (2011), "Clearance of platelet microparticles in vivo," *Platelets,* 22(2): 111-116.

Raposo, G., and Stoorvogel, W. (2013), "Extracellular vesicles: exosomes, microvesicles, and friends," *J Cell Biol,* 200(4): 373-383.

Raturi, A. *et al.* (2008), "Platelet microparticle-associated protein disulfide isomerase promotes platelet aggregation and inactivates insulin," *Biochim Biophys Acta,* 1778(12): 2790-2796.

Rauch, U.; Bonderman, D. *et al.* (2000), "Transfer of tissue factor from leukocytes to platelets is mediated by CD15 and tissue factor," *Blood,* 96(1): 170-175.

Rauch, U.; Crandall, J. *et al.* (2000), "Increased thrombus formation relates to ambient blood glucose and leukocyte count in diabetes mellitus type 2," *Am J Cardiol,* 86(2): 246-249.

Rautou, P. E. *et al.* (2011), "Microparticles from human atherosclerotic plaques promote endothelial ICAM-1-dependent monocyte adhesion and transendothelial migration," *Circ Res,* 108(3): 335-343.

Ravichandran, K. S., and Lorenz, U. (2007), "Engulfment of apoptotic cells: Signals for a good meal," *Nat Rev Immunol,* 7(12): 964-974.

Ray, K. K. *et al.* (2005), "Predictors of the rise in vWF after ST elevation myocardial infarction: implications for treatment strategies and clinical outcome: An ENTIRE-TIMI 23 substudy," Eur Heart J, 26(5): 440-446.

Rectenwald, J. E. *et al.* (2005), "D-dimer, P-selectin, and microparticles: novel markers to predict deep venous thrombosis. A pilot study," *Thromb Haemost,* 94(6): 1312-1317.

Regidor, E., and Gutiérrez-Fisac, J. (2013), "Patrones de Mortalidad en España 2010," *Ministry of Health, Social Services and Equality.*

Reichlin, T. *et al.* (2009), "Incremental value of copeptin for rapid rule out of acute myocardial infarction," *J Am Coll Cardiol,* 54(1): 60-68.

Reininger, A. J. *et al.* (2006), "Mechanism of platelet adhesion to von Willebrand factor and microparticle formation under high shear stress," *Blood,* 107(9): 3537-3545.

Rendu, F. *et al.* (2001), "Thiosulfinates inhibit platelet aggregation and microparticle shedding at a calpain-dependent step," *Thromb Haemost,* 86(5): 1284-1291.

Richardson, K. *et al.* (2013), "Gain-of-function lipoprotein lipase variant rs13702 modulates lipid traits through disruption of a microRNA-410 seed site," *Am J Hum Genet,* 92(1): 5-14.

Ridker, P. M. *et al.* (2000), "Plasma concentration of interleukin-6 and the risk of future myocardial infarction among apparently healthy men," *Circulation,* 101(15): 1767-1772.

Robert, S. *et al.* (2009), "Standardization of platelet-derived microparticle counting using calibrated beads and a Cytomics FC500 routine flow cytometer: a first step towards multicenter studies?," *J Thromb Haemost,* 7(1): 190-197.

Robertson, C. *et al.* (2006), "Cellular prion protein is released on exosomes from activated platelets," *Blood,* 107(10): 3907-3911.

Rogacev, K. S. *et al.* (2012), "CD14++CD16+ monocytes independently predict cardiovascular events: A cohort study of 951 patients referred for elective coronary angiography," *J Am Coll Cardiol,* 60(16): 1512-1520.

Ross, R. (1999), "Atherosclerosis--an inflammatory disease," *N Engl J Med,* 340(2): 115-126.

Rossig, L. *et al.* (2000), "Congestive heart failure induces endothelial cell apoptosis: protective role of carvedilol," *J Am Coll Cardiol,* 36(7): 2081-2089.

Rozmyslowicz, T. *et al.* (2003), "Platelet- and megakaryocyte-derived microparticles transfer CXCR4 receptor to CXCR4-null cells and make them susceptible to infection by X4-HIV," *Aids,* 17(1): 33-42.

Rubin, O. *et al.* (2010), "Pre-analytical and methodological challenges in red blood cell microparticle proteomics," *Talanta,* 82(1): 1-8.

Sabatier, F. *et al.* (2002), "Type 1 and type 2 diabetic patients display different patterns of cellular microparticles," *Diabetes,* 51(9): 2840-2845.

Sadallah, S. *et al.* (2011), "Microparticles (ectosomes) shed by stored human platelets downregulate macrophages and modify the development of dendritic cells," *J Immunol,* 186(11): 6543-6552.

Salic, K., and De Windt, L. J. (2012), "MicroRNAs as biomarkers for myocardial infarction," *Curr Atheroscler Rep,* 14(3): 193-200.

Sambola, A. *et al.* (2003), "Role of risk factors in the modulation of tissue factor activity and blood thrombogenicity," *Circulation,* 107(7): 973-977.

Sangiorgi, G. *et al.* (2006), "Plasma levels of metalloproteinases-9 and -2 in the acute and subacute phases of type A and type B aortic dissection," *J Cardiovasc Med (Hagerstown),* 7(5): 307-315.

Santos-Gallego, C. G.; Picatoste, B., and Badimon, J. J. (2014), "Pathophysiology of acute coronary syndrome," *Curr Atheroscler Rep,* 16(4): 401.

Sapet, C. *et al.* (2006), "Thrombin-induced endothelial microparticle generation: identification of a novel pathway involving ROCK-II activation by caspase-2," *Blood,* 108(6): 1868-1876.

Sarkar, A. *et al.* (2009), "Monocyte derived microvesicles deliver a cell death message via encapsulated caspase-1," PLoS One, 4(9): e7140.

Satta, N.; Freyssinet, J. M., and Toti, F. (1997), "The significance of human monocyte thrombomodulin during membrane vesiculation and after stimulation by lipopolysaccharide," *Br J Haematol,* 96(3): 534-542.

Satta, N. *et al.* (1994), "Monocyte vesiculation is a possible mechanism for dissemination of membrane-associated procoagulant activities and adhesion molecules after stimulation by lipopolysaccharide," *J Immunol,* 153(7): 3245-3255.

Scanu, A. *et al.* (2008), "Stimulated T cells generate microparticles, which mimic cellular contact activation of human monocytes: differential regulation of pro- and anti-inflammatory cytokine production by high-density lipoproteins," *J Leukoc Biol,* 83(4): 921-927.

Schlegel, R. A., and Williamson, P. (2001), "Phosphatidylserine, a death knell," *Cell Death Differ,* 8(6): 551-563.

Schoenwaelder, S. M. *et al.* (2009), "Two distinct pathways regulate platelet phosphatidylserine exposure and procoagulant function," *Blood,* 114(3): 663-666.

Sebbagh, M. *et al.* (2001), "Caspase-3-mediated cleavage of ROCK I induces MLC phosphorylation and apoptotic membrane blebbing," *Nat Cell Biol,* 3(4): 346-352.

Sekula, M. *et al.* (2011), "Endothelial microparticle formation in moderate concentrations of homocysteine and methionine in vitro," *Cell Mol Biol Lett,* 16(1): 69-78.

Sellam, J. *et al.* (2009), "Increased levels of circulating microparticles in primary Sjögren's syndrome, systemic lupus erythematosus and rheumatoid arthritis and relation with disease activity," *Arthritis Res Ther,* 11(5): R156.

Selvamani, A. *et al.* (2012), "An antagomir to microRNA Let7f promotes neuroprotection in an ischemic stroke model," *PLoS One,* 7(2): e32662.

Senzel, L.; Gnatenko, D. V., and Bahou, W. F. (2009), "The platelet proteome," *Curr Opin Hematol,* 16(5): 329-333.

Shai, E. *et al.* (2012), "Comparative analysis of platelet-derived microparticles reveals differences in their amount and proteome depending on the platelet stimulus," *J Proteomics,* 76 Spec No.: 287-296.

Shefler, I. *et al.* (2010), "T cell-induced mast cell activation: a role for microparticles released from activated T cells," *J Immunol,* 185(7): 4206-4212.

Sheremata, W. A. *et al.* (2008), "Evidence of platelet activation in multiple sclerosis," *J Neuroinflammation,* 5: 27.

Shet, A. S. *et al.* (2003), "Sickle blood contains tissue factor-positive microparticles derived from endothelial cells and monocytes," *Blood,* 102(7): 2678-2683.

Shouzu, A. *et al.* (2004), "Effect of ticlopidine on monocyte-derived microparticles and activated platelet markers in diabetes mellitus," *Clin Appl Thromb Hemost,* 10(2): 167-173.

Siljander, P.; Carpen, O., and Lassila, R. (1996), "Platelet-derived microparticles associate with fibrin during thrombosis," Blood, 87(11): 4651-4663.

Siljander, P. *et al.* (2001), "Platelet adhesion enhances the glycoprotein VI-dependent procoagulant response: Involvement of p38 MAP kinase and calpain," *Arterioscler Thromb Vasc Biol,* 21(4): 618-627.

Simak, J., and Gelderman, M. P. (2006), "Cell membrane microparticles in blood and blood products: potentially pathogenic agents and diagnostic markers," *Transfus Med Rev,* 20(1): 1-26.

Simak, J. et al. (2004), "Elevated circulating endothelial membrane microparticles in paroxysmal nocturnal haemoglobinuria," *Br J Haematol,* 125(6): 804-813.

Simak, J. et al. (2006), "Circulating endothelial microparticles in acute ischemic stroke: a link to severity, lesion volume and outcome," *J Thromb Haemost,* 4(6): 1296-1302.

Simak, J.; Holada, K., and Vostal, J. G. (2002), "Release of annexin V-binding membrane microparticles from cultured human umbilical vein endothelial cells after treatment with camptothecin," *BMC Cell Biol,* 3: 11.

Simoncini, S. *et al.* (2009), "TRAIL/Apo2L mediates the release of procoagulant endothelial microparticles induced by thrombin in vitro: a potential mechanism linking inflammation and coagulation," *Circ Res,* 104(8): 943-951.

Simons, M., and Raposo, G. (2009), "Exosomes--vesicular carriers for intercellular communication," *Curr Opin Cell Biol,* 21(4): 575-581.

Sims, P. J. *et al.* (1988), "Complement proteins C5b-9 cause release of membrane vesicles from the platelet surface that are enriched in the membrane receptor for coagulation factor Va and express prothrombinase activity," *J Biol Chem,* 263(34): 18205-18212.

Sims, P. J. *et al.* (1989), "Assembly of the platelet prothrombinase complex is linked to vesiculation of the platelet plasma membrane. Studies in Scott syndrome: an isolated defect in platelet procoagulant activity," *J Biol Chem,* 264(29): 17049-17057.

Sinauridze, E. I. *et al.* (2007), "Platelet microparticle membranes have 50- to 100-fold higher specific procoagulant activity than activated platelets," *Thromb Haemost,* 97(3): 425-434.

Sjouke, B. *et al.* (2011), "Familial hypercholesterolemia: present and future management," *Curr Cardiol Rep,* 13(6): 527-536.

Skog, J. *et al.* (2008), "Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers," *Nat Cell Biol,* 10(12): 1470-1476.

Smalley, D. M. *et al.* (2007), "Proteomic discovery of 21 proteins expressed in human plasma-derived but not platelet-derived microparticles," *Thromb Haemost,* 97(1): 67-80.

Soleti, R. *et al.* (2009), "Microparticles harboring Sonic Hedgehog promote angiogenesis through the upregulation of adhesion proteins and proangiogenic factors," *Carcinogenesis,* 30(4): 580-588.

Soleti, R., and Martinez, M. C. (2009), "Microparticles harbouring Sonic Hedgehog: role in angiogenesis regulation," *Cell Adh Migr,* 3(3): 293-295.

Sommeijer, D. W. *et al.* (2005), "Pravastatin reduces fibrinogen receptor gpIIIa on platelet-derived microparticles in patients with type 2 diabetes," *J Thromb Haemost,* 3(6): 1168-1171.

Soriano, A. O. *et al.* (2005), "Levels of endothelial and platelet microparticles and their interactions with leukocytes negatively correlate with organ dysfunction and predict mortality in severe sepsis," *Crit Care Med,* 33(11): 2540-2546.

Sossdorf, M. *et al.* (2011), "Cell-derived microparticles promote coagulation after moderate exercise," *Med Sci Sports Exerc,* 43(7): 1169-1176.

Stahl, A. L.; Sartz, L., and Karpman, D. (2011), "Complement activation on platelet-leukocyte complexes and microparticles in enterohemorrhagic Escherichia coli-induced hemolytic uremic syndrome," *Blood,* 117(20): 5503-5513.

Starega-Roslan, J. *et al.* (2011), "The role of the precursor structure in the biogenesis of microRNA," *Cell Mol Life Sci,* 68(17): 2859-2871.

Stary, H. C. *et al.* (1995), "A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association," *Circulation,* 92(5): 1355-1374.

Stenberg, P. E. *et al.* (1985), "A platelet alpha-granule membrane protein (GMP-140) is expressed on the plasma membrane after activation," *J Cell Biol,* 101(3): 880-886.

Steppich, B. *et al.* (2005), "Tissue factor pathway inhibitor on circulating microparticles in acute myocardial infarction," *Thromb Haemost,* 93(1): 35-39.

Stone, N. J. *et al.* (2013), "2013 ACC/AHA Guideline on the Treatment of Blood Cholesterol to Reduce Atherosclerotic Cardiovascular Risk in Adults: A Report of the American College of Cardiology/ American Heart Association Task Force on Practice Guidelines," *Circulation.*

Stoorvogel, W. *et al.* (2002), "The biogenesis and functions of exosomes," *Traffic,* 3(5): 321-330.

Street, J. M. *et al.* (2012), "Identification and proteomic profiling of exosomes in human cerebrospinal fluid," *J Transl Med,* 10: 5.

Suckau, L. *et al.* (2009), "Long-term cardiac-targeted RNA interference for the treatment of heart failure restores cardiac function and reduces pathological hypertrophy," *Circulation,* 119(9): 1241-1252.

Sugiyama, S. *et al.* (2004), "Hypochlorous acid, a macrophage product, induces endothelial apoptosis and tissue factor expression: involvement of myeloperoxidase-mediated oxidant in plaque erosion and thrombogenesis," *Arterioscler Thromb Vasc Biol,* 24(7): 1309-1314.

Sun, J. *et al.* (2007), "Mast cells promote atherosclerosis by releasing proinflammatory cytokines," *Nat Med,* 13(6): 719-724.

Szotowski, B. *et al.* (2007), "Antioxidative treatment inhibits the release of thrombogenic tissue factor from irradiation- and cytokine-induced endothelial cells," *Cardiovasc Res,* 73(4): 806-812.

Tabas, I.; Williams, K. J., and Boren, J. (2007), "Subendothelial lipoprotein retention as the initiating process in atherosclerosis: update and therapeutic implications," *Circulation,* 116(16): 1832-1844.

Takano, K. *et al.* (2004), "Collagen-induced generation of platelet-derived microparticles in whole blood is dependent on ADP released from red blood cells and calcium ions," *Platelets,* 15(4): 223-229.

Tan, K. T., and Lip, G. Y. (2005), "The potential role of platelet microparticles in atherosclerosis," *Thromb Haemost,* 94(3): 488-492.

Tang, K. *et al.* (2010), "Microparticles mediate enzyme transfer from platelets to mast cells: a new pathway for lipoxin A4 biosynthesis," *Biochem Biophys Res Commun,* 400(3): 432-436.

Tans, G. *et al.* (1991), "Comparison of anticoagulant and procoagulant activities of stimulated platelets and platelet-derived microparticles," *Blood,* 77(12): 2641-2648.

Tantawy, A. A. *et al.* (2010), "Platelet microparticles in immune thrombocytopenic purpura in pediatrics," *Pediatr Hematol Oncol,* 27(4): 283-296.

Tantawy, A. A. *et al.* (2013), "Flow cytometric assessment of circulating platelet and erythrocytes microparticles in young thalassemia major patients: relation to pulmonary hypertension and aortic wall stiffness," *Eur J Haematol.*

Taurino, C. *et al.* (2010), "Gene expression profiling in whole blood of patients with coronary artery disease," *Clin Sci (Lond),* 119(8): 335-343.

Terrisse, A. D. *et al.* (2010), "Internalization of microparticles by endothelial cells promotes platelet/ endothelial cell interaction under flow," *J Thromb Haemost,* 8(12): 2810-2819.

Tesse, A. *et al.* (2005), "Upregulation of proinflammatory proteins through NF-kappaB pathway by shed membrane microparticles results in vascular hyporeactivity," *Arterioscler Thromb Vasc Biol,* 25(12): 2522-2527.

Thery, C. *et al.* (1999), "Molecular characterization of dendritic cell-derived exosomes. Selective accumulation of the heat shock protein hsc73," *J Cell Biol,* 147(3): 599-610.

Thery, C. *et al.* (2001), "Proteomic analysis of dendritic cell-derived exosomes: a secreted subcellular compartment distinct from apoptotic vesicles," *J Immunol,* 166(12): 7309-7318.

THERY, C.; OSTROWSKI, M., AND SEGURA, E. (2009), "Membrane vesicles as conveyors of immune responses," *Nat Rev Immunol,* 9(8): 581-593.

THYGESEN, K. *et al.* (2007), "Universal definition of myocardial infarction," *Circulation,* 116(22): 2634-2653.

THYGESEN, K. *et al.* (2010), "Recommendations for the use of cardiac troponin measurement in acute cardiac care," *Eur Heart J,* 31(18): 2197-2204.

TIAN, T. *et al.* (2010), "Visualizing of the cellular uptake and intracellular trafficking of exosomes by live-cell microscopy," *J Cell Biochem,* 111(2): 488-496.

TIJSEN, A. J. *et al.* (2010), "MiR423-5p as a circulating biomarker for heart failure," *Circ Res,* 106(6): 1035-1039.

TOTH, B. *et al.* (2007), "Gender-specific and menstrual cycle dependent differences in circulating microparticles," *Platelets,* 18(7): 515-521.

TOTH, B. *et al.* (2008), "Platelet-derived microparticles and coagulation activation in breast cancer patients," *Thromb Haemost,* 100(4): 663-669.

Touyz, R. M. *et al.* (2011), "NOX isoforms and reactive oxygen species in vascular health," *Mol Interv,* 11(1): 27-35.

TRAJKOVIC, K. *et al.* (2008), "Ceramide triggers budding of exosome vesicles into multivesicular endosomes," *Science,* 319(5867): 1244-1247.

TRAMONTANO, A. F. *et al.* (2004), "Statin decreases endothelial microparticle release from human coronary artery endothelial cells: implication for the Rho-kinase pathway," *Biochem Biophys Res Commun,* 320(1): 34-38.

TSCHUOR, C. *et al.* (2008), "In vitro norepinephrine significantly activates isolated platelets from healthy volunteers and critically ill patients following severe traumatic brain injury," *Crit Care,* 12(3): R80.

TURCHINOVICH, A. *et al.* (2011), "Characterization of extracellular circulating microRNA," *Nucleic Acids Res,* 39(16): 7223-7233.

TUSHUIZEN, M. E. *et al.* (2006), "Two consecutive high-fat meals affect endothelial-dependent vasodilation, oxidative stress and cellular microparticles in healthy men," *J Thromb Haemost,* 4(5): 1003-1010.

UEBA, T. *et al.* (2010), "Plasma level of platelet-derived microparticles is associated with coronary heart disease risk score in healthy men," *J Atheroscler Thromb,* 17(4): 342-349.

ULLAL, A. J., and PISETSKY, D. S. (2010), "The release of microparticles by Jurkat leukemia T cells treated with staurosporine and related kinase inhibitors to induce apoptosis," *Apoptosis,* 15(5): 586-596.

UNDAS, A.; BRUMMEL-ZIEDINS, K. E., and MANN, K. G. (2005), "Statins and blood coagulation," *Arterioscler Thromb Vasc Biol,* 25(2): 287-294.

VALADI, H. *et al.* (2007), "Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells," *Nat Cell Biol,* 9(6): 654-659.

VALLES, J. *et al.* (1998), "Erythrocyte promotion of platelet reactivity decreases the effectiveness of aspirin as an antithrombotic therapeutic modality: the effect of low-dose aspirin is less than optimal in patients with vascular disease due to prothrombotic effects of erythrocytes on platelet reactivity," *Circulation,* 97(4): 350-355.

VALLES, J. *et al.* (2002), "Platelet-erythrocyte interactions enhance alpha(IIb)beta(3) integrin receptor activation and P-selectin expression during platelet recruitment: down-regulation by aspirin ex vivo," *Blood,* 99(11): 3978-3984.

VAN DEN AKKER, J. *et al.* (2012), "Transglutaminase 2 is secreted from smooth muscle cells by transamidation-dependent microparticle formation," *Amino Acids,* 42(2-3): 961-973.

VAN DER POL, E. *et al.* (2013), "Innovation in detection of microparticles and exosomes," *J Thromb Haemost,* 11 Suppl 1: 36-45.

Van Der Zee, P. M. *et al.* (2006), "P-selectin- and CD63-exposing platelet microparticles reflect platelet activation in peripheral arterial disease and myocardial infarction," *Clin Chem,* 52(4): 657-664.

Van Dommelen, S. M. et al. (2011), "Microvesicles and exosomes: opportunities for cell-derived membrane vesicles in drug delivery," *J Control Release,* 161(2): 635-644.

Van Niel, G. *et al.* (2006), "Exosomes: a common pathway for a specialized function," *J Biochem,* 140(1): 13-21.

Van Nispen Tot Pannerden, H. *et al.* (2010), "The platelet interior revisited: electron tomography reveals tubular alpha-granule subtypes," *Blood,* 116(7): 1147-1156.

Vanags, D. M. *et al.* (1996), "Protease involvement in fodrin cleavage and phosphatidylserine exposure in apoptosis," *J Biol Chem,* 271(49): 31075-31085.

Vanwijk, M. J. *et al.* (2002), "Isolated microparticles, but not whole plasma, from women with preeclampsia impair endothelium-dependent relaxation in isolated myometrial arteries from healthy pregnant women," *Am J Obstet Gynecol,* 187(6): 1686-1693.

Vasina, E. M. *et al.* (2011), "Microparticles from apoptotic platelets promote resident macrophage differentiation," *Cell Death Dis,* 2: e211.

Vickers, K. C. *et al.* (2011), "MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins," *Nat Cell Biol,* 13(4): 423-433.

Viemann, D. *et al.* (2007), "MRP8/MRP14 impairs endothelial integrity and induces a caspase-dependent and -independent cell death program," *Blood,* 109(6): 2453-2460.

Virmani, R. *et al.* (2000), "Lessons from sudden coronary death: a comprehensive morphological classification scheme for atherosclerotic lesions," *Arterioscler Thromb Vasc Biol,* 20(5): 1262-1275.

Virmani, R. *et al.* (2005), "Atherosclerotic plaque progression and vulnerability to rupture: angiogenesis as a source of intraplaque hemorrhage," *Arterioscler Thromb Vasc Biol,* 25(10): 2054-2061.

Vogl, T. *et al.* (2007), "Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock," *Nat Med,* 13(9): 1042-1049.

Wagner, G. M. *et al.* (1986), "Red cell vesiculation--a common membrane physiologic event," *J Lab Clin Med,* 108(4): 315-324.

Wagner, J. *et al.* (2013), "Characterization of levels and cellular transfer of circulating lipoprotein-bound microRNAs," *Arterioscler Thromb Vasc Biol,* 33(6): 1392-1400.

Wagner, D, F. *et al.*. (2010), "MicroRNAs: synthesis, mechanism, function, and recent clinical trials," *Biochim Biophys Acta,* 1803(11): 1231-1243.

Wang, G. K. *et al.* (2010), "Circulating microRNA: a novel potential biomarker for early diagnosis of acute myocardial infarction in humans," *Eur Heart J,* 31(6): 659-666.

Wang, J. *et al.* (2012), "Association of perfluorooctanoic acid with HDL cholesterol and circulating miR-26b and miR-199-3p in workers of a fluorochemical plant and nearby residents," *Environ Sci Technol,* 46(17): 9274-9281.

Wang, J. G. *et al.* (2011), "Monocytic microparticles activate endothelial cells in an IL-1beta-dependent manner," *Blood,* 118(8): 2366-2374.

Wang, J. M. *et al.* (2007), "C-Reactive protein-induced endothelial microparticle generation in HUVECs is related to BH4-dependent NO formation," *J Vasc Res,* 44(3): 241-248.

Wang, K. *et al.* (2010), "Export of microRNAs and microRNA-protective protein by mammalian cells," *Nucleic Acids Res,* 38(20): 7248-7259.

Wang, K. *et al.* (2012), "The complex exogenous RNA spectra in human plasma: an interface with human gut biota?," *PLoS One,* 7(12): e51009.

Wang, R. *et al.* (2011), "Circulating microRNAs are promising novel biomarkers of acute myocardial infarction," *Intern Med,* 50(17): 1789-1795.

Warkentin, T. E. *et al.* (1994), "Sera from patients with heparin-induced thrombocytopenia generate platelet-derived microparticles with procoagulant activity: an explanation for the thrombotic complications of heparin-induced thrombocytopenia," *Blood,* 84(11): 3691-3699.

Wassmer, S. C. *et al.* (2005), "Inhibition of endothelial activation: a new way to treat cerebral malaria?," *PLoS Med,* 2(9): e245.

Watanabe, J. *et al.* (2003), "Endotoxins stimulate neutrophil adhesion followed by synthesis and release of platelet-activating factor in microparticles," *J Biol Chem,* 278(35): 33161-33168.

Weber, J. A. *et al.* (2010), "The microRNA spectrum in 12 body fluids," *Clin Chem,* 56(11): 1733-1741.

Weber, M. *et al.* (2013), "Improved diagnostic and prognostic performance of a new high-sensitive troponin T assay in patients with acute coronary syndrome," *Am Heart J,* 162(1): 81-88.

Weerheim, A. M. *et al.* (2002), "Phospholipid composition of cell-derived microparticles determined by one-dimensional high-performance thin-layer chromatography," *Anal Biochem,* 302(2): 191-198.

Werner, N. *et al.* (2006), "Circulating CD31+/annexin V+ apoptotic microparticles correlate with coronary endothelial function in patients with coronary artery disease," *Arterioscler Thromb Vasc Biol,* 26(1): 112-116.

Wickman, G. R. *et al.* (2013), "Blebs produced by actin-myosin contraction during apoptosis release damage-associated molecular pattern proteins before secondary necrosis occurs," *Cell Death Differ,* 20(10): 1293-1305.

Wiedmer, T. *et al.* (1990), "Role of calcium and calpain in complement-induced vesiculation of the platelet plasma membrane and in the exposure of the platelet factor Va receptor.," *Biochemistry,* 29(3): 623-632.

Wiedmer, T., and Sims, P. J. (1991), "Participation of protein kinases in complement C5b-9-induced shedding of platelet plasma membrane vesicles," *Blood*, 78(11): 2880-2886.

Willekens, F. L. *et al.* (2005), "Liver Kupffer cells rapidly remove red blood cell-derived vesicles from the circulation by scavenger receptors," *Blood,* 105(5): 2141-2145.

Williams, J. C., and Mackman, N. (2011), "MPs or ICs?," *Blood,* 117(4): 1101-1102.

Williams, R. L., and Urbe, S. (2007), "The emerging shape of the ESCRT machinery," *Nat Rev Mol Cell Biol,* 8(5): 355-368.

Williamson, P. *et al.* (1995), "Continuous analysis of the mechanism of activated transbilayer lipid movement in platelets," *Biochemistry,* 34(33): 10448-10455.

Witztum, J. L. (1991), "The role of oxidized LDL in atherosclerosis," *Adv Exp Med Biol,* 285: 353-365.

Wu, Y.; Tibrewal, N., and Birge, R. B. (2006), "Phosphatidylserine recognition by phagocytes: a view to a kill," *Trens Cell Biol,* 16(4): 189-197.

Xiao, H. *et al.* (2002), "Thrombin-induced platelet microparticles improved the aggregability of cryopreserved platelets," *Cryobiology,* 44(2): 179-188.

Xu, Y. *et al.* (2012), "Prevalence, distribution, predictors, and outcomes of patients with calcified nodules in native coronary arteries: a 3-vessel intravascular ultrasound analysis from Providing Regional Observations to Study Predictors of Events in the Coronary Tree (PROSPECT)," *Circulation,* 126(5): 537-545.

Xu, Y.; Nakane, N., and Maurer-Spurej, E. (2011), "Novel test for microparticles in platelet-rich plasma and platelet concentrates using dynamic light scattering," *Transfusion,* 51(2): 363-370.

Yang, C. *et al.* (2008), "Lymphocytic microparticles inhibit angiogenesis by stimulating oxidative stress and negatively regulating VEGF-induced pathways," *Am J Physiol Regul Integr Comp Physiol,* 294(2): R467-476.

YANO, Y. *et al.* (1994), "The role of protein phosphorylation and cytoskeletal reorganization in microparticle formation from the platelet plasma membrane," *Biochem J,* 299 (Pt1): 303-308.

YUAN, G.; WANG, J., and HEGELE, R. A. (2006), "Heterozygous familial hypercholesterolemia: An underrecognized cause of early cardiovascular disease," *Cmaj,* 174(8): 1124-1129.

YUAN, X. L. *et al.* (2010), "Elevated expression of Foxp3 in tumor-infiltrating Treg cells suppresses T-cell proliferation and contributes to gastric cancer progression in a COX-2-dependent manner," *Clin Immunol,* 134(3): 277-288.

YUANA, Y.; BERTINA, R. M., and OSANTO, S. (2011), "Pre-analytical and analytical issues in the analysis of blood microparticles," *Thromb Haemost,* 105(3): 396-408.

YUN, C. H. *et al.* (2010), "Increased circulating endothelial microparticles and carotid atherosclerosis in obstructive sleep apnea," *J Clin Neurol,* 6(2): 89-98.

YUSUF, S. *et al.* (2004), "Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): case-control study," *Lancet,* 364(9438): 937-952.

ZEIGER, F. *et al.* (2000), "P-Selectin expression, platelet aggregates, and platelet-derived microparticle formation are increased in peripheral arterial disease," *Blood Coagul Fibrinolysis,* 11(8): 723-728.

ZERNECKE, A. *et al.* (2009), "Delivery of microRNA-126 by apoptotic bodies induces CXCL12-dependent vascular protection," *Sci Signal,* 2(100): ra81.

ZHANG, L. *et al.* (2012), "Exogenous plant MIR168a specifically targets mammalian LDLRAP1: evidence of cross-kingdom regulation by microRNA," *Cell Res,* 22(1): 107-126.

ZHANG, Y. *et al.* (2010), "Secreted monocytic miR-150 enhances targeted endothelial cell migration," *Mol Cell,* 39(1): 133-144.

ZHOU, X. *et al.* (2000), "Transfer of CD4(+) T cells aggravates atherosclerosis in immunodeficient apolipoprotein E knockout mice," *Circulation,* 102(24): 2919-2922.

ZWAAL, R. F.; COMFURIUS, P., and BEVERS, E. M. (2005), "Surface exposure of phosphatidylserine in pathological cells," *Cell Mol Life Sci,* 62(9): 971-988.

ZWAAL, R. F., and SCHROIT, A. J. (1997), "Pathophysiologic implications of membrane phospholipid asymmetry in blood cells," *Blood,* 89(4): 1121-1132.

ZWICKER, J. I. *et al.* (2011), "Tissue factor-bearing microparticles and thrombus formation," *Arterioscler Thromb Vasc Biol,* 31(4): 728-733.

2 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:

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

  The specific aims were:

  - To set up a flow cytometric method for measurement of plasma-derived microparticles, including pre-analytical sample procedure.
  - 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.
  - To determine the association between cMP phenotype and atherosclerotic lesion type.

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

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

The specific aims were:

- To evaluate the effect of high concentration of microparticles on coagulation, platelet function, and platelet deposition under flow conditions.
- To dissect the mechanisms by which microparticles enhance thrombus formation.
- To characterize the phenotype of circulating microparticles released from the growing thrombi under flow conditions and validate it to patients with acute coronary syndromes.

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

The specific aims were:

- To investigate the prothrombotic proteome of platelet-derived microparticles.
- To study the composition in microRNAs of circulating microvesicles and their relation with cardiovascular event presentation.

3 MATERIALS AND METHODS

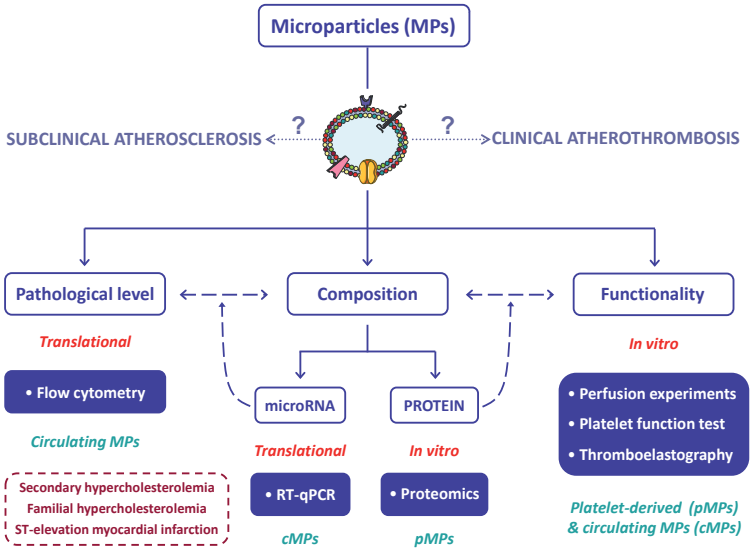
3.1. STUDY DESIGN

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

Figure 18

SCHEMATIC EXPERIMENTAL DESIGN



Note: cMPs indicates circulating microparticles; pMPs, platelet-derived microparticles; RT-qPCR, reverse transcription quantitative polymerase chain reaction.
Source: Own elaboration.

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.

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).*

3.2. 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.

3.2.1. 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 (Mata *et al.,* 2011). Specifically, the three groups of patients (n=37/group, Table 9) refer to:

■ HCVR patients with clinical and genetic diagnosis of heterozygous FH, which had been previously characterized by MRI-imaging (Caballero *et al.,* 2012) (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 (NCEP, 2002; Graham *et al.,* 2007; Wierzbicki, Humphries and Minhas, 2008; Civeira, 2004)

Table 9

CLINICAL CHARACTERISTICS OF GROUPS OF PATIENTS FROM THE SAFEHEART COHORT

	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/m2)	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%)	-

Notes: 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.
Source: Own elaboration.

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 (Pijlman *et al.,* 2010). All FH patients fulfilled the WHO criteria;

■ 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,

■ 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 (Gehi *et al.,* 2007). 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.

3.2.2. 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 (Terkelsen *et al.,* 2013). 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.

3.3. 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 (Mozas *et al.,* 2004).
- 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) (Friedewald, Levy and Fredrickson, 1972) 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) (Caballero *et al.,* 2012). MRI has been widely used in the last decade to evaluate atherosclerosis burden in high-risk patients including FH patients (Corti and Fuster, 2011; Vilades Medel *et al.,* 2013). 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.

3.4. MICROPARTICLE ISOLATION

3.4.1. 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 (Nieuwland *et al.,* 2000; Rank Liebhardt *et al.,* 2012; Rank Nieuwland *et al.,* 2012). 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.

3.4.2. 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 $4.0x10^6$ platelets/µL (Medonic hematologic analyzer) and incubated at 37ºC for 5 minutes without stirring to allow spontaneous pMP release (Aatonen, Gronholm and Siljander, 2012).

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) (Brill *et al.,* 2005) 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.

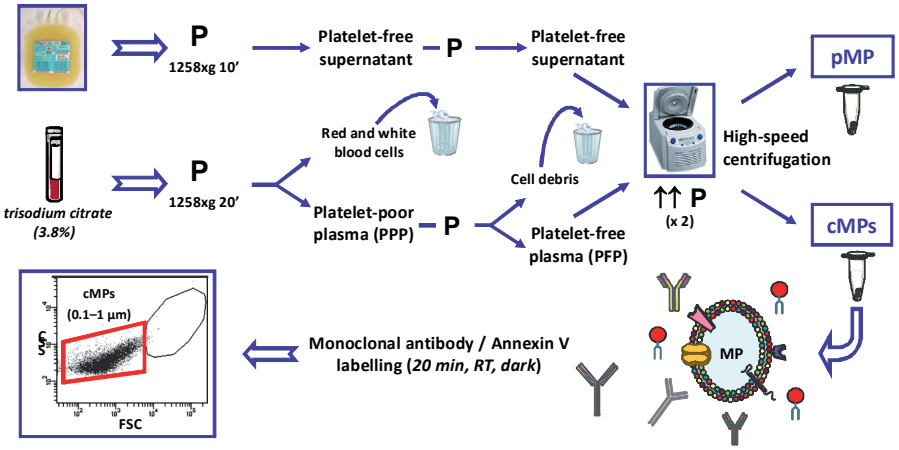
3.5. FLOW CYTOMETRY OF CIRCULATING MICROPARTICLES

Triple-label flow cytometric analysis was performed as described by Nieuwland et al with slight modifications (Nieuwland *et al.,* 2000). 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



Note: Major steps for platelet-derived (pMP) and circulating microparticle (cMP) isolation and labelling in flow cytometry analysis. FSC indicates forward scatter; SSC, side scatter.
Source: Own elaboration.

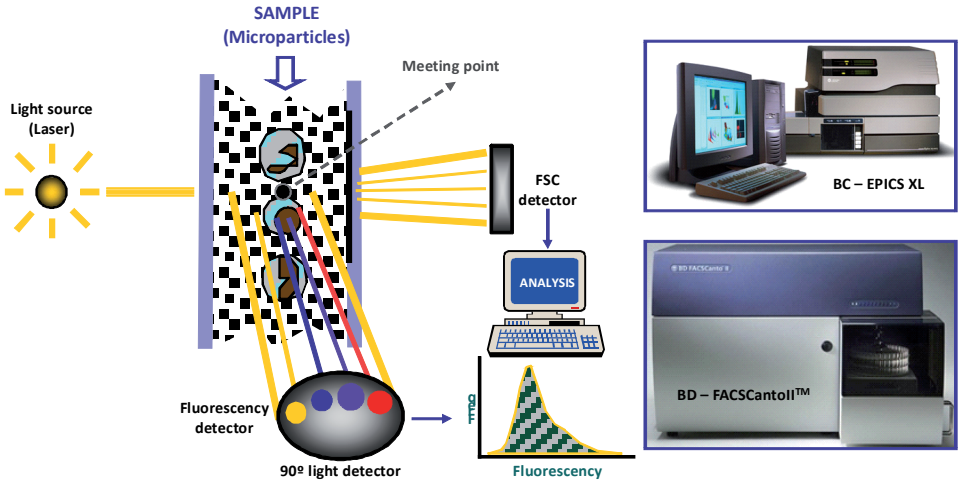
Acquisition was performed for 1 minute per sample. In flow cytometry technique, physical properties (size and internal complexity) and fluorescence characteristics of single particles are measured, as shown in Figure 20. Forward scatter (FSC), side scatters (SSC) and fluorescence data were obtained with 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) (Lacroix *et al.,* 2010) and (2) with an *in vitro* platelet-derived microparticle population as positive control (Biró *et al.,* 2005; Rank Nieuwland *et al.,* 2012) since calibration beads have different properties of FSC/SSC compared with biologic MPs (Williams and Mackman, 2011; van der Pol *et al.,* 2014). 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



Notes: 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.
Source: Own elaboration.

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 (Nieuwland *et al.,* 2000), based on sample's volume, flow cytometer's flow rate and the number of fluorescence-positive events (N), as follows *(Papers 2-6):*

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

[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.

Table 10

CELL SURFACE MOLECULES FOR CMP IDENTIFICATION AND CHARACTERIZATION

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
IgG1$\varkappa$	-	FITC / PE	MPOC21	1-6

Notes: ECs indicates endothelial cells; FITC, fluorescein isothiocyanate; PE, phycoerythrin.
Source: Own elaboration.

3.6. FUNCTIONAL STUDIES

3.6.1. Perfusion experiments

3.6.1.1. Experimental design

The effect of cMPs and pMPs on flow-induced platelet deposition was analyzed using the previously validated Badimon perfusion chamber (Badimon *et al.,* 1987, 1988) and the flat perfusion chamber (Molins *et al.,* 2010, 2011). 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) (Badimon *et al.,* 1987), 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.

3.6.1.2. 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 (Fernandez-Ortiz *et al.,* 1994). 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±0.26x10^6 ^{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.

3.6.1.3. 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.

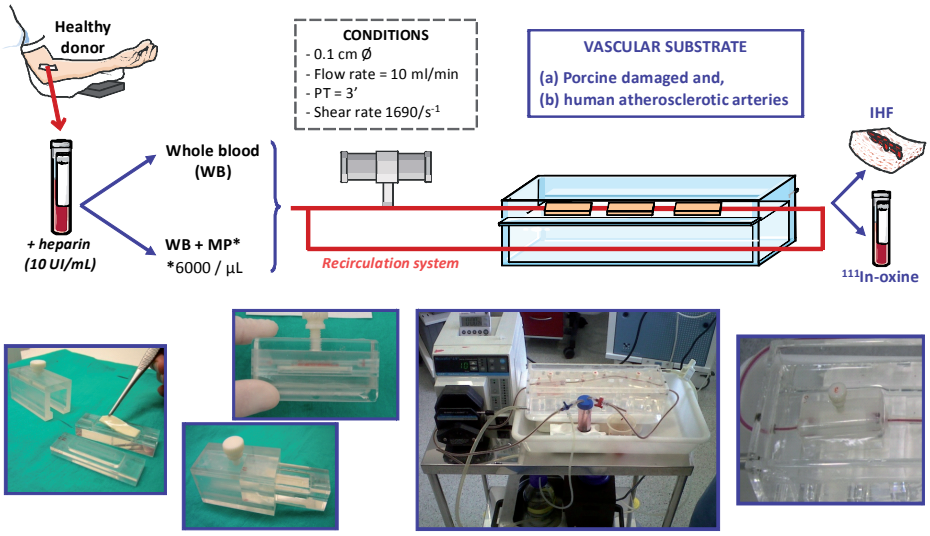
Before the experiments, the aortas were thawed in PBS at 4ºC, open longitudinally, and cut into 30x10mm segments. Segments of pig aorta were denuded (model of erosion). Human specimens composed of fatty streaks and atherosclerotic lesions (macroscopically characterized by raised yellow streaks and raised 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) (Badimon and Badimon, 1989; Badimon *et al.,* 1987). 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 $1690s^{-1}$ (Badimon *et al.,* 1988) 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 (Rand *et al.,* 2006). 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

Figure 21

BADIMON PERFUSION CHAMBER



Notes: 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.
Source: Own elaboration.

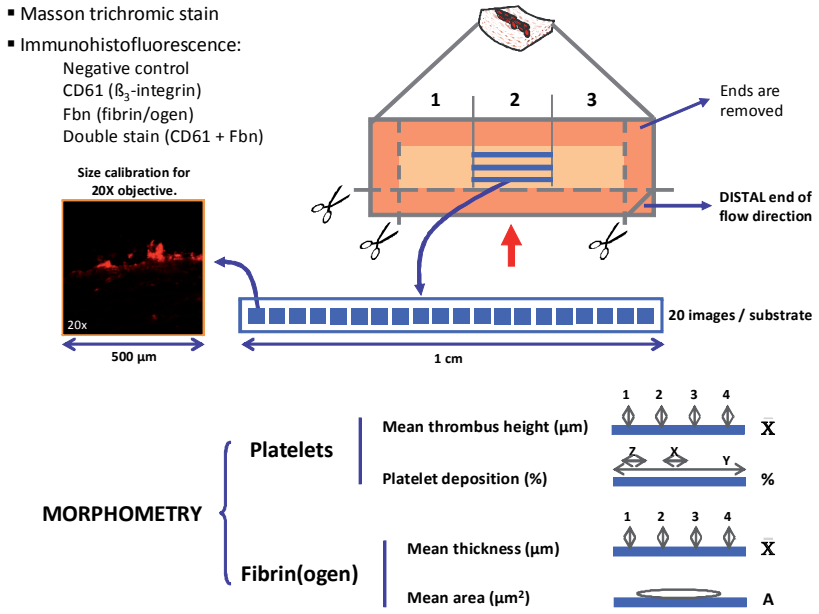
perfusions, blood samples collected from each donor and experimental condition were evaluated for hematologic counts and platelet indium-release. The number of platelets deposited on each specimen was calculated from the indium activity on the perfusion area and normalised by blood ^{111}In activity, platelet counts in blood, and area of exposed surface.

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

Figure 22

IMMUNOHISTOFLUORESCENCE



Note: Schematic representation of the immunohistofluorescence (IHF) and morphometric analysis of aortic substrates.
Source: Own elaboration.

platelet adhesion and mean thrombus height (Baumgartner, 1977). Fibrin deposition was calculated by the thickness of the protein layer and the total area covering the substrate.

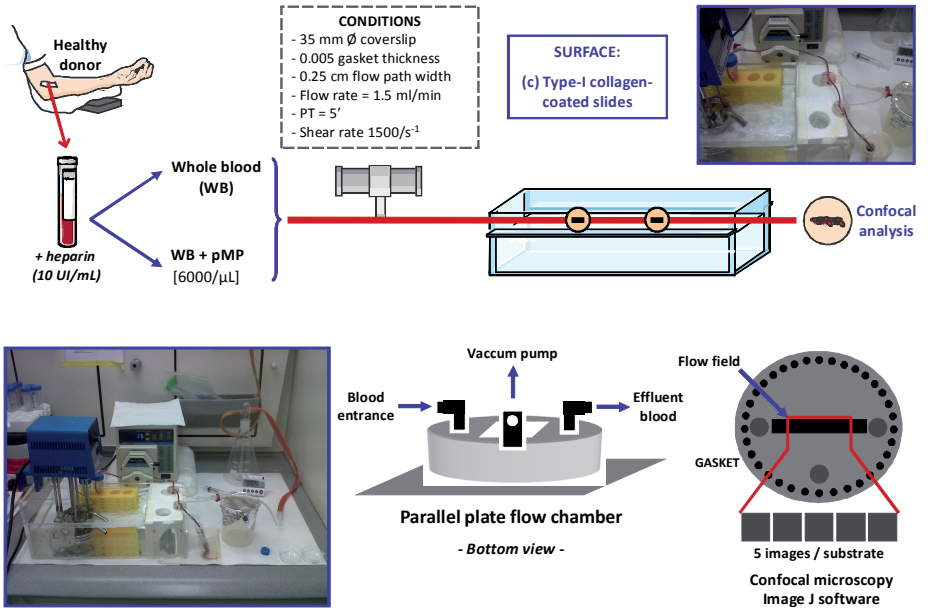
3.6.1.4. Flat perfusion chamber

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 (Molins *et al.,* 2010, 2011). 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

Figure 23

FLAT PERFUSION CHAMBER



Notes: 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.
Source: Own elaboration.

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.

Imaging of platelet thrombi: Platelet deposition on the collagen surface was analyzed with an inverted fluorescence confocal laser scanning microscope (Leica TCS SP2-AOBS) (Molins *et al.,* 2010, 2011). 488nm-Ar-Kr and 633nm He-Ne lasers were used as light source. Platelets and pMPs were viewed with a HCX PLAPO 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 (Arderiu *et al.,* 2011). 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.

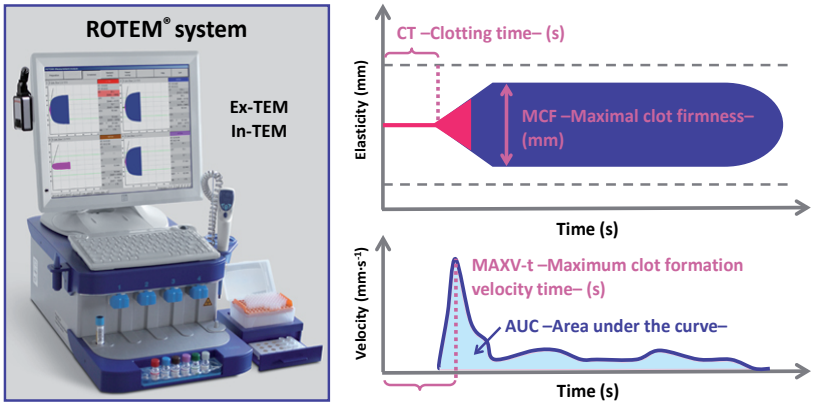
Fluorescence labeling of platelet-derived microparticles: For specific studies in the flat perfusion chamber in order to determine whether pMPs interfere with platelets during platelet adhesion, pMPs were labelled with 2.5 µmol/L fluorescence dye BODIPY™ 630/650-SE (Molecular Probes) for 20 minutes at 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.

3.6.2. Thromboelastography (Whole blood clotting model)

Thrombus dynamics was analyzed in a thromboelastography system (Figure 24) (Hartert, 1963) 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

Figure 24

THROMBOELASTOMETRY



Note: 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 ($MAXV\text{-}t$, 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.
Source: Own elaboration.

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.* (2003) 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.

3.6.3. Platelet function tests

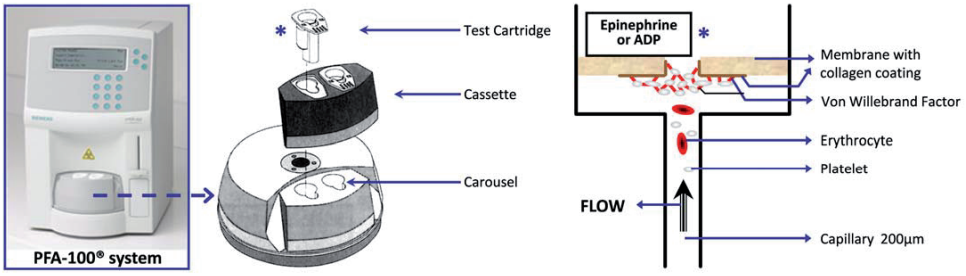
The ability of various agonists to induce *in vitro* activation and platelet-to-platelet activation was measured by two methodologies: (1) platelet function analyser system and (2) platelet aggregation testing.

3.6.3.1. 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



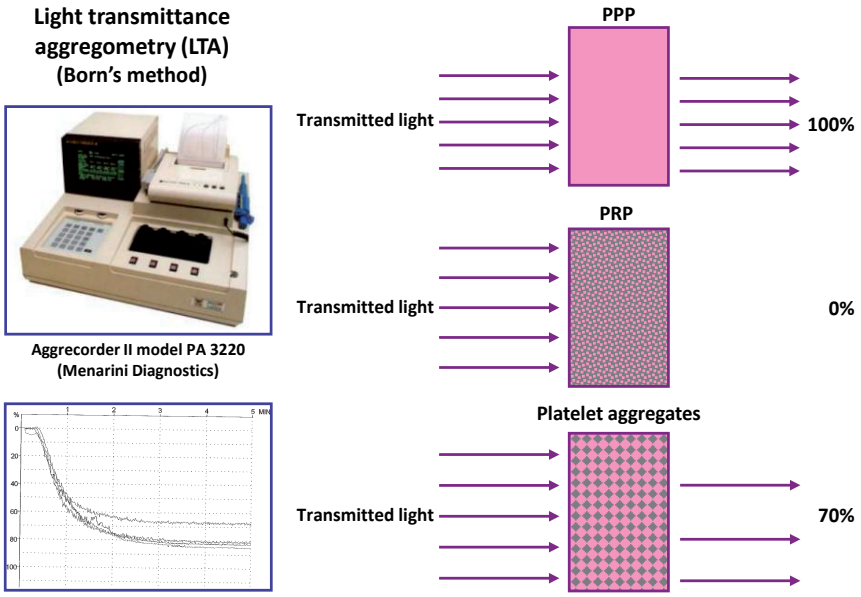
Note: Capillary closure time was measured with PFA-100 system with collagen/epinephrine and collagen/ADP cartridges. Schemes adapted from Dade Behring Siemens.
Source: Own elaboration.

3.6.3.2. 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 (Galvez *et al.,* 1986). 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



Notes: 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.
Source: Own elaboration.

3.7. 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).*

3.7.1. 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 4.0x106 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 (Fukami *et al.,* 1978; Karniguian, Zahraoui and Tavitian, 1993) 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) (Smalley *et al.,* 2007) 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.

3.7.2. 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).

3.7.3. Protein separation

3.7.3.1. 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 (Laemmli, 1970). 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).

3.7.3.2. Bidimensional electrophoresis (2-DE)

For 2-DE analysis, analytical and preparative gels were prepared (Figure 27) (Cubedo, Padro and Badimon, 2014). 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.

3.7.4. 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) (Cubedo, Padro and Badimon, 2014). 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).

3.7.5. MS identification

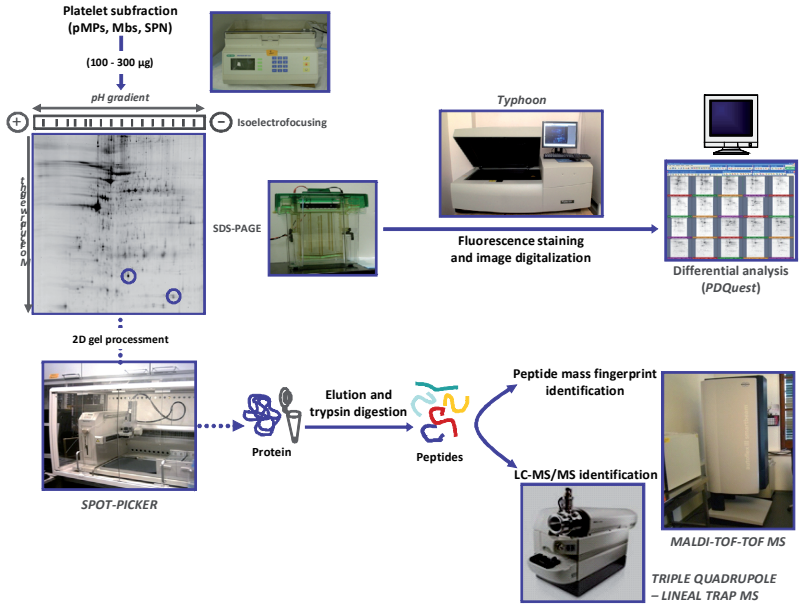
Protein bands (1-DE) or spots of interest (2-DE) were excised from gels and analyzed by (a) LC/MS/MS using a nano-HPLC and a triple-quadrupole associated to a lineal-trap mass spectrometer (Q-TRAP3200) or (b) MALDI-TOF/TOF mass spectrometry for protein identification, respectively, after in-gel tryptic digestion and extraction of peptides from the gel pieces, as previously described (Figure 27) (Cubedo, Padro and Badimon, 2014).

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,

Figure 27

PROTEOMIC ANALYSIS FLOWCHART



Note: Detailed sequence of two-dimensional gel electrophoresis, protein differential analysis and protein identification by mass spectrometry.
Source: Adapted from Cubedo, Padro and Badimon, 2014.

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.

3.8. MICRORNA ANALYSIS

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

3.8.1. 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,

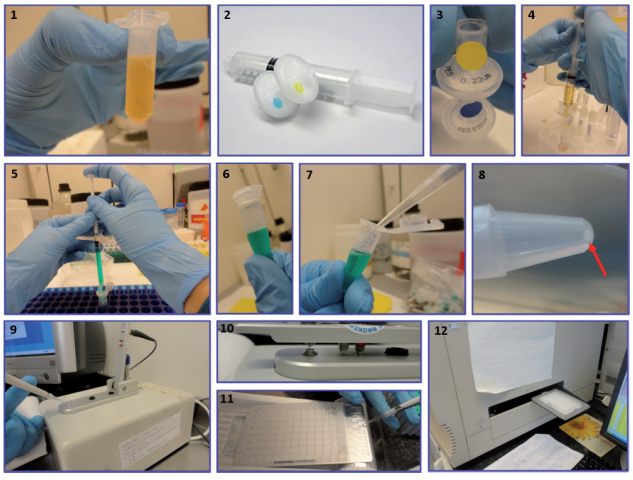
according to the manufacturer. Briefly, microparticles and exosomes were obtained by size exclusion filtration from EDTA-plasma samples thawed in melting ice. Briefly, a clarifying pre-spin was previously performed in order to ensure cell and lipoprotein depletion (Figure 28-1). Then, plasma samples 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.

3.8.2. 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

Figure 28

MICROVESICLE-ASSOCIATED MIRNA EXTRACTION AND ANALYSIS WORKFLOW



Notes: (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.
Source: Own elaboration.

Table 11

THERMAL CYCLING CONDITIONS OF PREAMPLIFICATION AND RT-QPCR OF MEGAPLEX TAQMAN ARRAY MICRORNA CARDS

	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 (12 cycles)	15 sec	95ºC
		4 min	60ºC
	Hold*	10 min	99.9ºC
	Hold	∞	4ºC
PCR	Hold	10 min	95ºC
	Cycle (40 cycles)	15 sec	95ºC
		60 sec	60ºC

Note: * Required for enzyme inactivation.
Source: Own elaboration.

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.

3.8.3. 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.

3.8.4. 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.

Table 12

THERMAL CYCLING CONDITIONS OF PREAMPLIFICATION AND RT-QPCR OF CUSTOM TAQMAN ARRAY MICRORNA CARDS

	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 (12 cycles)	15 sec	95ºC
		4 min	60ºC
	Hold*	10 min	99.9ºC
	Hold	∞	4ºC
PCR	Hold	10 min	95ºC
	Cycle (40 cycles)	15 sec	95ºC
		60 sec	60ºC

Note: * Required for enzyme inactivation.
Source: Own elaboration.

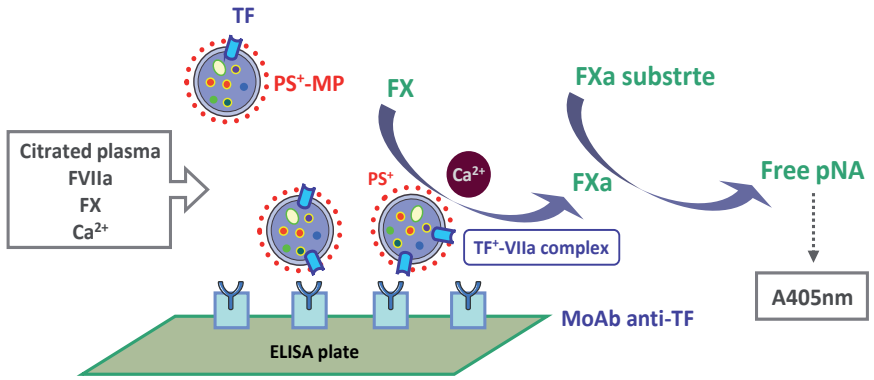
3.9. VALIDATION TECHNIQUES

3.9.1. 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



Source: Own elaboration.

3.9.2. 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.

3.10. *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.

3.11. 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.

REFERENCES

Aatonen, M.; Gronholm, M. and Siljander, P. R. (2012), "Platelet-derived microvesicles: multitalented participants in intercellular communication," *Semin Thromb Hemost,* 38(1): 102-113.

Arderiu, G. *et al.* (2011), "Tissue factor regulates microvessel formation and stabilization by induction of chemokine (C-C motif) ligand 2 expression," *Arterioscler Thromb Vasc Biol,* 31(11): 2607-2615.

Badimon, L. and Badimon, J. J. (1989), "Mechanisms of arterial thrombosis in nonparallel streamlines: platelet thrombi grow on the apex of stenotic severely injured vessel wall. Experimental study in the pig model," *J Clin Invest,* 84(4): 1134-1144.

Badimon, L. *et al.* (1987), "Characterization of a tubular flow chamber for studying platelet interaction with biologic and prosthetic materials: deposition of indium 111-labeled platelets on collagen, subendothelium, and expanded polytetrafluoroethylene," *J Lab Clin Med,* 110(6): 706-718.

Badimon, L. *et al.* (1988), "Platelet thrombus formation on collagen type I. A model of deep vessel injury. Influence of blood rheology, von Willebrand factor, and blood coagulation," *Circulation,* 78(6): 1431-1442.

Baumgartner, H. R. (1977), "Platelet interaction with collagen fibrils in flowing blood. I. Reaction of human platelets with alpha chymotrypsin-digested subendothelium," *Thromb Haemost,* 37(1): 1-16.

Biró, E. *et al.* (2005), "The phospholipid composition and cholesterol content of platelet-derived microparticles: a comparison with platelet membrane fractions," *J Thromb Haemost,* 3(12): 2754-2763.

Brill, A. *et al.* (2005), "Platelet-derived microparticles induce angiogenesis and stimulate post-ischemic revascularization," *Cardiovasc Res,* 67(1): 30-38.

Caballero, P. *et al.* (2012), "Detection of subclinical atherosclerosis in familial hypercholesterolemia using non-invasive imaging modalities," *Atherosclerosis,* 222(2): 468-472.

Civeira, F. (2004), "Guidelines for the diagnosis and management of heterozygous familial hypercholesterolemia," *Atherosclerosis,* 173(1): 55-68.

Corti, R. Badimon, L. *et al.* (1987), "Characterization of a tubular flow chamber for studying platelet interaction with biologic and prosthetic materials: deposition of indium 111-labeled platelets on collagen, subendothelium, and expanded polytetrafluoroethylene," *J Lab Clin Med,* 110(6): 706-718.

Corti, R. and Fuster, V. (2011), "Imaging of atherosclerosis: magnetic resonance imaging," *Eur Heart J,* 32(14): 1709-1719b.

Cubedo, J.; Padro, T. and Badimon, L. (2014), "Glycoproteome of human apolipoprotein A-I: N- and O-glycosylated forms are increased in patients with acute myocardial infarction," *Transl Res*.

Fernandez-Ortiz, A. *et al.* (1994), "Characterization of the relative thrombogenicity of atherosclerotic plaque components: implications for consequences of plaque rupture," *J Am Coll Cardiol,* 23(7): 1562-1569.

Friedewald, W. T.; Levy, R. I. and Fredrickson, D. S. (1972), "Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge," *Clin Chem,* 18(6): 499-502.

Fukami, M. H. *et al.* (1978), "An improved method for the isolation of dense storage granules from human platelets," *J Cell Biol,* 77(2): 389-399.

Galvez, A. *et al.* (1986), "Electrical aggregometry in whole blood from human, pig and rabbit," *Thromb Haemost,* 56(2): 128-132.

Gehi, A. K. *et al.* (2007), "Self-reported medication adherence and cardiovascular events in patients with stable coronary heart disease: the heart and soul study," *Arch Intern Med,* 167(16): 1798-1803.

Graham, I. *et al.* (2007), "European guidelines on cardiovascular disease prevention in clinical practice: executive summary: Fourth Joint Task Force of the European Society of Cardiology and Other Societies on Cardiovascular Disease Prevention in Clinical Practice

(Constituted by representatives of nine societies and by invited experts)," *Eur Heart J,* 28(19): 2375-2414.

Hartert, H. (1963), "[The current importance of thrombelastography]," *Minerva Med,* 54: 181-182.

Karniguian, A.; Zahraoui, A. and Tavitian, A. (1993), "Identification of small GTP-binding rab proteins in human platelets: thrombin-induced phosphorylation of rab3B, rab6, and rab8 proteins," *Proc Natl Acad Sci U S A,* 90(16): 7647-7651.

Lacroix, R. *et al.* (2010), "Standardization of platelet-derived microparticle enumeration by flow cytometry with calibrated beads: results of the International Society on Thrombosis and Haemostasis SSC Collaborative workshop," *J Thromb Haemost,* 8(11): 2571-2574.

Laemmli, U. K. (1970), "Cleavage of structural proteins during the assembly of the head of bacteriophage T4," *Nature,* 227(5259): 680-685.

Mata, N. *et al.* (2011), "Clinical characteristics and evaluation of LDL-cholesterol treatment of the Spanish Familial Hypercholesterolemia Longitudinal Cohort Study (SAFEHEART)," *Lipids Health Dis,* 10: 94.

Molins, B. *et al.* (2010), "Glucose-regulated protein 78 and platelet deposition: effect of rosuvastatin," *Arterioscler Thromb Vasc Biol,* 30(6): 1246-1252.

Molins, B. *et al.* (2011), "Monomeric C-reactive protein is prothrombotic and dissociates from circulating pentameric C-reactive protein on adhered activated platelets under flow," *Cardiovasc Res,* 92(2): 328-337.

Mozas, P. *et al.* (2004), "Molecular characterization of familial hypercholesterolemia in Spain: identification of 39 novel and 77 recurrent mutations in LDLR," *Hum Mutat,* 24(2): 187.

NCEP (2002), "Third Report of the National Cholesterol Education Program, Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report," *Circulation,* 106(25): 3143-3421.

Nieuwland, R. *et al.* (2000), "Cellular origin and procoagulant properties of microparticles in meningococcal sepsis," *Blood,* 95(3): 930-935.

Pijlman, A. H. *et al.* (2010), "Evaluation of cholesterol lowering treatment of patients with familial hypercholesterolemia: a large cross-sectional study in The Netherlands," *Atherosclerosis,* 209(1): 189-194.

Rand, M. L. *et al.* (2006), "Rapid clearance of procoagulant platelet-derived microparticles from the circulation of rabbits," *J Thromb Haemost,* 4(7): 1621-1623.

Rank, A..; Liebhardt, S. *et al.* (2012), "Circulating microparticles in patients with benign and malignant ovarian tumors," *Anticancer Res,* 32(5): 2009-2014.

Rank, A.; Nieuwland, R. *et al.* (2012), "Climacteric lowers plasma levels of platelet-derived microparticles: a pilot study in pre- versus postmenopausal women," *Acta Haematol,* 128(1): 53-59.

Smalley, D. M. *et al.* (2007), "Proteomic discovery of 21 proteins expressed in human plasma-derived but not platelet-derived microparticles," *Thromb Haemost,* 97(1): 67-80.

Sorensen, B. *et al.* (2003), "Whole blood coagulation thrombelastographic profiles employing minimal tissue factor activation," *J Thromb Haemost,* 1(3): 551-558.

Terkelsen, C. J. *et al.* (2013), "2012 ESC STEMI guidelines and reperfusion therapy: Evidence base ignored, threatening optimal patient management," *Heart,* 99(16): 1154-1156.

van der Pol, E. *et al.* (2014), "Particle size distribution of exosomes and microvesicles by transmission electron microscopy, flow cytometry, nanoparticle tracking analysis, and resistive pulse sensing," *J Thromb Haemost*.

VILADES MEDEL, D. *et al.* (2013), "Coronary computed tomographic angiographic findings in asymptomatic patients with heterozygous familial hypercholesterolemia and null allele low-density lipoprotein receptor mutations," *Am J Cardiol,* 111(7): 955-961.

WIERZBICKI, A. S.; HUMPHRIES, S. E. and MINHAS, R. (2008), "Familial hypercholesterolaemia: summary of NICE guidance," *Bmj,* 337: a1095.

WILLIAMS, J. C. and MACKMAN, N. (2011), "MPs or ICs?," *Blood,* 117(4): 1101-1102.

4 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): 1208-1219.

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

Rosa Suades[1,2,3]; Teresa Padró[1,2,3]; Gemma Vilahur[1,2,3]; Lina Badimon[1,2,3,4]

[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

Summary

Plaque rupture followed by thrombosis is the underlying cause of the majority of acute coronary syndromes. Circulating microparticles (cMPs), membrane blebs released into blood by activated cells, have been associated to vascular diseases. Specifically, high levels of platelet-derived microparticles (pMPs) have been found in patients with coronary disease. However, it is unknown whether microparticles have a contributing role to the development of damaged vessel wall-induced arterial thrombi. The aim of this proof of concept study was to investigate whether an increased number of cMPs and pMPs could functionally contribute to blood thrombogenicity on areas of arterial damage. Microparticles were isolated from blood of healthy volunteers and were characterised by flow cytometry. Effects of microparticles on platelet deposition were assessed under controlled flow conditions exposing damaged arterial wall in the Badimon perfusion chamber and collagen type-I in the flat perfusion chamber to human blood. Platelet deposition on damaged arteries was significantly increased in cMP- and pMP-enriched bloods ($p<0.05$). pMPs also induced increase in platelet ($p<0.05$) and fibrin ($p<0.05$) deposition on human atherosclerotic arteries and in platelet adhesion to purified collagen surfaces. pMP-enriched blood induced a dose-dependent shortening of epinephrine/collagen closure time evaluated by PFA-100 ($p<0.001$), increased low-dose ADP-induced platelet aggregation by LTA ($p<0.05$), and decreased clotting time by thromboelastography ($p<0.01$). In conclusion, an increased content of cMPs and pMPs, even in normal blood conditions, enhance platelet deposition and thrombus formation. This study shows for the first time that, beyond biomarkers of cell activation, blood microparticles have functional effects on cardiovascular atherothrombotic disease.

Keywords

Aggregation, atherothrombosis, coagulation, microparticles, platelets

Correspondence to:
Prof. Lina Badimon
Cardiovascular Research Center
c/Sant Antoni Mª Claret 167, 08025 Barcelona, Spain
Tel.: +34 935565880, Fax: +34 935565559
E-mail: lbadimon@csic-iccc.org

Financial support:
This work was supported by Spanish Science Ministry [PNS-SAF2010–16549 to LB], Tercel [RD06/0010/0017 to LB], CIBERObn [CB06/03 to LB], Instituto Carlos III-ISCIII [FIS-PI10–01115 to TP], and Fundación Jesús Serra. RS is a recipient of a predoctoral fellowship from Spanish Health Ministry (PFIS, ISCIII) and GV is a recipient of a contract from the Spanish Science Ministry (RyC).

Received: July 13, 2012
Accepted after minor revision: September 7, 2012
Prepublished online: November 8, 2012
doi:10.1160/TH12-07-0486
Thromb Haemost 2012; 108: 1208–1219

Introduction

Atherothrombosis is the major cause of cardiovascular disease. Platelets are key players in the pathogenesis of atherothrombotic processes. Platelet adhesion and aggregation at sites of atherosclerotic plaque rupture or vessel injury lead to the development of either mural or occlusive thrombus triggering acute coronary syndromes (ACS), peripheral cardiovascular disease and ischaemic cerebrovascular events (1, 2). The type and size of thrombotic mass developing during ACS, either mural or fully occlusive, has important clinical implications. However, the contributing factors beyond the underlying triggering atherosclerotic plaque are still not fully identified. It is our hypothesis that circulating microparticles (cMPs) are one of the factors contributing to thrombosis.

Microparticles (MPs), small phospholipid microvesicles, are usually shed by activated cells, lack a nucleus, contain a membrane skeleton and are defined by both size and expression of cell-specific antigens on their surface (3). It is generally accepted that microparticle size ranges from 0.1 to 1.0 μm in diameter; however, they are rather heterogeneous (up to 1.5 μm) and small MPs may overlap in size with exosomes (40–80 nm) (4, 5). MPs can originate from platelets, endothelial cells, leukocytes, erythrocytes, and smooth muscle cells, and are found in circulating blood at relative concentrations determined by the pathophysiological context. However, platelet-derived microparticles (pMPs) are by far the most abundant in the bloodstream, representing about 70–90% of all circulating MPs (6). There is evidence correlating the presence of cMPs in the human circulation with certain pathologic conditions (7, 8)

and elevated numbers of pMPs in blood are associated with arterial disease (9, 10), reasons why MPs are considered biomarkers of vascular disease. Furthermore, MPs have been described to take part actively in various disease models (11, 12).

The main variables regulating thrombosis include changes in the vessel wall (pathological substrate), alterations in blood flow parameters (local shear forces) and modifications in the thrombogenic potential of blood (13–15). cMPs and pMPs have structural features that may contribute to haemostasis, coagulation, and venous thrombosis (16). As such, it is well known that they have strong procoagulant properties due to exposure of procoagulant anionic phospholipids as phosphatidylserine (PS) in a similar fashion as activated platelets (17) and provide a catalytic surface that may promote coagulation since PS facilitates the binding of the coagulation factors and the assembly of the coagulation complexes, accelerating the formation of thrombin (18). Besides, cMPs may also be bioactive molecular carriers that can activate other cells and contribute to inflammatory processes (19, 20). In addition, tissue factor (TF), the principal initiator of haemostatic fibrin formation, has been demonstrated on procoagulant MPs (21). TF bearing MPs are likely to be mainly of monocyte origin (22–24). Falati et al., using a laser-induced vascular endothelial injury model in mice demonstrated that TF^+-MPs bind to activated platelets contributing to the thrombus formation in the microcirculation (25, 26). However, flow dynamics and vessel structure in the microcirculation are not representative of the situation that occurs in medium and large arteries as the coronary, carotid or aorta arteries, where rupture of atherosclerotic lesions is the clinical cause of thrombus formation. Up to now, platelet- and erythrocyte-derived MPs may initiate thrombin generation via the FXII-driven intrinsic pathway of coagulation (18); however, whether blood-derived MPs promote platelet adhesion and arterial thrombosis in areas of vascular injury has not yet been demonstrated. The aim of this proof-of-concept study was to investigate whether an increased number of cMPs and pMPs, obtained from blood of healthy donors, could functionally contribute to blood thrombogenicity on areas of arterial damage. A dose response study with PFA-100 helped to set a thrombogenic level of normal (non-cardiovascular disease donors) MPs that was afterwards tested on arterial wall under flow conditions. Our study shows that MPs enhance platelet deposition and thrombus formation. Thus, beyond biomarkers of cell activation, blood microparticles have functional effects on cardiovascular disease.

Methods

Blood collection

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 and functional experiments. 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.

MPs isolation

cMPs isolation

Isolation of cMPs was adapted from Berckmans et al. (27). Briefly, blood cells were removed from citrate-anticoagulated whole blood by low-speed centrifugation (1,258x g, 20 minutes [min], room temperature [RT]) in order to avoid *in vitro* platelet activation. After obtaining the platelet-poor plasma (PPP), a second equal centrifugation step was made to ensure the complete removal of cells and obtain the platelet free plasma (PFP). cMPs (particles less than approximately 1.0 µm) were isolated and washed from PFP (1 ml-aliquots) with two centrifugation steps (20,000 x g, 60 min, 10ºC).

pMPs isolation

Human platelets from fresh platelet concentrates were centrifuged (1,200 x g, 10 min, 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 x 10^6 platelets/µl (Medonic hematologic analyser, Medonic, Stockholm, Sweden) and incubated at 37ºC for 5 min without stirring to allow spontaneous pMP release (28). Platelets were then separated by a double centrifugation step (3,220 x g, 10 min, 20ºC) and the final suspension contained only pMPs. Thereafter, pMPs from 10 ml-aliquots were concentrated by ultracentrifugation at 150,000 x g (90 min, 10ºC) (29) and resuspended in HTB containing 0.105M trisodium citrate (citrate-HTB).

Due to different original sample volumes, the above described centrifugation settings were applied to obtain MPs from plasma and platelet concentrates in a similar size range (30), as determined by flow cytometry. Aliquots of cMPs and pMPs were snap-frozen in liquid nitrogen and stored at –80ºC until immunochemical characterisation and functional experiments were performed.

Flow cytometric analysis of cMPs and pMPs

MPs were identified based on their specific marker positivity, binding of annexin V and FSC/SSC characteristics based on their size, according to the methodology described by Nieuwland et al. (31). Briefly, frozen samples of cMPs and pMPs were thawed in melting ice for 1 hour (h). Aliquots of MPs (5 µl) were added to 45 µl of phosphate-buffered saline (PBS) solution (1.4 mM phosphate in 154 mM NaCl). For MP characterisation, samples were incubated with 5 µl of specific fluorochrome-conjugated anti-human monoclonal antibodies or 5 µl of isotype-matched control antibodies

Table 1: Flow cytometric characteristics of circulating microparticles (cMPs) isolated from blood of normal human subjects. Data are expressed as percentage of cMPs in blood of healthy donors that express each marker. LCA, leukocyte common antigen (n=3).

Marker	Epitope / cell origin	Circulating MPs (% of total cMPs)
Annexin V$^+$	Phosphatidylserine	74.7 (± 10.5)
CD45$^+$	LCA / Leukocytes	15.8 (± 6.1)
CD31$^+$ / CD41$^-$	Endothelial cells	2.6 (± 0.1)
CD41$^+$	Platelets	81.4 (± 2.0)
TSP1$^+$	Thrombospondin-1	21.9 (± 3.6)
CD62P$^+$	P-selectin	8.4 (± 2.1)
PAC1$^+$	Activated α_{IIb}/β_3-integrin	3.0 (± 1.1)
CD142$^+$	Tissue factor	22.0 (± 3.9)

(see ▶ Suppl. Table 1, available online at www.thrombosis-online.com) for 20 min at RT in the dark. Phosphatidylserine-positive MPs were determined by the positive binding of phycoerythrin-conjugated annexin V (Molecular Probes, Invitrogen, Carlsbad, CA, USA), in the presence of 5 mM $CaCl_2$. Calcium supplementation is essential for the annexin V conjugation. Annexin V measurements were corrected for autofluorescence by placing a threshold based on a MP sample prepared with the use of calcium-free buffer. For MP quantification, 5 µl of Flowcount beads (10 µm in diameter, Beckman Coulter, Brea, CA, USA) and 900 µl of PBS buffer were added to the samples and immediately analysed on an EPICS XL flow cytometer (Beckman Coulter) with Expo32 ADC analysis software. MP concentration was assessed by comparison with calibrator FlowCount beads in a predetermined concentration. Gate limits were established before analysis using a Flow Check YG Size Range Calibration Kit (Polysciences, Warrington, PA, USA). Acquisition was performed for 1 min/sample. Forward scatter (FSC), side scatter (SSC) and fluorescence data were obtained with gain settings in the logarithmic scale.

Experimental design

The effect of cMPs and pMPs on flow-induced platelet deposition was analysed using the previously validated Badimon perfusion chamber (32, 33) and the flat perfusion chamber, as previously described (34, 35). The thrombogenicity of MPs 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) (33), kept at 20ºC, and used within 2 h of collection. The haematologic 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 min with a suspension of MPs (adjusted to a final concentration of 6,000/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, 10 M, Sigma) or (b) CellTracing Calcein green AM (1 µg/ml, Invitrogen) to blood, before incubation with pMPs.

Radioactive labelling of human platelets

Platelets from healthy donor blood were isolated by differential centrifugations and labelled with ^{111}Indium-oxine (^{111}In) as previously described with slight modifications (36). 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 x 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 labelling procedure was 91.8 ± 0.3 % and the mean final activity was 5.9 ± 0.07 µCi.

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 (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 until needed. Before the experiments, the aortas were thawed in PBS at 4ºC, open longitudinally, and cut into 30 x 10 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 raised white or yellow-white plaques) were used as substrates for each experiment in a randomised fashion. Aorta substrates were mounted in the previously characterised Badimon perfusion chamber (13, 33). After a preperfusion period of 60 seconds (s) 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 1,690 s^{-1} (32), mimicking moderately stenotic coronary arteries. Perfusion period was 3 min, within the described time in which MPs are cleared from circulation (37). Finally, PBS was passed for 30 s to wash out the unattached cells. The perfused segments were fixed in 4% paraformaldehyde and counted in a gamma counter for quantitation of deposited platelets. After each sequence of perfusions, blood samples collected from each donor and experi-

mental condition were evaluated for haematologic 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.

Immunohistology

Perfused and fixed aorta substrates were cryoprotected with 2.3 M sucrose, frozen over dry ice and stored in optimal cutting temperature (OCT). Serial sections (5 µm) cut from the centerline of the vessel and longitudinal to the blood flow direction were analysed. Atherosclerotic lesions were microscopically characterised by Masson's Trichromic staining. A double immunohistofluorescence analysis was 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, Olympus, Center Valley, PA, USA) using fields at 20x magnification. Twenty serial images of the centerline segment of each substrate were taken with a digital Sony 3CCD (DXC-5500) camera (Sony, Tokyo, Japan) and were systematically analysed 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 (38). Fibrin deposition was calculated by the thickness of the protein layer and the total area covering the substrate.

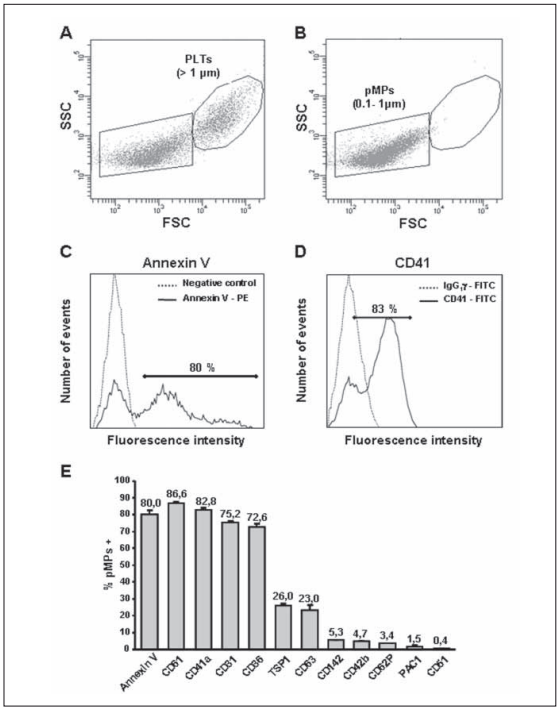


Figure 1: Characterisation of platelet-derived microparticles (pMPs) by flow cytometry. A-B) Determination of forward scatter (FSC) and side-scatter (SSC) characteristics of platelets and pMPs in suspension. B) The microparticle gate was established based on light scattering properties and size, using calibration microspheres and defining pMPs as events both smaller than 1 µm and smaller than unstimulated platelets. C-D) Representative histogram plots of size-selected events with expression of annexinV and platelet α_{IIb}-integrin (CD41) on pMP surface in the phycoerythrin (PE) gate (C) and fluorescein isothiocyanate (FITC) gate (D), respectively. Fluorescence intensity (x-axis) versus microparticle counts (y-axis) of each marker is shown with solid line histograms. Binding of the isotype-matched control antibody is also depicted with dotted line histograms (C-D). E) pMP characterisation by specific platelet markers and other monoclonal antibodies, expressed as labelling percentage for each one (n=6).

Fluorescence labelling of pMPs

For specific platelet adhesion studies in the flat perfusion chamber, pMPs were labelled with 2.5 μM fluorescence dye BODIPY™ 630/650-SE (Molecular Probes) for 20 min at RT in the dark, washed twice by centrifugation (20,000 x g, 30 min, RT) and then resuspended in citrate-HTB. Fluorescence-tagged pMPs were quantified by flow cytometry.

Flat perfusion chamber

Glass slides were coated with type-I collagen (10 μg/ml, 4ºC, overnight) and placed in a parallel plate chamber, as previously described (34, 35). After 1 min HTB preperfusion, blood was circulated through the chamber at a constant shear rate (1,500 s^{-1}, 5 min). Then, buffer was circulated for 1 min through the chamber. Thereafter, slides were carefully rinsed with PBS, fixed with 3.8% paraformaldehyde (15 min) and mounted on glass slides with Glycerol Mounting Medium.

Imaging of platelet thrombi

Platelet deposition on the collagen surface was analysed with an inverted fluorescence confocal laser scanning microscope (Leica TCS SP2-AOBS, Leica, Wetzlar, Germany) (34, 35). A 488 nm-Ar-Kr-laser and 633 nm He-Ne-laser 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 analysed field. Fluorescent images of platelet and pMPs were acquired in a scan format of 1,024 x 1,024 pixels in a spatial data set every 0.5 μm (xyz) and processed with the Leica Standard Software (39). Thresholds were applied to distinguish adhered platelets and pMPs from the background, and the same values were used for analysing all the stacks collected for a given experiment.

Platelet function analysis

Capillary closure time

Platelet reactivity was determined using a Platelet Function Analyser (PFA-100) with collagen-epinephrine and collagen-adenosine diphosphate (ADP) cartridges. Briefly, citrate-anticoagulated blood was incubated with various concentrations of pMPs or citrate-HTB for 3 min, and with 2,500 pMPs/μl at distinct time periods (3, 5, and 10 min), before closure time determination.

Light transmission aggregometry (LTA)

Platelet aggregation was analysed in blood samples incubated with 6,000 pMPs/μl for different periods of time and challenged with thrombin, collagen, ADP, and epinephrine, as previously reported (40). Maximal platelet aggregation was measured for 15 min after addition of the agonist in all cases.

Whole blood clotting model

Thrombus dynamics was analysed in a thromboelastography system (41), using extrinsically- and intrinsically-activated, and fibrin-based thromboelastometric assays (Ex-, In-, and Fib-TEM, respectively). The analyses were performed by adding 20 μl 0.2M $CaCl_2$ to 300 μl citrated whole blood with 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 for 30 min after blood withdrawal. The following parameters were recorded for each test: clotting time (CT; s), maximum clot formation velocity time (MAXV-t; s), maximum clot firmness (MCF; mm), and the area under the curve (AUC), as described by Sorensen et al. (42). ROTEM device was checked for proper functioning according to the manufacturer's recommendation using a control serum

Marker	Whole blood (nº of cMPs /μl PFP)	Whole blood + pMPs (nº of cMPs /μl PFP)	Statistics (Student t-test)
Total	1736 (± 293.4)	6795 (± 304.4)	$p < 0.0001$
Annexin V^+	1124 (± 489.3)	5316 (± 230.0)	$p < 0.0001$
$CD45^+$	364.8 (± 128.1)	322.5 (± 118.4)	ns
$CD31^+$ / $CD41^-$	14.8 (± 2.1)	12.5 (± 7.2)	ns
$CD41^+$	1257 (± 494.9)	5598.2 (± 306.3)	$p < 0.0001$
$TSP1^+$	485.8 (± 160.6)	1848.2 (± 303.5)	ns
$CD62P^+$	180.6 (± 96.3)	360.5 (± 100.2)	ns
$PAC1^+$	56.3 (± 8.4)	137.1 (± 25.5)	$p < 0.05$
$CD142^+$	356.3 (± 154.4)	635.7 (± 48.9)	$p < 0.001$

Table 2: Endogenous circulating microparticle characterisation before and after the enrichment of blood with platelet-derived microparticles. Data are expressed as number of circulating microparticles per microliter of platelet free plasma (PFP) (n=6). Annexin V^+, phosphatidylserine; $CD45^+$, LCA –common leukocyte antigen– (leukocyte origin); $CD31^+$/$CD41^-$, positive for PECAM-1 –platelet endothelial cell adhesion molecule-1– and not for $\alpha_{IIb}\beta_3$-integrin (endothelial origin); $CD41^+$, $\alpha_{IIb}\beta_3$-integrin (platelet origin); $TSP1^+$, thrombospondin 1; $CD62P^+$, P-selectin; $PAC1^+$, activated $\alpha_{IIb}\beta_3$-integrin; $CD142^+$, tissue factor.

(ROTROL). All reagents were purchased from Pentapharm GmbH (Basel, Switzerland).

Statistical analysis

Results are reported as mean ± standard error (SE), except when indicated. *n* indicates the number of experiments. Results were tested for normal distribution with Kolmogorov-Smirnov test. The statistical significance of the difference between group means was determined with the Student t-test, and analysis of variance (ANOVA) as needed, and when significant, Fisher's PSLD post-hoc analysis was used to assess intergroup differences. StatView software (Abacus Concept, Berkeley, CA, USA) was used for all the statistical tests, and a $p<0.05$ was considered significant.

Results

Flow cytometry characterisation of isolated MPs

Isolated MPs were identified by flow cytometry based on their light scattering properties (FSC and SSC) as events smaller than 1 µm. The centrifugation conditions used for their isolation provide an optimal yield of microparticles, while cell and platelet contamination is minimal (less than 0.1%), as shown in ▶Figure 1.

cMPs

Flow cytometric analysis of cMPs isolated from healthy individuals revealed that the majority of cMPs expressed phosphatidylserine and originated from platelets, as shown in ▶Table 1. Activation marker expression on circulating pMP surface ($PAC1^+$, $CD62P^+$) was relatively low, indicating the lack of platelet activation in the blood of healthy donors. Some cMPs were derived from endothelial cells ($CD31^+/CD41^-$) or leukocytes ($CD45^+$), and some stained positive for tissue factor ($CD142^+$).

pMPs

pMPs were isolated from normal platelets. ▶Figure 1A-B clearly depicts a pMP-rich fraction without platelet contamination after ultracentrifugation of the sample. pMPs showed binding capacity for annexin V (80%, ▶Fig. 1C). pMPs demonstrated the expected reactivity with antibodies to platelet α_{IIb}-integrin ($CD61^+$, 83% of labelling, ▶Fig. 1D). pMPs were also characterised based on their labelling properties for other specific platelet markers. Specifically they showed in their surface high levels of PECAM-1 ($CD31^+$), glycoprotein IV ($CD36^+$), $\alpha_{IIb}\beta_3$-integrin ($CD41a^+$), and low levels of activation markers as activated $\alpha_{IIb}\beta_3$-integrin ($PAC1^+$) and P-selectin ($CD62P^+$). pMPs also showed intermediate levels of thrombospondin-1 ($TSP1^+$) and lysosomal-associated membrane protein-3 ($CD63^+$), and low levels of tissue factor ($CD142^+$) and glycoprotein Ibα as surface markers ($CD42b^+$) (▶Fig. 1E).

pMPs derived from platelet concentrates presented the same antigenic characteristics as those isolated from human plasma (▶Table 1, ▶Fig. 1E).

Flow cytometry characterisation of cMPs preperfusion

cMP numbers as well as their antigenic characteristics were analysed in blood before and after adding the pMP concentrate (final concentration 6,000/µl) (▶Table 2). This concentration is in reference to the clinical situation based on the literature (43) and our own data on MPs levels of patients at high CVD risk (unpublished data). The enrichment of pMPs was significant and accounted for approximately about 76% increase in pMP over the basal level present in the whole blood. ▶Table 2 also shows that all platelet or activation markers increased with the addition of pMPs except for leukocyte- ($CD45^+$) and endothelial- ($CD31^+/CD41^-$) derived cMPs that did not change.

Effects of MPs on platelet adhesion and thrombus formation

Porcine arterial wall and human blood (Badimon perfusion chamber)

Blood enriched with circulating microparticles from healthy subjects increased significantly platelet deposition on damaged porcine vessel wall (eroded vessel wall) (two-fold, $p<0.05$, ▶Fig. 2A). When thrombogenicity under controlled flow conditions was assessed specifically with the addition of pMPs, obtained from normal human platelets, platelet deposition was significantly higher compared to blood without MPs (three-fold, $p<0.0001$) and even to blood enriched with similar number of cMPs (1.5-fold, $p<0.02$), as shown in ▶Figure 2A.

Human arterial wall and human blood (Badimon perfusion chamber)

Blood spiked with pMPs (6,000/µl) also showed increased thrombogenicity on human atherosclerotic vessel wall. Both platelet deposition and thrombus height on human atherosclerotic vessels were significantly increased (four- and three-fold, respectively, $p<0.05$) in the perfusions of normal blood added with pMPs (▶Fig. 2B).

Fibrin deposition on human atherosclerotic vessel wall was significantly increased ($p<0.05$) both in thickness (two-fold) and in area of coverage (2.5-fold) due to pMPs (▶Fig. 2B). Immunofluorescence staining (platelet, ▶Fig. 2C, I,III; fibrin, ▶Fig. 2C, II,IV) of perfused atherosclerotic lesions clearly showed the effects

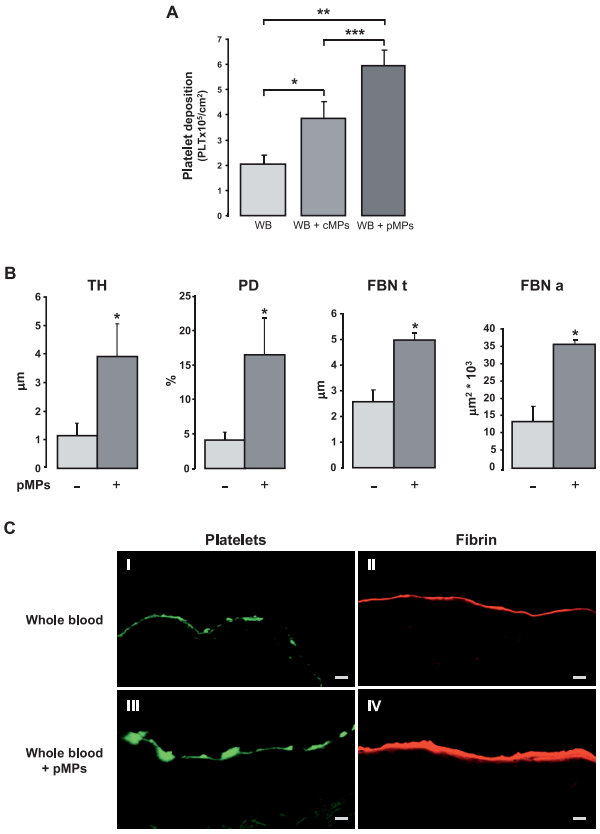


Figure 2: Effect of microparticles on thrombogenicity of eroded vessel wall and atherosclerotic plaques. A) Porcine substrates were perfused with blood without/with exogenous added cMPs or pMPs. Bar graphs of platelet deposition on porcine eroded vessel wall as determined by ^{111}Indium-oxine radioactive counts. Results are expressed as the mean ± SE values of platelet deposition (PLTs x 10^5/cm^2) (n=9). B) Bar graphs of platelet and fibrin-vessel wall interaction on human atherosclerotic lesions as determined by quantitative immunohistofluorescence analysis. Effect of pMPs on mean thrombus height (TH), mean platelet deposition (PD), mean fibrin thickness (FBNt) and mean fibrin area (FBNa) triggered by atherosclerotic lesions under high shear rate. Results are expressed as mean ± SE values of platelet deposition (%), thrombus height (µm), fibrin thickness (µm) and fibrin area (µm^2). A-B) Differences were analysed by ANOVA. *p<0.05 and **p<0.0001, significant versus control whole blood and, ***p<0.02, significant versus cMP-enriched whole blood (n=3). C) Representative immunophotographs of perfused human atherosclerotic substrates. Platelet (green) (I,III) and fibrin (red) (II,IV) deposition on human atherosclerotic vessels at a shear rate condition of 1,680 s^{-1} of whole blood (I-II) and of whole blood with 6,000 pMPs/µl (III-IV). Scale bar is 25 µm.

of adding pMPs to normal blood both in platelet and fibrinogen deposition.

Collagen type-I surface and human blood (flat perfusion chamber)

pMPs also increased platelet adhesion on type-I collagen-coated slides under high shear rate conditions (two-fold) (▶Fig. 3B). Representative confocal images of adhered platelets are shown in ▶Figure 3A.

pMPs bind to collagen and to adhered platelets

Exogenously-added pMPs (red labelled) colocalised with platelet aggregates (green labelled) on collagen surface (▶Fig. 4B). Confocal analysis of stack series (from the bottom to the upper side of the 5 μm-thick platelet aggregates) demonstrated that pMPs located within the platelet thrombi (▶Fig. 4C-F). Thus, serial cuts of the platelet thrombi gave evidence that pMPs were not present at the bottom (at the collagen surface), whereas positive labelled-pMPs were detected attached to the first layers of platelets and in middle sections. Endogenous shear stress-induced MPs could not be detected since by deriving from calcein-labelled platelets they would have been stained green.

Platelet function analysis

PFA-100 testing revealed a significant shortening of capillary closure time with increasing number of pMPs. The pMP effect was seen in epinephrine/collagen cartridges (27.2 ± 2.6% reduction over control, with 10,000 pMPs/μl, $p<0.001$) whereas closure time of ADP/collagen cartridges showed a shortening trend that did not reach significance (▶Fig. 5A-B). pMP-enriched blood had a significant time-dependent shortening of the closure time induced by both epinephrine/collagen (21.3 ± 4.5% reduction over control, with 10 min pMP incubation, $p<0.0005$) and adenosine diphosphate/collagen (16.8 ± 3.8% reduction over control, with 10 min pMP incubation, $p<0.005$) agonists (▶Fig. 5C-D).

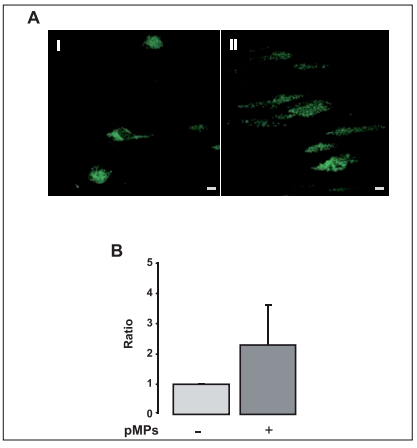


Figure 3: Contribution of platelet-derived microparticles to platelet deposition on collagen type-I surface. A) Photomicrographs show representative confocal images of adhered platelets to type-I collagen surface of whole blood (I) and of whole blood with 6,000 pMPs/μl (II). Platelets were immunostained with mepacrine. Scale bar is 5 μm. B) Mean platelet deposition on collagen surfaces at a shear rate condition of 1,500 s^{-1}. Results are expressed as ratio of percentage of mean values of surface covered by platelets per analysed field (μm^2/field). Differences were analysed by Student t-test. Not statistically significant versus control whole blood (n=3).

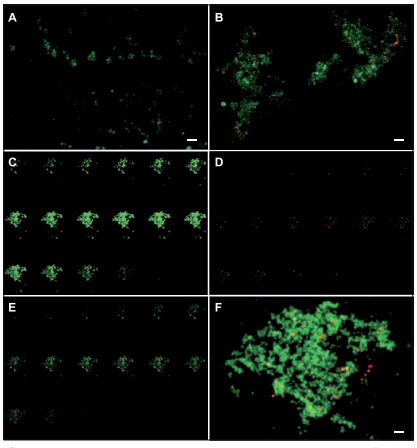


Figure 4: Fluorescence-tagged platelets and platelet-derived microparticles perfused over collagen type-I surface under flow conditions. Representative confocal microscopy photomicrographs of perfused (A) whole blood and (B-F) whole blood with pMPs in the flat perfusion chamber at high shear rate. C-E) z-stack series (showing 10 consecutive images obtained by optical sectioning); F) Maximal projection of the 10 z-stacks (step-size of 0.5 μm) of a selected platelet thrombi. Platelets were labelled by calcein (green) (A, C) and pMPs by BODIPY (red) (D). Colocalisation is shown in yellow (B, E, F – merged images). Photomicrographs were taken using a 20x-lens and are representative of five experiments. Scale bar is 20 μm (A-B) and 5 μm (F).

Standard LTA using ADP (1 μM) showed a significantly increased response in blood spiked with 6,000 pMPs/μl (37.0 ± 9.9% increase over control, with 5 min pMP incubation, $p<0.05$). However, the effect of MPs on platelet aggregation disappeared with higher doses of ADP and with stronger agonists such as thrombin and collagen (see ▶ Suppl. Table 2, available online at www.thrombosis-online.com).

Thromboelastometric coagulation analysis

Whole blood enriched with pMPs (6,000/μl) showed a significant decrease in clotting time when compared with whole blood without exogenous pMPs. An average reduction of 38.8 ± 8.5 ($p<0.05$) and 31.5 ± 6.4 ($p<0.01$) seconds in In-TEM and Ex-TEM tests, respectively, was obtained (▶ Fig. 6A). The addition of pMPs also increased MAXV-t in both Ex-TEM and In-TEM tests (▶ Fig. 6B), however, only in the In-TEM assay the effect reached significance (reduction of 38.8 ± 9.2 s, $p<0.05$). Supplementation with pMPs also led to an increase in the AUC parameter in Ex-TEM test (▶ Fig. 6C) and in the MCF difference between Ex-TEM and FibTEM tests (▶ Fig. 6D). Normally the clot is formed by the interaction of fibrin with activated platelets. MCF of Ex-TEM mainly depends on the activity of platelets and fibrinogen, whereas in FibTEM the platelets are inhibited. Therefore, the increase in the difference between both demonstrates platelet involvement in maximum clot firmness.

Discussion

Thrombosis on damaged and atherosclerotic arteries is the major cause of presentation of acute ischaemic syndromes and cardiovascular death (1, 2). An unresolved question in the presentation of ACS is that a similar type of underlying atherosclerotic lesion can trigger different types of ACS, either ST-elevation myocardial infarction (STEMI), non-STEMI or even unstable angina. Because the clinical implications of each type of ACS are quite different in morbidity and mortality (44), the mechanisms driving this outcome need to be investigated. Up till now the contribution of systemic factors to the thrombotic process has been mainly centred in characterising 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; thus their number is increased in patients with coronary artery disease (CAD) (8–10). However, whether circulating MPs had a role on the growth of the thrombotic mass triggered on top of atherosclerotic plaques had not been demonstrated. Here, in a proof-of-concept study based on blood-derived MPs purified from healthy donors, we show 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 pattern of thrombus formation on human atherosclerotic lesions is directly regulated by local rheological conditions and vessel wall composition (32, 33). Thus, in the present study we have

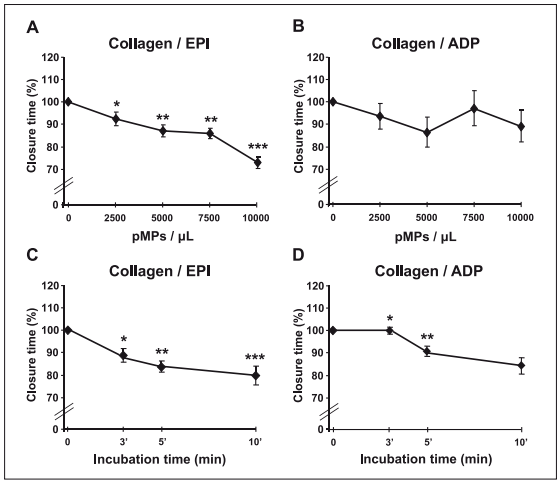


Figure 5: Effect of platelet-derived microparticles on platelet function. Closure time determination in PFA-100, using (A, C) epinephrine/collagen and (B, D) ADP/collagen cartridges. A-B) Different concentrations of pMPs were incubated with whole blood from healthy volunteers for 3 min and then closure time was measured. C-D) Incubation time effect on pMP-mediated closure time shortening. pMPs (2,500/l) were incubated with whole blood from healthy volunteers with different incubation times (3', 5' and 10'). Results are expressed as the mean ± SE percentage of closure time relative to that of the control. Differences were analysed by ANOVA. *$p<0.05$, **$p<0.005$, ***$p<0.0005$ significant versus control group (n=8). ADP, adenosine diphosphate; EPI, epinephrine.

used two well-characterised flow perfusion devices to rheologically model stenotic coronary blood conditions (32–35) and demonstrate that cMPs have a direct functional role in enhancing thrombus formation. When adjusted to an identical blood 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 (45), as well as to fibrinogen, von-Willebrand factor and surface immobilised platelets (46). Here, using fluorescence-tagged pMPs, we show that under high shear rate conditions pMPs also localise within the growing platelet thrombi on exposed collagen.

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 by pMPs in agreement with Kim et al. (47). In addition, we observed for the first time a pMP dose- and incubation time-dependent effect on closure time shortening. According to this assay, as well as to the reported level of circulating MPs in patients with different pathologies (43) and our own data on high CVD-risk patients (unpublished data), we selected 6,000 MPs/μl to investigate the effect of cMPs and pMPs on platelet adhesion and thrombosis under flow dynamic conditions. Interestingly, pMPs also shortened the clotting time of whole blood when analysed by thromboelastography and showed a proaggregatory effect on LTA when platelets were challenged with low ADP concentrations.

The pMPs used in our studies were fully characterised by flow cytometry showing high binding capacity for annexin V (phosphatidylserine) and carried platelet $\alpha_{IIb}\beta_3$-integrin, CD36, and PECAM-1. CD36 has been previously shown to associate to MPs in diabetics (48), and PECAM-1 has been found to be increased in cMPs of patients with different cardiovascular outcomes (49). pMPs also carried thrombospondin-1 and CD63, an antigen found to be increased in pMP subpopulations of peripheral arterial disease and myocardial infarction patients (43), and low levels of activation markers like P-selectin, activated $\alpha_{IIb}\beta_3$-integrin and tissue factor.

pMPs are generated not only during platelet activation or apoptosis, but also by strenuous blood flow conditions (50). In addition, pMPs might as well result from megakaryocytes or quiescent circulating platelets (51). Recently, it has been demonstrated that pMPs are associated with metabolic syndrome and the Framingham 10-year CHD risk score in healthy volunteers without signs, or symptoms, of cardio- or cerebrovascular disease (52, 53); and circulating pMPs in peripheral blood from healthy humans sup-

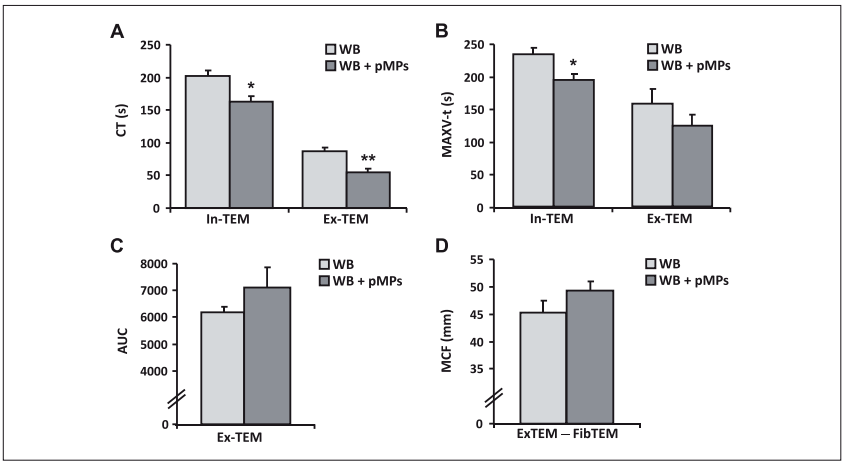


Figure 6: Effect of platelet-derived microparticles on clot formation and stability assessed by rotation thromboelastometry. A) Clotting time (CT; s) parameter measured in Ex-TEM and In-TEM assays. B) Maximum clot formation velocity time (MAXV-t; s) parameter measured in Ex-TEM and In-TEM assays. C) Area under the curve (AUC) parameter measured in Ex-TEM assay. D) Platelet involvement in maximum clot firmness (MCF) measured as the difference between MCF Ex-TEM and MCF Fib-TEM assays. Results are expressed as the mean ± SE percentage of measurements to that of the whole blood. Differences were analysed by ANOVA. A) *p<0.05, **p<0.001, and B) *p<0.05 significant versus control group (n=4).

What is known about this topic?

- Blood cells and platelets release microparticles with procoagulant activity.
- Until now the research regarding contribution of systemic factors to the mass/size of thrombus growing on top of the atherosclerotic plaques has mainly been focused on platelets and fibrin or inflammatory mediators.
- Circulating microparticles (cMPs) are increased in patients with coronary artery disease and with clustering of cardiovascular risk factors. However, their role on thrombosis has not been demonstrated.

What does this paper add?

- 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.
- Human blood pMPs enhance platelet adhesion and thrombus formation on human complex atherosclerotic lesions.
- Blood-cMPs seem to play a key role in the blood thrombogenic potential beyond being mere markers of cell activation.
- MPs reflect cell activation and serve as messengers and cellular crosstalk effectors in the systemic circulation.

port low-grade thrombin generation (27). Therefore, pMPs due to their protein content and characteristics might be potential procoagulant, proinflammatory and proatherogenic factors (17, 54, 55). As a proof-of-concept of our hypothesis, in the present study we have used cMPs from blood of healthy individuals and spontaneously generated pMPs in blood bank platelets concentrates. Studies with pMPs and cMPs from atherosclerotic patients are needed as a proof of principle after this study; indeed, not only the quantity but the quality of the circulating MPs may be key to their effects.

Our data 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. Because of this impact in thrombosis, MP proteomic studies may be interesting to understand the composition of MPs and reveal the potential mechanisms of cellular crosstalk and interactions. cMPs may also serve as pathogenic and prognostic markers not only for ACS-patients, but also for other patient groups, such as cancer patients, individuals with elevated cardiovascular risk factors and the elderly.

In summary, 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.

Acknowledgements

The authors thank Dr. J.Crespo, Dr. O.Juan, Dr. B.Molins, and Dr. E.Peña, for their insightful directions.

Conflicts of interest

None declared.

References

1. Fuster V, Badimon L, Cohen M, et al. Insights into the pathogenesis of acute ischemic syndromes. Circulation 1988; 77: 1213–1220.
2. Viles-Gonzalez JF, Fuster V, Badimon JJ. Atherothrombosis: a widespread disease with unpredictable and life-threatening consequences. Eur Heart J 2004; 25: 1197–1207.
3. Abrams CS, Ellison N, Budzynski AZ, et al. Direct detection of activated platelets and platelet-derived microparticles in humans. Blood 1990; 75: 128–138.
4. Working Group on Vascular Biology. 55th Annual Scientific and Standardization Committee Meeting. ISTH Scientific Subcommittee Minutes, Boston, USA; 2009. Available at http://c.ymcdn.com/sites/www.isth.org/resource/resmgr/yearly_subcommittee_minutes/2009_minutes.pdf.
5. Jy W, Horstman LL, Jimenez JJ, et al. Measuring circulating cell-derived microparticles. J Thromb Haemost 2004; 2: 1842–1851.
6. Burnier L, Fontana P, Kwak BR, et al. Cell-derived microparticles in haemostasis and vascular medicine. Thromb Haemost 2009; 101: 439–451.
7. Chirinos JA, Heresi GA, Velasquez H, et al. Elevation of endothelial microparticles, platelets, and leukocyte activation in patients with venous thromboembolism. J Am Coll Cardiol 2005; 45: 1467–1471.
8. Mallat Z, Benamer H, Hugel B, et al. Elevated levels of shed membrane microparticles with procoagulant potential in the peripheral circulating blood of patients with acute coronary syndromes. Circulation 2000; 101: 841–843.
9. Tan KT, Tayebjee MH, Lynd C, et al. Platelet microparticles and soluble P selectin in peripheral artery disease: relationship to extent of disease and platelet activation markers. Ann Med 2005; 37: 61–66.
10. Vidal C, Spaulding C, Picard F, et al. Flow cytometry detection of platelet procoagulation activity and microparticles in patients with unstable angina treated by percutaneous coronary angioplasty and stent implantation. Thromb Haemost 2001; 86: 784–790.
11. Mause SF, Ritzel E, Liehn EA, et al. Platelet microparticles enhance the vasoregenerative potential of angiogenic early outgrowth cells after vascular injury. Circulation 2010; 122: 495–506.
12. Rautou PE, Leroyer AS, Ramkhelawon B, et al. Microparticles from human atherosclerotic plaques promote endothelial ICAM-1-dependent monocyte adhesion and transendothelial migration. Circulation Res 2011; 108: 335–343.
13. Badimon L, Badimon JJ. Mechanisms of arterial thrombosis in nonparallel streamlines: platelet thrombi grow on the apex of stenotic severely injured vessel wall. Experimental study in the pig model. J Clin Invest 1989; 84: 1134–1144.
14. Badimon L, Badimon JJ, Galvez A, et al. Influence of arterial damage and wall shear rate on platelet deposition. Ex vivo study in a swine model. Arteriosclerosis 1986; 6: 312–320.
15. Spronk HM, van der Voort D, Ten Cate H. Blood coagulation and the risk of atherothrombosis: a complex relationship. Thrombosis J 2004; 2: 12.
16. Owens AP, 3rd, Mackman N. Microparticles in hemostasis and thrombosis. Circulation Res 2011; 108: 1284–1297.
17. Sinauridze EI, Kireev DA, Popenko NY, et al. Platelet microparticle membranes have 50– to 100-fold higher specific procoagulant activity than activated platelets. Thromb Haemost 2007; 97: 425–434.
18. Van der Meijden PE, Van Schilfgaarde M, Van Oerle R, et al. Platelet- and erythrocyte-derived microparticles trigger thrombin generation via factor XIIa. J Thromb Haemost 2012; 10: 1355–1362.
19. Nomura S, Tandon NN, Nakamura T, et al. High-shear-stress-induced activation of platelets and microparticles enhances expression of cell adhesion molecules in THP-1 and endothelial cells. Atherosclerosis 2001; 158: 277–287.
20. Shai E, Varon D. Development, cell differentiation, angiogenesis--microparticles and their roles in angiogenesis. Arterioscl Thromb Vasc Biol 2011; 31: 10–14.
21. Llorente-Cortes V, Otero-Vinas M, Camino-Lopez S, et al. Aggregated low-density lipoprotein uptake induces membrane tissue factor procoagulant activity and microparticle release in human vascular smooth muscle cells. Circulation 2004; 110: 452–459.
22. Del Conde I, Shrimpton CN, Thiagarajan P, et al. Tissue-factor-bearing microvesicles arise from lipid rafts and fuse with activated platelets to initiate coagulation. Blood 2005; 106: 1604–1611.

23. Nieuwland R, Berckmans RJ, McGregor S, et al. Cellular origin and procoagulant properties of microparticles in meningococcal sepsis. Blood 2000; 95: 930–935.
24. Shet AS, Aras O, Gupta K, et al. Sickle blood contains tissue factor-positive microparticles derived from endothelial cells and monocytes. Blood 2003; 102: 2678–2683.
25. Falati S, Liu Q, Gross P, et al. Accumulation of tissue factor into developing thrombi in vivo is dependent upon microparticle P-selectin glycoprotein ligand 1 and platelet P-selectin. J Exp Med 2003; 197: 1585–1598.
26. Thomas GM, Panicot-Dubois L, Lacroix R, et al. Cancer cell-derived microparticles bearing P-selectin glycoprotein ligand 1 accelerate thrombus formation in vivo. J Exp Med 2009; 206: 1913–1927.
27. Berckmans RJ, Nieuwland R, Boing AN, et al. Cell-derived microparticles circulate in healthy humans and support low grade thrombin generation. Thromb Haemost 2001; 85: 639–646.
28. Aatonen M, Gronholm M, Siljander PR. Platelet-derived microvesicles: multitalented participants in intercellular communication. Semin Thromb Hemost 2012; 38: 102–113.
29. Brill A, Dashevsky O, Rivo J, et al. Platelet-derived microparticles induce angiogenesis and stimulate post-ischemic revascularization. Cardiovasc Res 2005; 67: 30–38.
30. Jayachandran M, Miller VM, Heit JA, et al. Methodology for isolation, identification and characterization of microvesicles in peripheral blood. J Immunol Methods 2012; 375: 207–214.
31. Biro E, Akkerman JW, Hoek FJ, et al. The phospholipid composition and cholesterol content of platelet-derived microparticles: a comparison with platelet membrane fractions. J Thromb Haemost 2005; 3: 2754–2763.
32. Badimon L, Badimon JJ, Turitto VT, et al. Platelet thrombus formation on collagen type I. A model of deep vessel injury. Influence of blood rheology, von Willebrand factor, and blood coagulation. Circulation 1988; 78: 1431–1442.
33. Badimon L, Turitto V, Rosemark JA, et al. Characterization of a tubular flow chamber for studying platelet interaction with biologic and prosthetic materials: deposition of indium 111-labeled platelets on collagen, subendothelium, and expanded polytetrafluoroethylene. The J Lab Clin Med 1987; 110: 706–718.
34. Molins B, Pena E, Padro T, et al. Glucose-regulated protein 78 and platelet deposition: effect of rosuvastatin. Arterioscl Thromb Vasc Biol 2010; 30: 1246–1252.
35. Molins B, Pena E, Vilahur G, et al. C-reactive protein isoforms differ in their effects on thrombus growth. Arterioscl Thromb Vasc Biol 2008; 28: 2239–2246.
36. Fernandez-Ortiz A, Badimon JJ, Falk E, et al. Characterization of the relative thrombogenicity of atherosclerotic plaque components: implications for consequences of plaque rupture. J Am Coll Cardiol 1994; 23: 1562–1569.
37. Rand ML, Wang H, Bang KW, et al. Rapid clearance of procoagulant platelet-derived microparticles from the circulation of rabbits. J Thromb Haemost 2006; 4: 1621–1623.
38. Baumgartner HR. Platelet interaction with collagen fibrils in flowing blood. I. Reaction of human platelets with alpha chymotrypsin-digested subendothelium. Thromb Haemost 1977; 37: 1–16.
39. Arderiu G, Pena E, Aledo R, et al. Tissue factor regulates microvessel formation and stabilization by induction of chemokine (C-C motif) ligand 2 expression. Arterioscl Thromb Vasc Biol 2011; 31: 2607–2615.
40. Galvez A, Badimon L, Badimon JJ, et al. Electrical aggregometry in whole blood from human, pig and rabbit. Thromb Haemost 1986; 56: 128–132.
41. Hartert H. The current importance of thrombelastography. Minerva Medica 1963; 54: 181–182.
42. Sorensen B, Johansen P, Christiansen K, et al. Whole blood coagulation thrombelastographic profiles employing minimal tissue factor activation. J Thromb Haemost 2003; 1: 551–558.
43. van der Zee PM, Biro E, Ko Y, et al. P-selectin- and CD63-exposing platelet microparticles reflect platelet activation in peripheral arterial disease and myocardial infarction. Clin Chem 2006; 52: 657–664.
44. Badimon L, Badimon JJ, Vilahur G, et al. Pathogenesis of the acute coronary syndromes and therapeutic implications. Pathophysiol Haemost Thromb 2002; 32: 225–231.
45. Merten M, Pakala R, Thiagarajan P, et al. Platelet microparticles promote platelet interaction with subendothelial matrix in a glycoprotein IIb/IIIa-dependent mechanism. Circulation 1999; 99: 2577–2582.
46. Keuren JF, Magdeleyns EJ, Bennaghmouch A, et al. Microparticles adhere to collagen type I, fibrinogen, von Willebrand factor and surface immobilised platelets at physiological shear rates. Br J Haematol 2007; 138: 527–533.
47. Kim HK, Kim JE, Han KS, et al. The role of cellular microparticles in platelet-related hemostasis measured using Platelet Function Analyzer-100. Blood Coagul Fibrinolysis 2008; 19: 328–330.
48. Alkhatatbeh MJ, Mhaidat NM, Enjeti AK, et al. The putative diabetic plasma marker, soluble CD36, is non-cleaved, non-soluble and entirely associated with microparticles. J Thromb Haemost 2011; 9: 844–851.
49. Sinning JM, Losch J, Walenta K, et al. Circulating CD31+/Annexin V+ microparticles correlate with cardiovascular outcomes. Eur Heart J 2011; 32: 2034-2041.
50. Holme PA, Orvim U, Hamers MJ, et al. Shear-induced platelet activation and platelet microparticle formation at blood flow conditions as in arteries with a severe stenosis. Arterioscl Thromb Vasc Biol 1997; 17: 646–653.
51. Cramer EM, Norol F, Guichard J, et al. Ultrastructure of platelet formation by human megakaryocytes cultured with the Mpl ligand. Blood 1997; 89: 2336–2346.
52. Ueba T, Haze T, Sugiyama M, et al. Level, distribution and correlates of platelet-derived microparticles in healthy individuals with special reference to the metabolic syndrome. Thromb Haemost 2008; 100: 280–285.
53. Ueba T, Nomura S, Inami N, et al. Plasma level of platelet-derived microparticles is associated with coronary heart disease risk score in healthy men. J Atheroscl Thromb 2010; 17: 342–349.
54. Garcia BA, Smalley DM, Cho H, et al. The platelet microparticle proteome. J Proteome Res 2005; 4: 1516–1521.
55. Vasina EM, Cauwenberghs S, Feijge MA, et al. Microparticles from apoptotic platelets promote resident macrophage differentiation. Cell Death Dis 2011; 2: e211.

SUPLEMENTARY MATERIAL

Supplementary Table 1

CELL SURFACE MOLECULES FOR MICROPARTICLE IDENTIFICATION AND CHARACTERIZATION

Marker	Alternative name	Expression	Conjugation	Clone	Company
CD31	PECAM-1	Platelets and ECs	PE	1F11	BD Pharmingen
CD41	α_{IIb} integrin	Platelets	FITC	SZ22	Beckman Coulter
CD36	SCARB3	Widely expressed	PE	CB38	BD Pharmingen
CD42b	GPIbα	Platelets	FITC	HIP1	BD Pharmingen
CD45	LCA	Leukocytes	PE	Immu-19.2	Beckman Coulter
CD51	α_V integrin	Platelets	FITC	AMF7	Beckman Coulter
CD61	β_3 integrin	Platelets	PE	VI-PL2	BD Pharmingen
CD62P	P-selectin	Activated platelets	FITC	AK-4	BD Pharmingen
CD63	LAMP-3	Widely expressed	PE	H5C6	BD Pharmingen
CD142	Tissue Factor	Widely expressed	FITC	VD8	American Diagnostica
PAC1	$\alpha_{IIb}\beta_3$ integrin	Activated platelets	FITC	PAC1	BD Biosciences
TSP1	THBS-1	Platelets	PE	P10	Beckman Coulter
IgG1γ	-	-	FITC / PE	X40	BD Biosciences
IgG1κ	-	-	FITC/ PE	MPOC21	BD Pharmingen

Note: FITC indicates fluorescein isothiocyanate; PE, phycoerythrin; PECAM-1, platelet endothelial cell adhesion molecule-1; ECs, endothelial cells; SCARB3, Scavenger receptor class B member 3; LCA, leukocyte common antigen; LAMP-3, lysosomal-associated membrane protein 3; THBS1, thrombospondin 1.
Source: Own elaboration.

Supplementary Table 2

EFFECT OF PLATELET-DERIVED MICROPARTICLES ON PLATELET AGGREGATION *IN VITRO*

Agonist	Maximum aggregation (%)		
	PRP	PRP + pMPs 3'	PRP + pMPs 5'
T (0.2 uNIH/mL)	89.8 ± 0.25	84.8 ± 0.25	-
T (0.3 uNIH/mL)	92.0 ± 0	89.0 ± 0	-
COL (1 μg/mL)	92.4 ± 1.4	86.6 ± 2.4	91.4 ± 2.3
COL (3 μg/mL)	90.8 ± 1.1	92.8 ± 1.1	87.5 ± 1.2
ADP (1 μM)	35.1 ± 4.7	64.3 ± 16.7 *	68.8 ± 13.7 *
ADP (3 μM)	89.3 ± 1.0	88.3 ± 2.1	85.8 ± 6.5
EPI (3 μM)	82.0 ± 9.3	85.8 ± 4.4	87.3 ± 4.0
EPI (5 μM)	86.9 ± 2.1	85.3 ± 2.4	90.3 ± 0.8

Source: Own elaboration.

Thrombin, collagen, epinephrine, and ADP-induced platelet aggregation in platelet rich plasma (PRP). A pathologic concentration of pMPs (6000/μL) was incubated with human PRP from healthy volunteers for three or five minutes. Data are expressed as the mean ± SE percentage of maximum aggregation. *p<0.05, significant *versus* control PRP (results from four experiments). Differences were analysed by analysis of variance. T, thrombin; COL, collagen; ADP, adenosine diphosphate; EPI, epinephrine.

ARTICLE 2

Lipid-lowering therapy with statins reduces microparticle shedding from endothelium, platelets and inflammatory cells

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

Published – Thromb Haemost 2013; 110(2): 366-377.

OBJECTIVE

The aim of this study was to evaluate the effects of lipid-lowering therapy on circulating microparticle shedding in patients in primary prevention of atherosclerosis.

HIGHLIGHTS

- Patients with lipid-lowering therapy with statins have lower circulating microparticles numbers, especially of platelet, leukocyte and endothelial cell origin than blood from untreated patients with the same plasma lipid levels.
- At equal LDL-cholesterol levels, statin-treated patients have less cMPs carrying markers of parental cell activation.
- Beyond choleterol lowering, statins have direct effects on reducing activated cell mebrane shedding of cMPs, which is treatment time-dependent.

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): 366-377.

Lipid-lowering therapy with statins reduces microparticle shedding from endothelium, platelets and inflammatory cells

Rosa Suades[1,2]; Teresa Padró[1,2]; Rodrigo Alonso[3]; Pedro Mata[3]; Lina Badimon[1,2,4]

[1]Cardiovascular Research Center (CSIC-ICCC), IIB-Sant Pau, Barcelona, Spain; [2]CIBERobn – Pathophysiology of Obesity and Nutrition, Barcelona, Spain; [3]Jimenez Diaz Foundation, Madrid, Spain; [4]Cardiovascular Research Chair, UAB, Barcelona, Spain

Summary

Hyperlipidaemia is a causal factor in the ethiopathogenesis of atherosclerosis. Statins are the cornerstone drug therapy for LDL-cholesterol (LDL-c) lowering, that exert beneficial effects beyond lipid lowering. Circulating microparticles (cMPs), microvesicles released by activated cells into the bloodstream, are markers of vascular and inflammatory cell activation with tentative role in disease progression. However, the role of statins on cMPs seems controversial. We aimed at the evaluation of the effects of lipid-lowering treatment (LLT) on cMP generation in patients in primary prevention of atherosclerosis. A case-control study was conducted in hypercholesterolaemic patients receiving LLT with statins and normocholesterolaemic controls (LLT^+ and LLT^-, respectively, n=37/group), matched by age, gender and LDL-c levels. cMPs were characterised by flow cytometry using annexin-V and cell-specific antibodies. In LLT^+-patients overall numbers of cMPs ($p<0.005$) were lower than in controls. Levels of cMPs carrying parental cell markers from vascular and circulating cell origin (platelet, endothelial cell, pan-leukocyte and specific-leukocyte subsets) were significantly lower in blood of LLT^+ compared to LLT^-patients. Moreover, MPs from LLT^+-patients had reduced markers of activated platelets ($\alpha_{IIb}\beta_3$-integrin), activated inflammatory cells (α_M-integrin) and tissue factor. The effect of LLT on cMP shedding was found to be accumulative in years. cMP shedding associated to cardiovascular risk in LLT^+-patients. In summary, at similar plasma cholesterol levels patients on statin treatment had a significant lower number of cMPs carrying markers of activated cells. These findings indicate that statins protect against vascular cell activation.

Keywords

Atherosclerosis, circulating microparticles, inflammation, statins, thrombosis

Correspondence to:
Prof. Lina Badimon
Cardiovascular Research Center
c/Sant Antoni Mª Claret 167, 08025 Barcelona, Spain
Tel.: +34 935 565880, Fax: +34 935 565559
E-mail: lbadimon@csic-iccc.org

Financial support:
Funds were provided by PNS SAF2010–16549 (to LB) from the Spanish Ministry of Science and Innovation; and CNIC-082008 (to LB and PM), CIBEROBN- CB06/03 (to LB), TERCEL (to LB), FIS PI1001115 (to TP) and Fundación Investigación Cardiovascular – Fundación Jesús Serra. RS is a recipient of a predoctoral fellowship from Spanish Ministry of Health (PFIS-FI09/00092, ISCIII).

Received: March 18, 2013
Accepted after minor revision: May 17, 2013
Prepublished online: June 6, 2013
doi:10.1160/TH13-03-0238
Thromb Haemost 2013; 110: 366–377

Introduction

Effective treatment of hyperlipidaemia (HL) is of great importance in the overall management of vascular risk and prevention of cardiovascular disease (CVD). Statins, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) inhibitors, are the drug of choice for controlling lipid levels and reducing morbidity and mortality among patients with cardiovascular risk (1, 2). Beyond lipid-lowering effects, statins exert many pleiotropic effects, which involve anti-inflammatory, anti-atherogenic and anti-thrombotic effects (3-5). Pleiotropic effects of statins are mainly caused by inhibition of protein prenylation. Isoprenylation, a post-translation protein modification, adds intermediates of the mevalonate pathway (such as geranylgeranyl pyrophosphate –GGPP–) to small G proteins (Rho, Rac, Rab) and facilitates their cell membrane attachment and the subsequent activation of transmembrane signalling (6, 7). Thus, statins are involved in the modification of G protein functions, which mainly include cytoskeleton assembly, gene regulation, cell growth and motility, protein and lipid trafficking, and hence statins may affect membrane microparticle shedding. To evidence these tentative effects of statins in cell membranes in patients treated clinically we designed this study.

Circulating microparticles (cMPs) are submicron membrane vesicles shed from activated cells into the bloodstream (8). cMPs may originate from cells that are involved in the pathogenesis of atherothrombotic diseases and exposed to lipids in the circulation such as endothelial, leukocyte, and platelets (8-11). Indeed, it has been recently shown that circulating and platelet-derived microparticles enhance thrombosis on atherosclerotic plaques (12). Circulating MPs are also found in the plasma of healthy subjects

(13), but the number of cMPs increases in patients with existing cardiovascular disease (14-16) and with risk factors for CVD (17, 18). In fact, cMPs also seem to have substantial effects on early phases of atherogenesis (19, 20).

There are controversial results regarding the effects of lipid-lowering on cMP release (21). Diamant et al. reported that statins may stimulate endothelial-derived microparticle (eMP) release *in vitro* (22, 23). In contrast, other studies have shown that fluvastatin promotes inhibition of the release of eMPs *in vitro* (24). Atorvastatin has been reported to increase circulating eMPs *in vivo* (25) but also to reduce expression of activation markers on platelet-derived microparticles (pMPs) in patients with peripheral arterial occlusive disease (26, 27) and with type-1 diabetes and dyslipidaemia (28). Association of simvastatin with losartan decreased monocyte-, endothelial- and platelet-MP numbers in patients with hypertension and type-2 diabetes (29, 30) and pravastatin reduced β_3-integrin on pMPs in type-2 diabetes (31). Finally, pitavastatin could only decrease pMPs in association with eicosapentaenoic acid in the adiponectin-responder group of both hyperlipidaemic and diabetic patients (32). Recently, the increased plasma TF^+-MP activity shown in hyperlipidaemic mice and monkeys was found to be reduced by simvastatin treatment (33).

In general, the effects of statins on cMPs were associated to reduction in low-density-lipoprotein cholesterol (LDL-c) levels. To further understand whether the beneficial effects of statins on cardiovascular disease are also related to effects on cell activation independent of their lipid lowering effect, we designed this study analysing cMP numbers, cell source and their transported activation cell markers in blood of subjects with the same LDL-c levels with or without statin treatment.

Study design, materials and methods

Clinical study population

The present nested case-control study was part of the ongoing SAFEHEART study (34). Briefly, SAFEHEART is an open, multicentre, long-term prospective cohort study in a well-molecularly defined familial hypercholesterolaemia (FH) population. Demographic and clinical characteristics data, cardiovascular history, classic cardiovascular risk factors and current treatment for hypercholesterolaemia were obtained from all subjects using a standardised report form at the inclusion in the cohort. Data related to lipid-lowering treatment (LLT) included statin, dose, time of treatment and compliance. Adherence to lipid lowering treatment was assessed by indirect method with a single question, as previously described (35). Maximum statin dose was defined as previously described: simvastatin 80 mg/day, pravastatin 40mg/day, lovastatin 80 mg/day, fluvastatin 80 mg/day, atorvastatin 80 mg/day, rosuvastatin 20-40 mg/day (36). Cases were randomly selected from the group of hypercholesterolaemia and with negative genetic testing of FH that were receiving a stable LLT, at least one year before the inclusion, according to clinical guidelines (37-39). The control group included subjects from the same cohort with the same LDL-c levels that were not on LLT, matched by age, gender, and demographics. Neither patients nor control subjects present pregnancy, sepsis or infection or have history of cancer, since these conditions are known to independently impair MP number. The results of the study are presented in accordance with STROBE guidelines. The study was approved by the local ethics committee and was conducted according to the Declaration of Helsinki. A written informed consent was obtained from all participants prior to the study.

Blood sampling for analysis

Venous blood was withdrawn from the cubital vein without tourniquet using a 20-gauge needle after 10-14 hours (h) of fasting into serum, EDTA and 3.8% sodium citrate tubes, for biochemical, genotyping and microparticle analysis, respectively.

Serum (30 minutes [min] at 37ºC, 30 min at 4ºC, 1258 x g for 15 min at 4ºC) and EDTA-plasma (1258 x g for 20 min) were prepared for lipid profile and DNA analysis. Samples were processed for assay within first 2 h. Total cholesterol, triglycerides, and high-density-lipoprotein (HDL)-cholesterol were measured by standardised enzymatic methods. Serum LDL-cholesterol concentration was calculated using the Friedewäld formula (40). Lipoprotein (a) levels were measured using a turbidimetric method performed by suspension of latex particles coated with rabbit IgG anti-Lp (a) gene (Abbott Diagnostics, Abbott Park, IL, USA) in an Architect autoanalyzer c16000 (Abbot). For molecular genetic analysis, genomic DNA was isolated from whole blood using standard methods and the genetic diagnosis of FH made using a DNA-microarray (LIPOCHIP) in a central laboratory (Progenika, Bilbao, Spain) (41).

For flow cytometric analysis of cMPs, blood cells were removed from citrated blood by low-speed centrifugation at 1258 x g for 20 min at room temperature (RT) in order to avoid *in vitro* platelet activation, as previously described (42, 43). Platelet-poor plasma (PPP) was carefully aspirated, leaving about 0.1 cm undisturbed layer on top of the cells. A second centrifugation step (1258 x g for 10 min, RT) was made to ensure the complete removal of cells and obtain the platelet-free plasma (PFP). All samples were processed identically and within 60 min after extraction. Samples were tested with a cell counter for the absence of residual cells after centrifugation. PFP aliquots were snap-frozen in liquid nitrogen and stored at -80ºC until flow cytometric studies.

Circulating microparticle isolation

The cMP fraction was isolated from PFP by a two-step high speed centrifugation (42, 43). Briefly, frozen PFP aliquots were thawed on melting ice for 1 h and centrifuged for 30 min at 20,000 x g to pellet cMPs. Then, supernatant was 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 pellet was resuspended in citrate-PBS to a final volume of 100 µl.

Flow cytometric analysis of circulating microparticles

Triple-label flow cytometric analysis was performed as described by Nieuwland et al. (43, 44) with slight modifications. Briefly, washed cMP suspensions (5 µl) diluted in 30 µl phosphate-buffered saline (PBS) containing 2.5 mM $CaCl_2$ were incubated (20 min, RT, dark) with combinations of BD-horizon V450-conjugated annexin V (5 µl) with two specific monoclonal antibodies

	LLT⁻-patients	LLT⁺-patients	Statistics
Male /Female (n)	18/19	14/23	p = 0.35
Age (years, mean, range)	43.0 (41.0–51.0)	53.0 (38.0–62.0)	p = 0.30
Body mass index (kg/m²)	26.4 (23.1–29.4)	27.0 (22.8–30.7)	p = 0.52
Risk factor for CVD			
Diabetes mellitus (n, %)	0 (0%)	1 (2.7%)	p = 0.31
Hypertension (n, %)	1 (2.7%)	7 (18.9%)	p = 0.02
Tabacco consumption (n, %)			
• Current	10 (27%)	12 (32,4%)	p = 0.61
• Ex-smokers	6 (16.2%)	5 (13.5%)	p = 0.74
• Never	21 (56.8%)	20 (54.1%)	p = 0.82
Obesity (BMI > 30)	0 (0%)	7 (18.9%)	p = 0.005
Hypothyroidism	0 (0%)	1 (2.7%)	p = 0.31
Waist diameter (cm)	101.0 (98.0–106.0)	87.0 (78.0–99.0)	p < 0.0001
Corneal arcus	0 (0%)	12 (32.4%)	p = 0.0002
Total cholesterol (mg/dl)	203.0 (186.0–228.0)	210.0 (192.0–240.0)	p = 0.56
LDL-cholesterol (mg/dl)	130. (110.4–147.4)	134.2 (109.0–159.6)	p = 0.35
HDL-cholesterol (mg/dl)	55.0 (47.0–64.0)	55.0 (48.0–63.0)	p = 0.74
TC / HDL-C ratio	4.0 (3.3–4.5)	3.9 (3.1–4.5)	p = 0.88
Lp(a) (mg/dl)	14.8 (6.0–32.7)	21 (10.9–48.5)	p = 0.13
Triglyceride (mg/dl)	97.0 (67.0–124.0)	82.0 (64.0–120.0)	p = 0.50
High-sensitive CRP (mg/dl)	1.7 (1.0–2.7)	1.0 (0.7–2.4)	p = 0.04
Fasting plasma glucose (mg/dl)	85.5 (78.5–91.5)	86.0 (78.0–91.0)	p = 0.91
Medication			
ACEI	0 (0%)	3 (8.1%)	p = 0.08
ARB	0 (0%)	1 (2.7%)	p = 0.31
Anti-platelet drugs	0 (0%)	1 (2.7%)	p = 0.31
CCB	0 (0%)	1 (2.7%)	p = 0.31
Statins			
• Simvastatin	0 (0%)	10 (27%)	p = 0.0007
• Atorvastatin	0 (0%)	18 (48.6%)	p < 0.0001
• Rosuvastatin	0 (0%)	7 (18.9%)	p = 0.0054
• Fluvastatin	0 (0%)	2 (5.4%)	p = 0.15
Ezetimibe	0 (0%)	4 (10.8%)	p = 0.04
HRT	0 (0%)	1 (2.7%)	p = 0.31
Tiazidic diuretics	0 (0%)	1 (2.7%)	p = 0.31
Diurasa	0 (0%)	1 (2.7%)	p = 0.31
Metformin	0 (0%)	1 (2.7%)	p = 0.31
Glinidas	0 (0%)	1 (2.7%)	p = 0.31

Table 1: Clinical characteristics of untreated (LLT⁻) and treated (LLT⁺) patients. ACEI, angiotensin-converting-enzyme inhibitor; ARB, angiotensin-II receptor blocker; BMI, body mass index; CCB, calcium channel blocker; CRP, C-reactive protein; CVD, cardiovascular disease; HDL, high-density lipoprotein; HRT, hormone replacement therapy; LDL-c, low-density lipoprotein; LLT, lipid-lowering therapy; Lp(a), lipoprotein (a).

(mAbs, 5 µl each) labelled with fluorescein isothiocyanate and phycoerythrin, or with the isotype-matched control antibodies (see Suppl. Table 1, available online at www.thrombosis-online.com) and, then, diluted with 2.5 mM $CaCl_2$-PBS buffer before being analysed on a FACSCantoII™ flow cytometer (Becton Dickinson, San Diego, CA, USA). Acquisition was performed for 1 min 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. Granulocyte's percentage was inferred by subtracting agranulocytes (lymphocytes plus monocytes) from total leukocytes instead of labelling with specific mAb. Gate limits were established following two criteria: 1) calibration using a Flow Check YG Size Range Calibration Kit (Polysciences, Warrington, PA, USA) (45) and 2) with an *in vitro* platelet-derived microparticle population as positive control (46) (see Suppl. Figure 1A-D, available online at www.thrombosis-online.com) since cali-

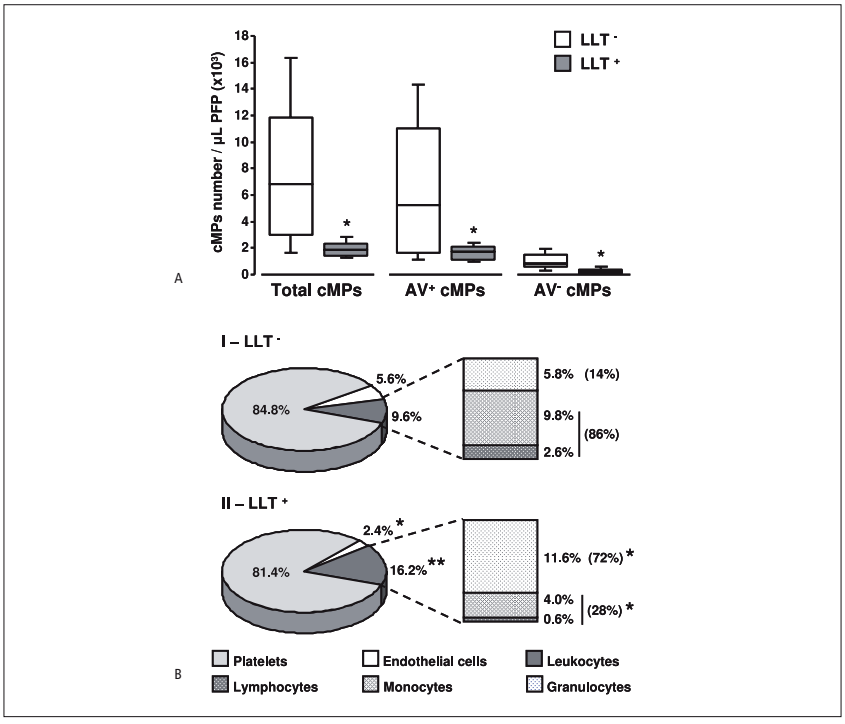


Figure 1: Comparison of cMP numbers and cellular origins between LLT⁻ and LLT⁺-patients. A) Box and whisker plots showing number of total, AV⁺, and AV⁻-cMPs per microliter of platelet-free plasma (cMPs/µl PFP), both in controls without LLT (n=37) and subjects under LLT (n=37). Lines within boxes represent median values, the upper and lower boxes lines represent the 25th and 75th percentiles, respectively, and the upper and lower bars outside the boxes represent the 10th and 90th percentiles, respectively. B) Pie charts showing distribution of cMPs from (I) controls and (II) subjects on LLT by cell origin, represented by the percentage of total cMPs from each cell type. Used markers were $CD41^+/CD61^+$ for platelet, $CD146^+/CD31^+$ for endothelial cell, $CD45^+$ for total leukocyte, $CD3^+$ for lymphocyte and $CD14^+$ for monocyte origins accounting for agranulocytes and, finally, granulocytes were inferred subtracting agranulocytes subpopulation from leukocytes fraction. Numbers indicate percentages of each marker relative to all cMP population and, percentages of leukocyte subpopulations relative to total leukocytes are expressed in brackets. *p<0.0001, **p<0.02 vs control group (U-Mann Whitney test).

bration beads have different properties of FSC/SSC compared with biologic MPs (47, 48). The lower detection limit was placed as a threshold above the electronic noise of our flow cytometer. 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. Phosphatidylserine-positive cMPs were labelled using annexin V in the presence of 2.5 mM $CaCl_2$, since calcium is essential for annexin V conjugation. Annexin V binding level was corrected for autofluorescence using fluorescence signals obtained with microparticles in a calcium-free buffer.

Data were analysed with FACSDiva™ software (version 6.1.3, Becton Dickinson). The concentration (number of cMPs per µl of plasma) was determined according to Nieuwland's procedure (44), based on sample's volume, flow cytometer's flow rate and the number of fluorescence-positive events (N), as follows: cMPs/µl = N x (Vf/Va) x (Vt/FR) x (1/Vi) [Vf(µl) = final volume of washed cMP suspension, Va(µl) = volume of washed cMP suspension used for each labelling analysis, Vt(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 analysed in 1 min), 1 is the µl unit of volume, and Vi(µl) = original volume of plasma used for microparticle isolation].

Flow rate was measured before each experiment. Intra-assay 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 0.2 µm pore size filters.

Statistical analysis

Sample size was selected based on the average of studies focusing on cMP analysis by FACS. All data are presented as medians (interquartile range), except when indicated. An initial descriptive analysis was carried out using number of cases and percentages for qualitative variables and median and interquartile range for quantitative variables. Frequencies of qualitative variables (risk factors and medications) were compared between two groups by using the Chi-square analysis. Median values of quantitative variables were compared with non-parametric tests. The statistical significances between patient and control groups were determined with U-Mann Whitney and multiple comparisons by Kruskal-Wallis, and when significant, Bonferroni post-hoc analysis was used to assess intergroup differences. StatView was used for all statistical tests and a $p<0.05$ was considered statistically significant.

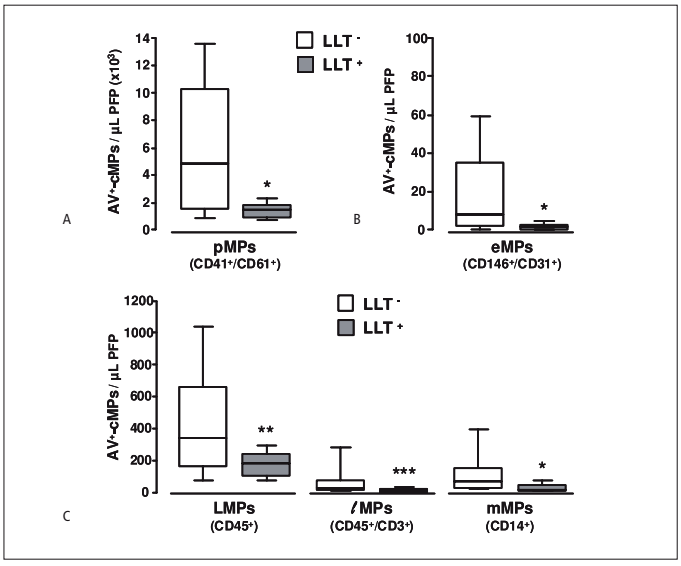


Figure 2: Annexin V-positive cMP characterisation in LLT⁻ and LLT⁺-patients. cMP cellular origin in non-LLT (n=37) and LLT (n=37) patients: A) platelet-derived ($CD41^+/CD61^+$), B) endothelial-derived ($CD146^+/CD31^+$), and C) leukocyte-derived ($CD45^+$), lymphocyte-derived ($CD3^+/CD45^+$), and monocyte-derived ($CD14^+$) AV^+-cMPs/µl of PFP. Data are given as a box and whisker plot as described in Figure 1A. *p<0.0001, **p<0.001, ***p<0.01 vs control group (U-Mann Whitney test).

Results

Clinical characteristics of the study population

A total of 37 untreated (LLT$^-$) and 37 statin-treated (LLT$^+$) non-FH subjects from the Spanish SAFEHEART cohort were included in the analysis. Demographic, biochemical, and clinical data of both groups as well as classical coronary risk factors are shown in ▶Table 1. The LLT$^+$-patient group was composed by 14 men and 23 women with a median age at inclusion of 53 years (range from 48 to 62 years) and the untreated group consisted of 18 men and 19 women with a median age of 43 years (range from 41 to 51 years). Total cholesterol, LDL-cholesterol, HDL-cholesterol, and triglycerides were similar in both groups. There were no differences either in gender, mean age, BMI, type 2 diabetes mellitus, current tobacco consumption, and Lp(a) levels, between the two groups. However, hypertension and obesity were more frequent in LLT$^+$ group. In LLT$^+$-patients, the dose of simvastatin use was 20 mg (n=10), atorvastatin 20 mg (n=18) and rosuvastatin 10 mg (n=7). Fluvastatin was not included in the statistical analysis of cMP reduction depending on LLT duration since only 5.4% of LLT$^+$-patients (2 out of 37) were taking this drug. Only one case (2.7% of LLT$^+$-patients) was receiving maximum statin dose. The mean duration of LLT was 7.5 ± 1.0 years (range 2-22 years).

Levels and cellular origin of circulating microparticle population

The total number of blood microparticles (based on FSC/SSC characteristics) was significantly lower in LLT$^+$-patients, as shown in ▶Figure 1A. Both phosphatidylserine (PS)-positive or annexin V-binding (AV$^+$) and PS negative (AV$^-$) microparticles were detected (▶Figure 1A); however, the majority of all circulating microparticles were AV$^+$ in both studied groups. Levels of AV$^+$-cMPs and AV$^-$-cMPs were significantly lower in blood of LLT$^+$ than in LLT$^-$-patients ($p<0.0001$ in all cases).

Circulating microparticles showed a different parental cell origin in LLT$^+$ and LLT$^-$-patients. ▶Figure 1B illustrates the pattern of circulating microparticles according to their cell source marker. Whilst percentages of total circulating platelet-derived (CD41$^+$/CD61$^+$; pMP) (84.8% LLT$^-$ / 81.8% LLT$^+$) microparticles did not significantly change, there were significant changes in the white cell type and vessel wall-derived cMPs between both groups (▶Figure 1B). Endothelial cell-derived cMPs (CD146$^+$/CD31$^+$; eMPs) were significantly lower in LLT$^+$-patients (▶Figure 1B). The pan-leukocyte-derived cMPs (CD45$^+$; LMPs) were higher in LLT$^+$-patients but there were significant differences within the LMPs. Pro-inflammatory cell-derived cMPs (lymphocyte- [CD45$^+$/CD3$^+$, ℓMPs] plus monocyte- [CD14$^+$, mMPs]) were significantly lower in LLT$^+$-patients (▶Figure 1B).

Annexin V-positive circulating microparticles

Total number of microparticles shed from the different parental cells is depicted in ▶Figure 2. Since most of all cMPs (> 86% in average) contained phosphatidylserine in their surface (annexin

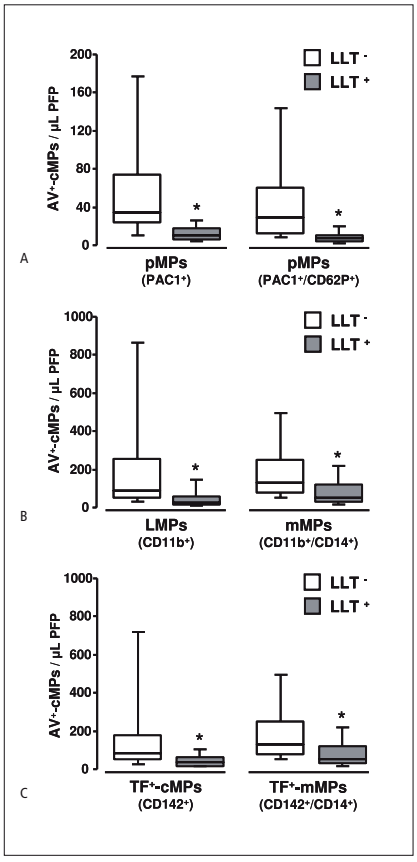


Figure 3: Activation status and tissue factor-bearing cMPs in LLT$^-$ and LLT$^+$ patients. Box and whisker plots of total AV$^+$-cMPs per µl of PFP bearing (A) activated $\alpha_{IIb}\beta_3$-integrin marker (PAC1$^+$) and both PAC1 and P-selectin markers (PAC1$^+$/CD62P$^+$) representing AV$^+$-cMPs from activated platelets, B) M-integrin marker (CD11b$^+$) and both α_M-integrin and monocyte markers (CD11b$^+$/CD14$^+$) corresponding to AV$^+$-cMPs from activated leukocytes (a-LMPs) and monocytes, and C) tissue factor (CD142$^+$; TF$^+$-cMPs) and both monocyte marker and TF (CD14$^+$/CD142$^+$; TF$^+$-mMPs) in non-LLT (n=37) and LLT (n=37) patients. Data are given as a box and whisker plot as described in Figure 1. *$p<0.0001$ vs control group (U-Mann Whitney test).

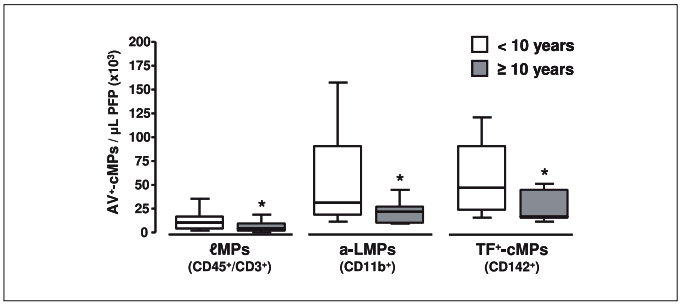


Figure 4: Annexin V-positive levels of cMPs according to time of LLT. Box and whisker plots showing number of AV$^+$-cMPs per µl of PFP corresponding to ℓMPs (CD3$^+$/CD45$^+$), activated-LMPs (CD11b$^+$) and TF$^+$-cMPs (CD142$^+$) related to exposure years to LLT (n=37). Data are given as a box and whisker plot as described in Figure 1. *p<0.05 (U-Mann Whitney test).

V$^+$), we specifically studied this subpopulation. LLT$^+$-patients were found to have significantly lower platelet-, endothelial cell-, leukocyte-, lymphocyte-, and monocyte-derived AV$^+$-cMP subsets than LLT$^-$-group (p<0.0001, p<0.0001, p<0.001, p<0.01 and p<0.0001, respectively) (▶Figure 2). There was a general lowering of activation of vascular and inflammatory cells.

Activation cell markers and tissue factor in circulating microparticles

AV$^+$-cMPs shed by activated platelets and activated leukocytes were significantly reduced in LLT$^+$-patients (▶Figure 3). Specifically, levels of AV$^+$-pMPs carrying the activated $\alpha_{IIb}\beta_3$ integrin marker PAC1$^+$ and both PAC1$^+$ and P-selectin markers together (PAC1$^+$/CD62P$^+$) were significantly lower in the LLT$^+$-patients (p<0.0001 and p<0.0001, respectively) (▶Figure 3A). cMPs derived from activated leukocytes (CD11b$^+$) and monocytes (CD11b$^+$/CD14$^+$) were also significantly lower in AV$^+$-cMPs of LLT$^+$-patients (▶Figure 3B).

Total levels of circulating MPs derived from activated tissue factor-bearing cells (TF; CD142$^+$-cMPs) were significantly lower in LLT$^+$-patients (▶Figure 3C). Thus, higher number of total annexin V-positive circulating (AV$^+$-cMPs) and monocyte-derived (AV$^+$-mMPs) microparticles had TF in LLT$^-$ than in LLT$^+$-patients (p<0.0001 in both cases) (▶Figure 3C). Therefore, platelet-derived MPs carrying $\alpha_{IIb}\beta_3$-integrin and P-selectin (PAC1$^+$/CD62P$^+$), leukocyte-derived MPs carrying MAC-1 (CD11b$^+$) and tissue factor (CD142$^+$)-bearing cMPs of LLT$^+$ group showed three-fold, three-fold and two-fold lower median values, respectively, than the LLT$^-$ group.

When focusing on relative amounts of cMPs bearing activation markers, AV$^+$-MPs carrying markers of platelet activation (PAC1$^+$ and PAC1$^+$/CD62P$^+$) and tissue factor from platelet origin (CD142$^+$/TSP1$^+$) given as percentage of the total AV$^+$-cMPs, were

Table 2: cMP profiling according to statin drugs in LLT$^+$ compared to LLT$^-$ patients. Numbers of AV$^+$-cMPs from total cMPs, platelets (pMPs, CD41$^+$/CD61$^+$), endothelial cells (eMPs, CD146$^+$/CD31$^+$), leukocytes (LMPs, CD45$^+$), lymphocytes (ℓMPs, CD45$^+$/CD3$^+$), monocytes (mMPs, CD14$^+$), activated platelets (PAC1$^+$) and leukocytes (CD11b$^+$) and bearing tissue factor (CD142$^+$) per µl of PFP (platelet-free plasma) in LLT$^-$ (n=37) and LLT$^+$-patients (n=35), in the latter classified according to the type of statin they were taken (simvastatin, atorvastatin or rosuvastatin). Data are given as median (interquartile range). *p<0.05 vs LLT$^-$ (Mann-Whitney). Differences in LLT$^+$ due to statin drugs were not significant (Kruskal-Wallis).

Annexin V$^+$ -cMPs	Marker	LLT$^-$	LLT$^+$		
			Simvastatin	Atorvastatin	Rosuvastatin
Total cMPs	cMPs (AV$^+$)	5238 (1773–10925)	1762 (1162–1877) *	1684 (1098–2082) *	1858 (1432–2062) *
Cell-derived MPs	pMPs (CD41$^+$/CD61$^+$/AV$^+$)	4844 (1682–10196)	1374 (977–1608) *	1458 (920–1806) *	1657 (1120–1926) *
	eMPs (CD146$^+$/CD31$^+$/AV$^+$)	8 (2–34)	0.34 (0.2–2) *	2 (0.25–4) *	2 (0.75–3) *
	LMPs (CD45$^+$/AV$^+$)	338 (164–660)	104 (78–187) *	202 (136–275) *	208 (124–240) *
	ℓMPs (CD45$^+$/CD3$^+$/AV$^+$)	16 (6–43)	5 (4–13)	6 (3–12) *	14 (14–17)
	mMPs (CD14$^+$/AV$^+$)	68 (23–147)	13 (8–31) *	15 (7–28) *	42 (15–62)
MPs carrying activation markers	pMPs (PAC1$^+$/AV$^+$)	34 (24–74)	14 (4–21) *	10 (6–14) *	10 (9–20.5) *
	LMPs (CD11b$^+$/AV$^+$)	90 (56–252)	22 (11–48) *	24 (20–48) *	53 (20–150)
	TF+-MPs (CD142$^+$/AV$^+$)	82 (54–174)	39 (24–68) *	35 (16–51) *	43 (17–94) *

significantly lower in LLT$^+$- compared to LLT$^-$-patients ($p<0.05$; see Suppl. Figure 3, available online at www.thrombosis-online.com). Similarly, AV$^+$-MPs bearing markers of inflammatory cell activation (CD11b$^+$) and tissue factor from monocyte origin (CD142$^+$/CD14$^+$) showed a reduction trend (see Suppl. Figure 3, available online at www.thrombosis-online.com). The percentage of AV$^+$-cMPs from activated inflammatory cells (CD11b$^+$) relative to all LMPs (CD45$^+$) was significantly lower in statin-treated patients than non-treated subjects (16.8 [8.7-36.9] vs 26.7 [13.5-51.3], $p<0.05$, respectively). Contrarily, percentage of total pMPs (CD41$^+$/CD61$^+$) and LMPs (CD45$^+$) did not differ between the statin and non-statin groups (►Figure 1B). No correlation was found between AV$^+$-cMPs numbers (TF$^+$-cMPs or activated cell-cMPs) and haematologic counts (platelet, leukocyte, lymphocyte and monocyte counts), showing that the effect of statins was on cell activation and not in cell numbers.

LLT-specific decrease in cMP levels is time-dependent

The decrease of cMP shedding driven by lipid-lowering treatment was directly associated with the duration of LLT treatment. Specifically, those patients who had received LLT during 10 or more years had significantly lower levels of AV$^+$-ℓMPs (CD45$^+$/CD3$^+$), activated AV$^+$-LMPs (CD11b$^+$) and tissue factor-bearing AV$^+$-cMPs (CD142$^+$) when compared to subjects under LLT for less than ten years ($p<0.05$ in all cases, ►Figure 4). The effects of statins on cMPs were not found different between statins (►Table 2). Therefore, differences due to different statin drugs were not significant (Kruskal-Wallis). The slight use of ezetimibe in LLT$^+$-patients (11%) did not exert any difference either (data not shown).

LLT-specific cMP profile is not determined by LDL-cholesterol levels

There was no correlation between lipid levels and activated cell-cMPs or TF$^+$-cMPs. Further, to identify whether LDL-c plasma levels were promoting MP shedding, the studied groups were classified in two groups using the cut-off in LDL-c levels of 130 mg/dl according to guideline target in primary prevention. A 51% of LLT$^-$ and 43% of LLT$^+$-patients were on-target LDL-c levels (►Figure 5). Significant lower values in cMP levels were detected in LLT$^+$-patients regardless of cholesterol levels. Therefore there is a

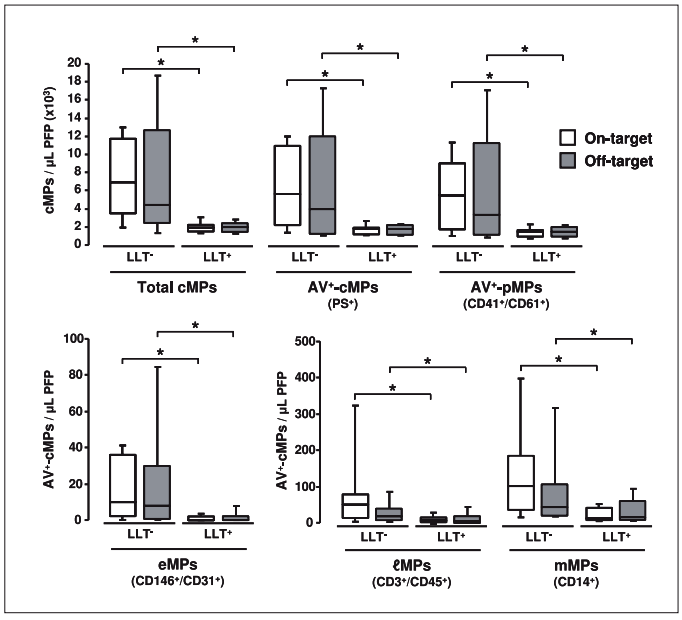


Figure 5: cMP profiling and LDL-cholesterol levels. Box and whisker plots of numbers of total cMPs, total AV$^+$-cMPs and AV$^+$-cMPs from platelets (pMPs, CD41$^+$/CD61$^+$), endothelial cells (eMPs; CD146$^+$/CD31$^+$), lymphocytes (ℓMPs, CD45$^+$/CD3$^+$) and monocytes (mMPs, CD14$^+$) per µl of PFP in LLT$^-$ (n=37) and LLT$^+$(n=37)-patients classified in two groups: on- and off-target LDL-c levels (with clinical guidelines target LDL-cholesterol levels, <130 mg/dl for primary prevention). Data are given as a box and whisker plot as described in Figure 1A. *$p<0.05$ (Mann-Whitney test). Non-significant differences due to LDL-c levels (between on-target and off-target) were found.

clear effect of statins on vascular and inflammatory cells activation independent of plasma cholesterol levels.

cMPs and cardiovascular risk

Since LLT$^-$-subjects are characterised by having almost no CV risk factors, only TF$^+$-cMPs significantly correlated with hs-CRP levels ($p<0.05$, $R^2=0.256$; see Suppl. Figure 2, available online at www.thrombosis-online.com).

On the other hand, levels of cMPs in LLT$^+$-subjects significantly correlated with CV risk scores (▶Figure 6). Specifically, Framingham risk scores (FRSs) for general cardiovascular disease at 10 years calculated using the National Cholesterol Education Program ranged from <5 to 48%. The mean FRSs did not differ among groups based on LLT treatment. However, within the range of FRSs in LLT$^+$-patients, significant differences were found in pMPs expressing activated $\alpha_{IIb}\beta_3$-integrin marker (PAC1$^+$) and tissue factor-bearing cMPs (CD142$^+$) levels between subjects with moderate to high Framingham risk and those with low Framingham risk (▶Figure 6A). The REGICOR (Registre Gironí del Cor) risk was also investigated. There was a significant increased activated pMPs, eMPs, LMPs, ℓMPs, and activated LMPs in LLT$^+$-pa-

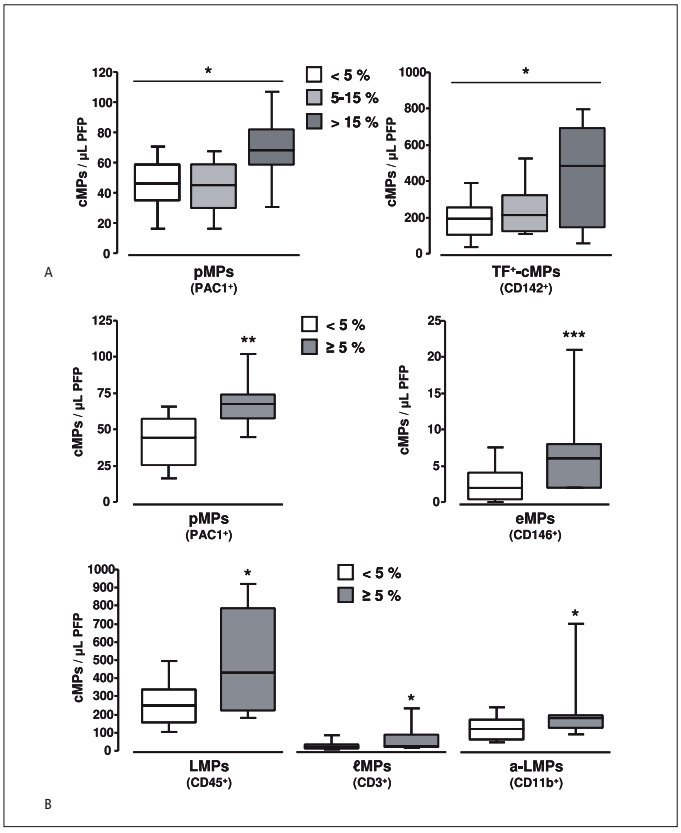


Figure 6: cMP profiling in LLT-subjects according to cardiovascular score risks. Box and whisker plots showing cMPs levels (per µl of PFP) depending on A) Framingham score risk (<5%, 5-15% or >15%) and B) REGICOR score risk (<5% or ≥5%). cMPs (A) exposing platelet activation markers (PAC1$^+$) and tissue factor (CD142$^+$) and (B) corresponding to activated pMPs (PAC1$^+$), eMPs (CD146$^+$), and pan-LMPs (CD45$^+$), ℓMPs (CD3$^+$), and activated LMPs (CD11b$^+$) in patients under LLT (n=37). Data are given as a box and whisker plot as described in Figure 1. *p<0.05, **p<0.005 and ***p<0.01 (U-Mann Whitney test).

tients with Regicor score risk of ≥5% compared to those who had lower score (<5%) (►Figure 6B).

Discussion

High cholesterol plasma levels are a causal factor in the ethiopathogenesis of atherosclerosis (49, 50). Hypercholesterolaemia induces entry of LDL-c in the intima, formation of modified LDL-c species and the triggering of an innate immunity response followed by acquired immunity complications (50). Induction of TF complicates the plaques with triggering of thrombotic events (50, 51). Statins are first-line drug therapy for LDL-c lowering in most patients (1, 2). Statins exert their beneficial effect by reducing cholesterol plasma levels, but other effects beyond lipid reduction have also been considered (3-5). We have recently found that cMPs and platelet-derived MPs enhance thrombosis on atherosclerotic plaques (12). Therefore, the inhibition of cMP release may have significant implications both in plaque identification and inhibition of plaque progression.

Here, we demonstrate that patients treated with LLT with statins have lower MP numbers, especially of platelet, leukocyte and endothelial cell-derived cMPs, than blood from untreated patients with the same plasma lipid levels. 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.

Circulating MPs and, specifically platelet-derived MPs, play an important role in mural thrombosis and also in coagulation (12). Therefore the effects detected may have implications in the protection against atherosclerosis exerted by statins. It has been described that some statins could improve plasma adiponectin levels, a circulating adipokine that suppresses the attachment of monocytes to endothelial cells (EC) and stimulates nitric oxide (NO) production in vascular ECs ameliorating endothelial function (32, 52, 53). These data indicate that the NO-dependent anti-platelet properties by adiponectin could contribute to the decrease of pMPs and to the anti-atherosclerotic effects of statins (32). Also cMPs from T cells and monocytes have been found to induce endothelial dysfunction, activation and subsequent amplification of inflammation (54, 55), therefore contributing to atherosclerosis (50, 56).

Interestingly, low numbers of cell-activation markers, such as $\alpha_{IIb}\beta_3$-integrin (PAC1$^+$), P-selectin (CD62P$^+$), α_M-integrin (Mac-1, CD11b$^+$) and tissue factor (TF$^+$, CD142$^+$) 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. This view is further supported by the statin-induced decrease in relative amounts of cMPs carrying markers of cell activation, especially evident in those of platelet origin.

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 HL. Because patients were treated with four commonly used statins at low dose, we have been able to show that all statins are effective in the modulation of cMP shedding in these asymptomatic hypercholesterolaemic patients. Therefore, the duration of the treatment more than the type of statin seems to influence these effects.

Results of large, well-controlled clinical trials have demonstrated that statins are effective in primary and secondary prevention of cardiovascular disease (1). In primary prevention results of clinical trials have shown less clear beneficial effects but yet reductions in coronary artery disease are evident (WOSCOP [57], AFCAP/TexCAP [58], ASCOT_LLA [59]) and even mortality was reduced in the JUPITER trial (60). However, the benefit on all-cause mortality has not been proved in a recent meta-analysis (61). For most hypercholesterolaemic patients unable to achieve recommended lipid level goals with therapeutic lifestyle changes, statins are considered first option for treatment.

The cardiovascular risk of each individual was calculated using the Framingham Heart Study equations for primary at 10 years as well as REGICOR risk charts (39, 62). Interestingly, cMP levels significantly correlated with Framingham CV risk score. In addition the REGICOR score (used to indentify high CV risk in the Spanish population) correlated significantly with the majority of specific cell-derived microparticles.

What is known about this topic?

- Blood cells and platelets release circulating microparticles (cMPs) with procoagulant activity.
- cMPs are increased in patients with coronary artery disease and with clustering of cardiovascular risk factors.
- Hypercholesterolaemia is a major risk factor for atherosclerosis and is associated with a pro-thrombotic state.
- Statins are hypolipidaemic drugs, which not only decrease plasma cholesterol levels but also exert beneficial effects in cardiovascular disease prevention due to their pleiotropic effects. However, there is very limited information about the effect of lipid-lowering therapy (LLT) with statins on cMPs.

What does this paper add?

- At equal LDL-cholesterol levels, patients treated with statins have less cMP levels and less cMPs carrying markers of parental cell activation (of platelet, lymphocyte, monocyte and endothelial cell origin).
- Beyond cholesterol lowering, statins have direct effects on reducing activated cell membrane shedding of cMPs.
- The effects of statins on cMP shedding are increased with years of treatment.
- Beyond cholesterol lowering the beneficial effects of statins are also due to vascular endothelial, platelet and inflammatory cell protection against activation.

This study has some characteristics and limitations that need to be discussed. First, this is a cross-sectional study from an observational (non-randomised) prospective clinical study and some bias related with the indication of treatment may have affected the results. However, all cases receiving LLT have been treated at least one year before the inclusion in the study with the same LLT. Indeed, LLT^+-patients had reduced their total cholesterol levels from 270 mg/dl (range 231-300 mg/dl) (maximum level that patients achieved before recruitment into SAFEHEART) to 210 mg/dl (range 192-240 mg/dl) by statin treatment. Our control group was matched to patients with similar cholesterol plasma levels but never-treated with statins. Interestingly, LLT^+-patients with higher exposure to lipids have fewer MPs, giving information of the protective role of statins in hypercholesterolaemic patients.

In conclusion, statins significantly reduce the shedding of blood cells and vascular cell MPs. The specific reduction of cMPs derived from activated parental cells demonstrates 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 (21). 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 cardiovascular disease by reducing vascular and inflammatory cell activation as detected by a reduced cMP shedding of the cell membranes. Further studies measuring changes in microparticle number and composition before and after LLT will help to complete our understanding of statin effects on the vessel wall and the role of microparticles as biomarkers of vascular disease.

Acknowledgements

The authors thank Dr. R Nieuwland and Chi M. Hau for the exceptional advice and expertise, Dr. J. Crespo for his help in flow cytometry, Marta Guindo for the technical support, and the Spanish Familial Hypercholesterolemia Foundation for the recruitment of participants. Authors are indebt to the FH patients and relatives for their valuable contribution and willingness to participate.

Conflicts of interest

None declared.

Abbreviations

AV, annexin V; BMI, body mass index; cMPs, circulating microparticles; CV, coefficient of variation; CVD, cardiovascular disease; DNA, deoxyribonucleic acid; EDTA, ethylenediaminetetraacetic acid; eMPs, endothelial-derived microparticles; FACS, fluorescence-activated cell sorting; FSC, forward scatter characteristics; GGPP, geranylgeranyl pyrophosphate; HDL, high-density lipoprotein; HL, hyperlipidaemia; HMG-CoA, 3-hydroxy-3-methylglutryl coenzyme A; Hs-CRP, high sensitive C-reactive protein; LDL-c, low-density-lipoprotein cholesterol; LLT, lipid-lowering therapy; ℓMPs, lymphocyte-derived microparticles; LMPs, leukocyte-derived microparticles; Lp(a), lipoprotein (a); mAb, monoclonal antibody; MPs, microparticles; mMPs, monocyte-derived microparticles; PS, phosphatidylserine; PFP, platelet-free plasma; pMPs, platelet-derived microparticles; SSC, side scatter characteristics; TC, total cholesterol; TF, tissue factor.

References

1. Baigent C, Keech A, Kearney PM, et al. Efficacy and safety of cholesterol-lowering treatment: prospective meta-analysis of data from 90,056 participants in 14 randomised trials of statins. Lancet 2005; 366: 1267-1278.
2. Law MR, Wald NJ, Rudnicka AR. Quantifying effect of statins on low density lipoprotein cholesterol, ischaemic heart disease, and stroke: systematic review and meta-analysis. Br Med J 2003; 326: 1423.
3. Landmesser U, Bahlmann F, Mueller M, et al. Simvastatin versus ezetimibe: pleiotropic and lipid-lowering effects on endothelial function in humans. Circulation 2005; 111: 2356-2363.
4. Sposito AC, Chapman MJ. Statin therapy in acute coronary syndromes: mechanistic insight into clinical benefit. Arterioscler Thromb Vasc Biol 2002; 22: 1524-1534.
5. Undas A, Brummel-Ziedins KE, Mann KG. Statins and blood coagulation. Arterioscler Thromb Vasc Biol 2005; 25: 287-294.
6. Kozai T, Eto M, Yang Z, et al. Statins prevent pulsatile stretch-induced proliferation of human saphenous vein smooth muscle cells via inhibition of Rho/Rho-kinase pathway. Cardiovasc Res 2005; 68: 475-482.
7. Takai Y, Sasaki T, Matozaki T. Small GTP-binding proteins. Physiol Rev 2001; 81: 153-208.
8. Montoro-Garcia S, Shantsila E, Marin F, et al. Circulating microparticles: new insights into the biochemical basis of microparticle release and activity. Basic Res Cardiol 2011; 106: 911-923.
9. Jy W, Horstman LL, Jimenez JJ, et al. Measuring circulating cell-derived microparticles. J Thromb Haemost 2004; 2: 1842-1851.
10. Boulanger CM, Dignat-George F. Microparticles: an introduction. Arterioscler Thromb Vasc Biol 2011; 31: 2-3.
11. Boulanger CM. Microparticles, vascular function and hypertension. Curr Opin Nephrol Hypertens 2010; 19: 177-180.
12. Suades R, Padro T, Vilahur G, et al. Circulating and platelet-derived microparticles in human blood enhance thrombosis on atherosclerotic plaques. Thromb Haemost 2012; 108: 1208-1219.
13. Berckmans RJ, Nieuwland R, Boing AN, et al. Cell-derived microparticles circulate in healthy humans and support low grade thrombin generation. Thromb Haemost 2001; 85: 639-646.
14. Amabile N, Guerin AP, Tedgui A, et al. Predictive value of circulating endothelial microparticles for cardiovascular mortality in end-stage renal failure: a pilot study. Nephrol Dial Transplant 2011; 27: 1873-1880.
15. Namba M, Tanaka A, Shimada K, et al. Circulating platelet-derived microparticles are associated with atherothrombotic events: a marker for vulnerable blood. Arterioscler Thromb Vasc Biol 2007; 27: 255-256.
16. Sinning JM, Losch J, Walenta K, et al. Circulating CD31+/Annexin V+ microparticles correlate with cardiovascular outcomes. Eur Heart J 2010; 32: 2034-2041.
17. Diamant M, Nieuwland R, Pablo RF, et al. Elevated numbers of tissue-factor exposing microparticles correlate with components of the metabolic syndrome in uncomplicated type 2 diabetes mellitus. Circulation 2002; 106: 2442-2447.
18. Koga H, Sugiyama S, Kugiyama K, et al. Elevated levels of VE-cadherin-positive endothelial microparticles in patients with type 2 diabetes mellitus and coronary artery disease. J Am Coll Cardiol 2005; 45: 1622-1630.
19. Hoyer FF, Giesen MK, Nunes Franca C, et al. Monocytic microparticles promote atherogenesis by modulating inflammatory cells in mice. J Cell Mol Med 2012; 16: 2777-2788.
20. Shantsila E, Kamphuisen PW, Lip GY. Circulating microparticles in cardiovascular disease: implications for atherogenesis and atherothrombosis. J Thromb Haemost 2010; 8: 2358-2368.

21. Montoro-Garcia S, Orenes-Pinero E, Marin F, et al. Pharmacological modulation of microparticle release: new strategies for the management of atherothrombotic vascular disorders. Curr Pharm Des 2012; 18: 840-849.
22. Diamant M, Tushuizen ME, Abid-Hussein MN, et al. Simvastatin-induced endothelial cell detachment and microparticle release are prenylation dependent. Thromb Haemost 2008; 100: 489-497.
23. Nomura S. Statin and endothelial cell-derived microparticles. Thromb Haemost 2008; 100: 377-378.
24. Tramontano AF, O'Leary J, Black AD, et al. Statin decreases endothelial microparticle release from human coronary artery endothelial cells: implication for the Rho-kinase pathway. Biochem Biophys Res Commun 2004; 320: 34-38.
25. Mobarrez F, Egberg N, Antovic J, et al. Release of endothelial microparticles in vivo during atorvastatin treatment; a randomized double-blind placebo-controlled study. Thromb Res 2012; 129: 95-97.
26. Montoro-Garcia S, Lip GY, Shantsila E. Atorvastatin and its collateral effects on microparticles. Thromb Haemost 2011; 106: 185-186.
27. Mobarrez F, He S, Broijersen A, et al. Atorvastatin reduces thrombin generation and expression of tissue factor, P-selectin and GPIIIa on platelet-derived microparticles in patients with peripheral arterial occlusive disease. Thromb Haemost 2011; 106: 344-352.
28. Tehrani S, Mobarrez F, Antovic A, et al. Atorvastatin has antithrombotic effects in patients with type 1 diabetes and dyslipidemia. Thromb Res 2010; 126: e225-231.
29. Nomura S, Shouzu A, Omoto S, et al. Losartan and simvastatin inhibit platelet activation in hypertensive patients. J Thromb Thrombolysis 2004; 18: 177-185.
30. Nomura S, Shouzu A, Omoto S, et al. Effects of losartan and simvastatin on monocyte-derived microparticles in hypertensive patients with and without type 2 diabetes mellitus. Clin Appl Thromb Hemost 2004; 10: 133-141.
31. Sommeijer DW, Joop K, Leyte A, et al. Pravastatin reduces fibrinogen receptor gpIIIa on platelet-derived microparticles in patients with type 2 diabetes. J Thromb Haemost 2005; 3: 1168-1171.
32. Nomura S, Inami N, Shouzu A, et al. The effects of pitavastatin, eicosapentaenoic acid and combined therapy on platelet-derived microparticles and adiponectin in hyperlipidemic, diabetic patients. Platelets 2009; 20: 16-22.
33. Owens AP, 3rd, Passam FH, Antoniak S, et al. Monocyte tissue factor-dependent activation of coagulation in hypercholesterolemic mice and monkeys is inhibited by simvastatin. J Clin Invest 2012; 122: 558-568.
34. Mata N, Alonso R, Badimon L, et al. Clinical characteristics and evaluation of LDL-cholesterol treatment of the Spanish Familial Hypercholesterolemia Longitudinal Cohort Study (SAFEHEART). Lipids Health Dis 2011; 10: 94.
35. Gehi AK, Ali S, Na B, et al. Self-reported medication adherence and cardiovascular events in patients with stable coronary heart disease: the heart and soul study. Arch Intern Med 2007; 167: 1798-1803.
36. Pijlman AH, Huijgen R, Verhagen SN, et al. Evaluation of cholesterol lowering treatment of patients with familial hypercholesterolemia: a large cross-sectional study in The Netherlands. Atherosclerosis 2010; 209: 189-194.
37. Graham I, Atar D, Borch-Johnsen K, et al. European guidelines on cardiovascular disease prevention in clinical practice: executive summary: Fourth Joint Task Force of the European Society of Cardiology and Other Societies on Cardiovascular Disease Prevention in Clinical Practice (Constituted by representatives of nine societies and by invited experts). Eur Heart J 2007; 28: 2375-2414.
38. Wierzbicki AS, Humphries SE, Minhas R. Familial hypercholesterolaemia: summary of NICE guidance. Br Med J 2008; 337: a1095.
39. Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report. Circulation 2002; 106: 3143-3421.
40. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clin Chem 1972; 18: 499-502.
41. Mozas P, Castillo S, Tejedor D, et al. Molecular characterization of familial hypercholesterolemia in Spain: identification of 39 novel and 77 recurrent mutations in LDLR. Hum Mutat 2004; 24: 187.
42. Nieuwland R, Berckmans RJ, McGregor S, et al. Cellular origin and procoagulant properties of microparticles in meningococcal sepsis. Blood 2000; 95: 930-935.
43. Rank A, Nieuwland R, Roesner S, et al. Climacteric lowers plasma levels of platelet-derived microparticles: a pilot study in pre- versus postmenopausal women. Acta Haematol 2012; 128: 53-59.
44. Biro E, Akkerman JW, Hoek FJ, et al. The phospholipid composition and cholesterol content of platelet-derived microparticles: a comparison with platelet membrane fractions. J Thromb Haemost 2005; 3: 2754-2763.
45. Lacroix R, Robert S, Poncelet P, et al. Standardization of platelet-derived microparticle enumeration by flow cytometry with calibrated beads: results of the International Society on Thrombosis and Haemostasis SSC Collaborative workshop. J Thromb Haemost 2010; 8: 2571-2574.
46. Nieuwland R, Berckmans RJ, Rotteveel-Eijkman RC, et al. Cell-derived microparticles generated in patients during cardiopulmonary bypass are highly procoagulant. Circulation 1997; 96: 3534-3541.
47. Williams JC, Mackman N. MPs or ICs? Blood 2011; 117: 1101-1102.
48. van der Pol E, van Gemert MJ, Sturk A, et al. Single vs. swarm detection of microparticles and exosomes by flow cytometry. J Thromb Haemost 2012; 10: 919-930.
49. Libby P, Ridker PM, Hansson GK. Progress and challenges in translating the biology of atherosclerosis. Nature 2011; 473: 317-325.
50. Badimon L, Storey RF, Vilahur G. Update on lipids, inflammation and atherothrombosis. Thromb Haemost 2011; 105 (Suppl 1): S34-42.
51. Badimon L, Vilahur G, Padro T. Lipoproteins, platelets and atherothrombosis. Rev Esp Cardiol 2009; 62: 1161-1178.
52. Nomura S, Omoto S, Yokoi T, et al. Effects of miglitol in platelet-derived microparticle, adiponectin, and selectin level in patients with type 2 diabetes mellitus. Int J Gen Med 2011; 4: 539-545.
53. Nomura S, Shouzu A, Omoto S, et al. Effects of eicosapentaenoic acid on endothelial cell-derived microparticles, angiopoietins and adiponectin in patients with type 2 diabetes. J Atheroscler Thromb 2009; 16: 83-90.
54. Martin S, Tesse A, Hugel B, et al. Shed membrane particles from T lymphocytes impair endothelial function and regulate endothelial protein expression. Circulation 2004; 109: 1653-1659.
55. Wang JG, Williams JC, Davis BK, et al. Monocytic microparticles activate endothelial cells in an IL-1beta-dependent manner. Blood 2011; 118: 2366-2374.
56. Topol EJ. Intensive statin therapy--a sea change in cardiovascular prevention. N Engl J Med 2004; 350: 1562-1564.
57. Shepherd J, Cobbe SM, Ford I, et al. Prevention of coronary heart disease with pravastatin in men with hypercholesterolemia. West of Scotland Coronary Prevention Study Group. N Engl J Med 1995; 333: 1301-1307.
58. Downs JR, Clearfield M, Weis S, et al. Primary prevention of acute coronary events with lovastatin in men and women with average cholesterol levels: results of AFCAPS/TexCAPS. Air Force/Texas Coronary Atherosclerosis Prevention Study. J Am Med Assoc 1998; 279: 1615-1622.
59. Sever PS, Dahlof B, Poulter NR, et al. Prevention of coronary and stroke events with atorvastatin in hypertensive patients who have average or lower-than-average cholesterol concentrations, in the Anglo-Scandinavian Cardiac Outcomes Trial--Lipid Lowering Arm (ASCOT-LLA): a multicentre randomised controlled trial. Lancet 2003; 361: 1149-1158.
60. Ridker PM, Danielson E, Fonseca FA, et al. Rosuvastatin to prevent vascular events in men and women with elevated C-reactive protein. N Engl J Med 2008; 359: 2195-2207.
61. Ray KK, Seshasai SR, Erqou S, et al. Statins and all-cause mortality in high-risk primary prevention: a meta-analysis of 11 randomized controlled trials involving 65,229 participants. Arch Intern Med 2010; 170: 1024-1031.
62. Marrugat J, Solanas P, D'Agostino R, et al. Coronary risk estimation in Spain using a calibrated Framingham function. Rev Esp Cardiol 2003; 56: 253-261.

SUPLEMENTARY MATERIAL

Supplementary Table 1

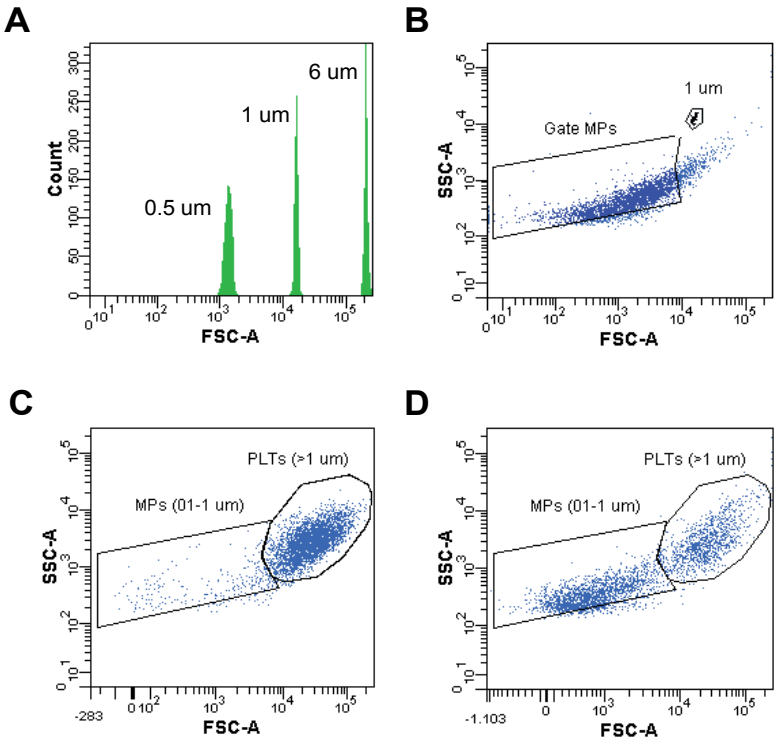
CELL SURFACE MOLECULES FOR CIRCULATING MICROPARTICLE IDENTIFICATION AND CHARACTERIZATION

Marker	Alternative name	Expression	Conjugation	Clone	Company
Annexin V	PS-binding protein	Widely expressed	BD HorizonV450	-	BD Biosciences
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
CD41	α_{IIb}-integrin	Platelets	FITC	SZ22	Beckman Coulter
CD45	LCA	Leukocytes	PE	Immu-19.2	Beckman Coulter
CD61	β_3-integrin	Platelets	PE	VI-PL2	BD Pharmingen
CD62P	P-selectin	Activated platelets	PE	AK-4	BD Pharmingen
CD142	Tissue factor	Widely expressed	FITC	VD8	Am. Diagnostica
CD146	MUC18	Endothelial cells	FITC	P1H12	BD Pharmingen
PAC1	$\alpha_{IIb}\beta_3$-integrin	Activated platelets	FITC	PAC1	BD Biosciences
TSP1	THBS-1	Platelets, megakaryocytes	PE	P10	Beckman Coulter
IgG1γ	-	-	FITC / PE	X40	BD Biosciences
IgG1κ	-	-	FITC / PE	MPOC21	BD Pharmingen

Notes: 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; THBS1, thrombospondin 1.
Source: Own elaboration.

Supplementary Figure 1

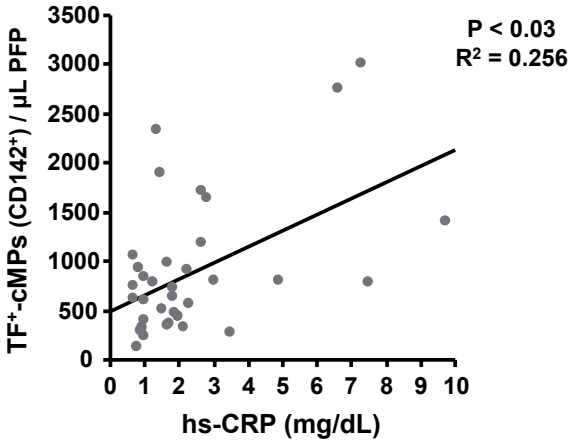
CHARACTERIZATION OF CIRCULATING MICROPARTICLES BY FLOW CYTOMETRY



Notes: The cell-microparticle gate was established based on light scattering properties and size, using calibration microspheres (A) and defining cMPs as events both <1 µm (B). The lower detection limit was placed as a threshold above the electronic noise of our flow cytometer. Further, in order to corroborate and validate established microparticle gate we determined the FSC/SSC characteristics of platelets and microparticles in suspension (C-D). Image C shows unstimulated platelets. Thereafter, platelets were stimulated with calcium ionophore A23187 (5 minute incubation, at 37ºC, 2.5µM) and microparticles generated were located within the established gate (D).
Source: Own elaboration.

Supplementary Figure 2

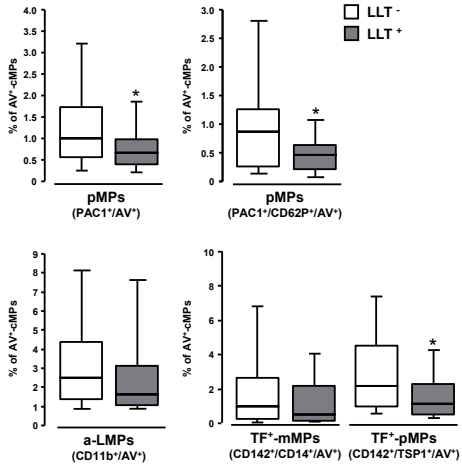
CMPS AND HS-CRP AS MARKER OF CARDIOVASCULAR RISK IN LLT⁻ PATIENTS



Notes: Correlation between tissue factor-bearing cMP levels (TF^+-cMPs, $CD142^+$) and high sensitive CRP in LLT^--subjects (n=37). *$p<0.05$ (Spearman correlation).
Source: Own elaboration.

Supplementary Figure 3

CMPS CARRYING MARKERS OF CELL ACTIVATION AND TISSUE FACTOR IN LLT^- AND LLT^+-PATIENTS



Notes: Box and whisker plots of relative amount of AV^+-cMPs bearing activated $\alpha_{IIb}\beta_3$-integrin ($PAC1^+$) and activated $\alpha_{IIb}\beta_3$-integrin / P-selectin markers ($PAC1^+/CD62P^+$) derived from activated platelets (pMPs) and α_M-integrin marker ($CD11b^+$) from activated leukocytes (a-LMPs), and tissue factor ($CD142^+$) together with a monocyte marker ($CD14^+$, TF^+-mMPs) and platelet marker ($TSP1^+$, TF^+-pMPs) in non-LLT (n=37) and LLT (n=37) patients. Data are given as percentage of each specific MP subpopulation related to total AV^+-cMPs, in a box and whisker plot as described in Figure 1. *$p<0.05$ vs. control group (U-Mann Whitney test).
Source: Own elaboration.

ARTICLE 3

Circulating $CD45^+/CD3^+$ lymphocyte-derived microparticles map lipid-rich atherosclerotic plaques in familial hypercholesterolemia patients

Rosa Suades, Teresa Padró, Rodrigo Alonso, José López-Miranda, Pedro Mata, Lina Badimon

Published – Thromb Haemost 2014; 111(1): 111-121.

OBJECTIVE

The aim of this study was to determine the abundance and pehnotype of circulating microparticles in stable statin-treated heterozigous familial hypercholesterolemia patients with life-long vascular exposure to high cholesterol levels, and whether circulating microparticle phenotype associate to the atherosclerotic lesion type in FH patients.

HIGHLIGHTS

- FH patients have higher number of overall cMPs and of those derived from endothelial cells, monocytes and lymphocytes than adult-onset non-FH hypercholesterolemic patients.
- Level of annexin V^+-total, pan-leukocyte and lymphocyte-derived microparticles are significantly associated to plaque burden in FH. Circulating $CD45^+/CD3^+$-lymphocyte microparticles are able to discriminate asymptomatic sublinical lipid-rich atherosclerotic plaques in FH patients.

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): 111-121.

Circulating CD45+/CD3+ lymphocyte-derived microparticles map lipid-rich atherosclerotic plaques in familial hypercholesterolaemia patients

Rosa Suades[1]; Teresa Padró[1]; Rodrigo Alonso[2]; José López-Miranda[3]; Pedro Mata[2]; Lina Badimon[1,4]

[1]Cardiovascular Research Center (CSIC-ICCC), IIB-Sant Pau, Barcelona, Spain; [2]Foundation Jimenez Diaz, Madrid, Spain; [3]Unidad de Lípidos y Arteriosclerosis, IMIBIC, Hospital Universitario Reina Sofia, Universidad de Córdoba, and CIBEROBN Fisiopatología Obesidad y Nutrición, Instituto de Salud Carlos III, Spain; [4]Cardiovascular Research Chair, UAB, Barcelona, Spain

Summary

Circulating microparticles (cMPs) seem to play important roles in vascular function. Beyond markers of activated cells, cMPs may have potential paracrine functions and influence atherosclerosis. Here, our objective was to characterise a) the abundance and phenotype of cMPs in stable statin-treated heterozygous familial hypercholesterolaemia (FH) patients exposed to life-long hypercholesterolaemia and b) the principal phenotype associated to lipid-rich atherosclerotic plaques in hFH-patients with significant atherosclerotic plaque burden. An age/gender/treatment-matched group of adult-onset non-FH hypercholesterolaemic patients (n=37/group) was comparatively analysed. cMPs were characterised by flow cytometry using annexin-V and cell surface-specific antibodies. Our study shows that LLT-FH patients had higher overall cMP-numbers ($p<0.005$) than LLT-non-FH patients. Endothelial cell-shed cMPs were also significantly higher in FH ($p<0.0005$). Within the leukocyte-derived cMP-subpopulations, FH-patients had significantly higher lymphocyte- and monocyte-derived cMP-numbers as well as cMPs carrying leukocyte-activation markers. Normalisation of cMPs by LDL levels did not affect cMP number or phenotype, indicating that the proinflammatory effect was derived from chronic vascular damage. Levels of AV^+-total, $CD45^+$-pan-leukocyte and $CD45^+/CD3^+$-lymphocyte-derived cMPs were significantly higher in FH-patients with subclinical lipid-rich atherosclerotic plaques than fibrous plaques. Levels of $CD45^+/CD3^+$-lymphocyte-MPs above 20,000/ml could differentiate between FH-patients with lipidic or non-lipidic plaques (area under the ROC curve of 0.803, 95%CI: 0.641–0.965, $p=0.008$). In summary, in this snapshot cross-sectional study cMP concentration and phenotype in FH differed markedly from non-FH hypercholesterolaemia. Patients with life-long high LDL exposure have higher endothelial activation and higher proinflammatory profile, even under current state-of-the-art LLT. cMPs carrying lymphocyte-epitopes appear as markers of lipid-rich atherosclerotic plaques in FH.

Keywords

Atherosclerosis, circulating microparticles, endothelial dysfunction, hypercholesterolaemia, inflammation

Correspondence to:
Prof. Lina Badimon
Cardiovascular Research Center,
c/Sant Antoni Mª Claret 167
08025 Barcelona, Spain
Tel.: +34 935565880, Fax: +34 935565559
E-mail: lbadimon@csic-iccc.org

Financial support:
This work was supported by the CNIC Foundation [CNIC-082008 to LB and PM]; ISCIII [TERCEL to LB]; SAFE-T project [Grant Agreement N° 115003, IMI-JU, to LB]; Spanish Ministry of Science [PNS SAF2010–16549 to LB]; Spanish Ministry of Health [FIS PI1001115 to TP]; and FIC-Fundación Jesús Serra. RS is a recipient of a predoctoral fellowship from Spanish Health Ministry (PFIS-FI09/00092, ISCIII).

Received: July 25, 2013
Accepted after minor revision: August 30, 2013
Prepublished online: October 2, 2013
doi:10.1160/TH13-07-0612
Thromb Haemost 2014; 111: 111–121

Introduction

Most cells in the circulatory system vesiculate and release membrane-shed circulating microparticles (cMPs) to the extracellular space in response to cell activation or apoptosis (1). cMPs are submicron vesicles (between 0.1-1 μm in size) that contain phosphatidylserine and distinct surface proteins depending on their parental cells (2). cMPs are found in the plasma of healthy subjects (3), but increase considerably in patients with diverse cardiovascular (CV) risk factors (4-6) and pathologies (7-11). However, whether individual cMP phenotypes may be markers of subclinical atherosclerotic lesion types remains unknown.

High plasma cholesterol has been found associated to high levels of leuko-endothelial-derived ($CD31^+/CD42^-$)-cMPs (12) in newly-diagnosed untreated hypercholesterolaemic subjects. Interestingly, pan-leukocyte-derived cMPs ($CD11a^+$) were found to correlate with number of atherosclerotic plaques in subjects with evidence of subclinical atherosclerosis by using microplate affinity capture assay and ultrasound (13). Recently, increased leukocyte-derived cMPs were detected in unstable plaques of patients with high-grade carotid stenosis (14). However, whether the proinflammatory state defined by microparticle shedding is associated with aortic atherosclerotic plaque burden and discriminate the type of atherosclerotic lesion is unknown.

Atherosclerosis is chronic inflammatory disease that is caused by high low-density lipoprotein (LDL) plasma levels. Lipid-rich plaques characterised by a thin fibrous cap and the presence of inflammatory cells are associated with the highest clinical risk because they are prone to rupture. A model to study cMPs and atherosclerosis is found in patients with familial hypercholesterolaemia (FH) who because of their life-long exposure to high LDL levels develop premature atherosclerosis. Familial hypercholesterolaemia is the most common monogenic hereditary disorder that affects 1 in 400-500 people in the general population (15). It is caused by mutations in the LDL receptor gene, leading to life-time exposure to increased LDL levels in plasma, early development of atherosclerosis and premature clinical cardiovascular disease (16). Life expectancy is shortened and fatal coronary events (sudden death and myocardial infarction) are the principal causes of death (17, 18). Despite the use of available lipid-lowering treatment (LLT) regimes and high dose statins, most of these patients do not achieve therapeutic goals and have a high risk for the development of coronary artery disease (CAD) (19). Indeed, using magnetic resonance imaging (MRI) we have recently demonstrated a significantly higher atherosclerotic burden in the aorta of asymptomatic middle-aged FH patients with long-term LLT (20). This microparticle study has been performed in blood collected from the FH-patients at the time of the MRI-analysis.

Despite lesion formation may be driven primarily by hypercholesterolaemia, other local and systemic factors may exert significant influences as to condition the risk of rupture. In the present study, we sought to determine whether treated hFH patients with a life-long vascular exposure to high cholesterol levels have the same abundance of cMPs and similar markers of vascular cell activation as treated adult-onset non-FH hypercholesterolaemic patients, and whether cMP phenotype may be able to discriminate the atherosclerotic lesion type of the FH patients.

Methods

Detailed methods are provided in the Supplementary Material online at www.thrombosis-online.com.

Table 1: Clinical characteristics of FH and non-FH LLT-treated patients.

	FH patients (n=37)	Non-FH patients (n=37)	P-value
N (n)	37	37	P = 0.99
Male /Female (n)	19/18	14/23	P = 0.35
Age (years, mean ± SE)	47.0 (41.0–54.0)	53.0 (38.0–62.0)	P = 0.51
Body mass index (kg/m^2)	25.8 (22.9–28.1)	27.0 (22.8–30.7)	P = 0.21
Risk factor for CAD			
Hyperlipidaemia (n, %)	37 (100%)	37 (100%)	P = 0.99
Diabetes mellitus (n, %)	0 (0%)	1 (2.7%)	P = 0.31
Systemic hypertension (n, %)	4 (10.8%)	7 (18.9%)	P = 0.33
Total cholesterol (mg/dl)	223.0 (185.8–257.0)	210.0 (192.0–240.0)	P = 0.48
Triglyceride (mg/dl)	82.0 (64.8–141.0)	82.0 (64.0–120.0)	P = 0.73
LDL-cholesterol (mg/dl)	145.0 (124.3–175.0)	134.2 (109.0–159.6)	P = 0.21
HDL-cholesterol (mg/dl)	49.0 (40.3–59.0)	55.0 (48.0–63.0)	P = 0.03
High-sensitive CRP (mg/dl)	1.5 (0.9–3.9)	1.0 (0.7–2.4)	P = 0.06
Lp(a) (mg/dl)	32.5 (14.8–55.2)	21 (10.9–48.5)	P = 0.33
Fasting plasma glucose (mg/dl)	85.5 (78.5–94.8)	86.0 (78.0–91.0)	P = 0.91
Gene mutation[a]	(21/17/1)	-	-
Lipid-lowering therapy (LLT)			
Statins	37 (100%)	37 (100%)	P = 0.99
• Atorvastatin	25 (67.6%)	18 (48.6%)	P = 0.10
• Fluvastatin	0 (0%)	2 (5.4%)	P = 0.15
• Rosuvastatin	1 (2.7%)	7 (18.9%)	P = 0.03
• Simvastatin	11 (29.7%)	10 (27%)	P = 0.80
Ezetimibe	23 (62.2%)	4 (10.8%)	P < 0.01
Time of LLT	12.1 ± 1.1	7.5 ± 1.0	P = 0.02

Values are median (interquartile range) except when indicated. [a]Gene mutation (null / defective / unknown). LDL, low-density lipoprotein; HDL, high-density lipoprotein.

Clinical study population

The present study (nested case-control) included 37 patients with clinical and genetic diagnosis of heterozygous FH and thus lifelong exposure to high LDL plasma levels and 37 non-FH patients with adult secondary hypercholesterolaemia, matched by age, gender, demographics, and LLT-treatment (►Table 1) from the SAFEHEART cohort (21). FH cases had been characterised by MRI-imaging (20) as having subclinical aortic atherosclerotic lesions. All patients were receiving lipid-lowering treatment according to clinical guidelines (22, 23). Only 11% of FH patients and 46% of non-FH patients achieved therapeutic LDL targets according to guidelines. Maximum statin dose was defined as previously described: simvastatin 40 mg/day, fluvastatin 80 mg/day, atorvastatin 80 mg/day, rosuvastatin 20-40 mg/day (24). Sociodemographic data, lifestyle, medical and therapeutic data, current LLT and classical risk factors were obtained from all subjects. 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, as previously described (25). Patients were also classified depending on the known residual activity of the LDL-r as null or defective mutations (►Table 1). No patient had past history of cancer, inflammatory disorders, and pregnancy because these conditions are known to independently affect cMP number. The results of the study are presented in accordance with STROBE guidelines. The study was approved by the local ethics committee and was conducted according to the Declaration of Helsinki. Written informed consent was obtained from all participants prior to the study.

Blood collection and sampling for analysis

Venous blood was withdrawn from cubital vein without tourniquet using a 20-gauge needle after 10-14 hours (h) of fasting into serum, EDTA and 3.8% sodium citrate tubes, for biochemical, genotyping and microparticle analysis, respectively. Samples were processed for assay within first 2 h. For lipid profile, total cholesterol, triglycerides, and HDL-c were measured by standardised enzymatic methods; serum LDL-c concentration was calculated using the Friedewäld formula (26); and lipoprotein (a) levels were measured using a turbidimetric method. Genetic diagnosis of FH was made using a DNA-microarray (LIPOCHIP) (27). For flow cytometric analysis of cMPs, blood cells were removed by low-speed centrifugation (1,258 × g, 20 minutes [min]) at room temperature (RT) (28, 29). Platelet-poor plasma (PPP) was carefully aspirated. A second centrifugation step was made to ensure the complete removal of cells and obtain the platelet-free plasma (PFP). All samples were processed identically and within 60 min after extraction. Samples were tested with a cell counter for the absence of residual cells after centrifugation. PFP aliquots were snap-frozen in liquid nitrogen and stored, for an identical time interval, at -80ºC until flow cytometric studies (1, 30).

Atherosclerotic plaque characterisation

Aortic MRI has been used in the last decade to evaluate atherosclerosis burden in high-risk patients including FH patients (31, 32). Aortic atherosclerotic plaque MRI evaluation and morphometric analysis were performed in the FH patients of this study at the level of the descending thoracic aorta by measuring mean arterial wall area (MAWA; mm^2) and plaque composition: lipidic, fibrous or mixed atherosclerotic plaques based on lipid or fibrocellular plaque component and also calcium content (presence or absence), as fully described (20). Inter-observer variability was low and the intra-class correlation coefficient was of 0.735 (95% confidence interval [CI]: 0.591-0.833).

Circulating microparticle isolation

The cMP-fraction was isolated from PFP frozen samples (33) by a two-step high-speed centrifugation (28, 29). Briefly, PFP aliquots were thawed on melting ice for 1 h and centrifuged (20,000 × g, 30 min) to pellet cMPs. The supernatants were discarded and the cMP-enriched pellet washed once with citrate-phosphate buffered saline solution (citrate-PBS) before a second equal centrifugation step was made. Finally, the remaining cMP-pellets were resuspended in citrate-PBS.

Flow cytometric analysis of circulating microparticles

Triple-label flow cytometric analysis was performed as described by Nieuwland et al (28, 29, 34). Briefly, washed cMP suspensions diluted in PBS buffer containing $CaCl_2$ were incubated (20 min, RT, dark) 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 (Suppl. Table 1, available online at www.thrombosis-online.com). Samples were diluted with $CaCl_2$-PBS buffer before being immediately analysed on a FACSCantoII™ flow cytometer. Acquisition was performed for 1 min 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. Granulocyte's percentage was inferred by subtracting agranulocytes (lymphocytes plus monocytes) from total leukocytes instead of labelling with specific mAb. Gate limits were established following two criteria: 1) calibration using a Flow-Check Size Range Calibration Kit (Polysciences) (35) and 2) using an *in vitro* platelet-derived microparticle population as positive control (Suppl. Fig. 1A-D) (36). 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 cor-

rected for autofluorescence using fluorescence signals obtained with microparticles in a calcium-free buffer.

Data were analysed with FACSDiva™ software (BD). The concentration (number of cMPs per µl of plasma) was determined according to Nieuwland's procedure (29, 34), 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

Sample size was selected according to (1) available patients with aortic-MRI detection, (2) average sample sizes of published flow cytometry studies on cMPs and (3) to provide sufficient statistical power for group comparisons. All data are presented as medians (interquartile range) except when indicated. An initial descriptive analysis was carried out using number of cases and percentages for qualitative variables and median and interquartile range for quantitative variables. Frequencies of qualitative variables (risk factors and medications) were compared between two groups by using the Chi-square analysis. Median values of quantitative variables were compared with non-parametric tests. The statistical significances between patient and control groups or comparison of two groups were determined with U-Mann Whitney and multiple comparisons by Kruskal Wallis, and when significant, Bonferroni post-hoc analysis was used to assess intergroup differences. To evaluate the prognostic value or information 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. A cut-off level of MPs was determined with the shortest distance from upper left corner of the ROC curve (minimizing $[(1\text{-sensitivity})^2 + (1\text{-specificity})^2]$. StatView software (Abacus Concepts) and SPSS Statistics Version 21.0.0 (SPSS, Chicago, IL, USA) were used for statistical analysis, and a p-value <0.05 was considered statistically significant.

Results

Clinical characteristics of the study population

The demographic, biochemical, and clinical data of the two groups of patients are shown in ▶Table 1. There were no differences in gender, mean age, BMI, type-2 diabetes mellitus, hypertension, current tobacco consumption, total cholesterol, LDL-cholesterol, non-high-density lipoprotein (non-HDL) cholesterol, triglycerides and Lp(a) levels, between the two groups. FH patients had significantly lower HDL-cholesterol levels ($p<0.05$) and a non-significant trend to higher hsCRP and Lp(a) levels (▶Table 1). All FH patients and controls were receiving LLT with statins. In non-FH patients the mean dose of simvastatin used was 20 mg, atorvastatin 20 mg and rosuvastatin 10 mg while in FH patients was 40 mg,

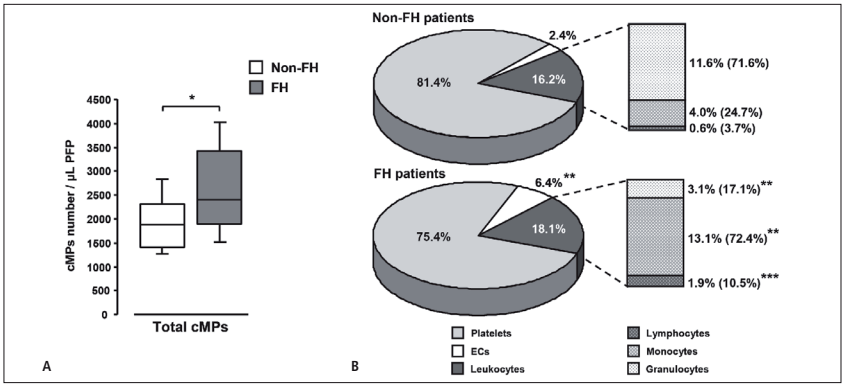


Figure 1: cMP numbers and cellular origin in non-FH and FH patients. A) Box and whisker plots showing total cMP numbers (cMPs per µl of platelet-free plasma, PFP), both in non-FH (n=37) and FH patients (n=37). Lines within boxes represent median values, the upper and lower boxes lines represent the 25th and 75th percentiles, respectively, and the upper and lower bars outside the boxes represent the 10th and 90th percentiles, respectively. B) Pie-charts showing distribution of cMPs from non-FH (n=37) and FH patients (n=37) by cell origin, indicated by percentages of each marker relative to cMPs and to leukocytes (in brackets). Used markers were $CD41^+/CD61^+$ for platelet, $CD146^+/CD31^+$ for endothelial cell, $CD45^+$ for pan-leukocyte, $CD3^+$ for lymphocyte and $CD14^+$ for monocyte origin accounting for agranulocytes and, finally, granulocytes were inferred subtracting agranulocytes subpopulation from leukocytes fraction. Comparison of the group's differences were analysed by non-parametric U-Mann Whitney test. *P<0.005, **P<0.0005, ***P<0.05 vs non-FH group.

40 mg, and 20 mg, respectively. More FH patients were receiving combined LLT with ezetimibe compared to non-FH subjects (62.2% vs 10.8%, respectively, p<0.05). Indeed, most of non-FH patients were receiving monotherapy with statins (89.2%). Contrarily, only 37.8% FH-patients were receiving monotherapy with statins or maximum statin dose, 40.5% combined therapy and 21.6% maximum combined therapy. The mean duration of LLT was 7.5 ± 1.0 years for non-FH patients and 12.1 ± 1.1 years for FH patients. There were no differences in usage of other cardiovascular drugs and tobacco consumption.

Levels and cellular origin of circulating microparticles

The total number of blood microparticles (based on FSC/SSC characteristics) was significantly higher in patients with FH than in non-FH patients (p<0.005, ▶Figure 1A). The circulating microparticle surface markers showed a different cell origin in FH and non-FH patients. ▶Figure 1B illustrates the pattern of cMPs according to their parental cell marker. Whilst percentages of total circulating platelet-derived (CD41$^+$/CD61$^+$; pMPs) (81.4% non-FH / 75.4% FH) and leukocyte-derived (CD45$^+$; LMPs) (16.2% controls / 18.1% FH) microparticles did not significantly change, there were significant changes in white cell type-derived and endothelial cell-derived cMPs between FH and non-FH patients (▶Figure 1B). Indeed, endothelial cell-derived (CD146$^+$/CD31$^+$; eMPs), lymphocyte-derived (CD45$^+$/CD3$^+$; ℓMPs), and monocyte-derived (CD14$^+$; mMPs) MPs were significantly higher in FH patients (▶Figure 1B).

Both phosphatidylserine (PS)-positive / annexin V-binding (AV$^+$) and PS-negative / non-annexin V-binding (AV$^-$) microparticles were detected. ▶Figure 2 shows the AV$^+$-cMPs carrying markers of specific cell types. FH patients were found to have significantly higher amounts of endothelial cell-derived AV$^+$-cMPs and of lymphocyte- and monocyte-derived AV$^+$-cMP subsets (▶Figure 2A) than non-FH patients (p<0.0001, p<0.0005 and p<0.0001, respectively). Suppl. Figure 2A (available online at www.thrombosis-online.com) shows similar results when total cMP numbers (AV$^+$- and AV$^-$-cMPs) were considered. Thus, LLT in hFH patients does not reduce endothelial nor inflammatory cell MP shedding as it does in non-FH hypercholesterolaemic patients showing that patients with long-life exposure to LDL-cholesterol have unabated chronic vascular damage and vascular inflammation.

Markers of cell activation in cMPs

In FH patients activated leukocytes shed significantly higher numbers of cMPs (▶Figure 2B). Specifically, levels of AV$^+$-MPs carrying the αM-integrin marker (CD11b$^+$, activated leukocytes) and both CD11b$^+$ and monocyte markers together (CD11b$^+$/CD14$^+$) were significantly increased in FH compared to non-FH patients (p<0.002 and p<0.0005, respectively, ▶Figure 2B). Suppl. Figure 2B (available online at www.thrombosis-online.com) shows similar results when total cMP numbers (both AV$^+$- and AV$^-$-cMPs) carrying markers of leukocyte activation were determined.

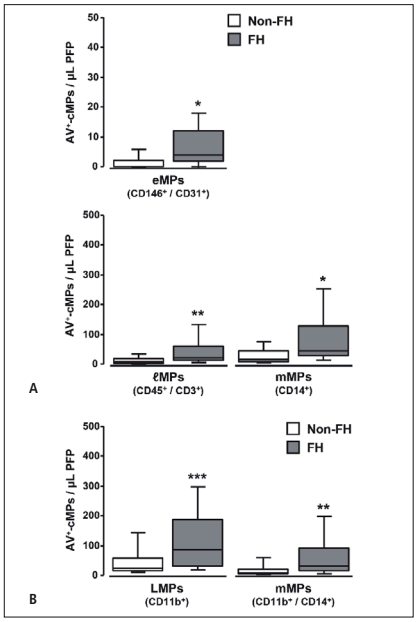


Figure 2: cMP characterisation in non-FH and FH patients. Annexin V$^+$-cMPs from non-FH (n=37) and FH patients (n=37) in function of (A) cellular origin: endothelial-(eMPs; CD146$^+$/CD31$^+$), leukocyte-(LMPs; CD45$^+$), lymphocyte-(ℓMPs; CD3$^+$/CD45$^+$), and monocyte-(mMPs; CD14$^+$) derived AV$^+$-cMPs and (B) activation status: AV$^+$-cMPs exposing αM-integrin marker (CD11b$^+$, MAC-1) corresponding to activated leukocytes. Data are given as a box and whisker plot as described in Figure 1A. Values (number/µl) for each kind of MP and patient group were as follows (median [IQR] of non-FH vs FH): eMPs (0.1 [0.0–2.0] vs 4.0 [2.0–12.0]), ℓMPs (7.2 [4.0–14.0] vs 18.0 [4.0–35.2]), mMPs (15.0 [7.0–42.5] vs 43.0 [28.0–127.0]), LMPs CD11b$^+$ (24.0 [16.0–54.0] vs 85.7 [32.0–186.0]) and LMPs CD11b$^+$/CD14$^+$ (8.0 [4.0–20.0] vs 32.0 [16.0–90.0]). *P<0.0001, **P<0.0005, ***P<0.002 vs. non-FH group (U-Mann Whitney).

FH-specific microparticle profile is not determined by LDL-cholesterol levels and systemic inflammation

Both groups of patients had similar on-treatment lipid levels, except for HDL (▶Table 1), and there was no correlation between lipid levels and cMPs levels. In order to further test whether patients with lower LDL plasma levels had different cell activation and AV$^+$-MP shedding, the results were analysed according to median LDL levels (below and above the median value of the entire study population, 141.5 mg/dl, ▶Figure 3). Circulating micro-

particle levels were significantly higher in FH patients independently of LDL-cholesterol levels for eMPs (▶Figure 3A), ℓMPs (▶Figure 3B) and mMPs and both LMPs / mMPs expressing the leukocyte activation marker CD11b$^+$ (▶Figure 3C). These results indicate a chronic vascular and inflammatory cell activation in the FH subjects. Similar results were obtained if data were analysed according to reaching or not target LDL levels (as per guidelines, <100 mg/dl and <130 mg/dl for FH and non-FH patients). Suppl. Figure 3 (available online at www.thrombosis-online.com) also shows that FH-specific cMP phenotype is independent of LDL levels if the total cMP are evaluated (both AV$^+$- and AV$^-$-cMPs). The cMP phenotype did not change with the different statin treatment either alone or combined with ezetimibe (data not shown).

We investigated whether there was an association of cMP phenotype with systemic inflammation measured by hsCRP. There were non-significantly higher levels of hsCRP in hFH patients (1.5 mg/dl) than in the non-FH hypercholesterolaemic patients (1.0 mg/dl) (▶Table 1). No correlation was found between hsCRP levels and cMPs and LDL levels.

Levels of Lp(a) were also non-significantly higher in non-FH patients. Interestingly, there was a correlation between annexin-V$^+$-cMPs and Lp(a) levels ($p<0.02$) in the hFH patients.

Atherosclerotic plaque composition and cMPs in FH patients

The MRI study performed in the group of patients with FH showed that thirty four cases (94% of studied group) had atherosclerotic plaques in the aorta and one third of these were lipidic (20). Calcium was present in 30% of aortic plaques (in ten FH patients) (20). Among FH patients, there were no differences between type of mutation (null/defective) and atherosclerotic plaque type. The presence of plaques with lipid-rich cores was significantly associated with increased levels of total AV$^+$-cMPs (▶Figure 4A), AV$^+$-LMPs (▶Figure 4C) and AV$^+$-ℓMPs (▶Figure 4E) ($p<0.05$ in all cases). There were no significant differences in endothelial AV$^+$-eMPs (▶Figure 4B) nor in monocyte AV$^+$-mMPs (▶Figure 4D). Thus, the discriminating factor mapping lipid-rich atherosclerotic plaques among FH patients was the presence of AV$^+$-phosphatidylserine-rich lymphocytic CD45$^+$/CD3$^+$-microparticles in circulating blood.

An associated ROC curve analysis, conducted to identify the threshold level of ℓMPs that was capable of predicting a lipidic atherosclerotic plaque, indicated that the cut-off point for lymphocyte-derived MPs that best predicted the presence of lipidic plaques was above 20,000 ℓMPs/ml PFP [CD45$^+$/CD3$^+$/AV$^+$] with a 77.8% sensitivity and 75% specificity. CD45$^+$/CD3$^+$/AV$^+$-ℓMPs displayed an AUC for the prediction of lipidic atherosclerotic plaques of 0.803 ± 0.08 (95% CI: 0.641-0.965, $p = 0.008$, ▶Figure 4F).

The cMPs in FH patients with calcified plaques had an opposed pattern as total levels of AV$^+$-cMPs were significantly lower in patients with calcified plaques. There was a significant difference in AV$^+$-cMPs, AV$^+$-LMPs, and activated leukocytes-derived (CD11b$^+$) AV$^+$-LMPs (▶Figure 5A), thus indicating a lower inflammation state.

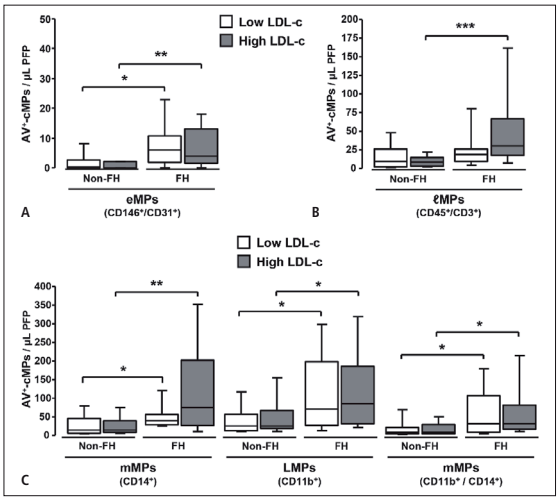


Figure 3: cMP profiling and LDL-cholesterol levels. AV$^+$-cMPs from (A) endothelial cells (eMPs; CD146$^+$/CD31$^+$), (B) lymphocytes (ℓMPs; CD45$^+$/CD3$^+$) and (C) leukocytes subsets: monocytes (mMPs; CD14$^+$), activated leukocytes (LMPs; CD11b$^+$) and activated monocytes (mMPs; CD14$^+$/CD11b$^+$) in the non-FH (n=37) and FH patients (n=37) classified in two groups: low and high LDL-c levels (with the median of LDL-cholesterol level of all population: 141.5 mg/dl). Data are given as a box and whisker plot as described in Figure 1A. *P<0.05, **P<0.005, ***P<0.0005 vs non-FH group (U-Mann Whitney). Differences due to LDL levels were not significant.

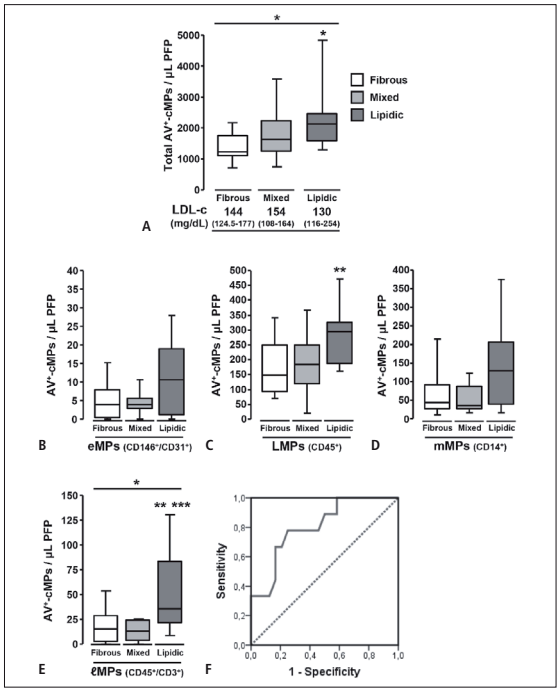


Figure 4: cMP levels according to atherosclerotic plaque composition in FH patients. Box and whisker plots showing AV+-cMPs in FH patients studied with magnetic resonance imaging (n=34) corresponding to (A) total cMPs, (B) eMPs (CD146+/CD31+), (C) LMPs (CD45+), (D) mMPs (CD14+), and (E) ℓMPs (CD45+/CD3+) related to atherosclerotic plaque characterisation: fibrous, lipidic and mixed. Data are given as a box and whisker plot as described in Figure 1A. *P<0.05 (Kruskal Wallis), **P<0.05 vs fibrous group, ***P<0.05 vs mixed group (U-Mann Whitney). F) ROC curve analysis was used to determine the threshold of lymphocyte-derived MPs (ℓMPs) that predicts a lipidic content with the highest sensitivity and specificity in these FH patients. Area under the curve (AUC) of CD45+/CD3+/AV+-MPs (the solid line): 0.803 ± 0.08 (95%CI: 0.641–0.965, P = 0.008) and of reference line (the dotted line): 0.5. The cut-off point for ℓMPs that best predicted the presence of lipidic content was above 20,000/ml PFP (77.8% sensitivity / 75% specificity).

In a similar fashion, mean aortic wall area (MAWA; ▶Figure 5B) inversely correlated with percentage of AV+-cMPs. A significant negative association between MAWA and AV+-LMPs, activated leukocyte-derived AV+-LMPs, AV+-ℓMPs, AV+-mMPs and activated monocyte-derived AV+-mMPs was found (▶Figure 5B).

Discussion

Long-term exposure to high plasma cholesterol levels is a risk factor for atherosclerosis and cardiovascular disease (37). This study shows that asymptomatic FH patients receiving long-term LLT, have significant higher number of overall circulating microparticles and in particular higher number of those derived from endothelial cells, monocytes and lymphocytes than non-FH hypercholesterolaemic patients. Important changes in MP phenotype (according to the cell origin) were found in comparison with LLT-treated hypercholesterolaemic non-FH patients. This study also shows that within the hFH patients, lymphocyte-derived cMPs (CD45+/CD3+) were significantly raised in patients with lipid-rich plaques. These data show that vascular inflammation is present in FH patients in comparison to non-FH hypercholesterolaemic patients, even though they are treated as per guideline.

Higher levels of endothelial-derived (CD146+) MPs were detected in FH than in non-FH patients. Higher plasma levels of endothelial-derived microparticles are an indicator of endothelial cell activation and dysfunction (38); indeed, high plasma cholesterol levels directly impair flow mediated dilatation and induce aortic stiffness, both associated with the onset and progression of atherosclerosis (39). eMPs were proposed as potential prognostic markers for future cardiovascular events in high CV risk patients (40). Pirro et al. showed high levels of leuko-eMPs (CD31+/CD42-) in newly-diagnosed never-LLT-treated hypercholesterolaemic subjects (12). Moreover, Huber et al. found that those microvesicles containing oxidised phospholipids, typically in lipid-related dis-

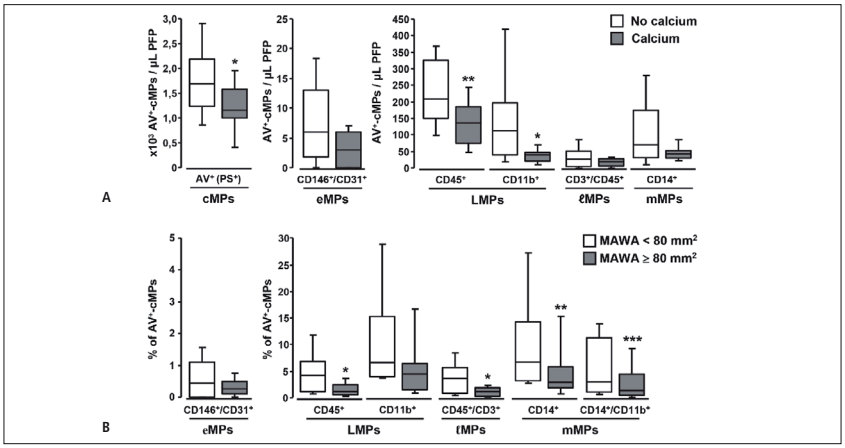


Figure 5: cMP number regarding atherosclerotic plaque burden (calcium and MAWA) in FH patients. Box and whisker plots showing AV⁺-cMPs levels (per µl of PFP) depending on (A) atherosclerotic plaque calcium content and (B) mean aortic wall area (MAWA; mm²) in FH patients (n=34). Data are given as a box and whisker plot as described in Figure 1A. *P<0.02 and **P<0.03 vs no calcium, *P<0.01, **P<0.03, and ***P<0.05 vs MAWA <80 mm² (U-Mann Whitney).

ease states, could induce monocyte-endothelial interactions (41), contributing to atherogenesis (42).

While the number of total non-activated leukocyte-derived MPs is similar in FH and in non-FH patients, a significantly higher number of monocyte-derived MPs was found in the FH patients, suggesting a link between cMPs, endothelial dysfunction and inflammation, in agreement to Wang et al. (43), who demonstrated that monocyte-cMPs might amplify inflammation by activation of endothelium. It was also described that atherosclerotic plaques contain large amounts of LMPs (44) and that plaque-MPs promote plaque progression by promoting inflammatory cells adhesion to ECs (45). In addition, the FH patients had significantly higher number of cMPs bearing the marker of leukocyte activation MAC-1 (α_M-integrin, CD11b⁺). The increase in cMPs carrying MAC-1 indicates that there is ongoing platelet interaction to leukocytes (9) 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. Previously, ℓMPs were shown to promote production of tumour necrosis factor (TNF) α and interleukin (IL)-1β by monocytes (46) and were proposed as markers of reduced vasculoprotective properties of endothelial cells (47) in diabetic or HIV-infected patients. Here we show that increased levels of ℓMPs are found in hFH patients being even higher in those with lipid-rich plaques. We show for the first time that circulating ℓMP levels could be a robust predictor of presence of lipidic plaques in FH. These results are in accordance with data recently obtained from patients with high-grade carotid stenosis and leukocyte MPs (14). It has been proposed that mMPs induce atherogenic effects (48), because a dysregulated sterol-responsive protein network can be released from cholesterol-loaded macrophages in the form of microvesicles (49) enhancing intercellular communication (50).

Because FH patients are at high CV risk, a <100 mg/dl cut-off point was selected as the therapeutic target according to clinical guidelines (22, 23). Only a low percentage of 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 (51) and vascular smooth muscle cells (52) trigger the release of procoagulant MPs. 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 patients seems to confer them a higher susceptibility to premature atherosclerosis and CAD. Although statins have been shown to affect microvesicle budding in control subjects (53, 54), the differences in cMP shedding detected between FH and non-FH patients may reflect this lifetime exposure to LDL-c that is not fully corrected by conventional LLT. Thus, our data indicates that longer time and further LDL-lowering strategies are needed to protect hFH patients. Once

an index case is indentified, all the family members should be scrutinised and treated as soon as possible. Our results also indicate that cMP determination may be helpful to improve risk assessment and define the optimal use of treatment in asymptomatic patients. Indeed, the fact that the gold standard inflammatory marker, hsCRP, did not correlate with cMP number in these well-treated patients indicate that although systemic inflammatory markers can be regulated by the statin treatment, as seen in the Jupiter trial (55), vascular cell activation evidenced by measuring cMP release is not.

Activated cells generate phosphatidylserine (PS)-positive cMPs, but also PS-negative cMPs can be found in the circulation (56). Surface exposure of PS on microparticles appears to confer them procoagulant activity, to regulate both their internalisation and clearance by phagocytosis, and be mediators of their own formation (57). Recent studies have correlated AV^--cMPs to clinical parameters in systemic diseases, which emphasise the importance of including characterisation of AV binding in cMPs (58). Although both types of cMPs were detected in this study, we have focused on AV^+-cMPs (and on total cMPs) because of their relevant association to atherothrombotic disease (10).

For the first time, we show a relation of the type of plaque, lipid-rich or calcified, with circulating microparticle number, phenotype and activation status of the source cell in the hFH patients. We have found that lipid-rich plaques correlate significantly with higher levels of total circulating microparticles and with cMPs carrying markers of activation of their mother-inflammatory cells even in the presence of LLT as compared to fibrous/mixed plaques. In contrast, lower levels of cMPs are found in hFH patients with calcified atherosclerotic plaques and large mean aortic wall area. High calcium content and large mean aortic wall area and volumes were found in fibrotic/mixed lower risk plaques (20). Although total calcium (measured by different scores) is indicative of atherosclerotic burden (presence of atherosclerosis), calcified plaques in itself are not markers of vulnerability. Indeed, the PROSPECT study showed that calcification per se may not cause coronary events (59) and other clinical studies have shown that, in fact, calcification might confer stability to the established atherosclerotic plaques (60). Of special interest is the highly significant increase in lymphocyte-derived $CD45^+/CD3^+$ microparticles found in patients with lipid-rich atherosclerotic plaques. Increasing evidence supports the concept that cMPs are not merely markers of cell activation but also could be causal effectors in atherosclerosis. We have recently reported that high cMP content in blood enhance platelet deposition on atherosclerotic plaques (61). Thus, all together these results sustain the concept of effects of cMPs beyond markers of vascular disease (62). The remarkable positive correlation found in atherosclerotic plaque type and microparticles expand the mechanistic insights that can be generated by MP characterisation. This study with statin-treated FH patients with aortic MRI has identified that the specific FH-cMP profile of $CD45^+/CD3^+$ microparticles maps aortic lipid-rich atherosclerotic plaques, highlighting the role of activated lymphocytes in atherosclerosis. Activated lymphocytes are considered effectors of atherogenesis by promoting lesion formation and exacerbating atherosclerotic disease (63). The ROC curve has indicated that > 20,000 ℓMPs/ml PFP are found in plasma of patients with lipid-rich atherosclerotic plaque, which deserves further proof-of-principle studies in other patients with CAD. However, prior to large-scale prospective studies aimed at the evaluation of cMPs as biomarkers, it would be desirable that the cMP quantification could be internationally standardised.

It may be considered a limitation that we did not include hFH patients without statin-treatment but this study is from an ongoing clinical cohort registry data of cardiovascular disease presentation in hFH and it is deemed unethical not to treat FH patients because the risk of long-life exposure to cholesterol found in different studies. However, inclusion of a control group of adult hypercholesterolaemic patients also LLT-treated has helped to exclude the impact of statins may have in cMP shedding. A specific analysis of cMPs has been only performed in relation to atherosclerotic plaques in hFH patients and conclusions can only be taken for these patients.

In summary, our results indicate that increased circulating microparticle release from different vascular resident and inflammatory cells is directly associated to the increased atherosclerosis burden in patients with FH. Altogether this study shows that cMPs are valuable biomarkers of subclinical atherosclerosis.

What is known about this topic?

- Circulating microparticles (cMPs) are small membranous vesicles released by activated blood cells that may be involved in biological intercellular communication.
- Elevated cMP levels are found in patients with cardiovascular risk factors and many pathological states.
- Familial hypercholesterolaemia (FH) is a major risk factor for atherosclerosis and is associated with early development of cardiovascular disease.

What does this paper add?

- FH patients have higher number of overall cMPs and of those derived from endothelial cells, monocytes and lymphocytes than non-FH hypercholesterolaemic patients.
- Circulating $CD45^+/CD3^+$-lymphocyte microparticles are biomarkers of asymptomatic subclinical lipid-rich atherosclerotic plaques in FH.

Acknowledgements

The authors thank Dr. R Nieuwland and Chi M. Hau for the exceptional expertise, Dr. J. Crespo for his help in flow cytometry,

Supplementary Material

Supplementary data to this article is available online at www.thrombosis-online.com.

Marta Guindo for the technical support, Maria Teresa Puig and Ignasi Gich for helpful advice in statistics, and the Spanish Familial Hypercholesterolaemia Foundation and all physicians participating in the cohort study for the assistance in the recruitment of participants. Authors are indebt to the patients for their valuable contribution and willingness to participate.

Conflicts of interest
None declared.

References

1. Jy W, Horstman LL, Jimenez JJ, et al. Measuring circulating cell-derived microparticles. J Thromb Haemost 2004; 2: 1842-1851.
2. Boulanger CM. Microparticles, vascular function and hypertension. Curr Opin Nephrol Hypertens 2010; 19: 177-180.
3. Berckmans RJ, Nieuwland R, Boing AN, et al. Cell-derived microparticles circulate in healthy humans and support low grade thrombin generation. Thromb Haemost 2001; 85: 639-646.
4. Diehl P, Aleker M, Helbing T, et al. Increased platelet, leukocyte and endothelial microparticles predict enhanced coagulation and vascular inflammation in pulmonary hypertension. J Thromb Thrombolysis 2010; 31: 173-179.
5. Koga H, Sugiyama S, Kugiyama K, et al. Elevated levels of VE-cadherin-positive endothelial microparticles in patients with type 2 diabetes mellitus and coronary artery disease. J Am Coll Cardiol 2005; 45: 1622-1630.
6. Tan KT, Tayebjee MH, Lim HS, et al. Clinically apparent atherosclerotic disease in diabetes is associated with an increase in platelet microparticle levels. Diabet Med 2005; 22: 1657-1662.
7. Amabile N, Guerin AP, Tedgui A, et al. Predictive value of circulating endothelial microparticles for cardiovascular mortality in end-stage renal failure: a pilot study. Nephrol Dial Transplant 2011; 27: 1873-1880.
8. Bulut D, Tuns H, Mugge A. CD31+/Annexin V+ microparticles in healthy offsprings of patients with coronary artery disease. Eur J Clin Invest 2009; 39: 17-22.
9. Chirinos JA, Heresi GA, Velasquez H, et al. Elevation of endothelial microparticles, platelets, and leukocyte activation in patients with venous thromboembolism. J Am Coll Cardiol 2005; 45: 1467-1471.
10. Mallat Z, Benamer H, Hugel B, et al. Elevated levels of shed membrane microparticles with procoagulant potential in the peripheral circulating blood of patients with acute coronary syndromes. Circulation 2000; 101: 841-843.
11. Sinning JM, Losch J, Walenta K, et al. Circulating CD31+/Annexin V+ microparticles correlate with cardiovascular outcomes. Eur Heart J 2010; 32: 2034-2041.
12. Pirro M, Schillaci G, Paltriccia R, et al. Increased ratio of CD31+/CD42- microparticles to endothelial progenitors as a novel marker of atherosclerosis in hypercholesterolaemia. Arterioscler Thromb Vasc Biol 2006; 26: 2530-2535.
13. Chironi G, Simon A, Hugel B, et al. Circulating leukocyte-derived microparticles predict subclinical atherosclerosis burden in asymptomatic subjects. Arterioscler Thromb Vasc Biol 2006; 26: 2775-2780.
14. Sarlon-Bartoli G, Bennis Y, Lacroix R, et al. Plasmatic level of leukocyte-derived microparticles is associated with unstable plaque in asymptomatic patients with high-grade carotid stenosis. J Am Coll Cardiol 2013; epub ahead of print.
15. Gill PJ, Harnden A, Karpe F. Familial hypercholesterolaemia. Br Med J 2012; 344: e3228.
16. Neefjes LA, Ten Kate GJ, Alexia R, et al. Accelerated subclinical coronary atherosclerosis in patients with familial hypercholesterolaemia. Atherosclerosis 2011; 219: 721-727.
17. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. N Engl J Med 2005; 352: 1685-1695.
18. Yuan G, Wang J, Hegele RA. Heterozygous familial hypercholesterolaemia: an underrecognized cause of early cardiovascular disease. Cmaj 2006; 174: 1124-1129.
19. Sjouke B, Kusters DM, Kastelein JJ, et al. Familial hypercholesterolaemia: present and future management. Curr Cardiol Rep 2011; 13: 527-536.
20. Caballero P, Alonso R, Rosado P, et al. Detection of subclinical atherosclerosis in familial hypercholesterolaemia using non-invasive imaging modalities. Atherosclerosis 2012; 222: 468-472.
21. Mata N, Alonso R, Badimon L, et al. Clinical characteristics and evaluation of LDL-cholesterol treatment of the Spanish Familial Hypercholesterolaemia Longitudinal Cohort Study (SAFEHEART). Lipids Health Dis 2011; 10: 94.
22. Wierzbicki AS, Humphries SE, Minhas R. Familial hypercholesterolaemia: summary of NICE guidance. Br Med J 2008; 337: a1095.
23. Graham I, Atar D, Borch-Johnsen K, et al. European guidelines on cardiovascular disease prevention in clinical practice: executive summary: Fourth Joint Task Force of the European Society of Cardiology and Other Societies on Cardiovascular Disease Prevention in Clinical Practice (Constituted by representatives of nine societies and by invited experts). Eur Heart J 2007; 28: 2375-2414.
24. Pijlman AH, Huijgen R, Verhagen SN, et al. Evaluation of cholesterol lowering treatment of patients with familial hypercholesterolaemia: a large cross-sectional study in The Netherlands. Atherosclerosis 2010; 209: 189-194.
25. Gehi AK, Ali S, Na B, et al. Self-reported medication adherence and cardiovascular events in patients with stable coronary heart disease: the heart and soul study. Arch Intern Med 2007; 167: 1798-1803.
26. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clin Chem 1972; 18: 499-502.
27. Mozas P, Castillo S, Tejedor D, et al. Molecular characterisation of familial hypercholesterolaemia in Spain: identification of 39 novel and 77 recurrent mutations in LDLR. Hum Mutat 2004; 24: 187.
28. Nieuwland R, Berckmans RJ, McGregor S, et al. Cellular origin and procoagulant properties of microparticles in meningococcal sepsis. Blood 2000; 95: 930-935.
29. Rank A, Liebhardt S, Zwirner J, et al. Circulating microparticles in patients with benign and malignant ovarian tumors. Anticancer Res 2012; 32: 2009-2014.
30. Yuana Y, Bertina RM, Osanto S. Pre-analytical and analytical issues in the analysis of blood microparticles. Thromb Haemost 2011; 105: 396-408.
31. Corti R, Fuster V. Imaging of atherosclerosis: magnetic resonance imaging. Eur Heart J 2011; 32: 1709-1719b.
32. Vilades Medel D, Leta Petracca R, Carreras Costa F, et al. Coronary computed tomographic angiographic findings in asymptomatic patients with heterozygous familial hypercholesterolaemia and null allele low-density lipoprotein receptor mutations. Am J Cardiol 2013; 111: 955-961.
33. Working Group on Vascular Biology. 55th Annual Scientific and Standardisation Committee Meeting. ISTH Scientific Subcommittee Minutes, Boston, USA; 2009.
34. Biro E, Akkerman JW, Hoek FJ, et al. The phospholipid composition and cholesterol content of platelet-derived microparticles: a comparison with platelet membrane fractions. J Thromb Haemost 2005; 3: 2754-2763.
35. Lacroix R, Robert S, Poncelet P, et al. Standardisation of platelet-derived microparticle enumeration by flow cytometry with calibrated beads: results of the International Society on Thrombosis and Haemostasis SSC Collaborative workshop. J Thromb Haemost 2010; 8: 2571-2574.
36. Nieuwland R, Berckmans RJ, Rotteveel-Eijkman RC, et al. Cell-derived microparticles generated in patients during cardiopulmonary bypass are highly procoagulant. Circulation 1997; 96: 3534-3541.
37. Hazen SL. Neutrophils, hypercholesterolaemia, and atherogenesis. Circulation 2010; 122: 1786-1788.
38. Dignat-George F, Boulanger CM. The many faces of endothelial microparticles. Arterioscler Thromb Vasc Biol 2011; 31: 27-33.
39. Alonso R, Mata P, De Andres R, et al. Sustained long-term improvement of arterial endothelial function in heterozygous familial hypercholesterolaemia patients treated with simvastatin. Atherosclerosis 2001; 157: 423-429.
40. Nozaki T, Sugiyama S, Koga H, et al. Significance of a multiple biomarkers strategy including endothelial dysfunction to improve risk stratification for cardiovascular events in patients at high risk for coronary heart disease. J Am Coll Cardiol 2009; 54: 601-608.
41. Huber J, Vales A, Mitulovic G, et al. Oxidized membrane vesicles and blebs from apoptotic cells contain biologically active oxidized phospholipids that induce monocyte-endothelial interactions. Arterioscler Thromb Vasc Biol 2002; 22: 101-107.
42. Hartvigsen K, Chou MY, Hansen LF, et al. The role of innate immunity in atherogenesis. J Lipid Res 2009; 50 (Suppl): S388-393.

43. Wang JG, Williams JC, Davis BK, et al. Monocytic microparticles activate endothelial cells in an IL-1beta-dependent manner. Blood 2011; 118: 2366-2374.
44. Leroyer AS, Isobe H, Leseche G, et al. Cellular origins and thrombogenic activity of microparticles isolated from human atherosclerotic plaques. J Am Coll Cardiol 2007; 49: 772-777.
45. Rautou PE, Leroyer AS, Ramkhelawon B, et al. Microparticles from human atherosclerotic plaques promote endothelial ICAM-1-dependent monocyte adhesion and transendothelial migration. Circ Res 2011; 108: 335-343.
46. Scanu A, Molnarfi N, Brandt KJ, et al. Stimulated T cells generate microparticles, which mimic cellular contact activation of human monocytes: differential regulation of pro- and anti-inflammatory cytokine production by high-density lipoproteins. J Leukoc Biol 2008; 83: 921-927.
47. Martin S, Tesse A, Hugel B, et al. Shed membrane particles from T lymphocytes impair endothelial function and regulate endothelial protein expression. Circulation 2004; 109: 1653-1659.
48. Hoyer FF, Giesen MK, Nunes Franca C, et al. Monocytic microparticles promote atherogenesis by modulating inflammatory cells in mice. J Cell Mol Med 2012; 16: 2777-2788.
49. Becker L, Gharib SA, Irwin AD, et al. A macrophage sterol-responsive network linked to atherogenesis. Cell Metab 2010; 11: 125-135.
50. Mause SF, Weber C. Microparticles: protagonists of a novel communication network for intercellular information exchange. Circ Res 2010; 107: 1047-1057.
51. Liu ML, Reilly MP, Casasanto P, et al. Cholesterol enrichment of human monocyte/macrophages induces surface exposure of phosphatidylserine and the release of biologically-active tissue factor-positive microvesicles. Arterioscler Thromb Vasc Biol 2007; 27: 430-435.
52. Llorente-Cortes V, Otero-Vinas M, Camino-Lopez S, et al. Aggregated low-density lipoprotein uptake induces membrane tissue factor procoagulant activity and microparticle release in human vascular smooth muscle cells. Circulation 2004; 110: 452-459.
53. Owens AP, 3rd, Passam FH, Antoniak S, et al. Monocyte tissue factor-dependent activation of coagulation in hypercholesterolaemic mice and monkeys is inhibited by simvastatin. J Clin Invest 2012; 122: 558-568.
54. Suades R, Padro T, Alonso R, et al. Lipid-lowering therapy with statins reduces microparticle shedding from endothelium, platelets and inflammatory cells indicating protection against cell activation. Thromb Haemost 2013; 110: 366-377.
55. Ridker PM, Danielson E, Fonseca FA, et al. Rosuvastatin to prevent vascular events in men and women with elevated C-reactive protein. N Engl J Med 2008; 359: 2195-2207.
56. Perez-Pujol S, Marker PH, Key NS. Platelet microparticles are heterogeneous and highly dependent on the activation mechanism: studies using a new digital flow cytometer. Cytometry A 2007; 71: 38-45.
57. Burger D, Schock S, Thompson CS, et al. Microparticles: biomarkers and beyond. Clin Sci 2013; 124: 423-441.
58. Nielsen CT, Ostergaard O, Johnsen C, et al. Distinct features of circulating microparticles and their relationship to clinical manifestations in systemic lupus erythematosus. Arthritis Rheum 2011; 63: 3067-3077.
59. Xu Y, Mintz GS, Tam A, et al. Prevalence, distribution, predictors, and outcomes of patients with calcified nodules in native coronary arteries: a 3-vessel intravascular ultrasound analysis from Providing Regional Observations to Study Predictors of Events in the Coronary Tree (PROSPECT). Circulation 2012; 126: 537-545.
60. Beckman JA, Ganz J, Creager MA, et al. Relationship of clinical presentation and calcification of culprit coronary artery stenoses. Arterioscler Thromb Vasc Biol 2001; 21: 1618-1622.
61. Suades R, Padro T, Vilahur G, et al. Circulating and platelet-derived microparticles in human blood enhance thrombosis on atherosclerotic plaques. Thromb Haemost 2012; 108: 1208-1219.
62. Mause SF. Platelet microparticles: reinforcing the hegemony of platelets in atherothrombosis. Thromb Haemost 2012; 109: 5-6.
63. Weber C, Noels H. Atherosclerosis: current pathogenesis and therapeutic options. Nat Med 2011; 17: 1410-1422.

SUPPLEMENTARY MATERIAL

SUPPLEMENTARY METHODS

Clinical study population

The present nested case-control study included 37 patients with clinical and genetic diagnosis of heterozygous FH and, thus life-long exposure to high LDL plasma levels, and 37 non-FH patients with adult hypercholesterolemia but with negative genetic testing, matched by age, gender, demographics, and lipid-lowering treatment (Table 1). FH cases had been previously characterized by MRI-imaging and non-FH cases were randomly selected, all from the SpAnish Familial hypErcHolEsterolaemiA cohoRt STudy (SAFEHEART), which is an open, multicenter, long-term prospective ongoing cohort study. Baseline characteristics of the cohort population have been previously described (Mata *et al.,* 2011). All patients were receiving lipid-lowering treatment according to clinical guidelines (Wierzbicki, Humphries and Minhas, 2008; Civeira, 2004; Graham *et al.,* 2007). A 11% of FH patients and 46% of non-FH patients achieved therapeutic LDL targets according to guidelines. Maximum statin dose was defined as previously described: simvastatin 40 mg/day, fluvastatin 80 mg/day, atorvastatin 80 mg/day, rosuvastatin 20-40 mg/day (Pijlman *et al.,* 2010). The study was approved by the local ethics committee and was conducted according to the Declaration of Helsinki. A written informed consent was obtained from all participants prior to the study. Sociodemographic data, lifestyle, medical and therapeutic data, current LLT and classical risk factors were obtained from all subjects. 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, as previously described (Gehi *et al.,* 2007). Physical examination included weight (kg), height (cm), body mass index (kg/m^2), waist circumference (cm), and blood pressure (Table 1). No patient had past history of cancer, inflammatory disorders, and pregnancy because these conditions are known to independently impair cMP number. The results of the study are presented in accordance with STROBE guidelines.

Biochemical and genotyping analysis

Blood samples were withdrawn by cubital venopuncture after 10-14 hours of fasting to obtain serum (30 minutes[min], 37ºC; 30 min, 4ºC; 1258×g, 15 min, 4ºC) and EDTA-plasma (1258×g, 20 min) for lipid profile and DNA analysis. Samples were processed for assay within first two hours. Total cholesterol (TC), triglycerides (TG), and HDL-cholesterol were measured by standardized enzymatic methods in a centralized routine laboratory. Serum LDL-cholesterol concentration was

calculated using the Friedewäld formula (Friedewald, Levy and Fredrickson, 1972). Lipoprotein (a) (Lp(a)) levels were measured using a turbidimetric method performed by suspension of latex particles coated with rabbit IgG anti-Lp (a) gene (Abbott Diagnostics) in an Architect autoanalyzer c16000 (Abbot, USA). For molecular genetic analysis, genomic DNA was isolated from whole blood samples using standard methods and the genetic diagnosis of FH made using a DNA-microarray (LIPOCHIP) in a central laboratory (Progenika, Bilbao, Spain) (Mozas *et al.,* 2004).

Atherosclerotic plaque characterization

Aortic MRI has been used in the last decade to evaluate atherosclerosis burden in high risk patients including FH patients (Caballero *et al.,* 2012; Corti and Fuster, 2011; Vilades Medel *et al.,* 2013). Atherosclerotic plaque evaluation and morphometric analysis were performed in the FH patients of this study using aortic MRI at the level of the descending thoracic aorta, as fully described (Caballero *et al.,* 2012). Briefly, atherosclerotic plaque was defined as a clearly identified luminal protrusion ≥1 mm with focal wall thickening. For aortic wall area, external and internal wall boundaries of the aorta were traced on each slice by manual planimetry and MAWA (mm^2) was determined by subtracting one from the other in each slice. Plaque characterization was based on the signal intensities of plaque on T1 and T2 weighted images. Using combined analyses of different tissue signal intensities generated by the application of these two parameters T1 (longitudinal relaxation time) and T2 (transverse relaxation time) it has been possible to determined plaque composition. MRI differentiates plaque components on the basis of physical and biochemical characteristics: the signal originating from a particular tissue partly depend on the amount of water and intrinsic magnetic properties within the tissue. Thus, in T1-weighted (T1W) images, tissues with short T1 times will have high signal intensity and appear bright on the image. Conversely, in T2-weighted (T2W) images, tissues with short T2 times will have low signal intensity in comparison with those with high T2. Therefore, the lipid component was identified as hyperintense on T1 and hypointense on T2 images (lipidic plaques) and the fibrocellular components were identified as hyperintense on both images (fibrotic plaques). Calcium deposits were identified as hypointense on all the images, and results were expressed as presence or absence (Nieuwland *et al.,* 2000; Rank, Liebhardt *et al.,* 2012; Rank, Nieuwland *et al.,* 2012). Inter-observer variability was low and the intra-class correlation coefficient was of 0.735 (95%CI: 0.591-0.833).

Blood sampling 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. Blood cells were removed by low-speed centrifugation (1258×g, 20 min) at room

temperature (RT) in order to avoid *in vitro* platelet activation as previously described (Gyorgy *et al.,* 2011; Jy *et al.,* 2004; Hohlfeld *et al.,* 2008; Yuana, Bertina and Osanto, 2011; Dey-Hazra *et al.,* 2010). Platelet poor plasma (PPP) was carefully aspirated, leaving about 0.1cm undisturbed layer on top of the cells. A second centrifugation step (1258×g, 10 min, RT) was made to ensure the complete removal of cells and obtain the platelet free plasma (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. PFP aliquots were snap-frozen in liquid nitrogen and stored, for an identical time interval, at -80ºC until flow cytometric studies.

Using frozen PFP for MP analysis has been previously validated (Working Group on Vascular Biology, 2008, 2009). What is really critical is that cells and platelets from biological fluids (plasma) must be pelleted after collection and before frozen stage. Indeed, snap-freezing and thawing on melting ice, recommended also by the report on standardization of cMP analysis from International Society on Thrombosis and Haemostasis (Scientific and Standardization Committee: Standardization of Pre-analytical Variables in Plasma Microparticle Determination) (Nieuwland *et al.,* 2000; Rank, Liebhardt *et al.,* 2012; Rank, Nieuwland *et al.,* 2012) ensure the best possible preservation of MP structure and function; thus it minimize the effect of pre-analytical variables.

Circulating microparticle isolation

The cMP-fraction was isolated from PFP by a two-step high-speed centrifugation (Nieuwland *et al.,* 2000; Rank, Liebhardt *et al.,* 2012; Rank, Nieuwland *et al.,* 2012; Biro *et al.,* 2005). Briefly, 250 µL-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 to a final volume of 100 µL.

Flow cytometric analysis of circulating microparticles

Triple-label flow cytometric analysis was performed as described by Nieuwland *et al.* (Williams and Mackman, 2011; van der Pol *et al.,* 2012) with slight modifications. Briefly, washed cMP suspensions (5µL) were diluted in 30µL PBS buffer containing 2.5mM $CaCl_2$. Thereafter, combinations of BD-horizon V450-conjugated annexin V (5µL) with two specific monoclonal antibodies (5µL each) labelled with fluorescein isothiocyanate and phycoerythrin, or the isotype-matched control antibodies were added (Suppl. Table 1). Samples were incubated (20 min, RT, dark) and diluted with

2.5mM $CaCl_2$-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. Granulocyte's percentage was inferred by subtracting agranulocytes (lymphocytes plus monocytes) from total leukocytes instead of labelling with specific mAb. Gate limits were established following two criteria: (Mata *et al.,* 2011) calibration using a Flow Check YG Size Range Calibration Kit (Polysciences) (Rank, Nieuwland *et al.,* 2012; Biro *et al.,* 2005) and (Wierzbicki, Humphries and Minhas, 2008) using an *in vitro* platelet-derived microparticle population as positive control *(Figure S1A-D),* as previously described (Rautou *et al.,* 2011), since calibration beads have different properties of FSC/SSC compared with biologic MPs . 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 CaCl2, since calcium is essential for annexin V conjugation. 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 (version 6.1.3, Becton Dickinson). The concentration (number of cMPs per µL of plasma) was determined according to Nieuwland's procedure , based on sample's volume, flow cytometer's flow rate and the number of fluorescence-positive events (N), as follows: cMPs/µL = N x (V_f/V_a) x (V_t/FR) x ($1/V_i$) [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 microparticle isolation]. Flow rate was measured before each experiment. Intra-assay 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 0.2 µm pore size filters.

Microparticles mediate intercellular transfer of bioactive molecules such as surface receptors. If the resulting membrane fusion distributes cell-specific markers from one MP to another, this may complicate identification of cMP origins. We have overcome this complex interaction determining cMP cell source by triple-staining (with two different markers of the same parent cell and phosphatidylserine). Additionally, an overlapping biophysical interaction between protein complexes and microparticles could affect the purity of isolated MP-preparations. However, in the present work, we used direct immunolabelling and we centrifuged the antibodies before their use in order to avoid immune complex formation. These complex interactions are a proof of the need for standardization of both microparticle isolation and analysis among different laboratories.

Statistical analysis

Sample size was selected according to (1) available patients with aortic-MRI detection, (2) average sample sizes of published flow cytometry studies on cMPs and (3) to provide sufficient statistical power for group comparisons. All data are presented as medians (interquartile range) except when indicated. An initial descriptive analysis was carried out using number of cases and percentages for qualitative variables and median and interquartile range for quantitative variables. Frequencies of qualitative variables (risk factors and medications) were compared between two groups by using the Chi-square analysis. Median values of quantitative variables were compared with nonparametric tests. The statistical significances between patient and control groups or comparison of two groups were determined with U-Mann Whitney and multiple comparisons by Kruskal Wallis, and when significant, Bonferroni post-hoc analysis was used to assess intergroup differences 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. A cut-off level of MPs was determined with the shortest distance from upper left corner of the ROC curve (minimizing $[(1\text{-sensitivity})^2 + (1\text{-specificity})^2]$. StatView software (Abacus Concepts) and SPSS Statistics Version 21.0.0 (SPSS, Chicago) were used for statistical analysis, and a $P<0.05$ was considered statistically significant.

SUPPLEMENTARY METHODS REFERENCES

Biró, E. *et al.* (2005), "The phospholipid composition and cholesterol content of platelet-derived microparticles: a comparison with platelet membrane fractions," *J Thromb Haemost,* 3(12): 2754-2763.

Caballero, P. *et al.* (2012), "Detection of subclinical atherosclerosis in familial hypercholesterolemia using non-invasive imaging modalities," *Atherosclerosis,* 222(2): 468-472.

Civeira, F. (2004), "Guidelines for the diagnosis and management of heterozygous familial hypercholesterolemia," *Atherosclerosis,* 173(1): 55-68.

Corti, R. and Fuster, V. (2011), "Imaging of atherosclerosis: magnetic resonance imaging," *Eur Heart J,* 32(14): 1709-1719b.

Dey-Hazra, E. *et al.* (2010), "Detection of circulating microparticles by flow cytometry: influence of centrifugation, filtration of buffer, and freezing," *Vasc Health Risk Manag,* 6: 1125-1133.

Friedewald, W. T.; Levy, R. I. and Fredrickson, D. S. (1972), "Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge," *Clin Chem,* 18(6): 499-502.

Gehi, A. K. *et al.* (2007), "Self-reported medication adherence and cardiovascular events in patients with stable coronary heart disease: the heart and soul study," *Arch Intern Med*, 167(16): 1798-1803.

Graham, I. *et al.* (2007), "European guidelines on cardiovascular disease prevention in clinical practice: executive summary: Fourth Joint Task Force of the European Society of Cardiology and Other Societies on Cardiovascular Disease Prevention in Clinical Practice (Constituted by representatives of nine societies and by invited experts)," *Eur Heart J,* 28(19): 2375-2414.

Gyorgy, B. *et al.* (2011), "Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles," *Cell Mol Life Sci,* 68(16): 2667-2688.

Hohlfeld, T. *et al.* (2008), "Pyrazolinone analgesics prevent the antiplatelet effect of aspirin and preserve human platelet thromboxane synthesis," *J Thromb Haemost,* 6(1): 166-173.

Jy, W. *et al.* (2004), "Measuring circulating cell-derived microparticles," *J Thromb Haemost,* 2(10): 1842-1851.

Mata, N. *et al.* (2011), "Clinical characteristics and evaluation of LDL-cholesterol treatment of the Spanish Familial Hypercholesterolemia Longitudinal Cohort Study (SAFEHEART)," *Lipids Health Dis,* 10: 94.

Mozas, P. *et al.* (2004), "Molecular characterization of familial hypercholesterolemia in Spain: identification of 39 novel and 77 recurrent mutations in LDLR," *Hum Mutat,* 24(2): 187.

Nieuwland, R. *et al.* (2000), "Cellular origin and procoagulant properties of microparticles in meningococcal sepsis," *Blood,* 95(3): 930-935.

Pijlman, A. H. *et al.* (2010), "Evaluation of cholesterol lowering treatment of patients with familial hypercholesterolemia: a large cross-sectional study in The Netherlands," *Atherosclerosis,* 209(1): 189-194.

Rank, A.; Liebhardt, S. *et al.* (2012), "Circulating microparticles in patients with benign and malignant ovarian tumors," *Anticancer Res,* 32(5): 2009-2014.

Rank, A.; Nieuwland, R. *et al.* (2012), "Climacteric lowers plasma levels of platelet-derived microparticles: a pilot study in pre- versus postmenopausal women," *Acta Haematol,* 128(1): 53-59.

Rautou, P. E. *et al.* (2011), "Microparticles, vascular function, and atherothrombosis," *Circ Res,* 109(5): 593-606.

Van Der Pol, E. *et al.* (2012), "Single vs. swarm detection of microparticles and exosomes by flow cytometry," *J Thromb Haemost,* 10(5): 919-930.

Vilades Medel, D. *et al.* (2013), "Coronary computed tomographic angiographic findings in asymptomatic patients with heterozygous familial hypercholesterolemia and null allele low-density lipoprotein receptor mutations," *Am J Cardiol,* 111(7): 955-961.

Wierzbicki, A. S.; Humphries, S. E. and Minhas, R. (2008), "Familial hypercholesterolaemia: summary of NICE guidance," *Bmj,* 337: a1095.

Williams, J. C. and Mackman, N. (2011), "MPs or ICs?," *Blood,* 117(4): 1101-1102.

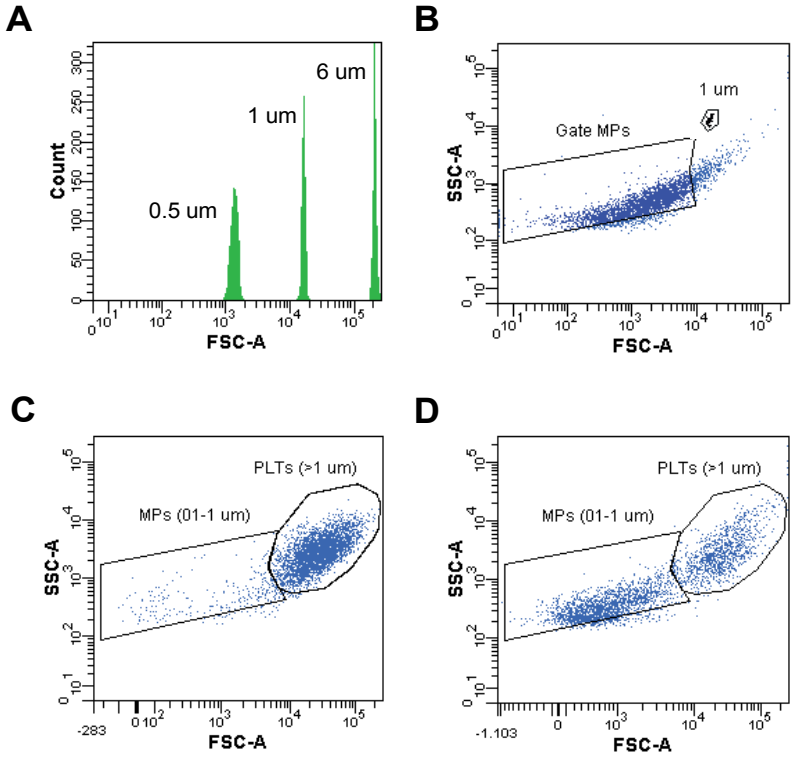
Working GROUP on Vascular Biology (2008), *54th Annual Scientific and Standardization Committee Meeting,* Vienna, Austria, ISTH Scientific Subcommittee Minutes.

— (2009), 55th Annual Scientific and Standardization Committee Meeting, Boston, USA, ISTH Scientific Subcommittee Minutes.

Yuana, Y.; Bertina, R. M. and Osanto, S. (2011), "Pre-analytical and analytical issues in the analysis of blood microparticles," *Thromb Haemost,* 105(3): 396-408.

Supplementary Figure 1

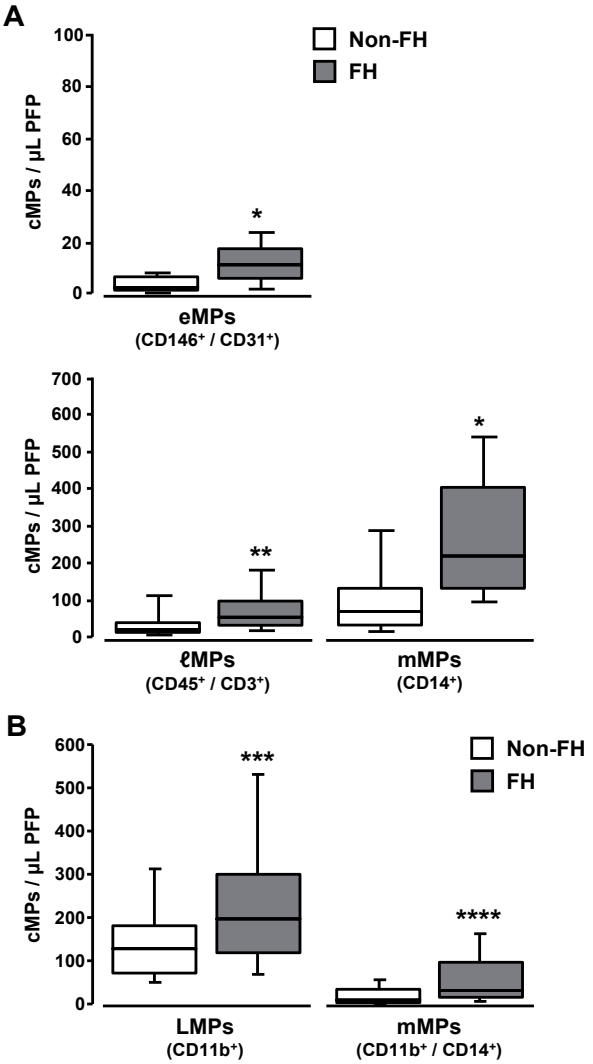
CHARACTERIZATION OF CIRCULATING MICROPARTICLES (CMPS) BY FLOW CYTOMETRY



Notes: The cell-microparticle gate was established based on light scattering properties and size, using calibration microspheres (A) and defining cMPs as events <1 µm (B). The lower detection limit was placed as a threshold above the electronic noise of our flow cytometer (B). Further, in order to corroborate and validate established microparticle gate we determined the forward scatter (FSC) and side scatter (SSC) characteristics of platelets and microparticles in suspension (C-D). Image C shows unstimulated platelets. Thereafter, platelets were stimulated with calcium ionophore A23187 (5 minute incubation, at 37°C, 2.5µM) and microparticles generated were located within the established gate (D).
Source: Own elaboration.

Supplementary Figure 2

CMP CHARACTERIZATION IN NON-FH AND FH PATIENTS

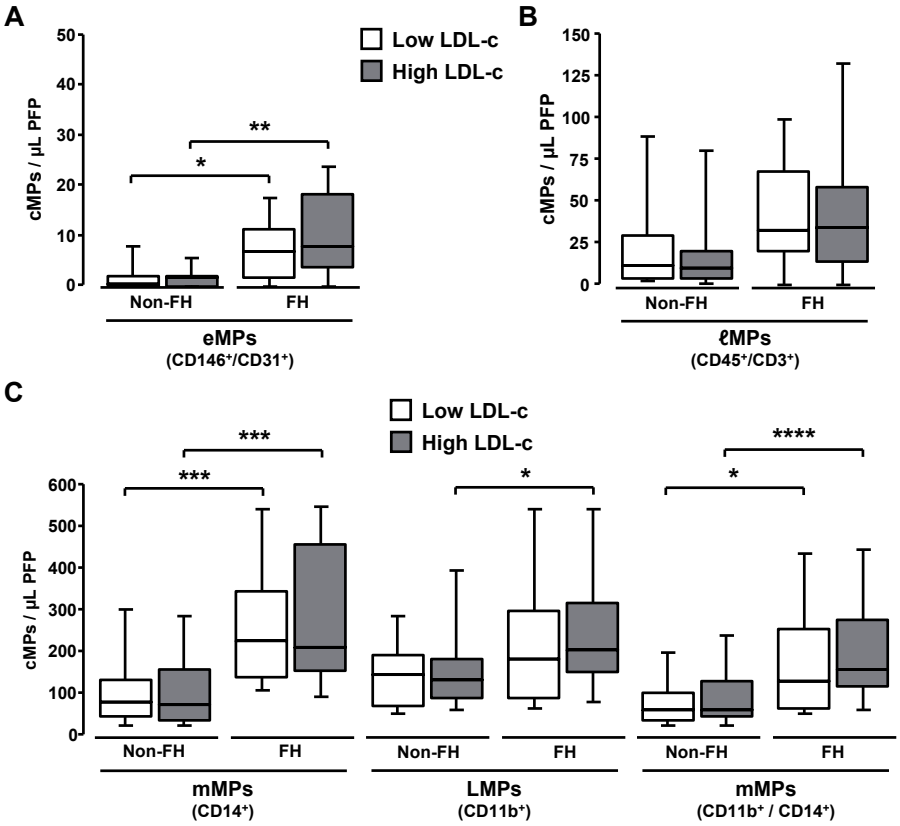


Notes: Total cMPs from non-FH (n=37) and FH patients (n=37) in function of (A) cellular origin: endothelial- (eMPs; $CD146^+/CD31^+$), leukocyte- (LMPs; $CD45^+$), lymphocyte- (ℓMPs; $CD3^+/CD45^+$), and monocyte- (mMPs; $CD14^+$) derived cMPs and (B) activation status: cMPs exposing α_M-integrin marker ($CD11b^+$) corresponding to activated leukocytes. Data are given as a box and whisker plot as described in Figure 1A. *P<0.0001, **P<0.0002, ***P<0.05, ****P<0.0005 vs. non-FH group (U-Mann Whitney).

Source: Own elaboration.

Supplementary Figure 3

CMP PROFILING AND LDL-CHOLESTEROL LEVELS



Notes: Total cMPs from (A) endothelial cells(eMPs; $CD146^+/CD31^+$), (B) lymphocytes (ℓMPs; $CD45^+/CD3^+$) and (C) leukocytes subsets: monocytes (mMPs; $CD14^+$), activated leukocytes (LMPs; $CD11b^+$) and activated monocytes (mMPs; $CD14^+/CD11b^+$) in the non-FH (n=37) and FH patients (n=37) classified in two groups: low and high LDL-c levels (with the median of LDL-cholesterol level of all population: 141.5 mg/dL). Data are given as a box and whisker plot as described in Figure 1A.*P<0.05, **P<0.005, ***P<0.01, ****P<0.0001 vs. non-FH group (U-Mann Whitney). Differences due to LDL levels were not significant.

Source: Own elaboration.

Supplementary Table 1

CELL SURFACE MOLECULES FOR CIRCULATING MICROPARTICLE IDENTIFICATION AND CHARACTERIZATION

Marker	Alternative name	Expression	Conjugation	Clone	Company
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
CD41	α_{IIb}-integrin	Platelets	FITC	SZ22	Beckman Coulter
CD45	LCA	Leukocytes	PE	Immu-19.2	Beckman Coulter
CD61	β_3-integrin	Platelets	PE	VI-PL2	BD Pharmingen
CD146	MYX18	Endothelial cells	FITC	P1H12	BD Pharmingen
IgG1γ	-	-	FITC / PE	X40	BD Biosciences
IgG1κ	-	-	FITC / PE	MPOC21	BD Pharmingen

Notes: 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.
Source: Own elaboration.

ARTICLE 4

High levels of TSP1^{+}/CD142^{+} platelet-derived microparticles characterise young patients with high cardiovascular risk and subclinical atherosclerosis

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

Published – Thromb Haemost 2015; 114(6): 1310-1321

OBJECTIVE

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; 114(6): 1310-1321.

High levels of TSP1+/CD142+ platelet-derived microparticles characterise young patients with high cardiovascular risk and subclinical atherosclerosis*

Rosa Suades[1]; Teresa Padró[1]; Rodrigo Alonso[2]; Pedro Mata[2]; Lina Badimon[1,3]

[1]Cardiovascular Research Center (CSIC-ICCC), IIB-Sant Pau, Barcelona, Spain; [2]Fundación Hipercolesterolemia Familiar, Madrid, Spain; [3]Cardiovascular Research Chair, UAB, Barcelona, Spain

Summary

Circulating microparticles (cMPs) play important roles in cellular crosstalk and are messengers of cell activation. We have previously reported that platelet-released microparticles (pMPs) stimulate thrombosis and that lipid-lowering treatment as per guidelines in patients with familial hypercholesterolaemia (FH) is not sufficiently effective in reducing pro-inflammatory cell activation and, consequently, $CD45^+/CD3^+$-lymphocyte-derived cMP shedding. FH patients, due to life-long vascular exposure to high LDL-cholesterol levels, are at high cardiovascular risk (HCVR) and develop premature coronary artery disease. Our objectives were to investigate a) whether patients with HCVR have cMPs with a prothrombotic phenotype, and b) whether patients with magnetic resonance imaging (MRI) evidence of lipid-rich atherosclerotic lesions have a specific cMP profile regarding prothrombotic protein cargos. cMPs were isolated from HCVR-patients and from age/gender/treatment-matched control patients. cMP phenotype was characterised by triple-labelling flow cytometry. HCVR-patients have higher numbers of pMPs derived from activated platelets as well as of tissue factor-rich microparticles (TF^+-cMPs) than controls ($p<0.0001$). TF^+-cMPs showed procoagulant activity, which associate with atherosclerotic plaque burden, indicating that TF in the cMPs is functional. In HCVR-patients, overall TF^+-cMPs (monocyte-derived [$CD142^+/CD14^+$] and platelet-derived [$CD142^+/TSP1^+$]) and activated pMPs directly correlate with MRI-detected lipid-rich atherosclerotic plaques while inversely correlate with MRI-detected calcified plaques. C-statistics analysis showed that prothrombotic cMPs add significant prognostic value to a risk factor model for the prediction of lipid-rich plaques. In conclusion, the activation status of blood cells in HCVR-patients differed markedly from controls as shown by higher circulating levels of prothrombotic and TF^+-cMPs. Prothrombotic cMP numbers identify subclinical atherosclerotic plaque burden.

Keywords

Atherothrombosis, circulating microparticles, platelets, risk factors, tissue factor

Correspondence to:
Prof. Lina Badimon
Cardiovascular Research Center
C/ Sant Antoni Mª Claret 167
08025 Barcelona, Spain
Tel.: +34 935565880, Fax: +34 935565559
E-mail: lbadimon@csic-iccc.org

Financial support:
This work was supported by SAFE-T–Nº115003 IMI-JU, Plan Estatal I+D+I 2013–2016 SAF2013–42962-R Plan Estatal de I+D+I 2013–2016 (SAF2013–42962-R) cofunded by Fondo Europeo de Desarrollo Regional (FEDER-"Una manera de hacer Europa"), AGAUR–2014SGR1303, and Tercel–RD/12/0019/0026 and RIC–RD12/0042/0027 (to LB), and FIS-PI13/02850 (to TP) from Instituto Salud Carlos III (ISCIII), Associació Esportiva Jordi Pitarque Ceprià and FIC-Fundación Jesús Serra to the Cardiovascular Research Chair-UAB (LB).

Received: April 20, 2015
Accepted after minor revision: June 5, 2015
Epub ahead of print: July 16, 2015
http://dx.doi.org/10.1160/TH15-04-0325
Thromb Haemost 2015; 114: ■■■

* Presented in abstract form at the XXIV Congress of the International Society of Thrombosis and Haemostasis, Amsterdam, July 2nd, 2013. Received a Young Investigator Award.

Introduction

High plasma cholesterol levels are a causal factor for endothelial dysfunction, vessel inflammation, early atherosclerotic disease, and ultimately for premature plaque rupture and thrombosis. Thus, patients with severe long-life exposure to hypercholesterolaemia are at high cardiovascular risk (HCVR) and particularly suited for the evaluation of HCVR markers, atherosclerotic triggers and biomarkers of vascular disease. Familial hypercholesterolaemia (FH) is caused by mutations in the low-density lipoprotein (LDL) receptor gene, causing very high levels of LDL-cholesterol (1) and premature atherosclerosis (2). Despite the use of statins these patients remain at risk for the development of coronary artery disease (CAD) (3). Indeed, we have previously shown using aortic-magnetic resonance imaging (MRI) a significantly higher atherosclerotic burden in asymptomatic FH-patients, when compared with age-matched non-FH subjects (4). Therefore, novel biomarkers and imaging methods must be investigated to identify HCVR determinants and refine cardiovascular risk prediction.

Thrombosis is a critical component of atherosclerosis and when it is totally occlusive leads to the presentation of the acute coronary syndromes (ACS). Tissue factor (TF) activation is one of the main triggers of thrombosis on atherosclerotic plaques (5) and it is present within the lipid-rich core components (6). In addition, activated platelets contribute to thrombogenicity triggering the coagulation cascade and the subsequent formation of fibrin (7, 8).

Circulating and vascular cells are capable of releasing vesicles to the extracellular medium in response to cell activation or cell apoptosis (9). Circulating microparticles (cMPs) are submicron extracellular vesicles of different cellular origin that contain phosphatidylserine and distinct proteins depending on their parental cells. These cMPs seem to confer risk through crosstalk with other vascular and circulating cells (9). cMPs are found in plasma of healthy subjects (10), and their numbers are considerably increased in patients with diverse cardiovascular risk factors (11, 12) and certain pathologies (13). Recently, we have demonstrated that FH patients, treated to guidelines and with subclinical atherosclerosis, have significant lymphocyte activation because $CD45^+/CD3^+/AV^+$-lymphocyte cMPs are found in large number and are able to predict subclinical atherosclerotic lesion type (12). However, in that study, thrombogenic cellular sources and prothrombotic characteristics of cMPs were not investigated. These thrombogenic cellular markers can provide novel insights into the

	HCVR patients (n=37)	Control patients (n=37)	P-value
Past medical history			
Cardiovascular disease (n, %)	5 (13.5 %)	0 (0 %)	P=0.021
Cancer (n, %)	0 (0 %)	0 (0 %)	P=0.999
Risk factors			
Hyperlipidemia (n, %)	37 (100 %)	37 (100 %)	P=0.999
Diabetes mellitus (n, %)	0 (0 %)	1 (2.7 %)	P=0.314
Systemic hypertension (n, %)	4 (10.8 %)	7 (18.9 %)	P=0.327
Current tobacco consumption (n, %)	13 (35.1 %)	12 (32.4 %)	P=0.806
Obesity (BMI>30) (n, %)	5 (13.5 %)	7 (18.9 %)	P=0.528
Waist diameter (cm) §Male (>102 cm) §Female (>88 cm)	2 (5.4 %) 5 (13.5 %)	5 (13.5 %) 8 (21.6 %)	 P=0.359 P=0.233
Gene mutation*	(21/15/1)	-	-
Biochemical data			
Total cholesterol (mg/dl)	223.0 (185.8–257.0)	210.0 (192.0–240.0)	P=0.482
Triglyceride (mg/dl)	82.0 (64.8–141.0)	82.0 (64.0–120.0)	P=0.729
LDL-cholesterol (mg/dl)	145.0 (124.3–175.0)	134.2 (109.0–159.6)	P=0.208
HDL-cholesterol (mg/dl)	49.0 (40.3–59.0)	55.0 (48.0–63.0)	P=0.031
Non-HDL-cholesterol (mg/dl)	168.0 (139.3–210.0)	159.0 (129.8–179.0)	P=0.200
Lp(a) (mg/dl)	32.5 (14.8–55.2)	21 (10.9–48.5)	P=0.326
TC/HDL-C ratio	4.5 (3.7–5.7)	3.9 (3.1–4.5)	P=0.021
High-sensitive C-reactive protein	1.5 (0.9–3.9)	0.5 (0.2–0.9)	P=0.028
Fasting plasma glucose (mg/dl)	85.5 (78.5–94.8)	86.0 (78.0–91.0)	P=0.914
Medication therapy			
ACEI	3 (8.1 %)	3 (8.1 %)	P=0.999
Angiotensin-II receptor blocker	1 (2.7 %)	1 (2.7 %)	P=0.999
Anti-platelet drugs	4 (10.8 %)	1 (2.7 %)	P=0.164
?-blockers	1 (2.7 %)	0 (0 %)	P=0.314
Statins	37 (100 %)	37 (100 %)	P=0.999
Ezetimibe	12 (32.4 %)	4 (10.8 %)	P=0.024
Lipid-lowering therapy time (years)	14 (6–17.5)	4 (3–10)	P=0.011
Target LDL-cholesterol†	4 (10.8 %)	17 (45.9 %)	P=0.001

Table 1: Clinical characteristics of HCVR and control patients. Values are given as median (IQR). *Gene mutation (null/defective/unknown), †Target LDL-cholesterol (FH≤100mg/dl, non-FH≤130mg/dl). HDL, high-density lipoprotein; ACEI, angiotensin-converting-enzyme inhibitor.

early prediction of cardiovascular disease presentation in HCVR patients. Therefore, the aim in this study was to investigate a) whether HCVR patients have increased prothrombotic cMPs; and, b) whether HCVR patients with atherosclerotic lipidic-rich lesions display a specific cMP phenotype in terms of TF and platelet activation markers.

Materials and methods

Clinical study population

The present nested case-control study comprised HCVR patients with clinical and genetic diagnosis of heterozygous FH (n=37) and non-FH adult secondary hypercholesterolaemic patients (n=37), matched by age, gender, demographics, and lipid-lowering therapy (LLT) with lower cardiovascular risk (►Table 1). Both groups were selected from the SAFEHEART cohort (12, 14). The subclinical atherosclerotic burden of FH cases had previously been characterised by aortic MRI (4). All selected subjects had been prescribed a LLT according to guidelines (15–17). Maximum statin dose was defined as previously described (18). Clinical data were obtained from all subjects using a standardised report form at the inclusion (►Table 1). Data related to LLT included statin type, dose, time and compliance. Adherence to LLT was assessed by indirect method with a single question (19). Patients were also classified depending on the known residual activity of the LDL-r as null or defective mutations. No patient present pregnancy or have past history of cancer, since these conditions are known to independently impair cMP number. The study was approved by the Local Ethics Committee of the Investigación Clínica Fundación Jimenez Diaz (CEIC-FJD) [protocol's number: 01/09], was conducted according to the Declaration of Helsinki and a written informed consent was obtained from all participants prior to the study. The results of the study are presented in accordance with STROBE guidelines.

Blood collection and sampling for analysis

Venous blood was withdrawn from the cubital vein without tourniquet using a 20-gauge needle after 10–14 hours (h) of fasting into serum, EDTA and 3.8%-sodium citrate tubes, for biochemical, genotyping and microparticle analysis, respectively.

Serum (30 minutes [min] at 37ºC, 30 min at 4ºC, 1258×g for 15 min at 4ºC) and EDTA-plasma (1258 ×g for 20 min) were prepared for lipid profile and DNA analysis, respectively. Samples were processed for assay within the first 2 h. Total cholesterol, triglycerides, and HDL-c were measured by standardised enzymatic methods; serum LDL-c concentration was calculated using the Friedewäld formula (20); lipoprotein (a) was measured using a turbidimetric method; and genetic diagnosis of FH was made using a DNA-microarray (LIPOCHIP) (21).

For flow cytometric analysis of cMPs, blood cells were removed by low-speed centrifugation at 1258×g for 20 min at room temperature (RT) (12, 22). Platelet poor plasma was carefully aspirated. A second centrifugation step was made to ensure the complete removal of cells and obtain the platelet-free plasma (PFP). All samples were processed identically and within 60 min after extraction. Samples were tested with a cell counter for the absence of residual cells after centrifugation. PFP aliquots were snap-frozen in liquid nitrogen and stored, for an identical time interval, at -80ºC until flow cytometric studies (12, 22).

Atherosclerotic plaque characterisation

Aortic MRI has been widely used to detect atherosclerotic burden in high-risk patients (23–26). The total burden of atherosclerosis measured by MRI had been investigated previously (4). Atherosclerotic plaque MRI evaluation and morphometric analysis was performed in FH cases at the level of the descending thoracic aorta by measuring mean arterial wall area (MAWA, mm^2) and plaque composition based on lipid plaque component and calcium content, as fully described (4). All HCVR patients that had atherosclerotic plaques in the carotid arteries (15% of the studied group) also had plaques in the aorta (4). The MRI study showed that (a) one third of atherosclerotic plaques detected in the HCVR patients were lipid-rich and (b) 30% of detected aortic plaques were also calcified (characterised by relevant calcium content) (4). Inter-observer variability was low and the intra-class correlation coefficient was of 0.735 (95% confidence interval [CI]: 0.591–0.833). This microparticle study has been performed in blood collected from the FH-patients at the time of the MRI analysis.

Circulating microparticle isolation

The cMP-fraction was isolated from PFP by a two-step high speed centrifugation (12, 22, 27). Briefly, PFP aliquots were thawed on melting ice for 1 h and centrifuged for 30 min (20,000×g, RT) to pellet cMPs. Then, supernatant was discarded and the cMP-pellet was washed once with citrate phosphate-buffered saline solution (citrate-PBS; 1.4mM phosphate, 154mM NaCl, 10.9mM trisodium citrate, pH 7.4) before a second equal centrifugation step was made. Finally, the remaining cMP-pellet was resuspended in citrate-PBS.

Flow cytometric analysis of circulating microparticles

Briefly, washed cMP suspensions diluted in $CaCl_2$-PBS buffer (2.5 mM) were incubated (20 min, RT, dark) with combinations of BD-horizon V450-conjugated annexin V with two specific monoclonal antibodies (MoAbs, see Suppl. Table 1, available online at www.thrombosis-online.com) before being analysed on a FACSCantoII™ flow cytometer (BD). Forward scatter (FSC), side scatter (SSC) and fluorescence data were obtained with gain settings in the logarithmic scale. cMPs were identified based on their FSC/SSC characteristics according to their size, binding to annexin V and reactivity to cell-specific MoAb. Gate limits were established using a Flow-Check YG Size-Range Calibration Kit (Polysciences, Eppelheim, Germany) and an *in vitro* platelet-derived microparticle population as positive control (Suppl. Figure 1, available online at www.thrombosis-online.com). 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. Annexin V binding level was corrected for autofluorescence using fluorescence signals obtained with microparticles in a calcium-free buffer. The lower detection limit was placed as a threshold above the electronic noise.

Data were analyzed with the FACSDiva™ software. The cMP concentration was determined according to Nieuwland's procedure (12, 22, 27), based on sample's volume, flow cytometer's flow rate and the number of fluorescence-positive events. Intra-assay coefficient of variation (CV) of cMP counts was 3.1%, while inter-assay CV was 5.4%.

MP-TF activity assay

TF-bearing microparticle procoagulant activity (PCA) potential was measured using a functional assay (Zymuphen MP-TF, Hyphen Biomed, Neuville-sur-Oise, France) with an automated microplate washer device (Revelation-Dsx 5.19, Dynex, Denkendorf, Germany). Briefly, TF^+-cMPs from citrated PFP were captured by a murine MoAb directed against the extracellular domain of TF. 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 FXa-specific substrate was added and absorbance was recorded at 405 nm. A lyophilised calibrator, containing recombinant relipidated TF with synthetic liposomes, enabled the standardisation of the assay. MP-TF concentration was established against an internal standard and expressed as TF antigen equivalent in pg/ml. Measurements were done in duplicate.

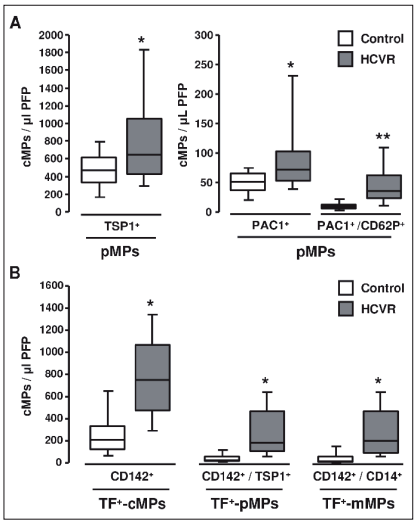


Figure 1: cMP activation and prothrombotic status in control and HCVR patients. Box and whisker plots of cMPs per µl PFP exposing (A) platelet activation markers and (B) TF-bearing cMPs in control and HCVR patients (n=37/group). Data are given as median (IQR). (A) *P<0.005, **P<0.001 and (B) *P<0.0001 vs control (U-Mann Whitney).

Statistical analysis

All data are presented as medians (interquartile range [IQR]) except when indicated. Frequencies of qualitative variables were compared between two groups by the Chi-square analysis and median values of quantitative variables with two-sided nonparametric tests. The statistical significances between two groups were determined with U-Mann Whitney and multiple comparisons by Kruskal Wallis, and when significant, Bonferroni post-hoc analysis was used to assess intergroup differences. Strength of the association between continuous variables was calculated by Spearman's correlation coefficients. To evaluate prognostic markers, associated receiver-operating characteristic (ROC) curve analyses and the corresponding areas under the curve (AUC) along with their 95% CI were calculated. Multivariable models for the prediction of lipidic plaques were performed with a binary logistic regression model with cMP levels and risk factor model by creating predicted probabilities, which then were transferred to the ROC curve algorithm to estimate the likelihood of a lipidic plaque by calculating the corresponding AUC along with their 95% CI. The comparison of different AUC from ROC analyses was carried out by applying the method previously described (28). StatView (5.0.1, Abacus Concepts, Picataway, NJ, USA) and SPSS Statistics (21.0.0, SPSS, IBM; Armonk, NY, USA) software were used for all tests and a value of $p<0.05$ was considered statistically significant.

Results

Clinical characteristics of the study population

Clinical characteristics of both HCVR and control patients are shown in ▶Table 1. Mean age at inclusion was 48.4 ± 1.7 years for HCVR patients (19 men / 18 women) and 49.1 ± 2.5 years for controls (14 men / 23 women). There were no significant differences in gender, mean age, body mass index (BMI), diabetes mellitus, hypertension, tobacco consumption, total, LDL- and non-HDL cholesterol, triglycerides, and Lp(a) levels. Control patients had significantly higher HDL-cholesterol levels ($p<0.05$). On the other hand, xanthomas and history of CVD were observed only in HCVR patients. Both groups were receiving LLT with statins according to guidelines (17).

Activated cell markers in cMPs

cMPs shed by activated platelets were significantly increased in HCVR patients (▶Figure 1A). Specifically, levels of platelet-de-

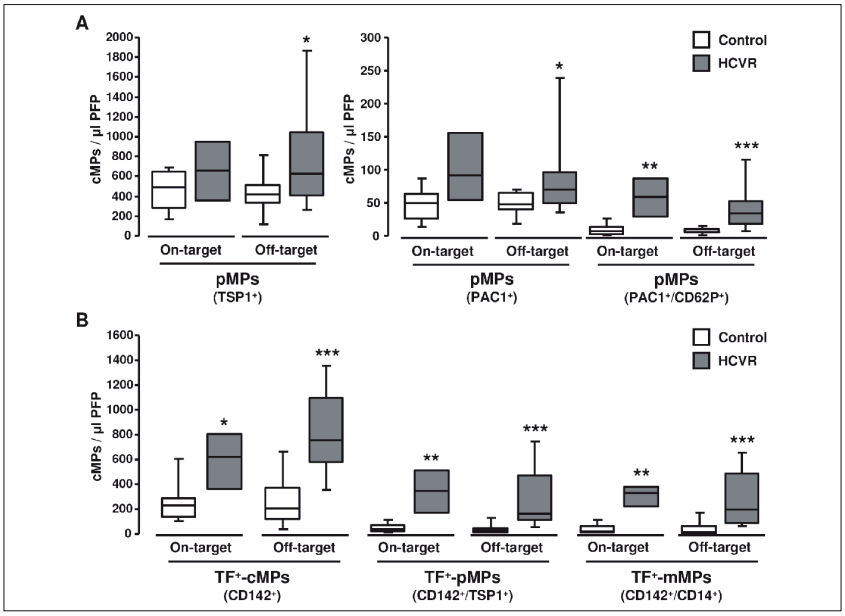


Figure 2: cMP profiling in regard to LDL-cholesterol levels. Box and whisker plots of (A) platelet activation markers and (B) TF+-cMPs (/µl PFP) in control and HCVR patients (n=37/group) classified in two groups: on- and off-target LDL-cholesterol levels (with clinical guidelines, 100 mg/dl and 130 mg/dl for control and HCVR patients, respectively). Data are given as median (IQR). *P<0.05, **P<0.0005, ***P<0.0001, vs control (U-Mann Whitney).

rived microparticles (pMPs) carrying thrombospondin-1 ($TSP1^+$), the activated $\alpha_{IIb}\beta_3$-integrin marker ($PAC1^+$) and both PAC1 and P-selectin markers together ($PAC1^+/CD62P^+$) were significantly increased in HCVR patients plasma compared to control patients ($p<0.005$, $p<0.005$ and $p<0.001$, respectively).

TF–bearing cMPs

cMPs carrying tissue factor ($CD142^+$-cMPs) were significantly higher in HCVR patients compared with controls (▶Figure 1B). Interestingly, higher number of activated platelet-derived (pMPs) and monocyte-derived (mMPs) cMPs were positive for TF in HCVR than in control patients ($p<0.0001$) (▶Figure 1B). No correlation was found between cMPs numbers and blood cell counts, implying higher cMP release per cell, and therefore, higher cell activation.

FH-specific microparticle profile is not related to LDL-cholesterol levels

There was no correlation between lipid levels at collection time and cMP numbers. Further, to identify whether LDL plasma levels were promoting MP shedding in HCVR patients, cMP results were analysed according to a) achievement of LDL target levels (▶Figure 2) and b) to median LDL levels (Suppl. Figure 2, available online at www.thrombosis-online.com). In HCVR patients, significant increases were found in cMPs carrying cell activation markers [thrombospondin-1 ($TSP1^+$), activated $\alpha_{IIb}\beta_3$-integrin marker ($PAC1^+$) and both $PAC1^+$ and P-selectin markers together ($PAC1^+/CD62P^+$)] (▶Figure 2A and Suppl. Figure 2A, available online at www.thrombosis-online.com) and TF^+-cMPs, TF^+-pMPs and TF^+-mMPs (▶Figure 2B and Suppl. Figure 2B, available online at www.thrombosis-online.com), independently of LDL-cholesterol. These results indicate that cMPs were not related to LDL-cholesterol levels at the moment of sampling but they are markers of cell damage and activation in the circulatory tree.

Circulating microparticle-associated tissue factor activity and atherosclerotic burden

Atherosclerotic plaques express TF that triggers coagulation and thrombosis. Therefore, we focused on the measurement of cMP-associated TF-PCA in these HCVR patients. We tested the isolated TF$^+$(CD142$^+$)-microparticle preparation and evidenced that these cMPs are able to trigger TF-FXa induced coagulation activity (0.9 ± 0.1 pg TF/ml). The presence of extended atherosclerotic burden (carotid and aortic plaques) correlated significantly with MP-TF-PCA (►Figure 3A). Accordingly, MP-associated TF-PCA correlated significantly to level of cMPs exposing TF antigen (CD142$^+$) in these patients (►Figure 3B).

Framingham Risk Score (FRS) for coronary heart disease (% of risk at 10 years) was calculated using the high-risk Framingham Heart Study equations of the NCEP (15). CD142$^+$-cMPs did not show a significant correlation with FRS (►Figure 3C). Patients were then stratified according to atherosclerotic plaque burden and FRS did not significantly differentiate between patients with only aortic and aortic plus carotid disease (►Figure 3D).

Atherosclerotic plaque composition and cMPs in HCVR patients

The presence of plaques with high lipid content was significantly and directly associated with increased levels of

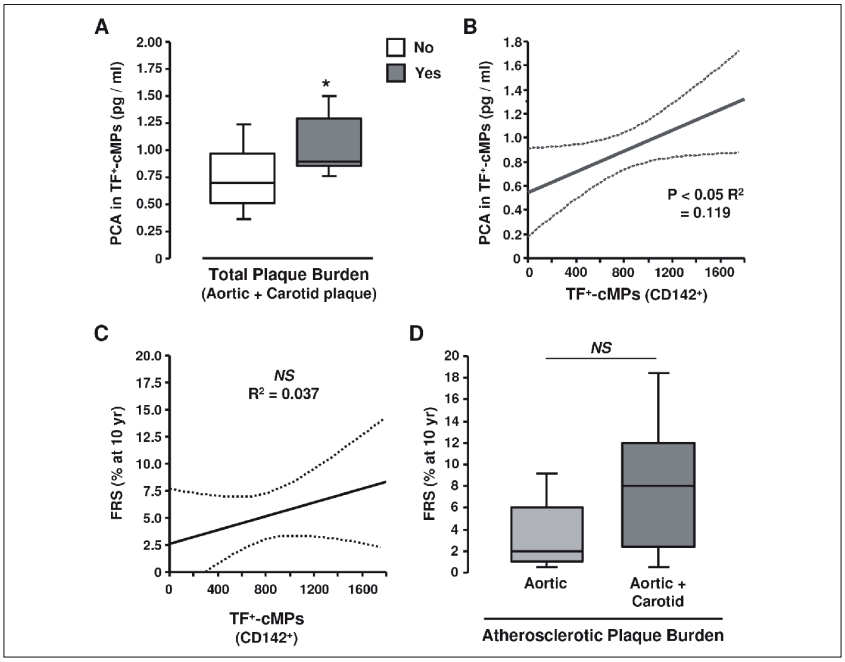


Figure 3: Prevalence of atherosclerotic plaque burden related to microparticle-associated TF activity and Framingham Risk Score in the HCVR patients. A) Box and whisker plot of PCA in TF$^+$-cMPs (pg TF/ml) corresponding to the MRI-studied HCVR population (n=36) in regard to total atherosclerotic plaque burden (presence of both carotid and aortic plaques, n=7). *P<0.05 (U-Mann Whitney). B) Linear regression plot of cMP levels (/µl PFP) carrying TF antigen (CD142$^+$) that correlated with MP-associated TF-PCA in HCVR patients (n=37) (Spearman correlation). C) Linear regression plot of TF$^+$-cMP levels (CD142$^+$-cMP /µl PFP) and Framingham Risk Score (FRS) at 10 years (%) (n=37) (Spearman correlation). D) Box and whisker plot of FRS at 10 years (%) associated to atherosclerotic plaque burden in HCVR patients (n=36). Data are given as median (IQR). NS, non-significant (U-Mann Whitney).

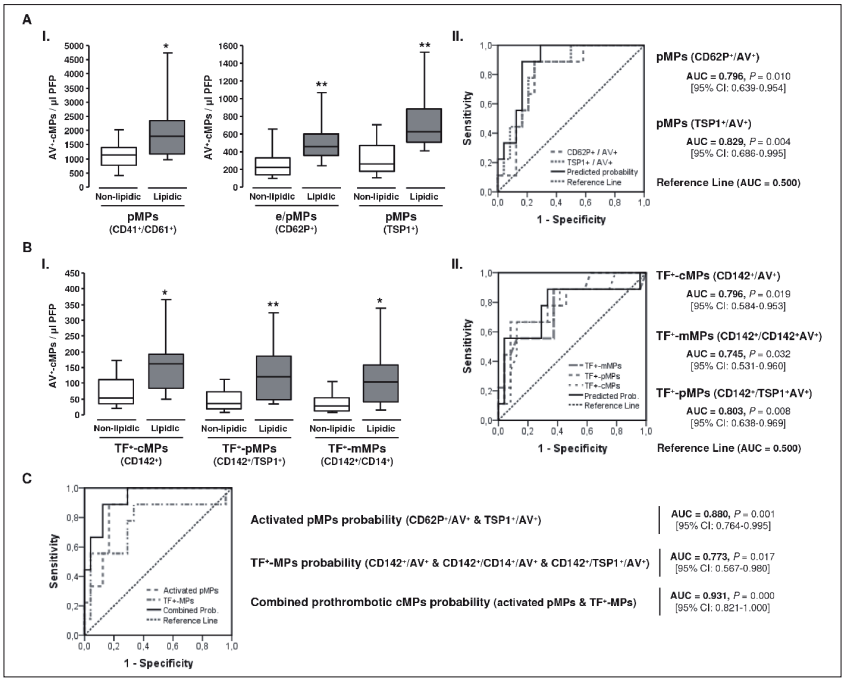


Figure 4: Annexin V-positive levels of cMPs according to atherosclerotic plaque composition in HCVR patients. (I) Box and whisker plots showing AV⁺-cMP numbers (/µl PFP) corresponding to (A) cMPs with platelet activation markers and (B) TF-bearing cMPs related to atherosclerotic plaque characterisation: non-lipidic and lipidic, in HCVR patients (n=36). Data are given as median (IQR). *P<0.05 and **P<0.01 vs non-lipidic group (U-Mann Whitney). (II) ROC curve analyses used to evaluate (A) activated pMPs, (B) TF+-cMPs and (C) combined effects as predictors of lipidic content in HCVR patients with atherosclerotic plaque burden with AUC indicated along with their 95 %CI (p<0.05).

AV⁺-pMPs from total platelets (CD41⁺/CD61⁺) and activated platelets (TSP1⁺ and CD62P⁺) when compared to non-lipidic plaques (p<0.05 in all cases, ▶Figure 4A-I). CD62P⁺-AV⁺-MPs are derived from both platelet and endothelial origin. Total endothelial-derived MPs were previously investigated and found to be significantly raised in FH compared to non-FH subjects and hence here are not addressed (12). Similarly, TF-rich AV⁺-cMPs (p<0.05) were higher in patients with lipid-rich than with non-lipid-rich atherosclerotic plaques (▶Figure 4B-I), both from platelet and monocyte origin (p<0.01 and p<0.05, respectively). Therefore, activated AV⁺-pMPs and AV⁺-TF⁺-cMPs in blood mapped the presence of lipid-rich plaques. Since these prothrombotic cMPs were the type of cMPs that most changed according to HCVR condition and lipid-rich plaques in the study, ROC curve analyses were conducted to identify the best cMP determination that was capable of predicting atherosclerotic plaque. ROC curves were determined for activated pMPs (CD62P⁺ and TSP1⁺) individually and in combination (▶Figure 4A-II) and for TF-rich cMPs, pMPs and mMPs (▶Figure 4B-II) also individually and in combination. Finally, a comparison of ROC curves for predicted probabilities of activated platelet-derived microparticles and TF-containing microparticles was performed (▶Figure 4C). cMPs derived from activated platelets exhibited a higher AUC (0.880 ± 0.059 [95 %CI: 0.764-.995]; p=0.001) than TF-rich cMPs. Moreover, among TF-rich MPs those derived from activated platelets gave the maximum AUC (0.0803 ± 0.084 [95 %CI: 0.638–0.969]; p=0.008). Finally, clustering all types of prothrombotic cMPs displayed the

highest prediction for lipid-rich plaques (AUC of 0.931 ± 0.04 [95%CI: 0.821–1.000], p< 0.001, ▶Figure 4C).

Levels of AV^+-cMPs were significantly lower in HCVR patients with calcified plaques (▶Figure 5A). There was a significant reduction in AV^+-pMPs ($CD41^+/CD61^+$), AV^+-e/pMPs expressing P-selectin ($CD62P^+$), and TF-bearing AV^+-cMPs ($CD142^+$) and AV^+-pMPs ($CD142^+/TSP1^+$). These data show that cMP shedding from activated platelets and vascular cells occurs when plaques are not yet calcified (less than type-VII following AHA) and hence at a higher risk of rupture. In a similar fashion, mean aortic wall area (MAWA; ▶Figure 5B) inversely correlated with percentage of AV^+-cMPs. A significant negative association between MAWA and AV^+-pMPs carrying activation epitopes of activated $\alpha_{IIb}\beta_3$-integrin and P-selectin ($PAC1^+/CD62P^+$) as well as TF-rich AV^+-mMPs ($CD142^+/CD14^+$) was found (▶Figure 5B).

Incremental prognostic value of prothrombotic cMPs for lipid-rich atherosclerotic plaque prediction

Since existing risk scores were not efficient in discriminating CV risk (▶Figure 3C and ▶Figure 6A), we hypothesise that the inclusion of cMPs could add value to currently used risk algorithms. To this aim, we performed ROC analysis with the combination of major risk factors normally used in risk prediction (LDL-cholesterol, CRP, LDL/HDL ratio and systolic blood pressure [SBP]) with and without prothrombotic MPs. Inclusion of the prothrombotic cMPs (AV^+-cMPs derived from activated platelets [$TSP1^+$, $CD62P^+$] and rich in TF [$CD142^+$, $CD142^+/TSP1^+$ and $CD142^+/CD14^+$]) into a risk factor cluster model for the prediction of lipid-rich plaques increased the AUC from 0.716 ± 0.097 [95%CI: 0.526–0.906] to 0.955 ± 0.037 [95%CI: 0.883–1.000] (p=0.0213, ▶Figure 6A), indicating that cMPs display a higher degree of discrimination for plaque type than the commonly used classical risk factor prediction parameters.

Discussion

High plasma cholesterol levels are a causal factor for atherothrombosis and CVD. This study demonstrates that HCVR patients have a significantly higher number of cMPs carrying markers of cell activation and TF than control patients. Indeed, HCVR patients with atherosclerotic plaque burden have cMPs rich in TF that is biologically active and capable of triggering procoagulant activity. Fur-

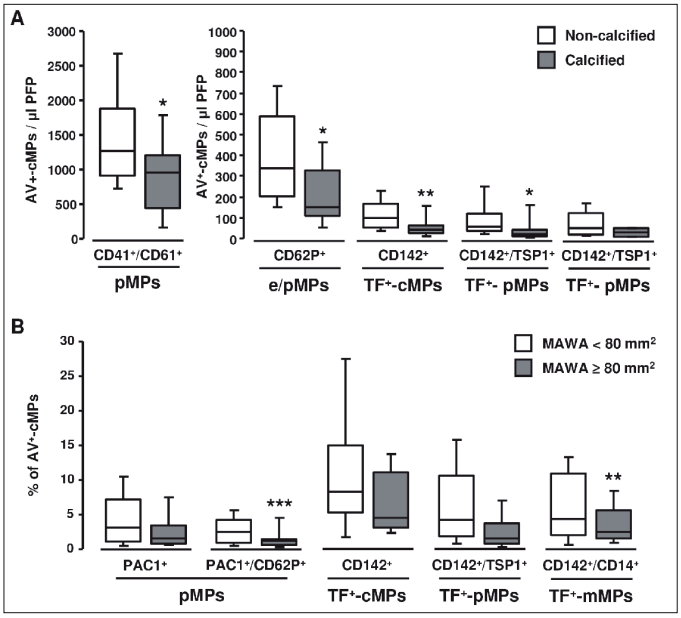


Figure 5: cMPs in HCVR according to aortic plaque characterisation. Box and whisker plots showing (A) AV^+-cMPs levels (/µl of PFP) depending on atherosclerotic plaque calcium content, (B) percentage of AV^+-cMPs depending on mean aortic wall area (MAWA), and (C) MAWA in association with LLT time in HCVR patients (n=36). Data are given as median (IQR). *P< 0.02, **P< 0.03, ***P< 0.05, and ****P< 0.001 (U-Mann Whitney).

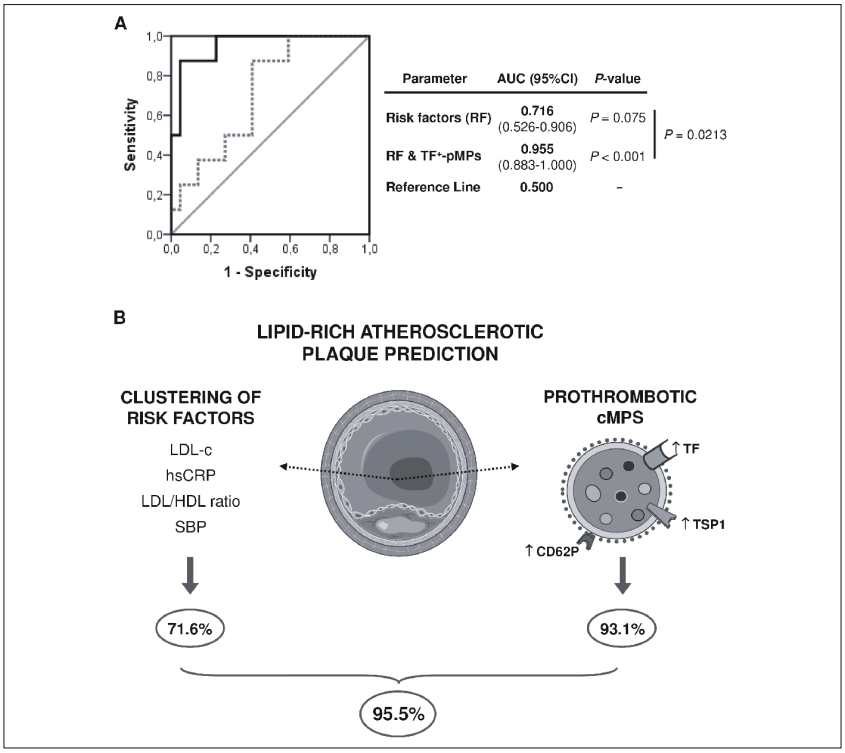


Figure 6: Prognostic value of prothrombotic cMPs in lipid-rich atherosclerotic plaque prediction. A) C-statistics of the incremental effect of: prothrombotic cMPs (TSP1$^+$, CD62P$^+$, CD142$^+$, CD142$^+$/TSP1$^+$, and CD142$^+$/CD14$^+$, solid line) in addition to the traditional risk factor model (including LDL-c, CRP, LDL/HDL ratio, and SBP; dotted lines) for the prediction of lipid-rich atherosclerotic plaques. CRP, C-reactive protein; SBP, systolic blood pressure. B) Representative figure showing that in HCVR patients, prothrombotic cMPs display a high discrimination power for the prediction of lipid-rich atherosclerotic plaques and add incremental value to the current risk prediction models based on risk factors. cMPs, circulating microparticles; HDL, high-density lipoprotein; LDL, low-density lipoprotein; hsCRP, high-sensitivity C-reactive protein; PS, phosphatidylserine; SBP, systolic blood pressure; TF, tissue factor; TSP, thrombospondin.

thermore, significantly elevated activated platelet-derived cMPs and TF-rich cMPs are found in the circulation of well-treated HCVR patients with lipid-rich plaques. This increased amount of cMPs in the HCVR patients indicates a chronic burden of vascular cell activation, subclinical atherosclerosis and premature CVD.

Extracellular vesicle (EV) nomenclature is still being defined and refined by the research community associated with their biogenesis, uptake and other processes. EVs are a heterogeneous group and the terminology used in this emerging field has included a variety of names. Fortunately, the recently launched International Society of Extracellular Vesicles seems to have reached a general consensus by using EVs to describe all classes of extracellular membrane vesicles including apoptotic bodies, microparticles/microvesicles, and exosomes. In this regard, shed particles originated by direct budding from the plasma membrane have been specifically designated as either "microvesicles", "ectosomes" or "microparticles". Over the past decades, these structures have been traditionally referred to as "microparticles" in most studies

within the cardiovascular field (29) and thus the term "microparticle" has been used throughout the manuscript.

Atherosclerosis is considered to be an inflammatory disease in which not only monocytes as a whole but also monocyte heterogeneity plays a key role in all stages of the disease (30). Different subsets of circulating monocytes can be distinguished into classical monocytes ($CD14^{++}CD16^{-}$), intermediate monocytes ($CD14^{++}CD16^{+}$) and non-classical monocytes ($CD14^{+}CD16^{++}$), the latter two being considered as pro-inflammatory cells and intermediate monocytes being independent predictors of cardiovascular events (31). Recently plasma levels of LDL particles have been associated with non-classical monocytes (32) and, thus, investigating cMPs-derived from these specific cells deserve further research. However, the present manuscript has focused on the study of prothrombotic rather than pro-inflammatory cMP characteristics, which were previously investigated in patients that are at particularly high-risk of atherothrombotic events (12).

HCVR patients show enhanced platelet activation as evidenced by significantly high numbers of pMPs carrying activated platelets markers, which 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 (33). Platelets become activated because of the chronic hypercholesterolaemia and the ensuing inflammatory processes (34). pMPs may contribute to the development or progression of atherosclerosis and premature CAD in HCVR patients by several mechanisms. Indeed, different reports have described that pMPs can enhance the expression of adhesion molecules on monocytes and endothelial cells (35), induce PCA (36), promote thrombin generation (37) and interact with leukocytes (38).

TF-positive microparticles were found in higher numbers in HCVR patients than in the control group. TF^{+}-cMPs are likely to have different activity depending on their cellular source. We have detected both monocyte and platelet-derived microparticles rich in TF. 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 (39), others reported that platelets express significant levels of TF mRNA and protein (40). In contrast, there is agreement on monocytes as the most important source of TF^{+}-cMPs (41). TF in platelets could be the result of plasma transfer, expression or even be acquired by internalisation of mMPs (42, 43). cMP-associated TF activity had been mainly attributed to mMPs (37, 44). Our data indicate that HCVR patients have an increased level of monocyte- but also platelet-derived TF^{+}-MPs, which are functional in terms of PCA and correlate with atherosclerotic plaque burden, suggesting that TF^{+}-cMPs might be causal effectors of atherothrombosis. Indeed, we show that levels of TF^{+}-MPs together with pMPs derived from activated platelets could predict the presence of lipidic plaques in asymptomatic HCVR patients.

The biological significance of TF^{+}-cMPs, further to their biomarker value, may be the amplification of TF effects by propagation to distant areas and vascular territories. Following this hypothesis activated cell released TF^{+}-cMPs may accumulate in the circulation and induce TF-driven functions (45), including TF-PCA and thrombosis in distant areas with vascular lesions (46). Recently, increased plasma TF^{+}-MPs were found in hyperlipidemic mice and monkeys as well as in severe FH patients who required LDL-aphaeresis (47). 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.

The continuous exposure to high LDL levels in FH patients confers them a very high susceptibility to premature CAD that is poorly detected in the existing risk scores. Here we have identified that MP assessment can help in the risk prediction. Indeed, TF^{+}-pMPs ($TSP^{+}/CD142^{+}$) showed to be useful predictors of lipid-rich atherosclerotic plaques by the area under the ROC curve. Interestingly, when c-statistics was measured combining predicted probabilities for TF-rich MPs and activated platelet-derived MPs together (best AUC of 0.931 ± 0.04) we improved the prediction of lipidic plaques and when these prothrombotic cMPs were added to a risk factor model the discrimination capacity significantly increased to 0.955 ± 0.04 (▶ Figure 6). Thus, MPs arise as promising predictors of subclinical atherosclerosis that may add incremental value to currently used risk prediction models.

To the best of our knowledge, this is the first study to identify a robust relationship between prothrombotic cMPs, TF content and

What is known about this topic?

- Platelet-derived microparticles enhance platelet adhesion and thrombus formation on both damaged vessel wall and atherosclerotic lesions.
- Patients with heterozygous familial hypercholesterolaemia (FH), despite being under lipid-lowering therapy as per guidelines, are at high cardiovascular risk (HCVR), develop premature coronary artery disease and display high shedding of circulating lymphocyte-derived $CD45^{+}/CD3^{+}$-microparticles.
- Although current cardiovascular risk algorithms such as Framingham risk score provide reliable information on cardiovascular risk, there is room for improvement and identification of novel biomarkers signalling for subclinical atherosclerosis are needed to allow the identification of patients at high cardiovascular risk.

What does this paper add?

- HCVR patients have higher number of cMPs from activated platelets and bearing tissue factor than controls, which are not dependent on plasma LDL levels but on life-long vascular exposure to high LDL-cholesterol levels and can influence the course of atherosclerotic disease.
- The specific HCVR-FH-cMPs displaying a prothrombotic phenotype directly associates to lipid-rich atherosclerotic plaques.
- Prothrombotic cMPs have an incremental prognostic value beyond a risk factor model for the prediction of lipid-rich atherosclerotic plaque burden and, hence, high cardiovascular risk, which should be expanded to prospective clinical studies and confirmed in a larger cohort of patients.

in vivo measured atherosclerotic burden. Lipidic plaques were found to correlate positively and significantly with high levels of TF^+-cMPs and pMPs carrying activated cell epitopes. In contrast, lower levels of cMPs are found in patients with calcified atherosclerotic plaques and large mean aortic wall area, which were found to be mainly fibrotic and characteristic of non-lipidic lower risk plaques (4), indicating the long evolution of atherosclerotic disease. In contrast, Jayachandran et al. (48) reported increased MPs in a low-risk population of menopausal women with high coronary artery calcification score. However, the intensities of calcification of these women were low, suggesting that cMPs might map early calcification in this context. When analysing the burden of calcium in the arteries we must bear in mind the multifactorial nature of the coronary calcification process. Indeed, a recent study has highlighted that MP-PCA was higher in moderate calcified plaque group of patients compared to non-calcified and totally calcified groups (49). Interestingly, the PROSPECT study showed that calcification *per se* may not cause coronary events and other clinical studies have shown that, in fact, calcification might confer stability to the established atherosclerotic plaques. Thus, calcified plaques in itself seem to be markers of stability rather than vulnerability.

Lipid-rich plaques accumulate lipids and inflammatory cells what may explain the changes observed in the activated cMPs. Additionally lipid infiltration in the vessel wall induces TF release from vascular resident cells (8). The state of activation of the cells of the blood-vascular interface amplifies the shedding of MPs that are markers of the high atherothrombotic risk of these patients. The burden in prothrombotic cMPs, such as TF-positive and activated pMPs, could 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. Accordingly, we have recently identified increased circulating lymphocyte-MPs in patients with atherosclerotic plaque burden (12), highlighting both their role in the early phases of atherogenesis and their potential use as biomarkers of subclinical atherosclerosis. Thus, thrombogenic cMP characterisation could be an easy method to predict thrombotic risk in asymptomatic patients. It is conceivable that cMPs are not merely markers of cell activation in the circulation but also direct inducers of atherothrombosis. Recently, we have reported that high levels of cMPs and, specifically, those derived from platelets, increase platelet deposition on atherosclerotic plaques (36, 50).

Taken together, this proof of principle study with patients from a large cohort study, and with demonstration of incremental value for specific cMPs even in a moderate number of patients, indicates that increased prothrombotic cMP release contribute to the increased atherothrombotic risk of HCVR patients and to atherosclerosis. In conclusion, cMPs may serve as potent biomarkers of silent atherothrombotic disease and help to predict cardiovascular risk (▶ Figure 6B).

Acknowledgements

The authors thank Dr. R Nieuwland and Chi M. Hau for the exceptional expertise, Dr. J. Crespo for his help in flow cytometry, Marta Guindo for the technical support, Maria Teresa Puig and Ignasi Gich for helpful advice in statistics, and the Spanish Familial Hypercholesterolaemia Foundation (FHF) and physicians that contribute to the FHF-patient cohort SAFEHEART recruitment. Authors are indebted to the FH patients and relatives for their valuable contribution and willingness to participate.

Conflict of Interest

None declared.

Abbreviations

ACS = acute coronary syndromes; AUC = area under the curve; AV = annexin V; CAC = coronary artery calcification; CAD = coronary artery disease; cMPs = circulating microparticles; CV = coefficient of variation; CVD = cardiovascular disease; FH = familial hypercholesterolaemia; FRS = Framingham risk score; HCVR = high cardiovascular risk; HDL = high-density lipoprotein; IQR = interquartile range; LDL = low-density lipoprotein; LDL-r = low-density lipoprotein receptor; LLT = lipid-lowering therapy; MAWA = mean arterial wall area; mMPs = monocyte-derived microparticles; MoAb = monoclonal antibody; MRI = magnetic resonance imaging; NCEP = National Cholesterol Education Program; PBS = phosphate-buffered saline; PCA = procoagulant activity; PFP = platelet-free plasma; pMPs = platelet-derived microparticles; ROC = receiver-operating characteristic curve; RT = room temperature; SAFEHEART = SpAnish Familial hypErcHolEsterolaemiA cohoRt sTudy; TF = tissue factor; TSP1 = thrombospondin-1.

References

1. Hovingh GK, Davidson MH, Kastelein JJ, et al. Diagnosis and treatment of familial hypercholesterolaemia. Eur Heart J 2013; 34: 962–971.
2. Neefjes LA, Ten Kate GJ, Alexia R, et al. Accelerated subclinical coronary atherosclerosis in patients with familial hypercholesterolaemia. Atherosclerosis 2011; 219: 721–727.
3. Gill PJ, Harnden A, Karpe F. Familial hypercholesterolaemia. Br Med J 2012; 344: e3228.
4. Caballero P, Alonso R, Rosado P, et al. Detection of subclinical atherosclerosis in familial hypercholesterolaemia using non-invasive imaging modalities. Atherosclerosis 2012; 222: 468–472.
5. Toschi V, Gallo R, Lettino M, et al. Tissue factor modulates the thrombogenicity of human atherosclerotic plaques. Circulation 1997; 95: 594–599.
6. Fernandez-Ortiz A, Badimon JJ, Falk E, et al. Characterisation of the relative thrombogenicity of atherosclerotic plaque components: implications for consequences of plaque rupture. J Am Coll Cardiol 1994; 23: 1562–1569.
7. Badimon L, Storey RF, Vilahur G. Update on lipids, inflammation and atherothrombosis. Thromb Haemost 2011; 105 (Suppl 1): S34–42.
8. Llorente-Cortes V, Otero-Vinas M, Camino-Lopez S, et al. Aggregated low-density lipoprotein uptake induces membrane tissue factor procoagulant activity and microparticle release in human vascular smooth muscle cells. Circulation 2004; 110: 452–459.
9. Boulanger CM. Microparticles, vascular function and hypertension. Curr Opin Nephrol Hypertens 2010; 19: 177–180.
10. Berckmans RJ, Nieuwland R, Boing AN, et al. Cell-derived microparticles circulate in healthy humans and support low grade thrombin generation. Thromb Haemost 2001; 85: 639–646.

11. Koga H, Sugiyama S, Kugiyama K, et al. Elevated levels of VE-cadherin-positive endothelial microparticles in patients with type 2 diabetes mellitus and coronary artery disease. J Am Coll Cardiol 2005; 45: 1622–1630.
12. Suades R, Padro T, Alonso R, et al. Circulating CD45+/CD3+ lymphocyte-derived microparticles map lipid-rich atherosclerotic plaques in FH patients. Thromb Haemost 2014; 111: 111–121.
13. Sinning JM, Losch J, Walenta K, et al. Circulating CD31+/Annexin V+ microparticles correlate with cardiovascular outcomes. Eur Heart J 2010; 32: 2034–2041.
14. Mata N, Alonso R, Badimon L, et al. Clinical characteristics and evaluation of LDL-cholesterol treatment of the Spanish Familial Hypercholesterolaemia Longitudinal Cohort Study (SAFEHEART). Lipids Health Dis 2011; 10: 94.
15. Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report. Circulation 2002; 106: 3143–3421.
16. Wierzbicki AS, Humphries SE, Minhas R. Familial hypercholesterolaemia: summary of NICE guidance. Br Med J 2008; 337: a1095.
17. Reiner Z, Catapano AL, De Backer G, et al. ESC/EAS Guidelines for the management of dyslipidaemias: the Task Force for the management of dyslipidaemias of the European Society of Cardiology (ESC) and the European Atherosclerosis Society (EAS). Eur Heart J 2011; 32: 1769–1818.
18. Pijlman AH, Huijgen R, Verhagen SN, et al. Evaluation of cholesterol lowering treatment of patients with familial hypercholesterolaemia: a large cross-sectional study in The Netherlands. Atherosclerosis 2010; 209: 189–194.
19. Gehi AK, Ali S, Na B, et al. Self-reported medication adherence and cardiovascular events in patients with stable coronary heart disease: the heart and soul study. Arch Intern Med 2007; 167: 1798–1803.
20. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clin Chem 1972; 18: 499–502.
21. Mozas P, Castillo S, Tejedor D, et al. Molecular characterisation of familial hypercholesterolaemia in Spain: identification of 39 novel and 77 recurrent mutations in LDLR. Hum Mutat 2004; 24: 187.
22. Suades R, Padro T, Alonso R, et al. Lipid-lowering therapy with statins reduces microparticle shedding from endothelium, platelets and inflammatory cells. Thromb Haemost 2013; 110: 366–377.
23. Corti R, Fuster V. Imaging of atherosclerosis: magnetic resonance imaging. Eur Heart J 2011; 32: 1709–1719b.
24. Vilades Medel D, Leta Petracca R, Carreras Costa F, et al. Coronary computed tomographic angiographic findings in asymptomatic patients with heterozygous familial hypercholesterolaemia and null allele low-density lipoprotein receptor mutations. Am J Cardiol 2013; 111: 955–961.
25. Teis A, Cadena A, Alomar X, et al. Quadricuspid pulmonic valve: diagnosis by MRI. Eur Heart J Cardiovasc Imaging 2013; 14: 398.
26. Carreras F, Garcia-Barnes J, Gil D, et al. Left ventricular torsion and longitudinal shortening: two fundamental components of myocardial mechanics assessed by tagged cine-MRI in normal subjects. Int J Cardiovasc Imaging 2012; 28: 273–284.
27. Rank A, Liebhardt S, Zwirner J, et al. Circulating microparticles in patients with benign and malignant ovarian tumors. Anticancer Res 2012; 32: 2009–2014.
28. Hanley JA, McNeil BJ. A method of comparing the areas under receiver operating characteristic curves derived from the same cases. Radiology 1983; 148: 839–843.
29. Loyer X, Vion AC, Tedgui A, et al. Microvesicles as cell-cell messengers in cardiovascular diseases. Circ Res 2014; 114: 345–353.
30. Hristov M, Weber C. Differential role of monocyte subsets in atherosclerosis. Thromb Haemost 2011; 106: 757–762.
31. Rogacev KS, Cremers B, Zawada AM, et al. CD14++CD16+ monocytes independently predict cardiovascular events: a cohort study of 951 patients referred for elective coronary angiography. J Am Coll Cardiol 2012; 60: 1512–1520.
32. Krychtiuk KA, Kastl SP, Pfaffenberger S, et al. Association of Small Dense LDL Serum Levels and Circulating Monocyte Subsets in Stable Coronary Artery Disease. PLoS One 2015; 10: e0123367.
33. Badimon L, Vilahur G. Thrombosis formation on atherosclerotic lesions and plaque rupture. J Intern Med 2014; 276: 618–632.
34. Chen K, Li W, Major J, et al. Vav guanine nucleotide exchange factors link hyperlipidemia and a prothrombotic state. Blood 2011; 117: 5744–5750.
35. Nomura S, Tandon NN, Nakamura T, et al. High-shear-stress-induced activation of platelets and microparticles enhances expression of cell adhesion molecules in THP-1 and endothelial cells. Atherosclerosis 2001; 158: 277–287.
36. Suades R, Padro T, Vilahur G, et al. Circulating and platelet-derived microparticles in human blood enhance thrombosis on atherosclerotic plaques. Thromb Haemost 2012; 108: 1208–1219.
37. Van Der Meijden PE, Van Schilfgaarde M, Van Oerle R, et al. Platelet- and erythrocyte-derived microparticles trigger thrombin generation via factor XIIa. J Thromb Haemost 2012; 10: 1355–1362.
38. Forlow SB, McEver RP, Nollert MU. Leukocyte-leukocyte interactions mediated by platelet microparticles under flow. Blood 2000; 95: 1317–1323.
39. Butenas S, Bouchard BA, Brummel-Ziedins KE, et al. Tissue factor activity in whole blood. Blood 2005; 105: 2764–2770.
40. Camera M, Brambilla M, Toschi V, et al. Tissue factor expression on platelets is a dynamic event. Blood 2010; 116: 5076–5077.
41. Osterud B, Bjorklid E. Sources of tissue factor. Semin Thromb Hemost 2006; 32: 11–23.
42. Camera M, Brambilla M, Facchinetti L, et al. Tissue factor and atherosclerosis: not only vessel wall-derived TF, but also platelet-associated TF. Thromb Res 2012; 129: 279–284.
43. Del Conde I, Shrimpton CN, Thiagarajan P, et al. Tissue-factor-bearing microvesicles arise from lipid rafts and fuse with activated platelets to initiate coagulation. Blood 2005; 106: 1604–1611.
44. Nieuwland R. Cellular origin of microparticles exposing tissue factor in cancer: a mixed double? J Thromb Haemost 2008; 6: 1514–1516.
45. Aberg M, Siegbahn A. Tissue factor non-coagulant signaling – molecular mechanisms and biological consequences with a focus on cell migration and apoptosis. J Thromb Haemost 2013; 11: 817–825.
46. Kleinjan A, Boing AN, Sturk A, et al. Microparticles in vascular disorders: how tissue factor-exposing vesicles contribute to pathology and physiology. Thromb Res 2012; 130 (Suppl 1): S71–73.
47. Owens AP, 3rd, Passam FH, Antoniak S, et al. Monocyte tissue factor-dependent activation of coagulation in hypercholesterolaemic mice and monkeys is inhibited by simvastatin. J Clin Invest 2012; 122: 558–568.
48. Jayachandran M, Litwiller RD, Owen WG, et al. Characterisation of blood borne microparticles as markers of premature coronary calcification in newly menopausal women. Am J Physiol Heart Circ Physiol 2008; 295: H931-H938.
49. Del Turco S, Basta G, Mazzarisi A, et al. Procoagulant activity of circulating microparticles is associated with the presence of moderate calcified plaque burden detected by multislice computed tomography. J Geriatr Cardiol 2014; 11: 13–19.
50. Mause SF. Platelet microparticles: reinforcing the hegemony of platelets in atherothrombosis. Thromb Haemost 2012; 109: 5–6.

1) Supplemental Methods

Clinical study population

The present nested case-control study comprised high cardiovascular risk (HCVR)-patients with clinical and genetic diagnosis of heterozygous familial hypercholesterolemia (FH) (n=37) and non-FH adult secondary hypercholesterolemic patients (n=37), matched by age, gender, demographics, and lipid-lowering therapy (LLT) with lower cardiovascular risk (Table 1). Both groups were selected from the SAFEHEART cohort (Mata *et al.,* 2011; Suades *et al.,* 2014). The subclinical atherosclerotic burden of FH cases had previously been characterized by aortic magnetic resonance imaging (MRI) (Caballero *et al.,* 2012). All selected subjects had been prescribed a LLT according to guidelines (NCEP, 2002; Wierzbicki, Humphries and Minhas, 2008; Reiner *et al.,* 2011). Maximum statin dose was defined as previously described (Pijlman *et al.,* 2010). Clinical data were obtained from all subjects using a standardized report form at the inclusion (Table 1). Data related to LLT included statin type, dose, time and compliance. Adherence to LLT was assessed by indirect method with a single question (Geji *et al.,* 2007). Patients were also classified depending on the known residual activity of the LDL-r as null or defective mutations. No patient present pregnancy or have past history of cancer, since these conditions are known to independently impair cMP number. The study was approved by the local ethics committee, was conducted according to the Declaration of Helsinki and a written informed consent was obtained from all participants prior to the study. The results of the study are presented in accordance with STROBE guidelines.

Blood collection and sampling for analysis

Venous blood was withdrawn from cubital vein without tourniquet using a 20-gauge needle after 10-14 hours of fasting into serum, EDTA and 3.8%-sodium citrate tubes, for biochemical, genotyping and microparticle analysis, respectively.

Serum (30 minutes [min] at 37ºC, 30 min at 4ºC, 1258×g for 15 min at 4ºC) and EDTA-plasma (1258 ×g for 20 min) were prepared for lipid profile and DNA and microRNA analysis, respectively. Samples were processed for assay within first 2 h. Total cholesterol, triglycerides, and HDL-c were measured by standardized enzymatic methods; serum LDL-c concentration was calculated using the Friedewäld formula; (Friedewald, Levy and Fredrickson, 1972) lipoprotein (a) was measured using a turbidimetric method; and genetic diagnosis of FH was made using a DNA-microarray (LIPOCHIP) (Mozas *et al.,* 2004).

For flow cytometric analysis of cMPs, blood cells were removed by low-speed centrifugation at 1258×g for 20 minutes at room temperature (RT) (Suades *et al.,* 2013, 2014). Platelet poor plasma was carefully aspirated. A second centrifugation step was made to ensure the complete removal of cells and obtain the platelet-free plasma (PFP). All samples were processed identically and within 60 min after extraction. Samples were tested with a cell counter for the absence of residual cells after centrifugation. PFP aliquots were snap-frozen in liquid nitrogen and stored, for an identical time interval, at -80ºC until flow cytometric studies.

Atherosclerotic plaque characterization

Aortic-MRI has been widely used to detect atherosclerotic burden in high-risk patients (Corti and Fuster, 2011; Vilades Medes *et al*., 2013; Teis *et al.,* 2013; Carreras *et al.,* 2012). The total burden of atherosclerosis measured by MRI had been investigated previously (Wierzbicki, Humphries and Minhas, 2008). Atherosclerotic plaque MRI-evaluation and morphometric analysis was performed in FH cases at the level of the descending thoracic aorta by measuring mean arterial wall area (MAWA, mm^2) and plaque composition based on lipid plaque component and calcium content, as fully described (Caballero *et al.,* 2012). All HCVR patients that had atherosclerotic plaques in the carotid arteries (15% of the studied group) also had plaques in the aorta (Wierzbicki, Humphries and Minhas, 2008). The MRI study showed that (a) one third of atherosclerotic plaques detected in the HCVR-patients were lipid-rich and (b) 30% of detected aortic plaques were also calcified (characterized by relevant calcium content) (Wierzbicki, Humphries and Minhas, 2008). Inter-observer variability was low and the intra-class correlation coefficient was of 0.735 (95% CI: 0.591-0.833). This microparticle study has been performed in blood collected from the FH-patients at the time of the MRI-analysis.

Circulating microparticle isolation

The cMP-fraction was isolated from PFP by a two-step high speed centrifugation (Suades *et al.,* 2013, 2014; Rank *et al.,* 2012). Briefly, PFP aliquots were thawed on melting ice for 1 hour and centrifuged for 30 min (20000×g, RT) to pellet cMPs. Then, supernatant was discarded and the cMP-pellet was washed once with citrate-phosphate buffered saline solution (citrate-PBS; 1.4mM phosphate, 154mM NaCl, 10.9mM trisodium citrate, pH 7.4) before a second equal centrifugation step was made. Finally, the remaining cMP-pellet was resuspended in citrate-PBS.

Flow cytometric analysis of circulating microparticles

Triple-label flow cytometric analysis was performed as described by Nieuwland *et al.* (Suades *et al.,* 2013, 2014; Rank *et al.,* 2012). Briefly, washed

cMP suspensions diluted in $CaCl_2$-PBS buffer (2.5 mM) were incubated (20 min, RT, dark) with combinations of BD-horizon V450-conjugated annexin V with two specific monoclonal antibodies (MoAbs, Supplemental Table 1) before being analyzed on a FACSCantoII™ flow cytometer (BD). Forward scatter (FSC), side scatter (SSC) and fluorescence data were obtained with gain settings in the logarithmic scale. cMPs were identified 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 YG Size-Range Calibration Kit (Polysciences) and an *in vitro* platelet-derived microparticle population as positive control (Figure S1). 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. Annexin V binding level was corrected for autofluorescence using fluorescence signals obtained with microparticles in a calcium-free buffer. The lower detection limit was placed as a threshold above the electronic noise.

Data were analyzed with the FACSDiva™ software. The cMP concentration was determined according to Nieuwland's procedure (Suades *et al.,* 2013, 2014; Rank *et al.,* 2012), based on sample's volume, flow cytometer's flow rate and the number of fluorescence-positive events. Intra-assay coefficient of variation (CV) of cMP counts was 3.1%, while inter-assay CV was 5.4%.

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. 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 FXa-specific substrate was added and absorbance was recorded at 405nm. A lyophilized calibrator, containing recombinant relipidated TF with synthetic liposomes, enabled the standardization of the assay. MP-TF concentration was established against an internal standard and expressed as TF antigen equivalent in pg/mL. Measurements were done in duplicate.

microRNA analysis

Total RNA was extracted from microparticles of frozen EDTA-plasma samples. To ensure that RNA was indeed from cMPs, the RNA was isolated with the Exo-MiR extraction kit (Bioo Scientific), according to the manufacturer's instructions and as previously described (Bryant et al., 2012). All samples were spiked with 25 fmol/µL of *Caenorhabditis elegans* miR-39 for use as a normaliser in downstream analyses. The ExoMir extraction method is designed to isolate RNA from the microparticle portions

of the sample. A pre-spin was performed in order to remove cellular fragments before the samples were pushed through the MP-specific sized filter (>200 nm) and then the RNA was isolated by eluting the sample off the filters with lysis solution. Briefly, RNA was reverse transcribed using the multi-scribe reverse transcriptase kit, cDNA was preamplified and, then miR-143 was measured using human Taqman miR Custom Array Cards (Life Technologies).

Statistical analysis

All data are presented as medians (interquartile range [IQR]) except when indicated. Frequencies of qualitative variables were compared between two groups by the Chi-square analysis and median values of quantitative variables with two-sided nonparametric tests. The statistical significances between two groups were determined with U-Mann Whitney and multiple comparisons by Kruskal Wallis, and when significant, Bonferroni post-hoc analysis was used to assess intergroup differences. Spearman's rank correlation coefficients were calculated to determine the strength of the association between the continuous variables. A binary logistic regression model with cMP levels and risk factor model was carried out to estimate the likelihood of a lipidic plaque by creating predicted probabilities. Then, to evaluate prognostic markers, an associated receiver operating characteristic (ROC) curve analyses for cMPs, predicted probabilities and/or miR-143 and the corresponding areas under the curve (AUC) along with their 95% CI were calculated. StatView (5.0.1, Abacus Concepts) and SPSS Statistics (21.0.0, SPSS) software were used for all tests and a $P<0.05$ was considered statistically significant.

SUPPLEMENTAL REFERENCES

Bryant, R. J. *et al.* (2012), "Changes in circulating microRNA levels associated with prostate cancer," *Br J Cancer,* 106(4): 768-774.

Caballero, P. *et al.* (2012), "Detection of subclinical atherosclerosis in familial hypercholesterolemia using non-invasive imaging modalities," *Atherosclerosis,* 222(2): 468-472.

Carreras, F. *et al.* (2012), "Left ventricular torsion and longitudinal shortening: two fundamental components of myocardial mechanics assessed by tagged cine-MRI in normal subjects," *Int J Cardiovasc Imaging,* 28(2): 273-284.

Corti, R. and Fuster, V. (2011), "Imaging of atherosclerosis: magnetic resonance imaging," *Eur Heart J,* 32(14): 1709-1719b.

Friedewald, W. T.; Levy, R. I. and Fredrickson, D. S. (1972), "Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge," *Clin Chem,* 18(6): 499-502.

Gehi, A. K. *et al.* (2007), "Self-reported medication adherence and cardiovascular events in patients with stable coronary heart disease: the heart and soul study," Arch Intern Med, 167(16): 1798-1803.

Mata, N. *et al.* (2011), "Clinical characteristics and evaluation of LDL-cholesterol treatment of the Spanish Familial Hypercholesterolemia Longitudinal Cohort Study (SAFEHEART)," *Lipids Health Dis,* 10: 94.

Mozas, P. *et al.* (2004), "Molecular characterization of familial hypercholesterolemia in Spain: identification of 39 novel and 77 recurrent mutations in LDLR," *Hum Mutat,* 24(2): 187.

NCEP (2002), "Third Report of the National Cholesterol Education Program, Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report," *Circulation,* 106(25): 3143-3421.

Pijlman, A. H. *et al.* (2010), "Evaluation of cholesterol lowering treatment of patients with familial hypercholesterolemia: a large cross-sectional study in The Netherlands," *Atherosclerosis,* 209(1): 189-194.

Rank, A. *et al.* (2012), "Circulating microparticles in patients with benign and malignant ovarian tumors," *Anticancer Res,* 32(5): 2009-2014.

Reiner, Z. *et al.* (2011), "ESC/EAS Guidelines for the management of dyslipidaemias: the Task Force for the management of dyslipidaemias of the European Society of Cardiology (ESC) and the European Atherosclerosis Society (EAS)," *Eur Heart J,* 32(14): 1769-1818.

Suades, R. *et al.* (2013), "Lipid-lowering therapy with statins reduces microparticle shedding from endothelium, platelets and inflammatory cells," *Thromb Haemost,* 110(2): 366-377.

Suades, R. *et al.* (2014), "Circulating CD45+/CD3+ lymphocyte-derived microparticles map lipid-rich atherosclerotic plaques in FH patients," *Thromb Haemost,* 111(1).

Teis, A. *et al.* (2013), "Quadricuspid pulmonic valve: diagnosis by MRI," *Eur Heart J Cardiovasc Imaging,* 14(4): 398.

Vilades Medel, D. *et al.* (2013), "Coronary computed tomographic angiographic findings in asymptomatic patients with heterozygous familial hypercholesterolemia and null allele low-density lipoprotein receptor mutations," *Am J Cardiol,* 111(7): 955-961.

Wierzbicki, A. S.; Humphries, S. E. and Minhas, R. (2008), "Familial hypercholesterolaemia: summary of NICE guidance," *Bmj,* 337: a1095.

2) Supplemental Tables

Supplemental Table 1

CELL SURFACE MOLECULES FOR CIRCULATING MICROPARTICLE IDENTIFICATION AND CHARACTERIZATION

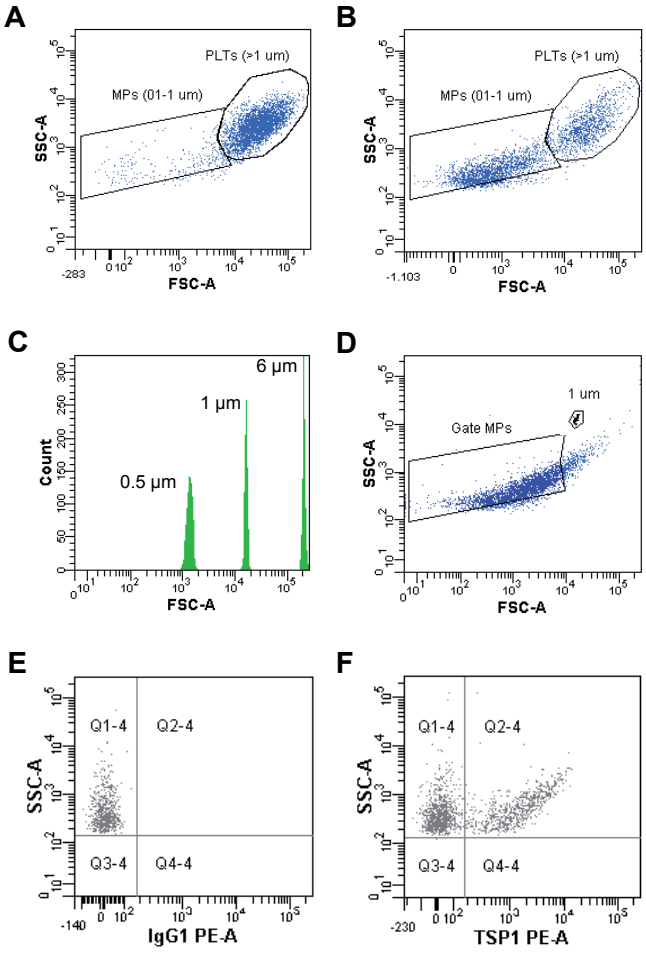
Marker	Alternative name	Expression	Conjugation	Clone	Company
Annexin V	PS-binding protein	Widely expressed	BD Horizon™ V450	-	BD Biosciences
CD14	LPS-receptor	Macrophages, monocytes	PE	M5E2	BD Pharmingen
CD41	$_{\alpha}$IIb-integrin	Platelets	FITC	SZ22	Beckman Coulter
CD61	β_3-integrin	Platelets	PE	VI-PL2	BD Pharmingen
CD62P	P-selectin	Activated platelets	PE	AK-4	BD Pharmingen
CD142	Tissue factor	Widely expressed	FITC	VD8	American Diagnostica
PAC1	$\alpha_{IIb}\beta_3$-integrin	Activated platelets	FITC	PAC1	BD Biosciences
TSP1	THBS-1	Platelets, megakaryocytes	PE	P10	Beckman Coulter
IgG1γ	-	-	FITC / PE	X40	BD Biosciences
IgG1κ	-	-	FITC / PE	MPOC21	BD Pharmingen

Notes: BD, Becton Dickinson; FITC indicates fluorescein isothiocyanate; PE, phycoerythrin; LPS, lipopolysaccharide; PS, phosphatidylserine; THBS-1, thrombospondin-1.
Source: Own elaboration.

3) Supplemental Figures

Supplemental Figure 1

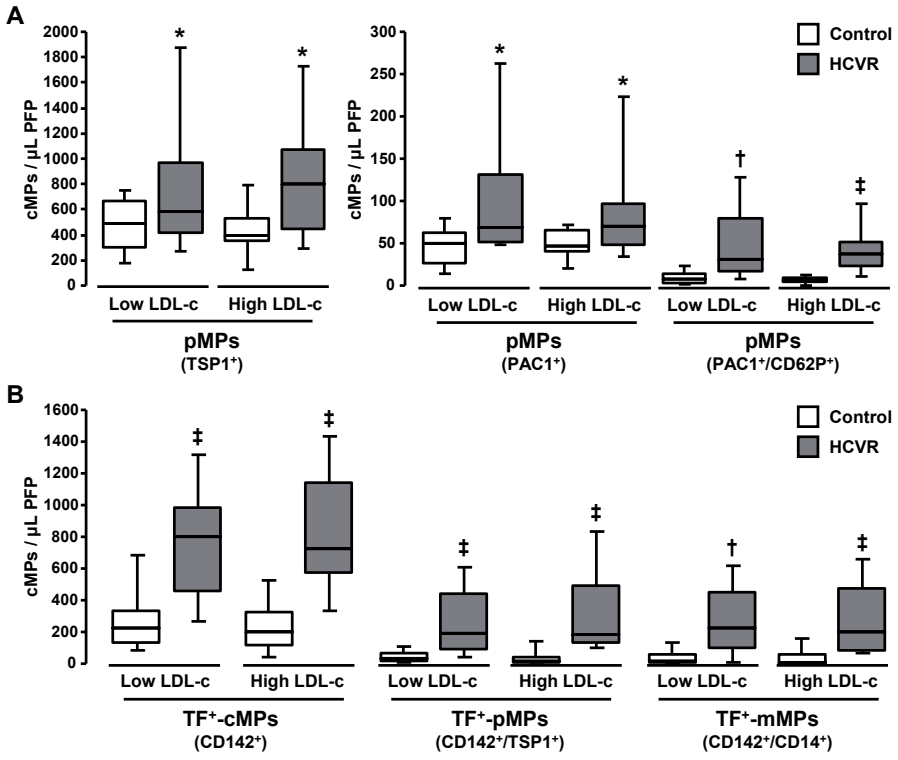
CHARACTERIZATION OF CIRCULATING MICROPARTICLES BY FLOW CYTOMETRY



Notes: The cell-microparticle gate was established based on light scattering properties and size, using calibration microspheres (C) and defining cMPs as events both smaller than 1 µm (D). Further, in order to corroborate and validate established microparticle gate we determined the forward scatter (FSC) and side-scatter (SSC) characteristics of platelets and microparticles in suspension (A-B). Image A shows unstimulated platelets. Thereafter, platelets were stimulated with calcium ionophore A23187 (5 minute incubation, at 37ºC, 2.5µM) and microparticles generated were located within the established gate (B). (E-F) Images illustrating the flow cytometric characterization of cMPs from FH patients from a specific cell type as an example: (E) platelet marker ($TSP1^{+}$) and (B) its respective isotype-matched control antibody, both with the demarcation of the event thresholds.
Source: Own elaboration.

Supplemental Figure 2

CMP PROFILING IN REGARD TO MEDIAN LDL-CHOLESTEROL LEVELS



Notes: Box and whisker plots of (A) platelet activation markers (PAC1$^+$, CD62P$^+$, TSP1$^+$) and (B) tissue factor (CD142$^+$) cMPs per µL of PFP in the non-FH and FH-patient populations classified in two groups: low and high LDL-cholesterol levels (below and above the median value of the entire study population, 141.5 mg/dL LDL-cholesterol). Data are given as a box and whisker plot as described in Figure 1A. **P*<0.05, †*P*<0.005, ‡*P*<0.0001, *vs.* non-FH group (U-Mann Whitney).
Source: Own elaboration.

ARTICLE 5

Growing thrombi release increased levels of CD235a+ microparticles and decreased levels of activated platelet-derived microparticles. Validation in ST-elevation myocardial infarction patients

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

Published Published – J. Thromb Haemost 2015; 13(10): 1776-1786

OBJECTIVE

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

Suades, R.; Padró, T.; Vilahur, G.; Martin-Yuste, V., Sabaté, M.; Sans-Roselló, J.; Sionis, A.; Badimon, L.

"Growing thrombi release increased levels of CD235a+ microparticles and decreased levels of activated platelet-derived microparticles. Validation in ST-elevation myocardial infarction patients."

J. Thromb Haemost 2015; 13(10): 1776-1786.

Journal of Thrombosis and Haemostasis, **13**: 1–11 DOI: 10.1111/jth.13065

ORIGINAL ARTICLE

Growing thrombi release increased levels of CD235a$^+$ microparticles and decreased levels of activated platelet-derived microparticles. Validation in ST-elevation myocardial infarction patients

R. SUADES,* T. PADRÓ,* G. VILAHUR,* V. MARTIN-YUSTE,† M. SABATÉ,† J. SANS-ROSELLÓ,‡ A. SIONIS‡ and L. BADIMON*§

**Cardiovascular Research Center, CSIC-ICCC, Biomedical Research Institute Sant Pau (IIB-Sant Pau); †Department of Interventional Cardiology, Hospital Clinic; ‡Acute and Intensive Cardiac Care Unit, Cardiology Department, Hospital Santa Creu i Sant Pau, Biomedical Research Institute Sant Pau (IIB-Sant Pau); and §Cardiovascular Research Chair, UAB, Barcelona, Spain*

To cite this article: Suades R, Padró T, Vilahur G, Martin-Yuste V, Sabaté M, Sans-Roselló J, Sionis A, Badimon L. Growing thrombi release increased levels of CD235a$^+$ microparticles and decreased levels of activated platelet-derived microparticles. Validation in ST-elevation myocardial infarction patients. *J Thromb Haemost* 2015; DOI: 10.1111/jth.13065.

Summary. *Background:* Local fluid dynamics and exposed atherosclerotic lesions regulate thrombus formation. Activated cells in the attached thrombi release microparticles to the circulation (circulating microparticles [cMPs]); however, their phenotype is unknown. *Objectives:* To investigate the specific phenotype of the cMPs released by growing thrombi. *Methods/patients:* cMPs released by thrombi growing in different well-characterized thrombogenic conditions were investigated. cMP contents just before and immediately after perfusion of the thrombogenic surfaces were analyzed by triple-labeling flow cytometry. cMPs were tested for their thrombin-generating capacity. The cMPs identified in the *ex vivo* perfusion experiments were validated in blood of ST-elevation myocardial infarction (STEMI) patients undergoing thrombectomy and percutaneous coronary intervention. Culprit coronary blood (STEMI-CCB) and peripheral artery blood (STEMI-PAB) were simultaneously analyzed and compared with peripheral artery blood from age-matched controls (C-PAB) and peripheral artery blood from patients who had recovered from acute coronary syndrome (ACS) (pSTEMI-PAB). *Results:* The levels of annexin V$^+$ cMPs significantly increased in blood collected after perfusion of the exposed thrombogenic surfaces. cMP release was directly related to the formed thrombus mass and the plasma procoagulant activity. Post-thrombus blood showed higher thrombin generation potential and contained higher levels of cMPs carrying glycophorin-A (CD235a$^+$; erythrocyte-derived microparticles [ErMPs]) than preperfusion blood ($P < 0.05$), whereas the levels of cMPs carrying activated and adhesion platelet markers were decreased. STEMI-CCB and STEMI-PAB had significantly higher ErMP levels than control blood ($P < 0.005$). ErMP levels were also significantly higher in STEMI-PAB than in pSTEMI-PAB, validating the experimental mechanistic studies and suggesting that ErMPs are markers of ongoing coronary thrombosis (*C*-statistics: 0.950; 95% confidence interval 0.889–1.000; $P < 0.001$). *Conclusion:* Glycophorin-A-rich microparticles are released from evolving growing thrombi into the distal perfusing blood, and can be measured in peripheral blood. CD235a$^+$ cMPs may constitute a novel systemic biomarker of ongoing thrombosis.

Keywords: blood platelets; cell-derived microparticles; erythrocytes; hemodynamics; thrombosis.

Correspondence: Lina Badimon, Cardiovascular Research Center, c/Sant Antoni Mª Claret 167, 08025 Barcelona, Spain.
Tel.: +34 935565880; fax: +34 935565559.
E-mail: lbadimon@csic-iccc.org

Received 20 February 2015
Manuscript handled by: D. Mezzano
Final decision: P. H. Reitsma, 20 July 2015

Introduction

Thrombus formation at sites of atherosclerotic plaque rupture or vessel injury leads to acute coronary syndromes (ACSs) [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]. Beyond platelets and blood components, other variables, such as local hemodynamic parameters or atherosclerotic plaque components, regulate arterial thrombosis [4–6]. Previous studies have demonstrated

that high shear stress (SS) induces platelet deposition, aggregation, and thrombus formation [7,8]. The identification of additional factors that may determine the thrombotic mass, and the likelihood of subsequent thrombosis and its influence on distal microvascular function, may lead to more effective strategies to better prevent ACS.

Circulating microparticles (cMPs) are small plasma membrane vesicles (0.1–1 μm in diameter) released from cells undergoing activation or apoptosis [9]. The primary hallmark of microparticles (MPs) is a phosphatidylserine-rich membrane with defined properties [10]; however, cMPs also carry epitopes that define their parental cell lineage and the level of activation/stress of that parental cell, constituting messengers of the cell to the microenvironment [11]. cMPs are present in the blood of healthy individuals [12], but their numbers and cellular sources are altered in pathologic states [13–15]. Recent research has shown that cMPs are not merely biomarkers of cell activation, but are also active players in the development of atherothrombotic disease [16]. Indeed, we have recently reported that high levels of blood cMPs and, specifically, platelet-derived MPs (pMPs) increase platelet deposition on the damaged arterial wall, contributing to thrombus formation [17]. 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 SS, a mechanical force generated by circulating blood against the vessel wall conduit, stimulates pMP formation [18], which can be further enhanced by stenosis [19], cytokines [20], or an activated endothelium [21]. Specifically, pMP generation in blood under the effect of SS was reported to be dependent on $\alpha_{IIb}\beta_3$ integrins [22], on von Willebrand factor–glycoprotein (GP)Ibα interactions [23], or on both of them [24]. Furthermore, SS related to aortic valve stenosis [25], strenuous exercise [26], surgery in upper gastrointestinal malignancy [27] and cyanotic congenital heart disease [28] also contributes to the release of procoagulant pMPs. However, it is not known whether MPs are released into the perfusing blood while the thrombus is formed, or whether occlusive coronary thrombi causing ST-elevation myocardial infarction (STEMI) may release MPs of a characteristic and determined phenotype.

Indeed, there is a lack of biomarkers in ischemic diseases to estimate thrombosis risk. Current markers for thrombosis are not well established, and do not yet have wide clinical applicability, because they are difficult to implement in daily practice in the case of platelet aggregation, or have not so far shown sufficient reliability to support their use, such as in point-of-care devices. One of the pitfalls of these devices in measuring thrombosis lies in the fact that they measure only one pathway (either platelets or coagulation) among the triggers of an occluding complex thrombus. Thus, it is crucial to look for a marker of the entire complex thrombus formation process, in order to identify those patients with large thrombotic burden.

It was our hypothesis that growing complex thrombi release cMPs that may be novel biomarkers of ongoing thrombosis. Thus, the aim of this study was to investigate: (i) the phenotype of MPs released by thrombi growing on substrates with different thrombogenic potential; and (ii) the relevance of these MPs in the coronary and peripheral circulation of patients with ongoing coronary thrombosis.

Materials and methods

Blood sampling

In the first part of the study, blood from non-smoking healthy volunteers was drawn by a cubital venopuncture to characterize the effects of SS and lesion type on cMP release. In the second part, and as a proof-of-concept validation study, both culprit coronary blood (STEMI-CCB) and peripheral artery blood (STEMI-PAB) of STEMI patients ($n = 40$) undergoing percutaneous coronary intervention (PCI) was collected in EDTA tubes. As controls, we used peripheral blood from healthy subjects without thrombosis ($n = 20$) and patients recovering from STEMI (72 h after the acute event, $n = 20$) (Table 1). Patients were treated according to guidelines. All STEMI patients were treated with unfractionated heparin, whereas GPIIb–IIIa antagonist was administered at the physician's discretion. 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. Reporting of the study conforms to the STROBE guidelines.

Experimental design

MP release from shear-induced growing thrombi was analyzed by exposing blood to damaged arterial substrates by use of the previously validated Badimon perfusion chamber [4,7] and to isolated type I collagen-coated surfaces in a flat perfusion chamber [29]. For the perfusion experiments, blood was collected in sodium heparin (10 IU mL^{-1}) [17]. In the flat chamber perfusion experiments, platelets were rendered fluorescent by the addition of mepacrine (quinacrine dihydrochloride) to blood. 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 − 80 °C [9,30] for cMP fluorescence-activated cell sorting analysis.

Table 1 Baseline clinical characteristics of the study population

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

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

Flow cytometric analysis of cMPs

Triple-label flow cytometric analysis was performed as previously described [31]. Briefly, the cMP fraction was washed and isolated from PFP by two-step high-speed centrifugation (20.000 x*g*, 30 min). Washed cMP suspensions diluted in $CaCl_2$ phosphate-buffered saline were incubated with combinations of BD Horizon V450-conjugated annexin V (AV) (Becton Dickinson, San Diego, CA, USA) and two specific conjugated mAbs, or the isotype-matched control antibodies (Table S1). cMPs were identified and quantified on the basis of their forward scatter/side scatter characteristics (logarithmic scale) according to their size, binding to AV, and reactivity to cell-specific mAbs. Gate limits were established with a Flow-Check Size-Range Calibration Kit (Polysciences, Eppelheim, Germany) and an *in vitro* pMP population as positive control. Flow cytometer photomultiplier tube voltage and compensation values were optimized in previous testing experiments prior to sample acquisition analysis. Phosphatidylserine-positive cMPs were labeled with AV in the presence of 2.5 mM $CaCl_2$. The AV-binding level was corrected for autofluorescence with MPs in a calcium-free buffer. Data were analyzed with BD-FACSDiva software (Becton Dickinson). The cMP concentration (cMP number per μL plasma) was determined according to Nieuwland's procedure and our previous studies [12,31,32]. The intra-assay coefficient of variation (CV) of cMP counts was 3.1%, and the interassay CV was 5.4%.

Thrombin generation (TG) assay

In vitro thrombin generation (TG) was determined with Hemker's method [32,33]. To prevent interference from heparin used for perfusion experiments, plasma samples were deheparinized with heparinase-I [34]. Then, cMPs were isolated and added to defibrinated normal human plasma containing Pefachrome chromogenic substrate (Pentapharm, Basel, Switzerland) and buffer A. After addition of 250 mmol L^{-1} $CaCl_2$, generation of *p*-nitroaniline was determined at a wavelength of 405 nm on a SpectraMax spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) every 60 s for 60 min. For quantitative analysis, results were determined as area under the TG curve (AUC) and thrombin activity (expressed as nM).

Statistical analysis

Results are reported as median (interquartile range [IQR]), except where indicated. An unpaired *t*-test was used to compare clinical characteristics between STEMI patients and controls. Frequencies for categorical data were compared by use of the chi-square test. Median values of quantitative variables were compared by the use of two-sided non-parametric tests, as the Kolmogorov–Smirnov test showed that the data were not normally distributed. Statistically significant differences between two variables were determined with the paired Wilcoxon signed-rank text and the unpaired Mann–Whitney *U*-test, and correlations were determined with the Spearman and simple regression tests. Analysis of variance, followed by Bonferroni correction, and the Kruskal–Wallis test were used in multiple comparisons. Analysis of covariance was used to test the effect of variables as covariates in the model. Receiver operating characteristic (ROC) curve analysis was performed to evaluate the prognostic value of erythrocyte-derived MPs (ErMPs). STATVIEW 5.0.1

(Abacus Concepts, Piscataway, NJ, USA) and SPSS 21.0.0 (IBM, Armonk, NY, USA) were used for all tests. A *P*-value of < 0.05 was considered to be statistically significant.

Results

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

Two characterized models of thrombus formation were used to investigate cMP shedding during ongoing thrombosis. MPs were analyzed by flow cytometry in blood taken immediately before and immediately after the growth of thrombus (Fig. 1). AV^+ cMP levels were significantly increased after perfusions in both the Badimon chamber (366.3 [IQR 335.7–396.5] versus 703.6 [IQR 507.8–1148.6] cMPs per μL of PFP, $P < 0.05$; Fig. 1A1) and the flat chamber (364.3 (IQR 327.4–389.7] versus 543.1 [IQR 453.9–566.2] cMPs per μL of PFP, $P < 0.05$; Fig. 1A2). Thus, thrombi attached to both the damaged atherosclerotic vessel wall and to the less thrombogenic type I collagen shed cMPs into perfusing blood. The level of cMP shedding was directly related to the mass of thrombus deposition. Thus, mural thrombosis with platelet aggregation and deposition on vascular substrates (Fig. 1A1/B1) resulted in a significantly higher number of cMPs than platelet adhesion on isolated type I collagen surfaces ($P < 0.05$; Fig. 1A2/B2).

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

Specific cell-derived markers were investigated in order to determine the cMP parental cells (Table 2). Cytofluorimetric analysis revealed that circulating ErMP levels were significantly increased in the perfusion effluents, depending on the type of thrombus (Fig. 1B1/C1 and B2/C2), whereas the levels of other cell-derived MPs did not drastically change. The total level of pMPs showed a non-significant increase (Table 2), but the level of cMPs carrying the erythrocyte protein glycophorin-A ($CD235a^+$) significantly increased after perfusion of the human damaged vascular wall (Badimon chamber) and after exposure to human type I collagen (flat chamber). Indeed, not only the level of cMPs (per μL of PFP) but also the percentage of $CD235a^+/AV^+$ cMPs increased after thrombus formation (Fig. 1C). $CD235a^+$ MP shedding was significantly higher after perfusion of damaged vessel in the Badimon chamber (Fig. 1C1), which induces a more complex thrombus than collagen type I. In order to discriminate the effects of the growing thrombus on the levels of released $CD235a^+$ cMPs from the possible chamber *ex vivo* effects on erythrocytes, we tested the perfusion system without substrate. There was no significant effect of the circuit. After normalization, the level of $CD235a^+/AV^+$-ErMPs was significantly increased owing to thrombus formation in direct relation to the growing thrombus mass (Table S2).

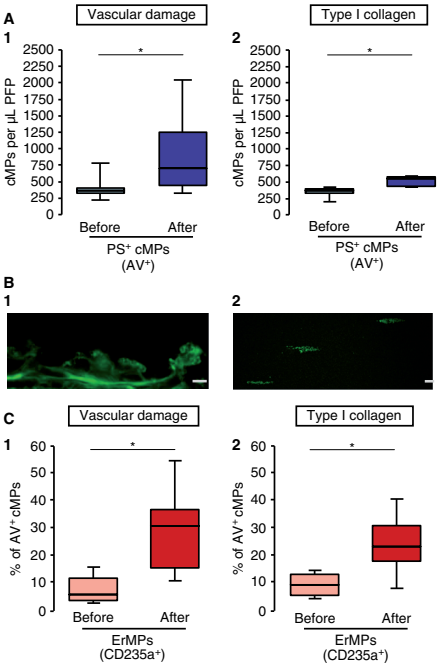


Fig. 1. Effects of blood rheology and vascular wall components on blood circulating microparticles (cMPs). (A) Total annexin V $(AV)^+$ cMP numbers (AV^+ cMPs per μL of platelet-free plasma [PFP]) before and after perfusion in (A1) the Badimon chamber ($n = 14$) and (A2) the flat chamber ($n = 6$) . Box and whisker plots represent median (interquartile range) values. $*P < 0.05$ versus the preperfusion group (Wilcoxon signed-rank test). (B) A representative immunophotograph of platelet staining on perfused human vascular substrates in the Badimon chamber (green: mepacrine) (B1) and a confocal image of platelets adhering to a type I collagen surface in the flat chamber (green: CD61) (B2). Scale bars: 25 μm and 5 μm, respectively. (C) Box and whisker plots showing erythrocyte-derived cMPs (ErMPs, $CD235a^+$) before and after exposure of blood to (C1) arterial substrates in the Badimon chamber ($n = 14$) and to (C2) collagen surfaces in the flat chamber ($n = 6$) under high shear rate flow conditions. Data are expressed as percentage of AV^+ cMPs. $*P < 0.05$ versus the preperfusion group (Wilcoxon signed-rank test). PS, phosphatidylserine.

Shedding of MPs bearing activation markers and adhesion surface receptors

cMPs bearing markers of cell activation and surface receptors involved in adhesion were analyzed in resting (pre-thrombus) and effluent (post-thrombus) perfused blood (Table 3; Figs 2 and 3). The levels of cMPs carrying MAC-1 ($CD11b^+$, a marker of activated leukocytes) and α_V integrin ($CD51^+$, an adhesion receptor for vitronectin) did not differ before and after perfusion (Table 3). The levels

Table 2 Shear stress effect on circulating microparticles (MPs) according to their cellular origin

		Vascular damage			Type I collagen		
Cell origin	Circulating MPs	Preperfusion	Postperfusion	Statistics (*P*)	Preperfusion	Postperfusion	Statistics (*P*)
ECs	eMPs ($CD31^+/CD41^-$)	3.1 (1.7–5.6)	1.9 (1.1–3.4)	0.18	4.5 (3.5–5.6)	3.1 (2.1–4.0)	0.25
Erythrocytes	ErMPs ($CD235a^+$)	6.0 (3.9–13.8)	31.1 (16.3–36.8)	0.001	8.7 (4.8–13.6)	21.8 (17.8–27.7)	0.03
PLTs	pMPs ($CD41a^+/CD61^+$)	78.3 (58.3–99.8)	61.8 (50.9–71.6)	0.04	88.9 (74.7–103.0)	91.7 (82.0–99.2)	0.92

Data are expressed as percentage of total annexin V+ circulating MPs (median [interquartile range]). EC, endothelial cell; eMP, endothelium-derived MP; ErMP, erythrocyte-derived MP; PLT, platelet; pMP, platelet-derived MP. The numbers in parentheses mean interquartile range.

Table 3 Effects of shear stress effect on circulating microparticles (cMPs) carrying markers of cell activation

		Vascular damage			Type I collagen		
Cell origin	Activation marker	Preperfusion	Postperfusion	Statistics (*P*)	Preperfusion	Postperfusion	Statistics (*P*)
WBCs	MAC-1 ($CD11b^+$)	9.2 (6.5–21.8)	6.3 (4.3–10.1)	0.14	21.6 (15.7–36.2)	18.8 (11.9–25.6)	0.12
Platelets	$\alpha_{IIb}\beta_3$ integrin ($PAC1^+$)	3.5 (2–9.6)	3.5 (2–6.6)	0.64	8.7 (7.3–9.6)	4.0 (2.9–6.1)	0.03
	Glycoprotein Ib ($CD42b^+$)	3.4 (1.8–4.0)	1.0 (0.6–2.9)	0.005	3.4 (2.1–6.7)	0.9 (0.4–2.1)	0.25
Multiple	Thrombospondin-1 ($TSP1^+$)	10.7 (6.2–13.4)	6.4 (5.2–9.8)	0.02	12.5 (9.8–16.7)	9.5 (4.0–11.8)	0.60
	α_V integrin ($CD51^+$)	2.9 (0.8–5.9)	1.7 (1.3–2.2)	0.14	4.9 (1.8–12.2)	2.2 (1.2–4.3)	0.07
	P-selectin ($CD62P^+$)	7.4 (6.5–10.3)	7.5 (5.5–12.3)	0.97	6.2 (4.4–9.3)	6.1 (3.2–9.0)	0.46
	Tissue factor ($CD142^+$)	37 (25.7–68.3)	41.2 (22.8–86.9)	0.25	30.8 (27.2–62.8)	36.0 (25.7–49.0)	0.60

Data expressed as percentage of total annexin V+ cMPs (median [interquartile range]). WBC, white blood cell. The numbers in parentheses mean interquartile range.

of cMPs carrying P-selectin ($CD62P^+$) and tissue factor (TF) ($CD142^+$) did not change either (Table 3). In contrast, the levels of AV^+ pMPs carrying GPIb ($CD42b^+$) and thrombospondin-1 were significantly reduced in blood after perfusion of severely damaged vessels ($P < 0.05$; Fig. 2A) but not in the flat chamber after exposure to type I collagen (Fig. 2B).

We further studied circulating pMP subpopulations (percentage of AV^+ pMPs) (Fig. 3; Tables 2 and 3). Specifically, the relative amount of total pMPs carrying β_3-integrin ($CD61^+$) was significantly decreased in the blood exposed to the damaged vascular wall, whereas it did not change when blood was exposed to collagen (Fig. 3). The relative amounts of pMPs carrying the platelet activation markers PECAM-1 ($CD31^+$) and activated $\alpha_{IIb}\beta_3$ integrin ($PAC1^+$) tended to decrease after thrombus formation on vascular substrates (Fig. 3A), whereas they were significantly reduced in the flat chamber after exposure of blood to collagen surfaces ($P < 0.05$; Fig. 3B).

Taken together, these data indicate that there were no major changes across and derived from the growing thrombi in pMPs able to become biomarkers. In contrast, glycophorin-A-rich ErMPs were good candidates to determine the ongoing formation of thrombi.

Thrombin generation by cMPs

Addition of cMPs from perfusion experiments to normal plasma supported blood coagulation as tested with a TG test (Fig. 4). cMPs released after perfusion of the human damaged vascular wall (Badimon chamber) were the most potent in stimulating TG. Indeed, postperfusion cMPs from vascular substrates were more efficient in supporting TG than preperfusion cMPs, resulting in the highest area under the TG curve (AUC) (Fig. 4AI) and the highest peak TG (Fig. 4AII). In contrast, postperfusion cMPs from collagen type I substrate (flat chamber) failed to trigger higher TG than preperfusion cMPs. Interestingly, ErMP levels after perfusion of vascular damaged substrates directly correlated with both thrombin activity (Fig. 4B) and peak TG (Fig. 4BII).

ErMPs in STEMI patients

We next investigated the systemic (STEMI-PAB) and intracoronary (STEMI-CCB) levels of pMPs and ErMPs in the blood of STEMI patients ($n = 40$). Blood from recovering STEMI patients (at day 3 postevent) ($n = 20$) and from control patients without thrombosis ($n = 20$) was also investigated (Table 1). The pattern of distribution of peripheral blood AV^+ cMPs in STEMI patients differed from that in healthy controls (Fig. 5). pMP levels were reduced in STEMI patients, and the levels were still reduced after 72 h of myocardial infarction (Fig. 5A). In contrast, $CD235a^+$ ErMP levels were significantly higher in STEMI-PAB than in peripheral artery blood from age-matched controls (C-PAB) ($P < 0.001$) and in peripheral artery blood from patients who had recovered from acute coronary syndrome (pSTEMI-PAB) ($P < 0.001$). Interestingly, 3-day pSTEMI-PAB had intermediate levels of ErMPs that were significantly different from those in STEMI patients and controls (Fig. 5B). When differences

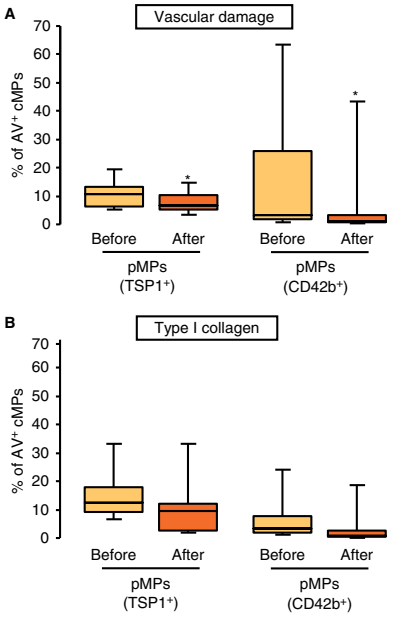


Fig. 2. Flow-dependent and substrate-dependent release of adhesion epitope-containing circulating microparticles (cMPs). Relative amounts of cMPs carrying molecules related to adhesion markers (thrombospondin 1 [TSP1$^+$] and glycoprotein Ibα [CD42b$^+$]) before and after exposure of blood to (A) arterial substrates in the Badimon chamber (n = 14) and to (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 annexin V (AV)$^+$ cMPs. *P < 0.05 versus the preperfusion group (Wilcoxon signed-rank test). pMP, platelet-derived microparticle.

attributable to the presence of risk factors (smoking, arterial hypertension, diabetes mellitus, dyslipidemia, obesity, and drugs of abuse) were statistically corrected for, the differences in pMP and ErMP levels between controls and STEMI patients remained statistically significant. We then investigated whether peripheral blood reflected coronary levels, and we found no significant differences between the levels of intracoronary and peripheral blood cell-derived MPs from platelets (CD61$^+$ pMPs) and erythrocytes (CD235a$^+$ ErMPs) (Fig. 5A,B). Interestingly, circulating ErMP levels did not correlate with established myocardial infarction biomarkers of STEMI patients. Neither troponin T nor creatinine kinase peak levels correlated with AV$^+$ ErMP levels. Similarly, no correlation was detected between the levels of cMPs derived from erythrocytes and major adverse cardiac events in STEMI patients, suggesting that ErMPs may serve as diagnostic rather than prognostic markers.

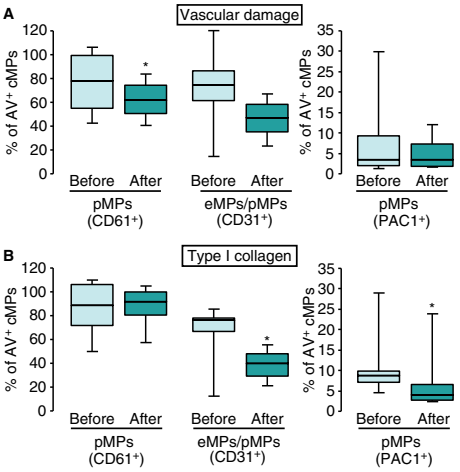


Fig. 3. Flow-dependent and substrate-dependent release of circulating microparticles (cMPs) carrying activation markers. Box and whisker plots show relative amounts of total platelet-derived microparticle (pMPs; CD61$^+$) and pMPs carrying activation markers, i.e. PECAM-1 (CD31$^+$) and activated $\alpha_{IIb}\beta_3$ integrin (PAC1$^+$), before and after exposure of blood to (A) arterial substrates in the Badimon chamber (n = 14) and to (B) collagen surfaces in the flat chamber (n = 6). Data are expressed as percentage of annexin V (AV)$^+$-cMPs. *P < 0.05 versus the preperfusion group (Wilcoxon signed-rank test). eMP, endothelium-derived microparticle.

We performed a ROC curve analysis to determine the potential of ErMPs as predictors of ongoing STEMI (Fig. 5C). The AUC of *C*-statistics measured with CD235a$^+$ cMPs from controls and ACS STEMI patients was 0.950 (95% confidence interval 0.889–1.000, P < 0.001), suggesting that ErMPs could significantly signal for the thrombotic mass in the coronary arteries of the STEMI patients. Additionally, a cut-off value of CD235a$^+$ cMPs for the prediction of total occlusion was obtained. A level of > 26% CD235a$^+$ cMPs (as a percentage of total AV$^+$ cMPs) predicted total occlusion of the culprit vessel with 85% sensitivity and 90% specificity. When *C*-statistics were measured with total circulating cMP numbers, a cut-off value of 133 AV$^+$/CD235a$^+$ cMPs per μL of PFP was a predictor of thrombosis with 62.5% sensitivity and 65% specificity.

Discussion

In the present study, we investigated the change in cMP levels in circulating blood induced by ongoing thrombus formation. Thrombosis was induced under controlled conditions of SS and exposure to atherosclerotic substrates of blood from healthy donors. Our results show that total cMP shedding is increased after thrombosis

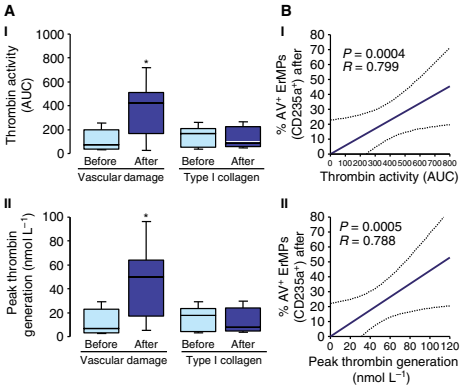


Fig. 4. Thrombin generation (TG) triggered by circulating microparticles (cMPs). cMPs from perfusion experiments were added to plasma, which was incubated with Pefachrome, as indicated. After 10 min of incubation, TG was triggered by the addition of $CaCl_2$. (A) Box and whisker plots showing (I) thrombin activity (area under the TG curve AUC) and (II) peak TG (nM) of cMPs from preperfusion and postperfusion experiments. $*P < 0.05$ versus the preperfusion group (Wilcoxon signed-rank test). (B) Correlation of erythrocyte-derived microparticles (ErMPs) obtained after perfusion of vascular damaged vessel wall with (I) thrombin activity (AUC) and (II) peak TG (nM) of postperfusion cMPs (simple regression). AV, annexin V.

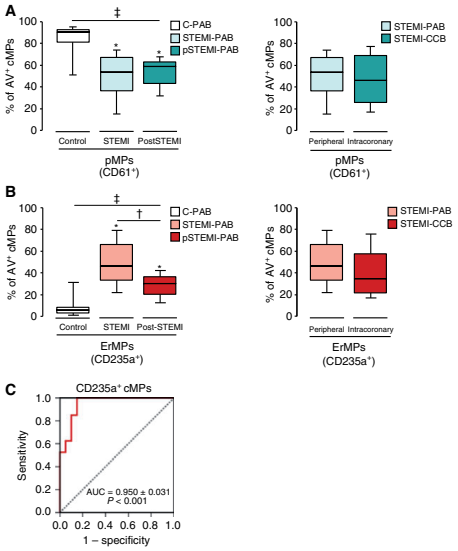


Fig. 5. Platelet-derived microparticle (pMP) and erythrocyte-derived microparticle (ErMP) levels in ST-elevation myocardial infarction (STEMI) patients. (A, B) Box and whisker plots showing (A) pMPs ($CD61^+$ pMPs) and (B) ErMPs ($CD235a^+$ ErMPs) from peripheral artery blood of control subjects (C-PAB) ($n = 20$), acute STEMI patients at baseline (STEMI-PAB) ($n = 40$) and patients recovering from STEMI (pSTEMI-PAB) ($*P < 0.0001$ versus controls and $\dagger P < 0.001$ versus STEMI [Mann–Whitney]; $\ddagger P < 0.05$ [Kruskal–Wallis]), and from both peripheral artery blood (STEMI-PAB) and culprit coronary blood (STEMI-CCB) of STEMI patients ($n = 40$) undergoing percutaneous coronary intervention at baseline (Wilcoxon signed-rank test). (C) To determine the discriminatory power of ErMPs in the prediction of STEMI, an associated receiver operating characteristic curve analysis was performed with $CD235a^+$ circulating microparticles (cMPs) of controls and STEMI patients ($n = 60$). The 95% confidence interval is shown ($P < 0.001$). AV, annexin V.

elicited by high shear and thrombogenic lesions, conditions that mimic stenotic coronary blood flow on the damaged vascular wall. In these conditions, there is an increase in circulating ErMP levels. Interestingly, TG potential was also increased in postperfusion blood when the damaged vessel wall was perfused. The levels of circulating pMPs bearing epitopes involved in adhesion were reduced after perfusion in both chambers, whereas the levels of pMPs carrying activation markers were found to be decreased after blood perfusion on collagen surfaces, which only support platelet adhesion, and not on the vascular wall, which anchors growing thrombi.

The identification of and best treatment for at-risk patients remain major issues in cardiology. Although some existing biomarkers have been associated with increased cardiovascular risk, few have been rigorously shown to provide discrimination with enough sensitivity and specificity to measure ongoing thrombosis. Recently, cMPs have emerged as potential novel markers for cardiovascular risk prediction. Here, we show that a specific type of cMP can significantly indicate thrombus formation.

The significant elevation of ErMP levels after exposure of human blood to damaged arterial substrates and collagen under high SS highlights the importance of erythrocytes in arterial thrombosis, an aspect that has been largely overlooked so far. Indeed, a very recent study revealed a previously unrecognized ability of erythrocytes to participate in thrombosis by mediating platelet adhesion to an intact endothelial surface in an $FeCl_3$-mediated thrombosis model [35]. ErMPs have also emerged as potential mediators of transfusion-related morbidity by thrombin-dependent activation of the complement system [36]. Moreover, $CD235a^+$ ErMPs are sensitive predictors of thrombotic events in thrombocytopenic HIV patients [37]. However, the physiologic role of ErMPs has not been fully elucidated. High ErMP levels have been detected in patients with hematologic disorders such as β-thalassemia [38], paroxysmal nocturnal hemoglobinuria [39], sickle cell anemia [40,41], severe pre-eclampsia [42], nephrotic syndrome [43], and Crohn's

disease [44], and in glucose-6-phosphate dehydrogenase-deficient patients [45]. Because of their hemoglobin content and phosphatidylserine exposure, ErMPs may account for nitric oxide scavenging and procoagulant activity enhancement [46]. In addition, the surfaces of ErMPs are also suitable for the anticoagulant reactions of the protein C system, owing to their ability to bind protein S [47], and this is important to balance *in vivo* coagulation. By enhancing both primary (platelet) and secondary (coagulation) hemostasis, ErMPs have been suggested as broad hemostatic agents [48]. The presence of ErMPs correlated with *in vivo* markers of increased coagulation [32]. In fact, ErMPs are able to support coagulation by decreasing the prothrombin time [49] and generating thrombin [50,51]. Together with pMPs, ErMPs have also been demonstrated to trigger TG in a factor XII-dependent/TF-independent manner (intrinsic coagulation pathway) [52]. TG and procoagulant activity in ErMPs can be further enhanced by either complement activation [39] or increased homocysteine plasma levels [53]. ErMP-induced TG and coagulation activation involvement was also corroborated in the clinical setting of sickle cell disease (SCD) [32,54,55]. Recently, ErMPs have shown to induce endothelial injury and facilitate vaso-occlusive events in a murine SCD model, connecting sickle cell anemia to vascular disease [56]. Indeed, ErMPs can concentrate cell-free heme from intravascular hemolysis and thus constitute a source of oxidant stress for the endothelium [57]. A recent study has supported the idea that ErMPs can also trigger increased release of local inflammatory cytokines [58]. Therefore, it seems plausible that oxidative stress and inflammation may also play a role in the effects of ErMPs. Here, we report on a clinical context in which elevated levels of circulating ErMPs are detected and can even become systemic biomarkers of thrombosis. Therefore, elevated ErMP levels triggered by incipient thrombus formation may also play a pathogenic role in the thrombotic profile of patients with vascular diseases. We have concentrated on arterial thrombosis, where shear forces have an important role; however, further studies should focus on evaluating ErMPs in venous thrombosis.

Here, we show that systemic blood of STEMI patients contains a high level of ErMPs. Indeed, an elevated heterogeneous population of erythrocyte-like vesicles was recently described in STEMI patients at the systemic level [59]; these vesicles were phenotyped directly from plasma, in which soluble antigens and potential immune complex formation might be present. By analyzing specifically isolated washed cMPs, we have additionally shown that peripheral artery blood of patients with coronary thrombus has a level of ErMPs that is similar to the level found in intracoronary blood, suggesting that circulating ErMPs may serve as a sensitive marker for an ongoing thrombotic event on the culprit coronary artery (Fig. 6). Although the peripheral venous blood MP level may also

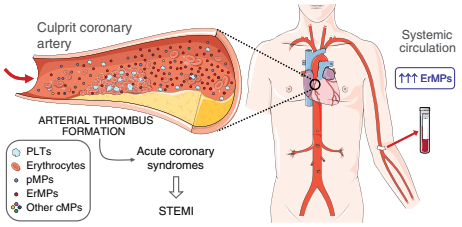


Fig. 6. Erythrocyte-derived microparticles (ErMPs) as indicators of ongoing thrombosis in ST-elevation myocardial infarction (STEMI) patients. Upon thrombus formation, the ErMP level increases significantly in the circulation. In STEMI patients, erythrocyte glycophorin-A ($CD235a^+$)-rich circulating microparticles (cMPs) are found at higher levels in both the culprit coronary and peripheral arteries. Thus, elevated ErMP levels in the systemic circulation might be considered as signals of ongoing evolving thrombosis. PLT, platelet; pMP, platelet-derived microparticle.

reflect systemic cell activation, the amount of released ErMPs has a substantial influence on the detected increase in the systemic circulation.

Additional cMPs can be released from the chronically diseased coronary arteries, which often have silent atherosclerosis until the thrombotic event is precipitated. Indeed, a recent study has shown that endothelium-derived MPs can be measured in the intracoronary circulation, and that systemic and intracoronary pMP level are significantly changed during PCI and affect the coronary microcirculation [60]. However, the importance of ErMPs is that they signal the specific growth of a thrombus. In our study, *C*-statistics revealed that ErMPs show a highly significant discriminatory power for the prediction of thrombus in STEMI patients. In contrast to a previous study that measured plasmatic ErMPs [59], we could not find a correlation between ErMPs and adverse clinical outcomes. This could be explained by differences in sampling and the type of MPs analyzed.

To validate the results, systemic blood of STEMI-recovered patients was tested, and showed lower levels of $AV^+/CD235a^+$ cMPs than those during the acute phase, because the activation trigger for ErMPs (occlusion-forming thrombi) had disappeared after in-hospital patient treatments according to guidelines. However, ErMP levels were still higher than in control blood that had never been exposed to thrombosis, probably because of uncompleted thrombus resolution and clearance. Therefore, ErMPs can be considered as a novel independent hemorheologic index to identify ongoing thrombosis.

pMPs are of great importance in the pathogenesis of cardiovascular disease [18–23,25–28]. pMPs provide the membrane surfaces that are necessary for assembly of the tenase and prothrombinase complexes. A number of studies have highlighted the fact that SS enhances their formation with a variety of stimuli and conditions [59].

Indeed, intracoronary pMP levels were higher than systemic levels in STEMI patients, suggesting their local production [17]. We have recently reported that cMPs and, in particular, pMPs enhance platelet and fibrin deposition on the atherosclerotic vessel wall [17]. Here, we have also found decreases in the levels of pMPs with surface markers of adhesion and activation in post-thrombus blood and in STEMI patients. Therefore, our data reinforce our previous results showing that pMPs bind to adhered platelets under high shear rate conditions stimulating further platelet deposition and thrombus growth [17]. Altogether, these results indicate that a high level of pMPs in blood promotes platelet adhesion, owing to a high tendency to adhere, as previously reported [61], and support their involvement in the atherothrombotic process.

Interestingly, our results also indicate that blood that has perfused thrombogenic vascular substrates contains procoagulant cMPs, which can activate blood coagulation, triggering TG. cMPs from collagen type I perfusion effluent bloods were not able to cause high TG, probably because of a less prothrombotic stimulus than provided by the damaged vascular wall. We performed TG assays in the absence of TF, to analyze the contribution of the intrinsic coagulation pathway; however, we cannot exclude the possibility that released cMPs may also contain TF and additionally activate the extrinsic pathway *in vivo*. As stated above, both pMPs and ErMPs trigger TG [52]; in accordance with this, we detected a correlation between thrombin activity and ErMP levels.

In the context of atherothrombosis, vascular and inflammatory cells help to establish the atherosclerotic milieu. Erythrocytes, by mechanical fluid dynamic forces, force 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 the progression of disease by crosstalk with other blood cells, inducing their activation and amplifying arterial thrombosis. It has been shown that P-selectin-containing MPs enhance leukocyte aggregation and accumulation on selectin-expressing substrates under high SS [62]. The specific cell-derived MPs found in the circulation could represent distinct biological vectors contributing to vascular disease.

Interestingly STEMI patients were treated with anticoagulant and antiplatelet therapies, so concomitant drug use might have influenced cMP release. However, the potent biomarker function of ErMPs has prevailed over the possible drug-induced variability, and ErMP experiments show similar results to those obtained in the variable-controlled experimental *ex vivo* setting. The influence of drugs was analyzed by statistical multivariate analysis, and was found to be negligible. Another important aspect of the study is that cMPs of patients recovering from an acute event were different from those analyzed during the acute event. This was a validation analysis of ErMPs in patients with a reduced burden of thrombosis after being aggressively treated for their STEMI episode. Results obtained with ErMPs therefore reflected a reduced thrombosis burden. Future follow-up clinical studies in patients with ACS (STEMI, non-STEMI, and unstable angina) and in patients with venous thrombosis are warranted.

Taken together, these data demonstrate that blood perfusing the thrombogenic damaged atherosclerotic vessel wall at high SS triggers platelet deposition and thrombus formation, which induce rapid cMP release into the circulation. The release of ErMPs has been shown to be an integral part of the thrombotic process, as erythrocytes are recruited to the growing thrombi by a rheologically dominant mechanism. Our data on ErMP release at sites of arterial thrombosis suggest that ErMP quantification could be a diagnostic biomarker of ongoing thrombotic mass (Fig. 6). This experimental and proof-of-concept study will require further validation in larger-scale studies aimed at evaluating the value of $CD235a^+$ ErMPs in the diagnosis of ongoing silent thrombosis, in order to enable early treatment initiation, with the ample armamentarium now available, in patients at risk.

Addendum

L. Badimon and R. Suades contributed to conception and the design of the study. R. Suades and G. Vilahur contributed to acquisition and collection of data. L. Badimon, T. Padró, G. Vilahur, and R. Suades analysed and interpreted data. V. Martin-Yuste, M. Sabaté, J. Sans-Roselló, and A. Sionis recruited and clinically diagnosed patients, and collected samples. L. Badimon and R. Suades wrote the manuscript. All authors revised the manuscript.

Acknowledgements

The authors are grateful to J. Crespo, O. Juan and E. Peña for their valuable technical advice. This work was supported by Spanish Ministry of Science Plan Estatal I+D+I 2013-2016 (SAF2013-42962-R) cofunded by Fondo Europeo de Desarrollo Regional (FEDER-'Una manera de hacer Europa'), Agència de Gestió d'Ajuts Universitaris i de Recerca (AGAUR-2014SGR1303), Red de Terapia Celular (RD/12/0019/0026) and Red Investigación Cardiovascular (RD/12/00420027) from Instituto Salud Carlos III (ISCIII), Associació Esportiva Jordi Pitarque Ceprià and Fundación Investigación Cardiovascular Jesús Serra to the Cardiovascular Research Chair-UAB to L. Badimon, Spanish Ministry of Science Plan Nacional I+D+I (SAF2012-40208) cofunded by FEDER to G. Vilahur, and Spanish Ministry of Health (FIS-PI13/02850) from ISCIII to T. Padró. G. Vilahur has a contract from the Spanish Ministry of Science and Innovation (RyC-2009-5495; MICINN).

Disclosure of Conflict of Interests

M. Sabaté is a consultant at Abbott and Medtronic outside the submitted work. The other authors state that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Cell surface molecules for circulating microparticle identification and characterization.
Table S2. High SS and vascular wall component effects on blood erythrocyte-derived circulating microparticles normalized by system hemolysis threshold.

References

1 Fuster V, Badimon L, Cohen M, Ambrose JA, Badimon JJ, Chesebro J. Insights into the pathogenesis of acute ischemic syndromes. *Circulation* 1988; **77**: 1213–20.
2 Viles-Gonzalez JF, Fuster V, Badimon JJ. Atherothrombosis: a widespread disease with unpredictable and life-threatening consequences. *Eur Heart J* 2004; **25**: 1197–207.
3 Badimon L, Fuster V, Corti R, Badimon JJ. *Coronary Thrombosis: Local and Systemic Factors*. New York: McGraw Hill, 2004.
4 Badimon L, Badimon JJ. Mechanisms of arterial thrombosis in nonparallel streamlines: platelet thrombi grow on the apex of stenotic severely injured vessel wall. Experimental study in the pig model. *J Clin Invest* 1989; **84**: 1134–44.
5 Badimon L, Badimon JJ, Galvez A, Chesebro JH, Fuster V. Influence of arterial damage and wall shear rate on platelet deposition. Ex vivo study in a swine model. *Arteriosclerosis* 1986; **6**: 312–20.
6 Spronk HM, van der Voort D, Ten Cate H. Blood coagulation and the risk of atherothrombosis: a complex relationship. *Thromb J* 2004; **2**: 12.
7 Badimon L, Badimon JJ, Turitto VT, Vallabhajosula S, Fuster V. Platelet thrombus formation on collagen type I. A model of deep vessel injury. Influence of blood rheology, von Willebrand factor, and blood coagulation. *Circulation* 1988; **78**: 1431–42.
8 Badimon L, Badimon JJ, Vilahur G, Segales E, Llorente V. Pathogenesis of the acute coronary syndromes and therapeutic implications. *Pathophysiol Haemost Thromb* 2002; **32**: 225–31.
9 Jy W, Horstman LL, Jimenez JJ, Ahn YS, Biro E, Nieuwland R, Sturk A, Dignat-George F, Sabatier F, Camoin-Jau L, Sampol J, Hugel B, Zobairi F, Freyssinet JM, Nomura S, Shet AS, Key NS, Hebbel RP. Measuring circulating cell-derived microparticles. *J Thromb Haemost* 2004; **2**: 1842–51.
10 Sinauridze EI, Kireev DA, Popenko NY, Pichugin AV, Panteleev MA, Krymskaya OV, Ataullakhanov FI. Platelet microparticle membranes have 50- to 100-fold higher specific procoagulant activity than activated platelets. *Thromb Haemost* 2007; **97**: 425–34.
11 Suades R, Padro T, Badimon L. The role of blood-borne microparticles in inflammation and haemostasis. *Semin Thromb Hemost* 2015. (In press). doi: 10.1055/s-0035-1556591 [Epub ahead of print].
12 Berckmans RJ, Nieuwland R, Boing AN, Romijn FP, Hack CE, Sturk A. Cell-derived microparticles circulate in healthy humans and support low grade thrombin generation. *Thromb Haemost* 2001; **85**: 639–46.
13 Montoro-Garcia S, Shantsila E, Tapp LD, Lopez-Cuenca A, Romero AI, Hernandez-Romero D, Orenes-Pinero E, Manzano-Fernandez S, Valdes M, Marin F, Lip GY. Small-size circulating microparticles in acute coronary syndromes: relevance to fibrinolytic status, reparative markers and outcomes. *Atherosclerosis* 2013; **227**: 313–22.
14 Namba M, Tanaka A, Shimada K, Ozeki Y, Uehata S, Sakamoto T, Nishida Y, Nomura S, Yoshikawa J. Circulating platelet-derived microparticles are associated with atherothrombotic events: a marker for vulnerable blood. *Arterioscler Thromb Vasc Biol* 2007; **27**: 255–6.
15 Sinning JM, Losch J, Walenta K, Bohm M, Nickenig G, Werner N. Circulating CD31+/Annexin V+ microparticles correlate with cardiovascular outcomes. *Eur Heart J* 2010; **32**: 2034–41.
16 Mause SF. Platelet microparticles: reinforcing the hegemony of platelets in atherothrombosis. *Thromb Haemost* 2013; **109**: 5–6.
17 Suades R, Padro T, Vilahur G, Badimon L. Circulating and platelet-derived microparticles in human blood enhance thrombosis on atherosclerotic plaques. *Thromb Haemost* 2012; **108**: 1208–19.
18 Miyazaki Y, Nomura S, Miyake T, Kagawa H, Kitada C, Taniguchi H, Komiyama Y, Fujimura Y, Ikeda Y, Fukuhara S. High shear stress can initiate both platelet aggregation and shedding of procoagulant containing microparticles. *Blood* 1996; **88**: 3456–64.
19 Holme PA, Orvim U, Hamers MJ, Solum NO, Brosstad FR, Barstad RM, Sakariassen KS. Shear-induced platelet activation and platelet microparticle formation at blood flow conditions as in arteries with a severe stenosis. *Arterioscler Thromb Vasc Biol* 1997; **17**: 646–53.
20 Nomura S, Imamura A, Okuno M, Kamiyama Y, Fujimura Y, Ikeda Y, Fukuhara S. Platelet-derived microparticles in patients with arteriosclerosis obliterans: enhancement of high shear-induced microparticle generation by cytokines. *Thromb Res* 2000; **98**: 257–68.
21 Macey MG, Wolf SI, Lawson C. Microparticle formation after exposure of blood to activated endothelium under flow. *Cytometry A* 2010; **77**: 761–8.
22 Haga JH, Slack SM, Jennings LK. Comparison of shear stress-induced platelet microparticle formation and phosphatidylserine expression in presence of alphaIIbbeta3 antagonists. *J Cardiovasc Pharmacol* 2003; **41**: 363–71.
23 Reininger AJ, Heijnen HF, Schumann H, Specht HM, Schramm W, Ruggeri ZM. Mechanism of platelet adhesion to von Willebrand factor and microparticle formation under high shear stress. *Blood* 2006; **107**: 3537–45.
24 Forestier M, Resendiz JC, Pontiggia L, Lassila R, Beer JH. Platelet microparticle suppressing antibody against GP Ibalpha acts independently of the filamin cleavage and increases protein tyrosine phosphorylation. *Blood Coagul Fibrinolysis* 2008; **19**: 801–6.
25 Diehl P, Nagy F, Sossong V, Helbing T, Beyersdorf F, Olschewski M, Bode C, Moser M. Increased levels of circulating microparticles in patients with severe aortic valve stenosis. *Thromb Haemost* 2008; **99**: 711–19.
26 Chen YW, Chen JK, Wang JS. Strenuous exercise promotes shear-induced thrombin generation by increasing the shedding of procoagulant microparticles from platelets. *Thromb Haemost* 2010; **104**: 293–301.
27 Ikeda M, Iwamoto S, Imamura H, Furukawa H, Kawasaki T. Increased platelet aggregation and production of platelet-derived microparticles after surgery for upper gastrointestinal malignancy. *J Surg Res* 2003; **115**: 174–83.
28 Horigome H, Hiramatsu Y, Shigeta O, Nagasawa T, Matsui A. Overproduction of platelet microparticles in cyanotic congenital heart disease with polycythemia. *J Am Coll Cardiol* 2002; **39**: 1072–7.
29 de la Torre R, Pena E, Vilahur G, Slevin M, Badimon L. Monomerization of C-reactive protein requires glycoprotein IIb–IIIa activation: pentraxins and platelet deposition. *J Thromb Haemost* 2013; **11**: 2048–58.

30 Yuana Y, Bertina RM, Osanto S. Pre-analytical and analytical issues in the analysis of blood microparticles. *Thromb Haemost* 2011; **105**: 396–408.

31 Suades R, Padro T, Alonso R, Lopez-Miranda J, Mata P, Badimon L. Circulating CD45+/CD3+ lymphocyte-derived microparticles map lipid-rich atherosclerotic plaques in familial hypercholesterolaemia patients. *Thromb Haemost* 2014; **111**: 111–21.

32 van Beers EJ, Schaap MC, Berckmans RJ, Nieuwland R, Sturk A, van Doormaal FF, Meijers JC, Biemond BJ. Circulating erythrocyte-derived microparticles are associated with coagulation activation in sickle cell disease. *Haematologica* 2009; **94**: 1513–19.

33 Kessels H, Beguin S, Andree H, Hemker HC. Measurement of thrombin generation in whole blood – the effect of heparin and aspirin. *Thromb Haemost* 1994; **72**: 78–83.

34 Rotteveel RC, Roozendaal KJ, Weijers RN, Eijsman L. Influence of heparin, protamine and polybrene on the time integral of thrombin generation (endogenous thrombin potential). *Haemostasis* 1996; **26**: 1–10.

35 Barr JD, Chauhan AK, Schaeffer GV, Hansen JK, Motto DG. Red blood cells mediate the onset of thrombosis in the ferric chloride murine model. *Blood* 2013; **121**: 3733–41.

36 Zecher D, Cumpelik A, Schifferli JA. Erythrocyte-derived microvesicles amplify systemic inflammation by thrombin-dependent activation of complement. *Arterioscler Thromb Vasc Biol* 2014; **34**: 313–20.

37 El-Menshawy N, Eissa M, Farag R, Aboalyazed A. CD235a (glycophorin-A) is the most predictive value among different circulating cellular microparticles in thrombocytopenic human immunodeficiency virus type 1. *J Clin Lab Anal* 2015. doi: 10.1002/jcla.21842 [Epub ahed of print].

38 Tantawy AA, Adly AA, Ismail EA, Habeeb NM. Flow cytometric assessment of circulating platelet and erythrocyte microparticles in young thalassemia major patients: relation to pulmonary hypertension and aortic wall stiffness. *Eur J Haematol* 2013; **90**: 508–18.

39 Kozuma Y, Sawahata Y, Takei Y, Chiba S, Ninomiya H. Procoagulant properties of microparticles released from red blood cells in paroxysmal nocturnal haemoglobinuria. *Br J Haematol* 2011; **152**: 631–9.

40 Nebor D, Romana M, Santiago R, Vachiery N, Picot J, Broquere C, Chaar V, Doumdo L, Odievre MH, Benkerrou M, Elion J. Fetal hemoglobin and hydroxycarbamide modulate both plasma concentration and cellular origin of circulating microparticles in sickle cell anemia children. *Haematologica* 2013; **98**: 862–7.

41 Tantawy AA, Adly AA, Ismail EA, Habeeb NM, Farouk A. Circulating platelet and erythrocyte microparticles in young children and adolescents with sickle cell disease: relation to cardiovascular complications. *Platelets* 2013; **24**: 605–14.

42 Marques FK, Campos FM, Filho OA, Carvalho AT, Dusse LM, Gomes KB. Circulating microparticles in severe preeclampsia. *Clin Chim Acta* 2012; **414**: 253–8.

43 Gao C, Xie R, Yu C, Wang Q, Shi F, Yao C, Xie R, Zhou J, Gilbert GE, Shi J. Procoagulant activity of erythrocytes and platelets through phosphatidylserine exposure and microparticle release in patients with nephrotic syndrome. *Thromb Haemost* 2012; **107**: 681–9.

44 Leonetti D, Reimund JM, Tesse A, Viennot S, Martinez MC, Bretagne AL, Andriantsitohaina R. Circulating microparticles from Crohn's disease patients cause endothelial and vascular dysfunctions. *PLoS One* 2013; **8**: e73088.

45 Nantakomol D, Palasuwan A, Chaowanathikhom M, Soogarun S, Imwong M. Red cell and platelet-derived microparticles are increased in G6PD-deficient subjects. *Eur J Haematol* 2012; **89**: 423–9.

46 Rubin O, Canellini G, Delobel J, Lion N, Tissot JD. Red blood cell microparticles: clinical relevance. *Transfus Med Hemother* 2012; **39**: 342–7.

47 Koshiar RL, Somajo S, Norstrom E, Dahlback B. Erythrocyte-derived microparticles supporting activated protein C-mediated regulation of blood coagulation. *PLoS One* 2014; **9**: e104200.

48 Jy W, Johansen ME, Bidot C Jr, Horstman LL, Ahn YS. Red cell-derived microparticles (RMP) as haemostatic agent. *Thromb Haemost* 2013; **110**: 751–60.

49 Rubin O, Crettaz D, Canellini G, Tissot JD, Lion N. Microparticles in stored red blood cells: an approach using flow cytometry and proteomic tools. *Vox Sang* 2008; **95**: 288–97.

50 Horne MK 3rd, Cullinane AM, Merryman PK, Hoddeson EK. The effect of red blood cells on thrombin generation. *Br J Haematol* 2006; **133**: 403–8.

51 Westerman M, Pizzey A, Hirschman J, Cerino M, Weil-Weiner Y, Ramotar P, Eze A, Lawrie A, Purdy G, Mackie I, Porter J. Microvesicles in haemoglobinopathies offer insights into mechanisms of hypercoagulability, haemolysis and the effects of therapy. *Br J Haematol* 2008; **142**: 126–35.

52 van der Meijden PE, van Schilfgaarde M, van Oerle R, Renne T, ten Cate H, Spronk HM. Platelet- and erythrocyte-derived microparticles trigger thrombin generation via factor XIIa. *J Thromb Haemost* 2012; **10**: 1355–62.

53 Xie R, Jia D, Gao C, Zhou J, Sui H, Wei X, Zhang T, Han Y, Shi J, Bai Y. Homocysteine induces procoagulant activity of red blood cells via phosphatidylserine exposure and microparticle generation. *Amino Acids* 2011; **46**: 1997–2004.

54 Gerotziafas GT, van Dreden P, Chaari M, Galea V, Khaterchi A, Lionnet F, Stankovic-Stojanovic K, Blanc-Brude O, Woodhams B, Maier-Redelsperger M, Girot R, Hatmi M, Elalamy I. The acceleration of the propagation phase of thrombin generation in patients with steady-state sickle cell disease is associated with circulating erythrocyte-derived microparticles. *Thromb Haemost* 2012; **107**: 1044–52.

55 Camus SM, Gausseres B, Bonnin P, Loufrani L, Grimaud L, Charue D, De Moraes JA, Renard JM, Tedgui A, Boulanger CM, Tharaux PL, Blanc-Brude OP. Erythrocyte microparticles can induce kidney vaso-occlusions in a murine model of sickle cell disease. *Blood* 2012; **120**: 5050–8.

56 Giannopoulos G, Oudatzis G, Paterakis G, Synetos A, Tampaki E, Bouras G, Hahalis G, Alexopoulos D, Tousoulis D, Cleman MW, Stefanadis C, Deftereos S. Red blood cell and platelet microparticles in myocardial infarction patients treated with primary angioplasty. *Int J Cardiol* 2014; **176**: 145–50.

57 Camus SM, De Moraes JA, Bonnin P, Abbyad P, Le Jeune S, Lionnet F, Loufrani L, Grimaud L, Lambry JC, Charue D, Kiger L, Renard JM, Larroque C, Le Clesiau H, Tedgui A, Bruneval P, Barja-Fidalgo C, Alexandrou A, Tharaux PL, Boulanger CM, *et al*. Circulating cell membrane microparticles transfer heme to endothelial cells and trigger vaso-occlusions in sickle cell disease. *Blood* 2015; **125**: 3805–14.

58 Xiong Z, Cavaretta J, Qu L, Stolz DB, Triulzi D, Lee JS. Red blood cell microparticles show altered inflammatory chemokine binding and release ligand upon interaction with platelets. *Transfusion* 2011; **51**: 610–21.

59 Porto I, Biasucci LM, De Maria GL, Leone AM, Niccoli G, Burzotta F, Trani C, Tritarelli A, Vergallo R, Liuzzo G, Crea F. Intracoronary microparticles and microvascular obstruction in patients with ST elevation myocardial infarction undergoing primary percutaneous intervention. *Eur Heart J* 2012; **33**: 2928–38.

60 Tan KT, Lip GY. The potential role of platelet microparticles in atherosclerosis. *Thromb Haemost* 2005; **94**: 488–92.

61 Forlow SB, McEver RP, Nollert MU. Leukocyte–leukocyte interactions mediated by platelet microparticles under flow. *Blood* 2000; **95**: 1317–23.

62 Suades R, Padro T, Alonso R, Mata P, Badimon L. Lipid-lowering therapy with statins reduces microparticle shedding from endothelium, platelets and inflammatory cells. *Thromb Haemost* 2013; **110**: 366–77.

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 (Badimon *et al.,* 1987) and a flat perfusion chamber, as previously described (Molins *et al.,* 2008, 2010). 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) (Badimon *et al.,* 1988), 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 (Badimon *et al.,* 1987; Badimon and Badimon, 1989), 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} (Badimon *et al.,* 1988), 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 (Suades *et al.,* 2012). Fibrinogen and platelet deposition on the substrate were also morphometrically evaluated by immunohistology and fluorescence microscopy as reported (Suades *et al.,* 2012).

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 (Molins *et al.,* 2008, 2010, 2011; Suades *et al.,* 2012; de la Torre *et al.,* 2013). 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 (Molins *et al.,* 2008; Suades *et al.,* 2012).

Flow cytometric analysis of circulating microparticles

Triple-label flow cytometric analysis was performed as previously described (Suades *et al.,* 2013, 2014). 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 (Rank *et al.,* 2012), 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.

SUPPLEMENTAL METHODS REFERENCES

Badimon, L. and Badimon, J. J. (1989), "Mechanisms of arterial thrombosis in nonparallel streamlines: platelet thrombi grow on the apex of stenotic severely injured vessel wall. Experimental study in the pig model," *J Clin Invest,* 84(4): 1134-1144.

Badimon, L. *et al.* (1987), "Characterization of a tubular flow chamber for studying platelet interaction with biologic and prosthetic materials: deposition of indium 111-labeled platelets on collagen, subendothelium, and expanded polytetrafluoroethylene," *J Lab Clin Med,* 110(6): 706-718.

Badimon, L. *et al.* (1988), "Platelet thrombus formation on collagen type I. A model of deep vessel injury. Influence of blood rheology, von Willebrand factor, and blood coagulation," *Circulation,* 78(6): 1431-1442.

de la Torre *et al.,* (2013), "Monomerization of C-reactive protein requires glycoprotein IIb-IIa activation: pentraxins and platelet deposition," *J Thromb Haemost,* 11(11): 2048-2058.

Molins, B. *et al.* (2008), "C-reactive protein isoforms differ in their effects on thrombus growth," *Arterioscler Thromb Vasc Biol,* 28(12): 2239-2246.

Molins, B. *et al.* (2010), "Glucose-regulated protein 78 and platelet deposition: effect of rosuvastatin," *Arterioscler Thromb Vasc Biol,* 30(6): 1246-1252.

Molins, B. *et al.* (2011), "Monomeric C-reactive protein is prothrombotic and dissociates from circulating pentameric C-reactive protein on adhered activated platelets under flow," *Cardiovasc Res,* 92(2): 328-337.

Rank, A. *et al.* (2012), "Circulating microparticles in patients with benign and malignant ovarian tumors," *Anticancer Res,* 32(5): 2009-2014.

Suades, R. *et al.* (2012), "Circulating and platelet-derived microparticles in human blood enhance thrombosis on atherosclerotic plaques," *Thromb Haemost,* 108(6): 1208-1219.

Suades, R. *et al.* (2013), "Lipid-lowering therapy with statins reduces microparticle shedding from endothelium, platelets and inflammatory cells," *Thromb Haemost,* 110(2): 366-377.

Suades, R. *et al.* (2014), "Circulating $CD45^{+}/CD3^{+}$ lymphocyte-derived microparticles map lipid-rich atherosclerotic plaques in FH patients," *Thromb Haemost,* 111(1).

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

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

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

Notes: AV^+ indicates annexin V-positive; cMPs, circulating-derived microparticles; ErMPs indicates erythrocyte-derived microparticles.
Source: Own elaboration.

ARTICLE 6

Circulating microparticle signature in coronary and peripheral blood of ST elevation myocardial infarction patients in relation to pain-to-PCI elapsed time

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

Published Published – Int. J. Cardiol 2016; 202: 378-387

OBJECTIVE

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.

Suades, R.; Padró, T.; Crespo, J.; Ramaiola, ll., Martin-Yuste, V.; Sabaté, M.; Sans-Roselló, J.; Sionis, A.; Badimon, L.

"Circulating microparticle signature in coronary and peripheral blood of ST elevation myocardial infarction patients in relation to pain-to-PCI elapsed time."

J. Cardiol 2016; 202: 378-387.

International Journal of Cardiology 202 (2016) 378–387

Contents lists available at ScienceDirect

International Journal of Cardiology

journal homepage: www.elsevier.com/locate/ijcard

Circulating microparticle signature in coronary and peripheral blood of ST elevation myocardial infarction patients in relation to pain-to-PCI elapsed time

R. Suades [a,1], T. Padró [a,1], J. Crespo [a,1], I. Ramaiola [a,1], V. Martin-Yuste [b,1], M. Sabaté [b,1], J. Sans-Roselló [c,1], A. Sionis [c,1], L. Badimon [a,d,*,1]

[a] Cardiovascular Research Center, CSIC-ICCC, IIB-Sant Pau, Barcelona, Spain
[b] Department of Interventional Cardiology, Hospital Clinic, Barcelona, Spain
[c] Acute and Intensive Cardiac Care Unit, Cardiology Department, Hospital Santa Creu i Sant Pau, Biomedical Research Institute Sant Pau (IIB Sant Pau), Barcelona, Spain
[d] Cardiovascular Research Chair, UAB, Barcelona, Spain

ARTICLE INFO

Article history:
Received 16 June 2015
Received in revised form 29 July 2015
Accepted 8 September 2015
Available online 12 September 2015

Keywords:
Biomarkers
Circulating cell-derived microparticles
Myocardial revascularization
Myocardial infarction
Thrombosis

ABSTRACT

Background: Circulating microparticle (cMP) levels are increased in the acute phase of ST-elevation myocardial infarction (STEMI) and associate with microvascular obstruction; however, the precise cMP-parental cell signature and activation level are not elucidated. Here, we aimed to study the cMP signature in STEMI-patients and whether cMP phenotype changes in relation to onset of pain-to-PCI [ischemic time (IT)]-elapsed time.
Methods: Blood was taken at PCI from the culprit coronary and the peripheral circulation in STEMI-patients ($N = 40$). Two control groups were included: peripheral blood of age-matched patients recovering from STEMI [after 72 h] and of control individuals ($N = 20$/group). cMP-parental origin and activation level were characterized by triple-labeling flow cytometry.
Results: Procoagulant annexin V-positive cMPs bearing parental cell markers as well as markers of activated cells displayed a significantly different profile in STEMI-patients, in control individuals and in patients recovering from STEMI. cMPs derived from monocytes, endothelium, and activated vascular cells were higher in the culprit coronary artery than in peripheral blood in STEMI-patients, especially in patients intervened at short IT. Indeed, cMP levels in coronary blood were inversely related to IT duration (more abundant in thrombi with pain-to-PCI time <180 min).
Conclusions: A characteristic [$CD66b^+/CD62E^+/CD142^+$] cMP signature in the systemic circulation reflects the formation of coronary thrombotic occlusions in STEMI-patients. Changes in the cMP signature in the culprit coronary artery blood reveal the sensitivity of MPs to detect the ischemia-elapsed time. Interestingly, cMPs in peripheral blood may be sensitive markers of the thrombo-occlusive vascular process developing in the coronary arteries of STEMI-patients.

1. Introduction

Acute coronary syndromes (ACSs) are the leading cause 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 (PCI) represents the reperfusion strategy of choice in patients with STEMI, provided it is delivered in a timely fashion [6].

The mechanisms of thrombus formation on disrupted or eroded atherosclerotic plaques have been partly elucidated, including both the concept of an arterial wall-driven and of a vulnerable blood-driven effect [7–9]. Indeed, we have recently demonstrated that circulating and, specifically, platelet-derived microparticles increase the

Abbreviations: ACS, acute coronary syndrome; AUC, area under the curve; AV, annexin V; CAD, coronary artery disease; cMP, circulating microparticle; CV, cardiovascular; ECs, endothelial cells; eMPs, endothelial-derived microparticles; ErMPs, erythrocyte-derived microparticles; FH, familial hypercholesterolemia; gMPs, granulocyte-derived microparticles; IQR, interquartile range; IT, ischemic time; LMPs, leukocyte-derived microparticles; /MPs, lymphocyte-derived microparticles; mAbs, monoclonal antibodies; MI, myocardial infarction; mMPs, monocyte-derived microparticles; PCI, percutaneous coronary intervention; PFP, platelet-free plasma; pMPs, platelet-derived microparticles; ROC, receiver operating characteristic curve; STEMI, ST elevation myocardial infarction; TF, tissue factor.

* Corresponding author at: Cardiovascular Research Center, c/Sant Antoni Mª Claret 167, 08025 Barcelona, Spain. Tel.: +34 935565880; fax: +34 935565559.
E-mail address: lbadimon@csic-iccc.org (L. Badimon).

[1] All the authors take responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.

http://dx.doi.org/10.1016/j.ijcard.2015.09.011
0167-5273/

thrombogenic potential of underlying triggering atherosclerotic plaques by modifying the composition of the growing thrombus and contributing to thrombosis [10,11].

Circulating microparticles (cMPs) are small phospholipid microvesicles shed into blood from activated or apoptotic cells of the vascular compartment with an uncertain commitment [12]. Whether they are residual debris with biomarker potential or have special functions is not fully known. However, they seem to be able to exert cell–cell communication [13] with special importance in atherothrombosis [14]. High levels of procoagulant microparticle subpopulations have been reported in the circulation of patients with ACS [15], including STEMI [16,17], even when compared to stable angina [18], reflecting both platelet and cell activation status [19,20]. Platelet and endothelial cell-derived MPs also correlate with the size of myocardium at risk in STEMI patients [21], indicating their involvement in disease severity. Local elevation of procoagulant MPs has also been detected within the occluded coronary artery of patients with STEMI [22,23] showing again that they might play a role in the formation of intracoronary thrombi and on microembolization [24].

Interestingly, several studies have investigated cMPs as novel biomarkers at different stages of cardiovascular disease progression [25–27]. 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: (1) whether the systemic blood cMP signature, in terms of parental cell origin and activation status, discriminates acute STEMI patients, (2) whether the cMP-STEMI phenotype differs in relation to ischemic time (pain-to-PCI-evolved time), and (3) whether the cMP signature predicts clinical evolution in the STEMI patients.

2. Methods

2.1. Clinical population

Forty STEMI patients treated by PCI with thrombus aspiration were included. PCI was performed according to guidelines [28]. All patients were treated with unfractionated heparin while glycoprotein IIb/IIIa antagonist was administered at physician's discretion. A group of control subjects ($N = 20$) matched by age, gender, risk factors and pharmacological treatments was included for comparative purposes. A second control group of patients recovering from STEMI ($N = 20$) was also included. Demographic and clinical characteristics of the STEMI and control groups are provided in Table S1. Pharmacological treatment of both STEMI and post-MI patients is summarized in Table S2. To investigate the effect of ischemic time (from onset-of-pain-to-PCI time) on changing patterns in cMP-signature, patients were categorized into two groups: ≤3 h ($N = 24$) or >3 h ($N = 16$). Main clinical characteristics of these groups are listed in Table 1. 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 the presence of sepsis, infectious disease, cancer or pregnancy, since these conditions may influence MP measurement. Patients undergoing rescue PCI were not included in the study. The results are presented in accordance with STROBE guidelines.

2.2. Blood sampling

Blood was withdrawn from the culprit coronary (obtained from the aspiration catheter during manual thrombectomy) arteries and simultaneously from a peripheral artery of the STEMI patients undergoing PCI. Peripheral artery blood was obtained from controls and from post-MI patients. Blood was collected in EDTA tubes for cMP characterization. Within 2 h of collection blood cells were removed by a double low-speed centrifugation to obtain the platelet-free plasma (PFP), which was snap-frozen in liquid nitrogen and stored at −80 °C until flow cytometric studies were performed, as we have previously described [26,29].

2.3. 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 [26,29,30]. Briefly, washed cMPs 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 S3). Gate limits were established as described [26,29]. 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. 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. Intra-assay CV of cMP counts was 3.1%, while inter-assay CV was 5.4%.

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 (83.3%)	14 (87.5%)	$P = 0.7177$
Drug-eluting stenting	4 (16.6%)	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 (83.3%)	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%)	$P = 0.9999$
Lipid-lowering therapy-statins	8 (33%)	8 (50%)	$P = 0.2918$

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; PCI, primary percutaneous coronary intervention; RCA, right coronary artery; SE, standard error; STEMI, ST-segment elevation myocardial infarction; TIMI, thrombolysis in myocardial infarction.

2.4. 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 (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, comparison between control and STEMI patients for variables with Mann–Whitney U test, and relationships between two studied parameters with Spearman correlation. Analysis of variance, followed by Bonferroni correction, and Kruskal–Wallis test were used in multiple comparisons. In order to test the effect of confounding factors in the studied variables, a bivariate analysis (correlation or t-test) followed by a multiple linear regression model (stepwise selection of variables) was performed for each comparison, including those variables statistically significant in the bivariate analysis to assess the most parsimonious model. To determine the prognostic value of cMPs, a binary logistic regression model (evaluated by Hosmer–Lemeshow goodness-of-fit test) was carried out to estimate the likelihood of STEMI by creating predicted probabilities in accordance with Peduzzi's recommendations [31] and receiver operating characteristic (ROC) curve analyses and the corresponding area 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 statistical tests and a $P < 0.05$ was considered statistically significant.

3. Results

3.1. Clinical characteristics

Baseline clinical characteristics of STEMI-patients undergoing PCI 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 6 h. Optimal PCI 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 h and >3 h. Clinical characteristics of STEMI-patients compared to 72 h post-STEMI patients and controls are listed in Table S1. Baseline characteristics were not different between the two groups.

3.2. Cell origin of circulating microparticles in the systemic circulation of STEMI patients

Results are expressed as percentage of annexin V-positive circulating microparticles (AV^+-cMPs). The pattern of distribution of peripheral blood annexin V^+-cMPs in STEMI-patients differed from controls (Figs. 1 and 2). Specifically, AV^+-cMPs derived from leukocytes (LMPs) (including monocytes [mMPs] and lymphocytes [↙MPs]) and endothelial cells (eMPs) were significantly increased in the systemic circulation of STEMI-patients compared to controls (Fig. 1A–B). In patients recovering from MI at day 3, the number of mMPs, ↙MPs, and eMPs in peripheral blood was lower than in the acute phase (Fig. 1A–B). Similarly, 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]) and were significantly reduced after 72 h post-MI but without reaching the low level of controls (29.9 [16.4] % of AV^+-cMPs), as previously reported [32]. On the contrary, pMPs were reduced in the peripheral arteries of STEMI patients (53.5 [30.0] vs. 90.7 [10.1] % of AV^+-cMPs, median [IQR]) and remained in reduced amounts after 72 h of MI (59.0 [18.4] % of AV^+-cMPs) as previously shown [32]. In contrast, LMPs remained increased after 72 h of MI compared to controls (Fig. 1A).

Finally, to investigate the potential role of systemic blood cMPs as biomarkers of STEMI, subsequent ROC analyses were performed in controls and STEMI-patients data ($N = 20$ C, $N = 40$ STEMI). There was an increase in LMPs in STEMI and post-MI patients over controls, and the ROC curve analysis of LMPs resulted in an AUC of 0.809 ± 0.064 [95% CI: 0.685–0.934] ($P < 0.001$) showing a high diagnostic power for STEMI. Then, a selected panel of cMPs derived from specific parental cells and composed of ↙MPs ($CD45^+/CD3^+$), mMPs ($CD14^+$), and eMPs ($CD146^+$) produced an AUC of 0. 898 ± 0.041 [95% CI: 0.818–0.978] ($P < 0.001$), showing a very high discriminatory power (Fig. 1C). pMPs were excluded from the ROC curve analysis as STEMI-patients are regularly under in-hospital anti-thrombotic treatment and hence pMPs could be affected by this regimen. Differences in cell origin-specific cMPs between acute STEMI patients and controls (both post-MI patients and controls) were still observed after adjustment for confounding factors such as classical CV risk factors, age and previous statin treatment in a multivariate regression model (Table S4).

3.3. Circulating microparticles in peripheral blood identify the activated parental cell in STEMI patients

Not only annexin V^+-cMPs bearing parental cell markers significantly changed but also those AV^+-cMPs carrying markers of activated parental cells (Fig. 2). Indeed, the percentage of AV^+-cMPs from activated granulocytes ($CD66b^+$-gMPs; Fig. 2A) was significantly increased in the peripheral arteries of STEMI patients compared to controls. Similarly AV^+-cMPs from endothelial cells (ECs) bearing E-selectin ($CD62E^+$-eMPs; Fig. 2B) and from activated platelets carrying activated $\alpha_{IIb}\beta_3$-integrin ($PAC1^+$-pMPs; Fig. 2D) were significantly higher in the peripheral circulation of STEMI-patients. On the contrary, the fraction of AV^+-cMPs carrying $CD31^+$ (from platelets and ECs) (PECAM-1 or $CD31^+$-pMPs; Fig. 2C) was significantly decreased ($P < 0.0001$). Interestingly, annexin V^+-cMPs rich in tissue factor (TF; $CD142^+$) were also significantly higher in the systemic circulation of STEMI-patients than in controls (Fig. 2E). In the peripheral blood of patients recovering from MI cMPs showed lower levels of inflammatory and endothelial cell as well as platelet activation than in the STEMI phase but higher than in control patients (Fig. 2A–D). To evaluate the discriminatory power of activated-cMPs to detect STEMI, a comparison by ROC curve analysis was performed between e/pMP and the combination of gMPs ($CD66b^+$), eMPs ($CD62E^+$), and TF^+-cMPs ($CD142^+$) [controls ($N = 20$) and STEMI patients ($N = 40$)]. An AUC of 0.843 ± 0.054 [95% CI: 0.737–0.949] ($P < 0.001$) was obtained for e/pMP and an AUC of 0.975 ± 0.019 [95% CI: 0.937–1.000] ($P < 0.001$) for gMPs/eMPs/TF^+-cMPs (Fig. 2F). Differences in activated cell-specific cMPs between acute STEMI patients and controls (both post-MI patients and controls) were still observed after adjustment for confounding factors such as classical CV risk factors, age and previous statin treatment in a multivariate regression model (Table S4).

3.4. Circulating microparticles in peripheral blood and intracoronary blood in STEMI patients

AV^+-cMPs derived from activated cells were significantly higher in intracoronary blood than in peripheral blood. Specifically, AV^+-cMPs from monocytes ($CD14^+$-mMPs) and from activated monocytes ($CD14^+/CD11b^+$-mMPs; Fig. 3A), activated platelets ($PAC1^+$ and $PAC1^+/CD62P^+$; pMPs; Fig. 3B), and ECs ($CD146^+$-eMPs) and activated ECs ($CD62E^+$; eMPs; Fig. 3C) were significantly higher in intracoronary blood. In addition, TF-bearing AV^+-cMPs were also higher in culprit coronary artery blood than in peripheral blood: total ($CD142^+$; TF^+-cMPs) and endothelial-derived ($CD62E^+/CD142^+$; TF^+-eMPs; Fig. 3D; $P < 0.05$ in both cases). Cell-derived MPs from leukocytes ($CD45^+$-LMPs), lymphocytes ($CD3^+$-↙MPs), platelets ($CD61^+$-pMPs), and erythrocytes ($CD235a^+$-ErMPs) were similar in the culprit coronary artery blood and in peripheral blood (Fig. 4A). These combined four cMPs were able to predict STEMI when c-statistics was applied to control subjects and STEMI patients with an AUC of 0.979 ± 0.014 [95% CI: 0.951–1.000] ($P < 0.0001$, Fig. 4B). Since ErMPs alone have been proved to significantly predict ongoing thrombosis [32], we performed the same analysis taking into account the combined predicted probabilities of $CD45^+$-LMPs, $CD3^+$-↙MPs, and $CD61^+$-pMPs. In this case, the

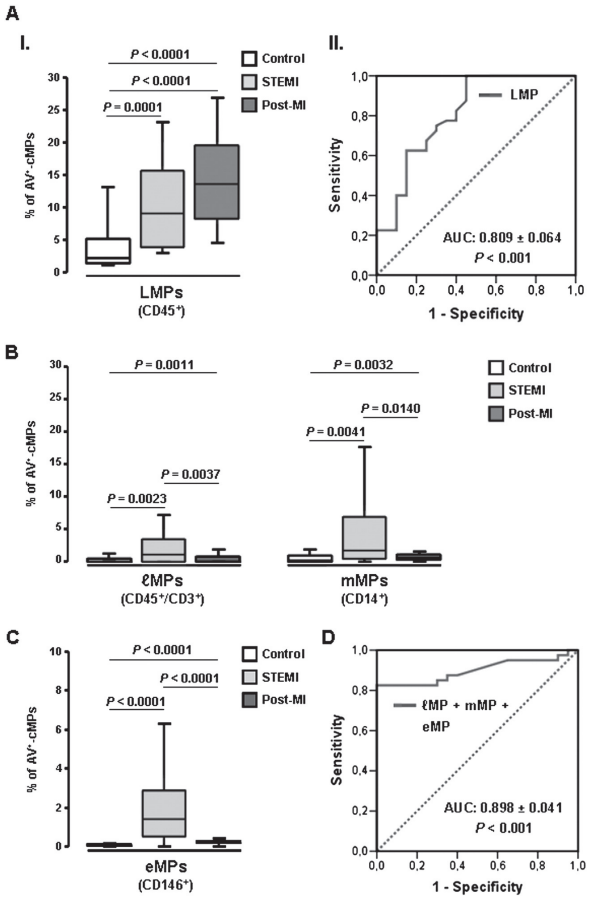


Fig. 1. Cell origin distribution of circulating microparticles in STEMI-patients. Box and whisker plots of AV^+-cMPs from (A–I) leukocytes, (B) lymphocytes and monocytes, and (C) endothelial cells in control subjects ($N = 20$), STEMI-patients ($N = 40$) and post-MI patients ($N = 20$). Data are expressed as median [IQR] (non-parametric U-Mann Whitney and Kruskal Wallis tests). ROC curve analyses were used to evaluate LMPs (A-II) and combination of cMPs (D) to predict STEMI with AUC indicated along its 95% CI ($P < 0.001$).

predictive value depicted an AUC of 0.913 ± 0.040 [95% CI: 0.835–0.990] ($P < 0.0001$, Fig. 4B).

3.5. Circulating microparticles and elapsed ischemic time (pain-to-PCI) — change in culprit site reactivity

The levels and phenotype of intracoronary AV^+-cMP discriminated between short and long ischemic times (IT; time from the onset of chest pain to PCI) ($P < 0.05$). AV^+-cMPs derived from monocytes ($CD14^+$-mMPs) and activated monocytes ($CD14^+/CD11b^+$-mMPs) (Fig. 5A), endothelial cells ($CD146^+$-eMPs; Fig. 5B), and TF-rich monocytes and platelets ($CD142^+/CD14^+$-TF^+-mMPs and $CD142^+/CD61^+$-TF^+-pMPs; Fig. 5C) were significantly higher in patients with an IT ≤3 h than in those with IT >3 h. The combined effect of mMPs ($CD14^+$), eMPs ($CD146^+$), and TF^+-pMPs ($CD142^+$) was also able to predict STEMI, when determined by ROC curve analysis. Specific c-statistics AUC was 0.920 ± 0.036 [95% CI: 0.850–.0990] ($P < 0.001$, Fig. 5D).

In the peripheral blood of STEMI patients, AV^+-cMP levels were increased but similar in the IT ≤3 h group to those observed in the IT >3 h group.

In the IT ≤3 h group, monocyte-derived AV^+-mMPs ($CD14^+$ and $CD14^+/CD11b^+$; Fig. S1A), endothelial-derived AV^+-eMPs ($CD146^+$ and $CD146^+/CD31^+$; Fig. S1B), activated EC-derived AV^+-eMPs ($CD62E^+$ and $CD62E^+/CD142^+$; Fig. S1C), and activated platelet-derived AV^+-pMPs ($PAC1^+$ and $PAC1^+/CD62P^+$; Fig. S1D) were significantly elevated in culprit coronary artery compared to peripheral artery, indicating the active focus of vascular injury, inflammation and thrombosis. In contrast, no significant differences, in terms of all type of analyzed AV^+-cMPs among peripheral versus coronary blood were observed in >3 h, except for AV^+-cMPs derived from activated platelets carrying the activated

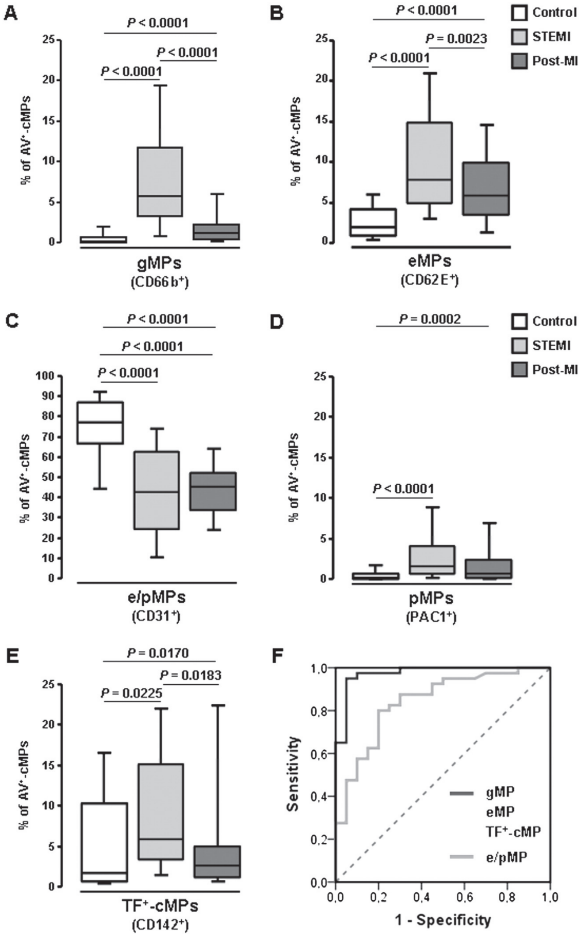


Fig. 2. Circulating microparticles from activated cells in STEMI-patients. Box and whisker plots of AV^+-cMPs from (A) activated leukocytes, (B) activated endothelial cells, (C) both platelets and endothelial cells, (D) activated platelets, and (E) tissue factor-positive cells in control subjects ($N = 20$), STEMI-patients ($N = 40$) and post-MI patients ($N = 20$). Data are expressed as median [IQR] (non-parametric U-Mann Whitney and Kruskal Wallis tests). (F) ROC curve analyses used to evaluate e/pMP with an AUC of 0.843 ± 0.054 along its 95% CI [0.737–0.949] and combination of cMPs with an AUC of 0.975 ± 0.019 [95% CI: 0.949–1.000] to predict STEMI (both $P < 0.001$).

$\alpha_{IIb}\beta_3$-integrin ($PAC1^+$-pMPs), which were also elevated in the culprit intracoronary blood in the IT >3 h group (Fig. S1D). Interestingly, intracoronary and peripheral levels of each phenotype were directly and positively related to each other (Table S5). Besides, cMP numbers did not correlate with Troponin T (Table S6) nor with CK peak levels (Table S7) in either acute STEMI or in the post-STEMI control group, indicating that they are measuring distinct mechanisms of injury.

3.6. Impact of clinical parameters on circulating microparticles

cMPs were able to discriminate the presence of multivessel disease. AV^+-eMPs ($CD62E^+$) and TF^+-eMPs ($CD62E^+/CD142^+$) were higher in the systemic circulation of patients with multivessel atherosclerotic disease (Fig. 6).

4. Discussion

The present study demonstrates a significant shift in the profile of cMPs in patients suffering ST-elevation myocardial infarction in comparison to two control groups, control subjects and patients recovering from MI (day 3). Changes in cMPs shed from cells involved in prothrombotic and proinflammatory activities and in endothelial dysfunction are found raised in the systemic circulation, reflecting the sensitivity of cMPs as markers of the atherothrombotic event. When these

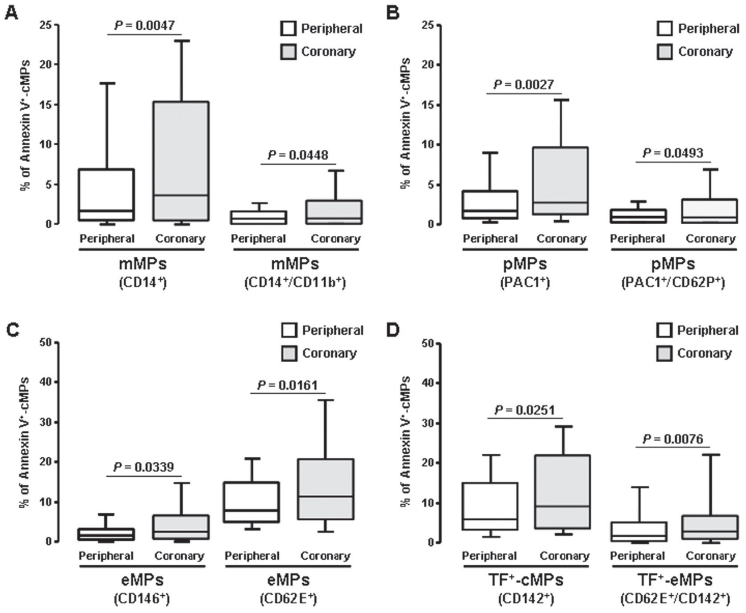


Fig. 3. Distribution of cell-derived circulating microparticles bearing markers of cell activation in peripheral and intracoronary blood of STEMI-patients. Box and whisker plots of AV^+-cMPs from (A) total and activated monocytes, (B) activated platelets, (C) total and activated endothelial cells, and (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] (non-parametric Wilcoxon-Signed Rank test).

changes in cMPs in controls and acute STEMI patients were analyzed by c-statistics, the area under the ROC curves for the prediction of STEMI was highly significant [0.898 for cMP-cell origin and 0.975 for cMPs carrying markers of cell activation].

High levels of cMPs in STEMI patients have been described [15–20], mainly procoagulant annexin V^+-MPs from endothelial ($CD31^+/CD42^-$; $CD146^+$) and platelet ($CD62P^+$; $CD63^+$; $CD31^+/CD42^+$) origin. Upon coronary plaque rupture, extracellular microvesicles are released from platelets ($CD31^+/CD42^+$) and apoptotic endothelial cells ($CD31^+/CD42^-$; $CD144^+$) which are indeed associated to the area at risk during ST-elevation myocardial infarction [21,33]. Additionally, here we have detected changes in the pattern of distribution of cMPs derived from the activated endothelium ($CD146^+$- and $CD62E^+$-eMPs), inflammatory cells ($CD45^+$-LMPs, $CD45^+/CD3^+$-↙MPs, $CD66b^+$-gMPs, and $CD14^+$-mMPs), activated platelets ($PAC1^+$-pMPs), erythrocytes ($CD235a^+$-ErMPs) as well as tissue factor-rich cMPs ($CD142^+$-cMPs) in the acute phase of STEMI. In the group of patients recovering from MI we observed a global decrease in cMPs, in accordance with the successful intervention procedures (revascularization plus pharmacology). Interestingly, in patients 3 days after MI the levels of activated pMPs, eMPs, and tissue factor-rich MPs were still high, likely being involved in the modulation of the post-ACS reparative response to injury [34]. Pan-LMPs ($CD45^+$-MPs) were kept increased after 72 h of the acute infarction, likely because the inflammatory burst occurred at STEMI onset. Indeed, not all subpopulations of LMPs are equally raised; post-STEMI cMPs from monocytes (mMPs) and lymphocytes (↙MPs) return to proportions of controls, a decrease that is not blunted after the acute phase. These results seem to indicate a selective targeting of leukocyte subsets to the damaged myocardium, possibly reflecting that specific monocyte types exert reparative roles in MI, as previously reported [35], while others participate in the cross-talk platelet-monocytes, which indicates a complex interplay of these cells in the post-MI state. The high levels of LMPs after 3 days of the ischemic event suggest that plaque atheromatous core may act as a reservoir of MPs within the coronary artery. In agreement, Leroyer et al. found that plaque MPs were mostly of leukocyte origin [36]. It is known that thrombus is not always fully resolved and the plaques are active for some time after the main event. Whether LMP release is a consequence of an active process with specific commitment in worsening of disease progression or is a passive reaction to the MI-associated inflammatory reaction remains to be determined. This point will require further study.

Levels of eMPs ($CD42^-CD31^+$) and pMPs ($CD42^+CD31^+$) have been found elevated in intracoronary blood of STEMI patients [24]. Here, we evaluated annexin V^+-circulating MPs with a more sophisticated methodology because we have isolated cMPs instead of measuring them directly from plasma, where there is potential immune complex formation. Here, in addition to $CD42^-CD31^+$-eMPs and $CD42^+CD31^+$-pMPs, we have investigated cMPs from total and activated monocytes ($CD14^+$- and $CD14^+/CD11b^+$-mMPs), activated platelets ($PAC1^+$ and $PAC1^+/CD62P^+$-pMPs), total and activated endothelial cells ($CD146^+$ and $CD62E^+$-eMPs), and those carrying tissue factor ($CD142^+$-TF^+cMPs and $CD62E^+/CD142^+$-TF^+eMPs) and found that they 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 from activated cells in the leading edge of growing thrombus. Indeed, patients with MI registered higher expression of VCAM-1 on eMPs from the site of coronary artery plaque during PCI compared to patients with stable CAD [37]. Likewise, intracoronary cMPs are directly related to ischemic time, being more prominent at the earlier time after symptom onset,

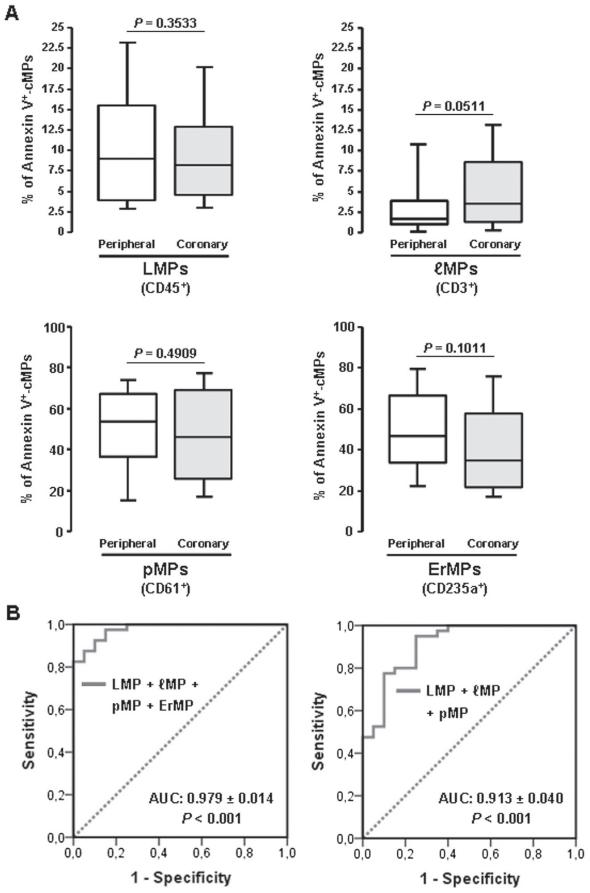


Fig. 4. Circulating microparticles identified by parental cell markers in peripheral blood of STEMI-patients as markers of culprit coronary lesion. (A) Box and whisker plots of AV^+-cMPs from leukocytes, lymphocytes, platelets, and erythrocytes in peripheral and intracoronary blood of STEMI-patients ($N = 40$). Data are expressed as median [IQR] (non-parametric Wilcoxon-Signed Rank test). (B) ROC curve analyses used to evaluate combination of cMPs to predict STEMI with AUC indicated along its 95% CI ($P < 0.001$).

reflecting active release of MPs at active sites. 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 h after symptom onset but not at later times, suggesting their local release and contribution to intracoronary thrombus. Leukocyte- $CD45^+$-LMPs, lymphocyte- $CD3^+$-ℓMPs, platelet- $CD61^+$-pMPs, and erythrocyte-derived $CD235a^+$-ErMPs levels did not change between intracoronary and peripheral artery blood and their combination at the systemic level was found to predict STEMI in a c-statistics model with an AUC of 0.979 (and of 0.913 without ErMPs). Interestingly, systemic circulating eMPs were associated to multivessel atherosclerotic disease patients.

Acute coronary events resulting in STEMI can lead to a high complex succession of processes such as release of inflammatory cytokines, oxidative burst of ROS from neutrophils, hypoxia and cardiomyocyte necrosis [38], which can also influence MP shedding. Indeed, the main MPs that are changed in STEMI are from circulating cells known to play a direct effect on atherothrombosis upon activation. In particular, endothelial-derived MPs have a role in endothelial dysfunction [39]; and $CD31^+/AV^+$-cMPs have been proposed as independent predictors of CV events in stable CAD patients [25] which may be useful for risk stratification. A recent study has shown increased eMPs in sudden cardiac death compared to STEMI without rhythmic disturbances suggesting different patterns of acute coronary occlusion [40]. We have found higher numbers of activated pMPs in STEMI patients that have exacerbated platelet activation and thrombus formation reinforcing our previous results [10,30]; and, pMPs carrying activation epitopes were higher both in intracoronary and systemic blood. Interestingly, MPs have emerged as signaling components favoring cell interaction and cell-to-cell communication [41]. 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 [42]. In addition,

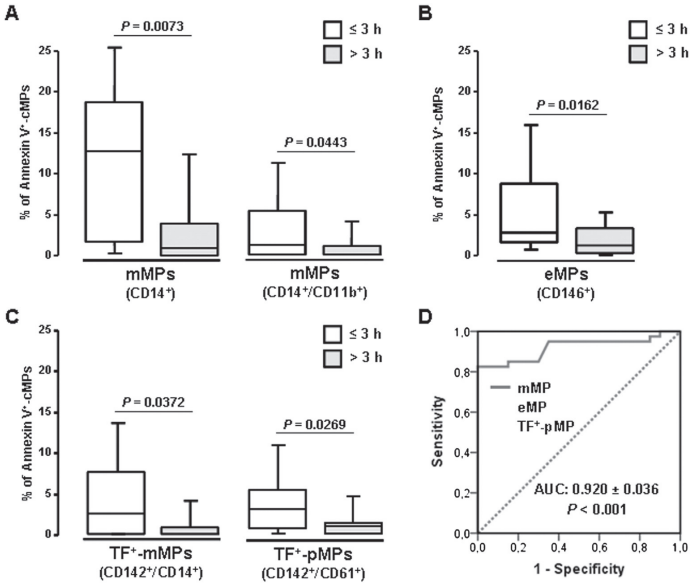


Fig. 5. Intracoronary circulating microparticles in association to ischemic time of STEMI-patients. Box and whisker plots of AV^+-cMPs from (A) monocytes and activated monocytes, (B) endothelial cells, and (C) tissue factor-positive monocytes and platelets in intracoronary blood of STEMI-patients ($N = 40$). Data are expressed as median [IQR] (non-parametric U-Mann Whitney test). (D) ROC curve analyses used to evaluate combination of cMPs to predict STEMI with AUC indicated along its 95% CI ($P < 0.0001$).

human atherosclerotic plaques contain high levels of MPs expressing CD40L and bearing TF [43]. 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 [44]. Therefore, the different types of cMPs act at various stages of the atherothrombotic disease, the major cause of ACS. cMPs from myocardial cells were out of the scope of this study due to a lack of reliable and cell specific surface markers. Recently signal regulatory protein α (SIRPα, CD172a) has been proposed as a suitable marker for human stem cells derived cardiomyocytes [45]; however, this is true within cardiovascular cell populations but not at the systemic level since it can also recognize $CD34^+$ progenitor cells, monocytes, granulocytes, macrophages, dendritic cells, among others. Thus, cardiomyocyte-MPs were not measured although they may also be contributors to the pathophysiology of STEMI patients and deserve further investigation.

In the present manuscript we evaluated cMPs as biomarkers of STEMI either alone or combined and data indicates that combining distinct cMP subsets is significantly superior in predicting STEMI than one type of cMPs alone and the combination adds value to the prognostic

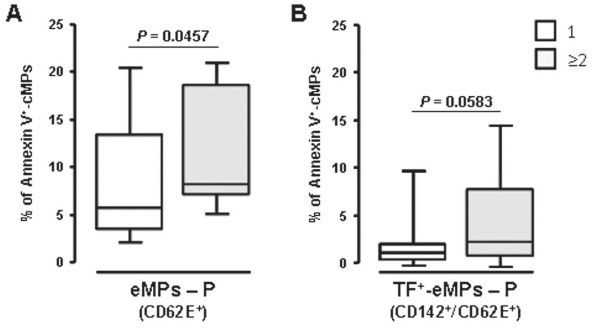


Fig. 6. Circulating microparticles of STEMI-patients related to number of diseased vessels. Box and whisker plots of AV^+-cMPs in association with number of diseased vessels (one versus two or more treated vessels). AV^+-cMPs from (A) endothelial cells and (B) tissue factor-positive endothelial cells in the peripheral blood of STEMI-patients ($N = 40$). Data are expressed as median [IQR] (non-parametric U-Mann Whitney test).

capacity of circulating MPs. Taken together, results suggest that a multi-panel approach could outperform the prediction of STEMI by adding complemented mechanistic information (with causal involvement) in the predictive value of cMPs for cardiovascular risk stratification.

5. Study limitations

A limitation of this study is that the use of concomitant drugs may affect cMP formation [29,46]; however, this possible influence has been evaluated by statistical multivariate analysis and found to be negligible.

6. Conclusions

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 PCI nor have approached a comparison between coronary and peripheral blood cMPs. In conclusion, our results give a profile signature of cMPs at both systemic and coronary levels in STEMI patients undergoing PCI, which is associated to duration of pain-to-PCI ischemic time. Taken together, these data provide information of cells involved in atherothrombosis, support a role for MPs in thrombus formation and propagation and indicate that MPs are sensitive to disease severity, reflecting the temporal evolution of disease. These new findings may in the long run improve the differential diagnosis in STEMI.

Conflict of interest

M.S. is a consultant at Abbott and Medtronic outside the submitted work.

Acknowledgments

This work was supported by the Spanish Ministry of Science Plan Estatal I+D+I 2013–2016 [SAF2013-42962-R to LB] cofunded by Fondo Europeo de Desarrollo Regional (FEDER-"Una manera de hacer Europa"), AGAUR (Agència de Gestió d'Ajuts Universitaris i de Recerca) [2009SGR826 to LB], TerCel (Red de Terapia Celular) [RD/12/0019/0026 to LB], Red de Investigación Cardiovascular (RIC) [RD12/0042/0027 to LB] and Spanish Ministry of Health [FIS-PI13/02850 to TP] from Instituto de Salud Carlos III (ISCIII) and Fundación de Investigación Cardiovascular (FIC)-Fundación Jesús Serra to the Cardiovascular Research Chair-UAB (LB).

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijcard.2015.09.011.

References

[1] J. Sanz, P.R. Moreno, V. Fuster, The year in atherothrombosis, J. Am. Coll. Cardiol. 62 (2013) 1131–1143.
[2] R. Lozano, M. Naghavi, K. Foreman, S. Lim, K. Shibuya, V. Aboyans, et al., Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010, Lancet 380 (2012) 2095–2128.
[3] E. Falk, M. Nakano, J.F. Bentzon, A.V. Finn, R. Virmani, Update on acute coronary syndromes: the pathologists' view, Eur. Heart J. 34 (2013) 719–728.
[4] V. Fuster, L. Badimon, J.J. Badimon, J.H. Chesebro, The pathogenesis of coronary artery disease and the acute coronary syndromes (2), N. Engl. J. Med. 326 (1992) 310–318.
[5] V. Fuster, L. Badimon, J.J. Badimon, J.H. Chesebro, The pathogenesis of coronary artery disease and the acute coronary syndromes (1), N. Engl. J. Med. 326 (1992) 242–250.
[6] P.W. Armstrong, A.H. Gershlick, P. Goldstein, R. Wilcox, T. Danays, Y. Lambert, et al., Fibrinolysis or primary PCI in ST-segment elevation myocardial infarction, N. Engl. J. Med. 368 (2013) 1379–1387.
[7] G. Cimmino, P. Golino, J.J. Badimon, Pathophysiological role of blood-borne tissue factor: should the old paradigm be revisited? Intern. Emerg. Med. 6 (2011) 29–34.
[8] L. Badimon, G. Vilahur, Thrombosis formation on atherosclerotic lesions and plaque rupture, J. Intern. Med. 276 (2014) 618–632.
[9] I. Ramaiola, T. Padro, E. Pena, O. Juan-Babot, J. Cubedo, V. Martin-Yuste, et al., Changes in thrombus composition and profilin-1 release in acute myocardial infarction, Eur. Heart J. 36 (16) (2014) 965–975, http://dx.doi.org/10.1055/s-0035-1556591.
[10] R. Suades, T. Padro, G. Vilahur, L. Badimon, Circulating and platelet-derived microparticles in human blood enhance thrombosis on atherosclerotic plaques, Thromb. Haemost. 108 (2012) 1208–1219.
[11] S.F. Mause, Platelet microparticles: reinforcing the hegemony of platelets in atherothrombosis, Thromb. Haemost. 109 (2013) 5–6.
[12] R. Suades, T. Padró, L. Badimon, The role of blood-borne microparticles in inflammation and hemostasis, Semin. Thromb. Hemost. 41 (6) (2015) 590–606, http://dx.doi.org/10.1055/s-0035-1556591.
[13] E. van der Pol, A.N. Boing, P. Harrison, A. Sturk, R. Nieuwland, Classification, functions, and clinical relevance of extracellular vesicles, Pharmacol. Rev. 64 (2012) 676–705.
[14] P.E. Rautou, A.C. Vion, N. Amabile, G. Chironi, A. Simon, A. Tedgui, et al., Microparticles, vascular function, and atherothrombosis, Circ. Res. 109 (2011) 593–606.
[15] Z. Mallat, H. Benamer, B. Hugel, J. Benessiano, P.G. Steg, J.M. Freyssinet, et al., Elevated levels of shed membrane microparticles with procoagulant potential in the peripheral circulating blood of patients with acute coronary syndromes, Circulation 101 (2000) 841–843.
[16] O. Morel, B. Hugel, L. Jesel, Z. Mallat, F. Lanza, M.P. Douchet, et al., Circulating procoagulant microparticles and soluble GPV in myocardial infarction treated by primary percutaneous transluminal coronary angioplasty. A possible role for GPIb-IIIa antagonists, J. Thromb. Haemost. 2 (2004) 1118–1126.
[17] G. Giannopoulos, G. Oudatzis, G. Paterakis, A. Synetos, E. Tampaki, G. Bouras, et al., Red blood cell and platelet microparticles in myocardial infarction patients treated with primary angioplasty, Int. J. Cardiol. 176 (2014) 145–150.
[18] L.M. Biasucci, I. Porto, L. Di Vito, G.L. De Maria, A.M. Leone, G. Tinelli, et al., Differences in microparticle release in patients with acute coronary syndrome and stable angina, Circ. J. 76 (2012) 2174–2182.
[19] E. Stepien, E. Stankiewicz, J. Zalewski, J. Godlewski, K. Zmudka, I. Wybranska, Number of microparticles generated during acute myocardial infarction and stable angina correlates with platelet activation, Arch. Med. Res. 43 (2012) 31–35.
[20] P.M. van der Zee, E. Biro, Y. Ko, R.J. de Winter, C.E. Hack, A. Sturk, et al., P-selectin- and CD63-exposing platelet microparticles reflect platelet activation in peripheral arterial disease and myocardial infarction, Clin. Chem. 52 (2006) 657–664.
[21] C. Jung, P. Sorensson, N. Saleh, H. Arheden, L. Ryden, J. Pernow, Circulating endothelial and platelet derived microparticles reflect the size of myocardium at risk in patients with ST-elevation myocardial infarction, Atherosclerosis 221 (2012) 226–231.
[22] P.K. Min, J.Y. Kim, K.H. Chung, B.K. Lee, M. Cho, D.L. Lee, et al., Local increase in microparticles from the aspirate of culprit coronary arteries in patients with ST-segment elevation myocardial infarction, Atherosclerosis 227 (2013) 323–328.
[23] O. Morel, B. Pereira, G. Averous, A. Faure, L. Jesel, P. Germain, et al., Increased levels of procoagulant tissue factor-bearing microparticles within the occluded coronary artery of patients with ST-segment elevation myocardial infarction: role of endothelial damage and leukocyte activation, Atherosclerosis 204 (2009) 636–641.
[24] I. Porto, L.M. Biasucci, G.L. De Maria, A.M. Leone, G. Niccoli, F. Burzotta, et al., Intracoronary microparticles and microvascular obstruction in patients with ST elevation myocardial infarction undergoing primary percutaneous intervention, Eur. Heart J. 33 (2012) 2928–2938.
[25] J.M. Sinning, J. Losch, K. Walenta, M. Bohm, G. Nickenig, N. Werner, Circulating $CD31^+$/Annexin V^+ microparticles correlate with cardiovascular outcomes, Eur. Heart J. 32 (2011) 2034–2041.
[26] R. Suades, T. Padro, R. Alonso, J. Lopez-Miranda, P. Mata, L. Badimon, Circulating $CD45^+/CD3^+$ lymphocyte-derived microparticles map lipid-rich atherosclerotic plaques in familial hypercholesterolaemia patients, Thromb. Haemost. 111 (2014) 111–121.
[27] G. Sarlon-Bartoli, Y. Bennis, R. Lacroix, M.D. Piercecchi-Marti, M.A. Bartoli, L. Arnaud, et al., Plasmatic level of leukocyte-derived microparticles is associated with unstable plaque in asymptomatic patients with high-grade carotid stenosis, J. Am. Coll. Cardiol. 62 (2013) 1436–1441.
[28] C.J. Terkelsen, D.S. Pinto, H. Thiele, P. Clemmensen, K. Nikus, J.F. Lassen, et al., 2012 ESC STEMI guidelines and reperfusion therapy: evidence base ignored, threatening optimal patient management, Heart 99 (2013) 1154–1156.
[29] R. Suades, T. Padro, R. Alonso, P. Mata, L. Badimon, Lipid-lowering therapy with statins reduces microparticle shedding from endothelium, platelets and inflammatory cells, Thromb. Haemost. 110 (2013) 366–377.
[30] R. Suades, T. Padro, R. Alonso, P. Mata, L. Badimon, High levels of $TSP1^+/CD142^+$ platelet-derived microparticles characterise young patients with high cardiovascular risk and subclinical atherosclerosis, Thromb. Haemost. 114 (2015), http://dx.doi.org/10.1160/TH15-04-0325.
[31] P. Peduzzi, J. Concato, A.R. Feinstein, T.R. Holford, Importance of events per independent variable in proportional hazards regression analysis. II. Accuracy and precision of regression estimates, J. Clin. Epidemiol. 48 (1995) 1503–1510.
[32] R. Suades, T. Padró, G. Vilahur, V. Martin-Yuste, M. Sabaté, J. Sans-Roselló, et al., Growing thrombi release increased levels of $CD235^+$-microparticles and decreased levels of activated platelet-derived microparticles. Validation in STEMI patients, J. Thromb. Haemost. (2015), http://dx.doi.org/10.1111/jth.13065.
[33] J. Jeanneteau, P. Hibert, M.C. Martinez, S. Tual-Chalot, S. Tamareille, A. Furber, et al., Microparticle release in remote ischemic conditioning mechanism, Am. J. Physiol. 303 (2012) H871–H877.

[34] S. Montoro-Garcia, E. Shantsila, L.D. Tapp, A. Lopez-Cuenca, A.I. Romero, D. Hernandez-Romero, et al., Small-size circulating microparticles in acute coronary syndromes: relevance to fibrinolytic status, reparative markers and outcomes, Atherosclerosis 227 (2013) 313–322.
[35] E. Shantsila, L.D. Tapp, B.J. Wrigley, S. Montoro-Garcia, G.Y. Lip, Receptors to interleukin-6 and adhesion molecules on circulating monocyte subsets in acute myocardial infarction, Thromb. Haemost. 110 (2013) 340–348.
[36] A.S. Leroyer, H. Isobe, G. Leseche, Y. Castier, M. Wassef, Z. Mallat, et al., Cellular origins and thrombogenic activity of microparticles isolated from human atherosclerotic plaques, J. Am. Coll. Cardiol. 49 (2007) 772–777.
[37] C.E. Radecke, A.E. Warrick, G.D. Singh, J. Rogers, S.I. Simon, E.J. Armstrong, Coronary artery endothelial cells and microparticles increase expression of VCAM-1 in myocardial infarction, Thromb. Haemost. 113 (2014) 605–616.
[38] D. Tousoulis, T. Psaltopoulou, E. Androulakis, N. Papageorgiou, S. Papaioannou, E. Oikonomou, et al., Oxidative stress and early atherosclerosis: novel antioxidant treatment, Cardiovasc. Drugs Ther. 29 (2015) 75–88.
[39] N. Werner, S. Wassmann, P. Ahlers, S. Kosiol, G. Nickenig, Circulating $CD31^+$/annexin V^+ apoptotic microparticles correlate with coronary endothelial function in patients with coronary artery disease, Arterioscler. Thromb. Vasc. Biol. 26 (2006) 112–116.
[40] J.P. Empana, C.M. Boulanger, M. Tafflet, J.M. Renard, A.S. Leroyer, O. Varenne, et al., Microparticles and sudden cardiac death due to coronary occlusion. The TIDE (Thrombus and Inflammation in sudden DEath) study, Eur. Heart J. Acute Cardiovasc. Care 4 (1) (2014) 28–36.
[41] M. Aatonen, M. Gronholm, P.R. Siljander, Platelet-derived microvesicles: multitalented participants in intercellular communication, Semin. Thromb. Hemost. 38 (2012) 102–113.
[42] J.I. Zwicker, C.C. Trenor 3rd, B.C. Furie, B. Furie, Tissue factor-bearing microparticles and thrombus formation, Arterioscler. Thromb. Vasc. Biol. 31 (2011) 728–733.
[43] A.S. Leroyer, P.E. Rautou, J.S. Silvestre, Y. Castier, G. Leseche, C. Devue, et al., CD40 ligand + microparticles from human atherosclerotic plaques stimulate endothelial proliferation and angiogenesis a potential mechanism for intraplaque neovascularization, J. Am. Coll. Cardiol. 52 (2008) 1302–1311.
[44] J. Habersberger, F. Strang, A. Scheichl, N. Htun, N. Bassler, R.M. Merivirta, et al., Circulating microparticles generate and transport monomeric C-reactive protein in patients with myocardial infarction, Cardiovasc. Res. 96 (2012) 64–72.
[45] N.C. Dubois, A.M. Craft, P. Sharma, D.A. Elliott, E.G. Stanley, A.G. Elefanty, et al., SIRPA is a specific cell-surface marker for isolating cardiomyocytes derived from human pluripotent stem cells, Nat. Biotechnol. 29 (2011) 1011–1018.
[46] J.J. Cha, J.Y. Kim, E.Y. Choi, P.K. Min, M. Cho, D.L. Lee, et al., Effect of abciximab on the levels of circulating microparticles in patients with acute myocardial infarction treated by primary angioplasty, Korean Circ. J. 43 (2013) 600–606.

SUPPLEMENTAL MATERIAL

Supplemental Table I

BASELINE CLINICAL CHARACTERISTICS OF STUDY POPULATION

				Statistics (*P*-value)		
	STEMI (n=40)	**Post-MI 72h (n=20)**	**Controls (n=20)**	**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

Notes: MI, myocardial infarction; SE, standard error; STEMI, ST-segment elevation myocardial infarction.
Source: Own elaboration.

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

Notes: SE, standard error; STEMI, ST-segment elevation myocardial infarction; TIMI, thrombolysis in myocardial infarction.
Source: Own elaboration.

Supplemental Table III

CELL SURFACE MOLECULES FOR CIRCULATING MICROPARTICLE IDENTIFICATION AND CHARACTERIZATION

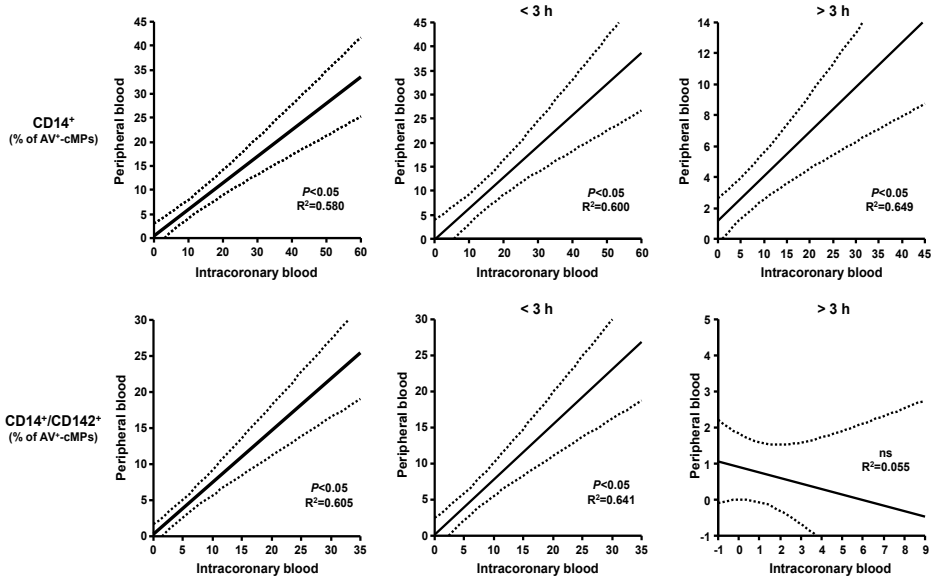
Marker	Alternative name	Expression	Conjugation	Clone	Company
Annexin V	PS-binding protein	Widely expressed	BD Horizon V450	-	BD Pharmingen
CD3	T3	Lymphocytes	FITC	HIT3a	BD Pharmingen
CD11b	MAC-1	Neutrophils, monocytes	FITC	VIM12	Molecular Probes
CD14	LPS-receptor	Macrophages, monocytes	PE	M5E2	BD Pharmingen
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

Notes: 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.
Source: Own elaboration.

SUPPLEMENTAL FIGURE

Supplemental Figure 1

RELATIONSHIP BETWEEN PERIPHERAL AND INTRACORONARY BLOOD



Notes: 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.
Source: Own elaboration.

ARTICLE 7

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

Rosa Suades, Teresa Padró, Lina Badimon

Manuscript under revision

OBJECTIVE

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

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

[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

Manuscript word count: 6758 words
Abstract word count: 329 words
Total tables and figures: 6 figures + 4 tables

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 (Boulanger, 2010, 2011; Jy *et al.,* 2004). pMPs constitute the majority of the circulating microparticles in the bloodstream (Flaumenhaft *et al.,* 2009; Piccin, Murphy and Smith, 2007; Smalley *et al.,* 2007). Although microparticles are present in the blood of healthy individuals (Berckmans *et al.,* 2001), elevation of platelet-derived microparticle levels occurs in cardiovascular disorders such as atherosclerosis and acute coronary syndromes (Namba *et al.,* 2007; Sinning *et al.,* 2010; Skeppholm *et al.,* 2012). pMPs are produced in different amounts and with differences in their proteome depending on the platelet stimulus (Shai *et al.,* 2012). 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 (Flaumenhaft, Mairuhu and Italiano, 2010) and as carriers of bioactive messages (Mause and Weber, 2010). Indeed, we have recently shown that platelet-derived microparticles enhance arterial thrombosis on atherosclerotic plaques (Suades *et al.,* 2012).

Platelets are major triggers of thrombosis on atherosclerotic plaques, the leading cause of cardiovascular disease (Fuster *et al.,* 1988; Viles-Gonzalez *et al.,* 2004). 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 (Badimon, Storey and Vilah, 2011). In atherosclerotic plaque-triggered thrombosis the main initiator is the tissue factor present in the disrupted atherosclerotic plaque (Fernandez-Ortiz *et al.,* 1994; Toschi *et al.,* 1997; Vilahur *et al.,* 2004) 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 (Suades *et al.,* 2012) 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 (Molins *et al.,* 2010; Di Michele, Van Geet and Freson, 2012; Pena *et al.,* 2011). 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 (Garcia *et al.,* 2005) and recently amplified (Capriotti *et al.,* 2013). Then, a comparative

proteome of plasma-derived with platelet-derived microparticles was described (Smalley *et al.,* 2007). Other studies have studied in-depth the plasma microparticle proteome from healthy donors (Jin *et al.,* 2005; Ostergaard *et al.,* 2011) or from different pathologies (Chaichompoo *et al.,* 2011; Choi *et al.,* 2011; Ramacciotti *et al.,* 2010; Watts *et al.,* 2011; Abdullah *et al.,* 2009). The protein composition of plasma microparticles from healthy donors shows high variability (Bastos-Amador *et al.,* 2011); however, a core set of plasma MP proteins found across population rather than in a subset of individuals has been reported (Little *et al.,* 2010). To increase the complexity it has been shown that different size classes of pMPs contain different protein components (Dean *et al.,* 2009) and that the different stimulation conditions (shear and shear plus thrombin) induce very different types of pMPs (Shai *et al.,* 2012). Releasates (soluble fraction or full secretome without removing MPs) from platelets activated by thrombin or TRAP was characterized (Coppinger *et al.,* 2004; Della Corte *et al.,* 2008; Piersma *et al.,* 2009), 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 (Suades *et al.,* 2012), 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 xg, 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 xg, 10 minutes, 20ºC) and stored at -80ºC for further studies. The supernatant was centrifuged once more (3220 xg, 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 xg, 90 minutes, 10ºC) with a 50.2Ti rotor

(Beckman Coulter) (Smalley et al., 2007). 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).

Two-dimensional gel electrophoresis (2-DE): 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).

Protein Identification by Maldi-ToF-MS Analysis: 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 (Garcia *et al.,* 2005). 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 (Suades *et al.,* 2012).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 (Benes *et al.,* 2012). 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 (Svensson *et al.,* 2009). 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 (Cselenyi and Lee, 2008; Mao *et al.,* 2002; Niehrs, 2006) which recently has been identified on platelets and may enhance the amplification of platelet activation (Steele *et al.,* 2009; Ueland *et al.,* 2009).

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) (Holbrook *et al.,* 2012; Wu *et al.,* 2012). 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 (Vilahur *et al.,* 2007). 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 (Cho *et al.,* 2008; Holbrook *et al.,* 2009; Reinhardt *et al.,* 2008). 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 (Exxex and Li, 1999).

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 (Crespin *et al.,* 2009). 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 (Little *et al.,* 2010). 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.

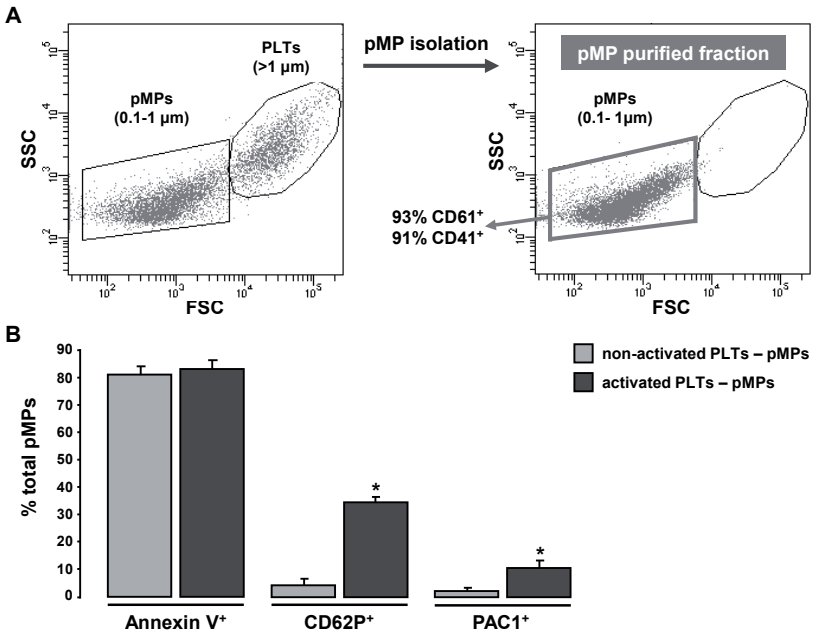
The differential analysis of thrombin-induced platelet microparticle subproteome allowed us to investigate the effects of platelet activation on microparticle shedding during platelet activation since a potent agonist such as thrombin could modify the expression pattern of some proteins related to thrombosis. 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

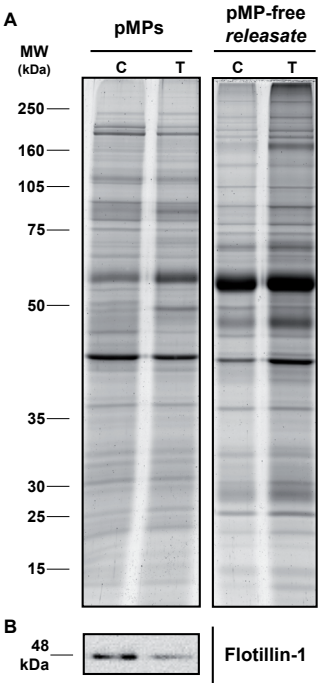
FLOW CYTOMETRIC CHARACTERIZATION OF PLATELET-DERIVED MICROPARTICLES



Notes: (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.
Source: Own elaboration.

Figure 2

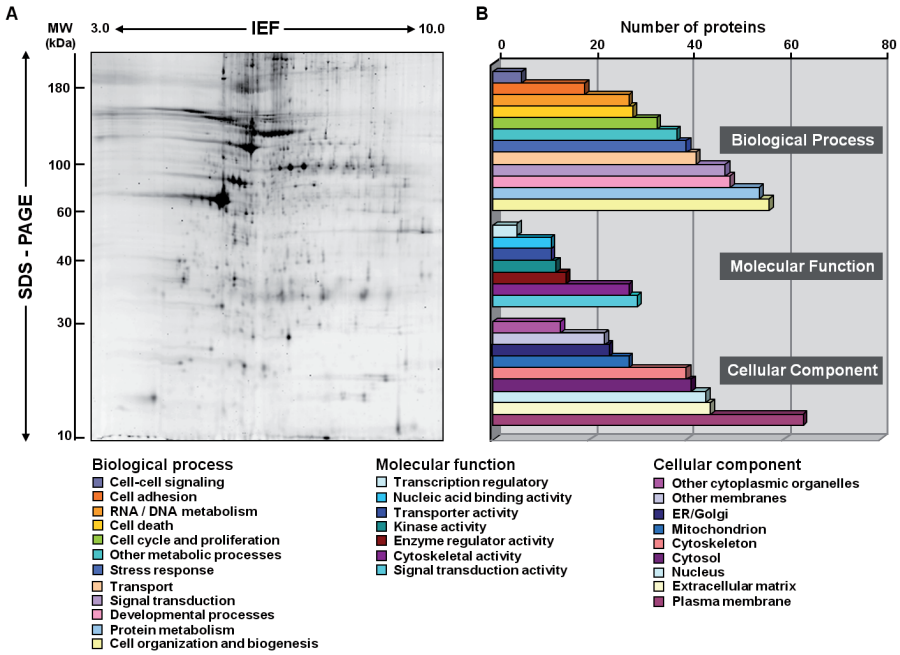
COMPARISON OF PLATELET-DERIVED SUBFRACTIONS



Notes: (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. *Source:* Own elaboration.

Figure 3

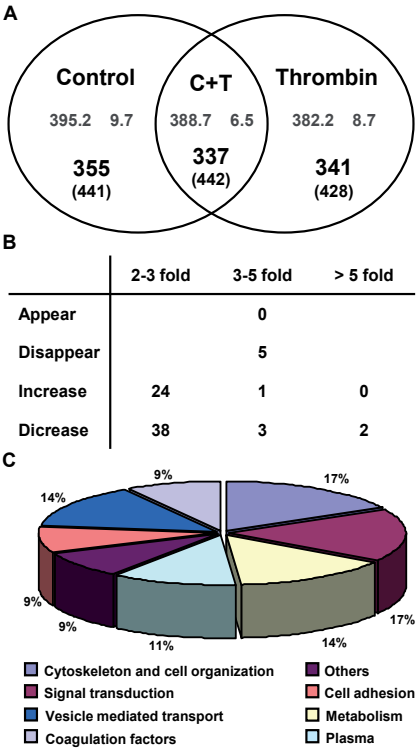
(A) REPRESENTATIVE 2D PROTEOME MAP OF PROTEINS IN PMPS



Notes: 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.
Source: Own elaboration.

Figure 4

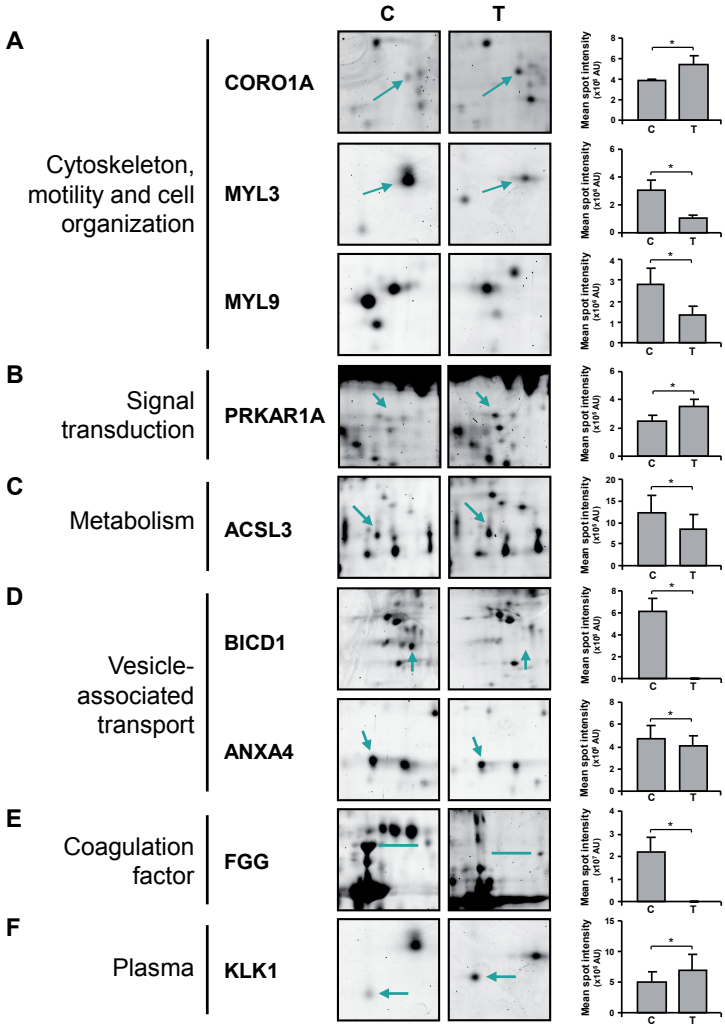
THROMBIN-INDUCED EFFECTS ON PMPS DERIVED FROM CONTROL PLATELETS AND THROMBIN-INDUCED PLATELETS



Notes: (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.
Source: Own elaboration.

Figure 5

THROMBIN-INDUCED EFFECTS ON PMPS DERIVED FROM CONTROL PLATELETS AND THROMBIN-STIMULATED PLATELETS

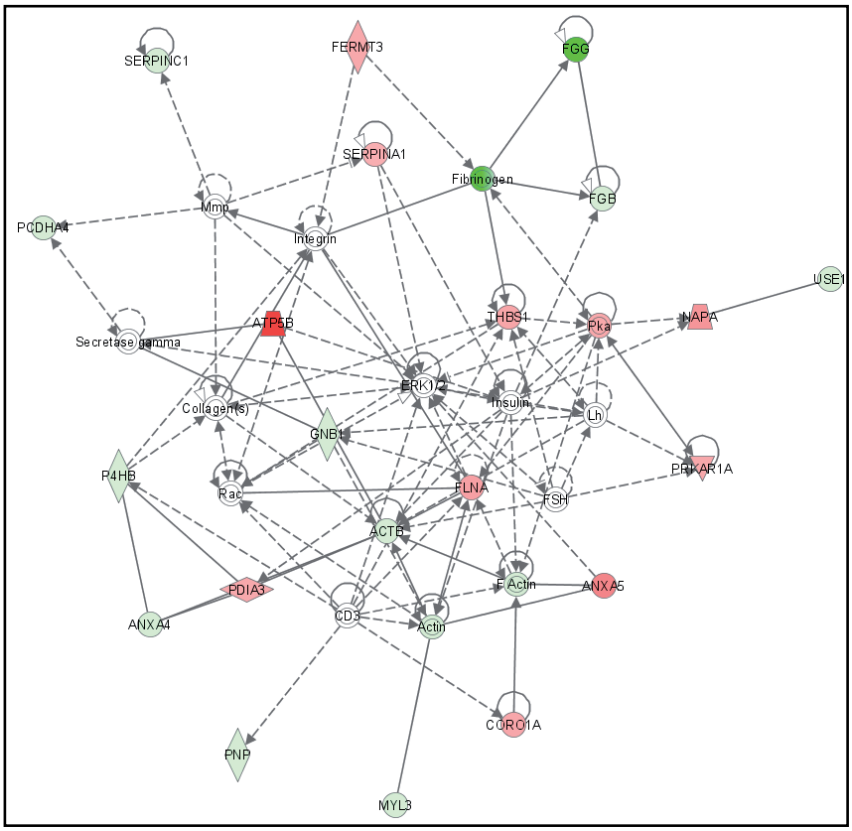


Notes: Selection of proteins differentially regulated between control and thrombin-induced platelets and, specifically, enlargement of representative 2-DE images corresponding to spots for (A) cytoskeleton, motility and cell organization, (B) signal transduction, (C) metabolism, (D) vesicle-associated 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).

Source: Own elaboration.

Figure 6

ANALYSIS OF DIFFERENTIALLY REGULATED PROTEINS BY INGENUITY PATHWAYS ANALYSIS CORE ANALYSIS



Notes: 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.
Source: Own elaboration.

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

Source: Own elaboration.

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

Source: Own elaboration.

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

Notes: 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.
Source: Own elaboration.

Table 4

IPA ANALYSIS

	T vs C	P-value / Score
Top canonical pathways	Intrinsic prothrombin activation pathway	$3.2 \cdot 10^{-7}$
	Coagulation system	$8.1 \cdot 10^{-7}$
	Extrinsic prothrombin activation pathway	$4.8 \cdot 10^{-6}$
	Regulation of actin-based motility by Rho	$8.5 \cdot 10^{-6}$
	RhoA signalling	$2.8 \cdot 10^{-5}$
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

Source: Own elaboration.

REFERENCES

Abdullah, N. M. *et al.* (2009), "Microparticle surface protein are associated with experimental venous thrombosis: a preliminary study," *Clin Appl Thromb Hemost,* 15(2): 201-208.

Badimon, L.; Storey, R. F. and Vilahur, G. (2011), "Update on lipids, inflammation and atherothrombosis," *Thromb Haemost,* 105 Suppl 1: S34-42.

Bastos-Amador, P. *et al.* (2011), "Proteomic analysis of microvesicles from plasma of healthy donors reveals high individual variability," *J Proteomics,* 75(12): 3574-3584.

Benes, C. H. *et al.* (2012), "The SRC-associated protein CUB Domain-Containing Protein-1 regulates adhesion and motility," *Oncogene,* 31(5): 653-663.

Berckmans, R. J. *et al.* (2001), "Cell-derived microparticles circulate in healthy humans and support low grade thrombin generation," *Thromb Haemost,* 85(4): 639-646.

Boulanger, C. M. (2010), "Microparticles, vascular function and hypertension," *Curr Opin Nephrol Hypertens,* 19(2): 177-180.

Boulanger, C. M. and Dignat-George, F. (2011), "Microparticles: an introduction," *Arterioscler Thromb Vasc Biol,* 31(1): 2-3.

Capriotti, A. L. *et al.* (2013), "Proteomic characterization of human platelet-derived microparticles," *Anal Chim Acta,* 776: 57-63.

Chaichompoo, P. *et al.* (2011), "Characterizations and proteome analysis of platelet-free plasma-derived microparticles in beta-thalassemia/hemoglobin E patients," *J Proteomics.*

Cho, J. *et al.* (2008), "A critical role for extracellular protein disulfide isomerase during thrombus formation in mice," *J Clin Invest,* 118(3): 1123-1131.

Choi, D. S. *et al.* (2011), "Proteomic analysis of microvesicles derived from human colorectal cancer ascites," *Proteomics,* 11(13): 2745-2751.

Coppinger, J. A. *et al.* (2004), "Characterization of the proteins released from activated platelets leads to localization of novel platelet proteins in human atherosclerotic lesions," *Blood,* 103(6): 2096-2104.

Crespin, M. *et al.* (2009), "Activation of PAK1/2 during the shedding of platelet microvesicles," *Blood Coagul Fibrinolysis,* 20(1): 63-70.

Cselenyi, C. S. and Lee, E. (2008), "Context-dependent activation or inhibition of Wnt-beta-catenin signaling by Kremen," *Sci Signal,* 1(8): pe10.

Dean, W. L. *et al.* (2009), "Proteomic and functional characterisation of platelet microparticle size classes," *Thromb Haemost,* 102(4): 711-718.

Della Corte, A. *et al.* (2008), "Application of 2-dimensional difference gel electrophoresis (2D-DIGE) to the study of thrombin-activated human platelet secretome," *Platelets,* 19(1): 43-50.

Di Michele, M.; Van Geet, C. and Freson, K. (2012), "Recent advances in platelet proteomics," *Expert Rev Proteomics,* 9(4): 451-466.

Essex, D. W. and Li, M. (1999), "Protein disulphide isomerase mediates platelet aggregation and secretion," *Br J Haematol,* 104(3): 448-454.

Fernandez-Ortiz, A. *et al.* (1994), "Characterization of the relative thrombogenicity of atherosclerotic plaque components: implications for consequences of plaque rupture," *J Am Coll Cardiol,* 23(7): 1562-1569.

Flaumenhaft, R. *et al.* (2009), "Megakaryocyte-derived microparticles: direct visualization and distinction from platelet-derived microparticles," *Blood,* 113(5): 1112-1121.

Flaumenhaft, R.; Mairuhu, A. T. and Italiano, J. E. (2010), "Platelet- and megakaryocyte-derived microparticles," *Semin Thromb Hemost,* 36(8): 881-887.

Fuster, V. *et al.* (1988), "Insights into the pathogenesis of acute ischemic syndromes," *Circulation,* 77(6): 1213-1220.

Garcia, B. A. *et al.* (2005), "The platelet microparticle proteome," *J Proteome Res,* 4(5): 1516-1521.

Holbrook, L. M. *et al.* (2009), "Platelets release novel thiol isomerase enzymes which are recruited to the cell surface following activation," *Br J Haematol,* 148(4): 627-637.

Holbrook, L. M. *et al.* (2012), "The platelet-surface thiol isomerase enzyme ERp57 modulates platelet function," *J Thromb Haemost,* 10(2): 278-288.

Jin, M. *et al.* (2005), "Distinct proteome features of plasma microparticles," *Proteomics,* 5(7): 1940-1952.

Jy, W. *et al.* (2004), "Measuring circulating cell-derived microparticles," *J Thromb Haemost,* 2(10): 1842-1851.

Little, K. M. *et al.* (2010), "The plasma microparticle proteome," *Semin Thromb Hemost,* 36(8): 845-856.

Mao, B. *et al.* (2002), "Kremen proteins are Dickkopf receptors that regulate Wnt/beta-catenin signalling," *Nature,* 417(6889): 664-667.

Mause, S. F. and Weber, C. (2010), "Microparticles: protagonists of a novel communication network for intercellular information exchange," *Circ Res,* 107(9): 1047-1057. and

Molins, B. *et al.* (2010), "Glucose-regulated protein 78 and platelet deposition: effect of rosuvastatin," *Arterioscler Thromb Vasc Biol,* 30(6): 1246-1252.

Namba, M. *et al.* (2007), "Circulating platelet-derived microparticles are associated with atherothrombotic events: a marker for vulnerable blood," *Arterioscler Thromb Vasc Biol,* 27(1): 255-256.

Niehrs, C. (2006), "Function and biological roles of the Dickkopf family of Wnt modulators," *Oncogene,* 25(57): 7469-7481.

Ostergaard, O. *et al.* (2011), "Quantitative proteome profiling of normal human circulating microparticles," *J Proteome Res,* 11(4): 2154-2163.

Pena, E. *et al.* (2011), "Proteomic signature of thrombin-activated platelets after in vivo nitric oxide-donor treatment: coordinated inhibition of signaling (phosphatidylinositol 3-kinase-gamma, 14-3-3zeta, and growth factor receptor-bound protein 2) and cytoskeleton protein translocation," A*rterioscler Thromb Vasc Biol,* 31(11): 2560-2569.

Piccin, A.; Murphy, W. G. and Smith, O. P. (2007), "Circulating microparticles: pathophysiology and clinical implications," *Blood Rev,* 21(3): 157-171.

Piersma, S. R. *et al.* (2009), "Proteomics of the TRAP-induced platelet releasate," *J Proteomics,* 72(1): 91-109.

Ramacciotti, E. *et al.* (2010), "Proteomics of microparticles after deep venous thrombosis," *Thromb Res,* 125(6): e269-274.

Reinhardt, C. *et al.* (2008), "Protein disulfide isomerase acts as an injury response signal that enhances fibrin generation via tissue factor activation," *J Clin Invest,* 118(3): 1110-1122.

Shai, E. *et al.* (2012), "Comparative analysis of platelet-derived microparticles reveals differences in their amount and proteome depending on the platelet stimulus," *J Proteomics,* 76 Spec No.: 287-296.

Sinning, J. M. *et al.* (2010), "Circulating CD31+/Annexin V+ microparticles correlate with cardiovascular outcomes," *Eur Heart J,* 32(16): 2034-2041.

Skeppholm, M. *et al.* (2012), "Platelet-derived microparticles during and after acute coronary syndrome," *Thromb Haemost,* 107(6): 1122-1129.

Smalley, D. M. *et al.* (2007), "Proteomic discovery of 21 proteins expressed in human plasma-derived but not platelet-derived microparticles," *Thromb Haemost,* 97(1): 67-80.

Steele, B. M. *et al.* (2009), "Canonical Wnt signaling negatively regulates platelet function," *Proc Natl Acad Sci U S A,* 106(47): 19836-19841.

Suades, R. *et al.* (2012), "Circulating and platelet-derived microparticles in human blood enhance thrombosis on atherosclerotic plaques," *Thromb Haemost,* 108(6): 1208-1219.

Svensson, L. *et al.* (2009), "Leukocyte adhesion deficiency-III is caused by mutations in KINDLIN3 affecting integrin activation," *Nat Med,* 15(3): 306-312.

Toschi, V. *et al.* (1997), "Tissue factor modulates the thrombogenicity of human atherosclerotic plaques," *Circulation,* 95(3): 594-599.

Ueland, T. *et al.* (2009), "Dickkopf-1 enhances inflammatory interaction between platelets and endothelial cells and shows increased expression in atherosclerosis," *Arterioscler Thromb Vasc Biol,* 29(8): 1228-1234.

Vilahur, G. *et al.* (2004), "Antithrombotic effects of saratin on human atherosclerotic plaques," *Thromb Haemost,* 92(1): 191-200.

Vilahur, G. *et al.* (2007), "Protein disulphide isomerase-mediated LA419- NO release provides additional antithrombotic effects to the blockade of the ADP receptor," *Thromb Haemost,* 97(4): 650-657.

Viles-Gonzalez, J. F.; Fuster, V. and Badimon, J. J. (2004), "Atherothrombosis: a widespread disease with unpredictable and life-threatening consequences," *Eur Heart J,* 25(14): 1197-1207.

Watts, J. A. *et al.* (2011), "Proteomics of microparticles after experimental pulmonary embolism," Thromb Res, 130(1): 122-128.

Wu, Y. *et al.* (2012), "The disulfide isomerase ERp57 mediates platelet aggregation, hemostasis, and thrombosis," *Blood,* 119(7): 1737-1746.

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 under revision

OBJECTIVE

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[1,3]

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

[2] Jimenez Diaz Foundation, Madrid, Spain

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

By

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

Manuscript word count: 5605 words
Abstract word count: 241 words
Total tables and figures: 7 figures + 3 tables

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 (Sanz, Moreno and Fuster, 2013). 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 (Arbab-Zadeh *et al.*, 2012). 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 (Gill, Harnden and Karpe, 2012; Hansson, 2005; Neefjes *et al.,* 2011; Sjouke *et al.,* 2011; Yuan, Wang and Hegele, 2006). 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 (Camaioni *et al.,* 2011; Contu, Latronico and Condorelli, 2010; Creemers, Tijsen and Pinto, 2012). Recently, miRNAs have been found in stable circulating form in blood and other body fluids mostly packed into microvesicles such as exosomes (Mitchell *et al.,* 2008; Gallo *et al.,* 2012). 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 (Thery, 2011). 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 (Lotvall and Valadi, 2007). 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 (Bang *et al.,* 2014).

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 (Tijsen, Pinto and Creemers, 2012; Turchinovich *et al.,* 2011). 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 (Mata *et al.,* 2011). 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 (Civeira, 2004; Graham *et al.,* 2007; Wierzbicki, Humphries and Minhas, 2008). 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 (Pijlman *et al.,* 2010).

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 (Ingenuity Systems I). 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 (Ingenuity Systems I). 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 (Contu, Latronico and Condorelli, 2010; Creemers, Tijsen and Pinto, 2012). 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 (Neefjes *et al.,* 2011) 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 (Suades *et al.,* 2012). 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 (Dangwal and Thum, 2013). and miR-425-5p to atrial natriuretic peptide regulation in arterial hypertension (Arora *et al.,* 2013). In addition, the myomiR-133a (cardiac-enriched miRNA) has recently been related to myocardial infarction and coronary artery disease diagnosis (Bostjancic *et al.*, 2010; Wang *et al.,* 2013) and miR-324-5p has also been involved in heart failure diagnosis (Ellis *et al.,* 2013). 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 (Bala *et al.,* 2012; Beaumont *et al.,* 2014). 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 (D'Alessandra *et al.,* 2010). 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 (Simons and Raposo, 2009). 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 (Komatsu and Node, 2010). 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 (Takano and Komuro, 2009). Interestingly, miR-130b is directly involved in the PPARγ regulation (Pan *et al.,* 2014). 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 (Wang *et al.,* 2013). Indeed, microvesicle-shuttled miR-130b has been shown to reduce adipogenesis and lipogenesis, and fat deposition in adypocytes (Pan *et al.,* 2014). 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) (Loirand, Guerin and Pacaud, 2006).

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.

Funding sources

This work was supported by Spanish Ministry of Science [PNS SAF2013-42962-R to LB], AGAUR (Agència de Gestió d'Ajuts Universitaris i de Recerca) [2009SGR826 to LB], TerCel (Red de Terapia Celular) [RD/12/0019/0026 to LB],

Spanish Ministry of Health [FIS-PI13/002850 to TP] and FIC (Fundación Investigación Cardiovascular)-Fundación Jesús Serra. RS is a recipient of a grant from Spanish Health Ministry [PFIS-FI09/00092, ISCIII].

Disclosures: None.

REFERENCES

Arbab-Zadeh, A. *et al.* (2012), "Acute coronary events," *Circ Cardiovasc Genet,* 125(9): 1147-1156.

Arora, P. *et al.* (2013), "Atrial natriuretic peptide is negatively regulated by microRNA-425," J Clin Invest, 123(8): 3378-3382.

Bala, S. *et al.* (2012), "Circulating microRNAs in exosomes indicate hepatocyte injury and inflammation in alcoholic, drug-induced, and inflammatory liver diseases," *Hepatology,* 56(5): 1946-1957.

Bang, C. *et al.* (2014), "Cardiac fibroblast-derived microRNA passenger strand-enriched exosomes mediate cardiomyocyte hypertrophy," *J Clin Invest,* 124(5): 2136-2146.

Beaumont, J. *et al,* (2014), "microRNA-122 down-regulation may play a role in severe myocardial fibrosis in human aortic stenosis through TGF-beta1 up-regulation," *Clin Sci* (Lond), 126(7): 497-506.

Bostjancic, E. *et al.* (2010), "MicroRNAs miR-1, miR-133a, miR-133b and miR-208 are dysregulated in human myocardial infarction," *Cardiology,* 115(3): 163-169.

Camaioni, C. *et al.* (2011), "Microparticles and microRNAs: new players in the complex field of coagulation," *Intern Emerg Med.*

Civeira, F. (2004), "Guidelines for the diagnosis and management of heterozygous familial hypercholesterolemia," *Atherosclerosis,* 173(1): 55-68.

Contu, R.; Latronico, M. V. and Condorelli, G. (2010), "Circulating microRNAs as potential biomarkers of coronary artery disease: a promise to be fulfilled?," *Circ Res,* 107(5): 573-574.

Creemers, E. E.; Tijsen, A. J. and Pinto, Y. M. (2012), "Circulating microRNAs: novel biomarkers and extracellular communicators in cardiovascular disease?," *Circ Res,* 110(3): 483-495.

D'Alessandra, Y. *et al.* (2010), "Circulating microRNAs are new and sensitive biomarkers of myocardial infarction," *Eur Heart J,* 31(22): 2765-2773.

Dangwal, S. and Thum, T. (2012), "MicroRNAs in platelet biogenesis and function," *Thromb Haemost,* 108(4): 599-604.

Ellis, K. L. *et al.* (2013), "Circulating microRNAs as candidate markers to distinguish heart failure in breathless patients," *Eur J Heart Fail,* 15(10): 1138-1147.

Gallo, A. *et al.* (2012), "The majority of microRNAs detectable in serum and saliva is concentrated in exosomes," *PLoS One,* 7(3): e30679.

Gill, P. J.; Harnden, A. and Karpe, F. (2012), "Familial hypercholesterolaemia," *Bmj,* 344: e3228.

Graham, I. *et al.* (2007), "European guidelines on cardiovascular disease prevention in clinical practice: executive summary: Fourth Joint Task Force of the European Society of Cardiology and Other Societies on Cardiovascular Disease Prevention in Clinical Practice (Constituted by representatives of nine societies and by invited experts)," Eur Heart J, 28(19): 2375-2414.

Hansson, G. K. (2005), "Inflammation, atherosclerosis, and coronary artery disease," *N Engl J Med,* 352(16): 1685-1695.

Ingenuity Systems I, Available at: http://www.ingenuity.com/.

Komatsu, A. and Node, K. (2010), [Effects of PPARgamma agonist on dyslipidemia and atherosclerosis]. *Nihon Rinsho,* 68(2): 294-298.

Loirand, G.; Guerin, P. and Pacaud, P. (2006), "Rho kinases in cardiovascular physiology and pathophysiology," *Circ Res*, 98(3): 322-334.

Lotvall, J. and Valadi, H. (2007), "Cell to cell signalling via exosomes through esRNA," *Cell Adh Migr,* 1(3): 156-158.

Mata, N. *et al.* (2011), "Clinical characteristics and evaluation of LDL-cholesterol treatment of the Spanish Familial Hypercholesterolemia Longitudinal Cohort Study (SAFEHEART)," *Lipids Health Dis,* 10: 94.

Mitchell, P. S. *et al.* (2008), "Circulating microRNAs as stable blood-based markers for cancer detection," *Proc Natl Acad Sci U S A,* 105(30): 10513-10518.

Neefjes, L. A. *et al.* (2011), "Accelerated subclinical coronary atherosclerosis in patients with familial hypercholesterolemia," *Atherosclerosis,* 219(2): 721-727.

Pan, S. *et al.* (2014), "Microvesicle-shuttled miR-130b reduces fat deposition in recipient primary cultured porcine adipocytes by inhibiting PPAR-g expression," *J Cell Physiol,* 229(5): 631-639.

Pijlman, A. H. *et al.* (2010), "Evaluation of cholesterol lowering treatment of patients with familial hypercholesterolemia: a large cross-sectional study in The Netherlands," *Atherosclerosis,* 209(1): 189-194.

Sanz, J.; Moreno, P.R. and Fuster, V. (2013), "The year in atherothrombosis," *J Am Coll Cardiol,* 62(13): 1131-1143.

Simons, M. and Raposo, G. (2009), "Exosomes--vesicular carriers for intercellular communication," *Curr Opin Cell Biol,* 21(4): 575-581.

Sjouke, B. *et al.* (2011), "Familial hypercholesterolemia: present and future management," *Curr Cardiol Rep,* 13(6): 527-536.

Suades, R. *et al.* (2012), "Circulating and platelet-derived microparticles in human blood enhance thrombosis on atherosclerotic plaques," Thromb Haemost, 108(6): 1208-1219.

Takano, H. and Komuro, I. (2009), "Peroxisome proliferator-activated receptor gamma and cardiovascular diseases," *Circ J,* 73(2): 214-220.

Thery, C. (2011), "Exosomes: secreted vesicles and intercellular communications," *F1000 Biol Rep,* 3: 15.

Tijsen, A. J.; Pinto, Y. M. and Creemers, E. E. (2012), "Circulating microRNAs as diagnostic biomarkers for cardiovascular diseases," *Am J Physiol Heart Circ Physiol,* 303(9): H1085-1095.

Turchinovich, A. *et al.* (2011), "Characterization of extracellular circulating microRNA," *Nucleic Acids Res,* 39(16): 7223-7233.

Wang, F. *et al.* (2013), "Plasma microRNA-133a is a new marker for both acute myocardial infarction and underlying coronary artery stenosis," *J Transl Med,*11: 222.

Wang, Y. C. *et al.* (2013), "Circulating miR-130b mediates metabolic crosstalk between fat and muscle in overweight/obesity," *Diabetologia,* 56(10): 2275-2285.

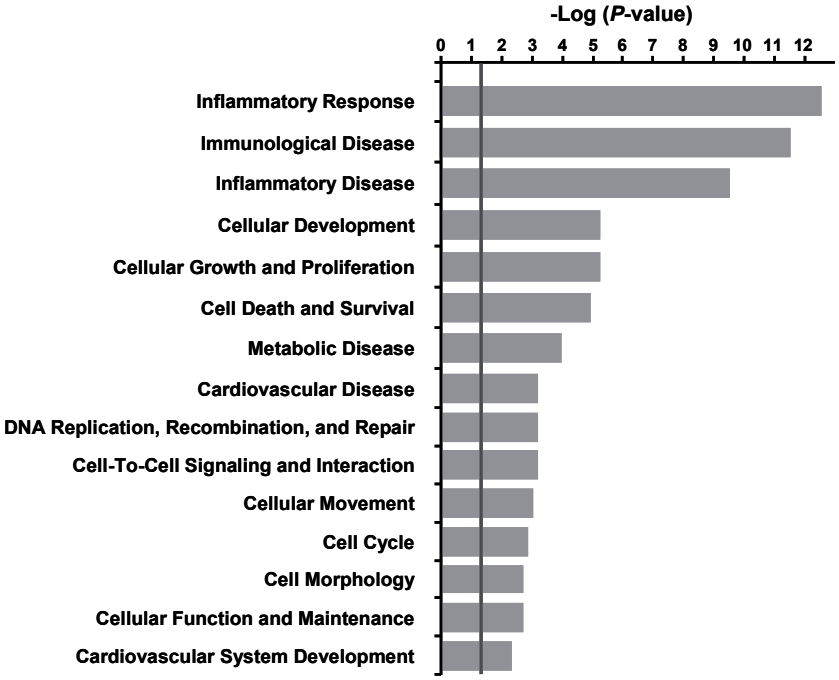
Wierzbicki, A. S.; Humphries, S. E. and Minhas, R. (2008), "Familial hypercholesterolaemia: summary of NICE guidance," *Bmj,* 337: a1095.

Yuan, G.; Wang, J. and Hegele, R. A. (2006), "Heterozygous familial hypercholesterolemia: an underrecognized cause of early cardiovascular disease," *Cmaj,* 174(8): 1124-1129.

FIGURE LEGENDS

Figure 1

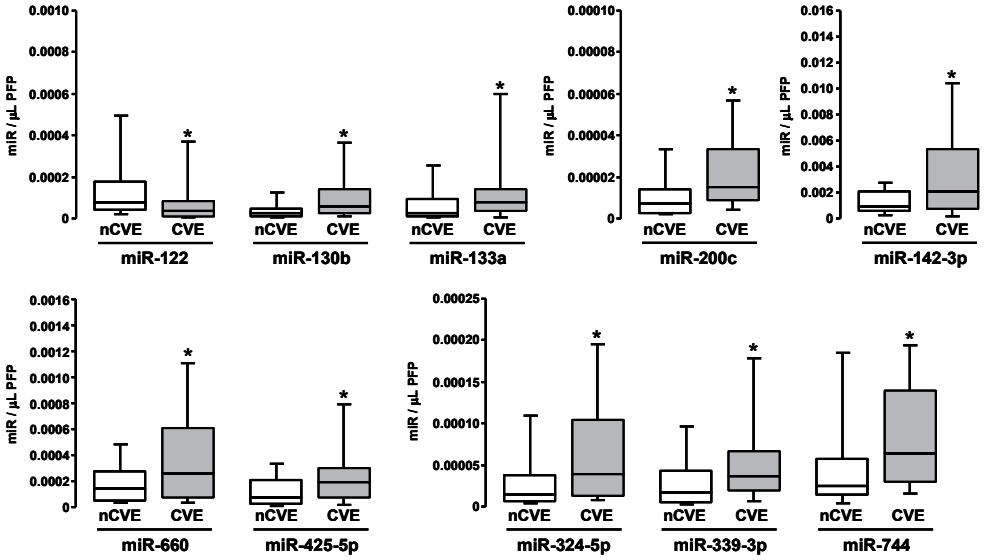
FUNCTIONAL ANALYSIS IDENTIFIED BY IPA ASSOCIATED WITH DIFFERENTIAL EXPRESSED MIRNAS IN THE PROFILING PHASE



Notes: 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.
Source: Own elaboration.

Figure 2

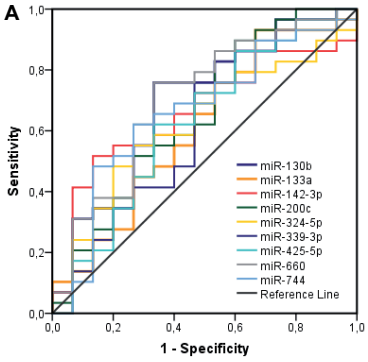
EXOSOMAL MICRORNA VALIDATION PHASE



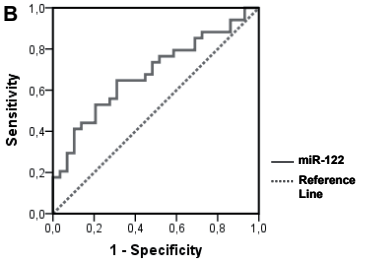
Notes: Box and whisker plots showing levels (per µL of PFP) of specific miRNAs: miR-122, miR-130b, miR-133a, miR-142-3p, miR-200c, miR-324-5p, miR-339-3p, miR-425-5p, miR-660, and miR-744 in FH patients who had an ischemic event 3 years after their inclusion (CVE, N=42) compared with non-FH hypercholesterolemic 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.
Source: Own elaboration.

Figure 3

DISCRIMINATORY POWER OF MIRNAS FOR THE PREDICTION OF ISCHEMIC CV EVENTS



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**	-	-

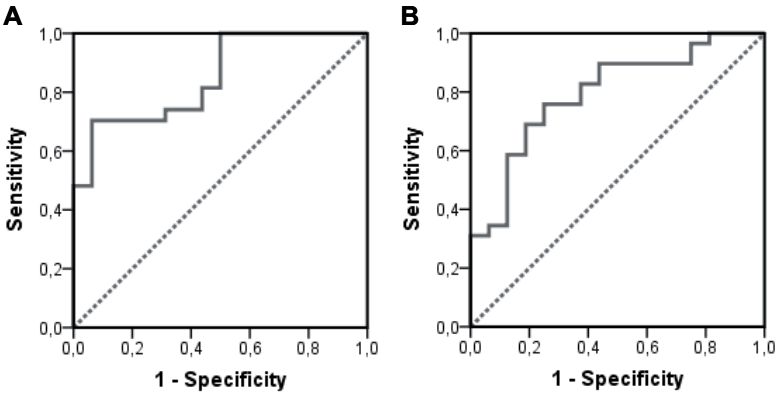


Exosomal microRNA	AUC (95% CI)	SE	Significance
hsa-miR-122	**0.682** (0.550-0.813)	0.067	*P* = 0.014
Reference Line	**0.500**	-	-

Notes: 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).
Source: Own elaboration.

Figure 4

DISCRIMINATORY POWER OF THE NINE- AND FIVE-MIRNA SIGNATURES FOR THE PREDICTION OF ISCHEMIC CARDIOVASCULAR EVENTS

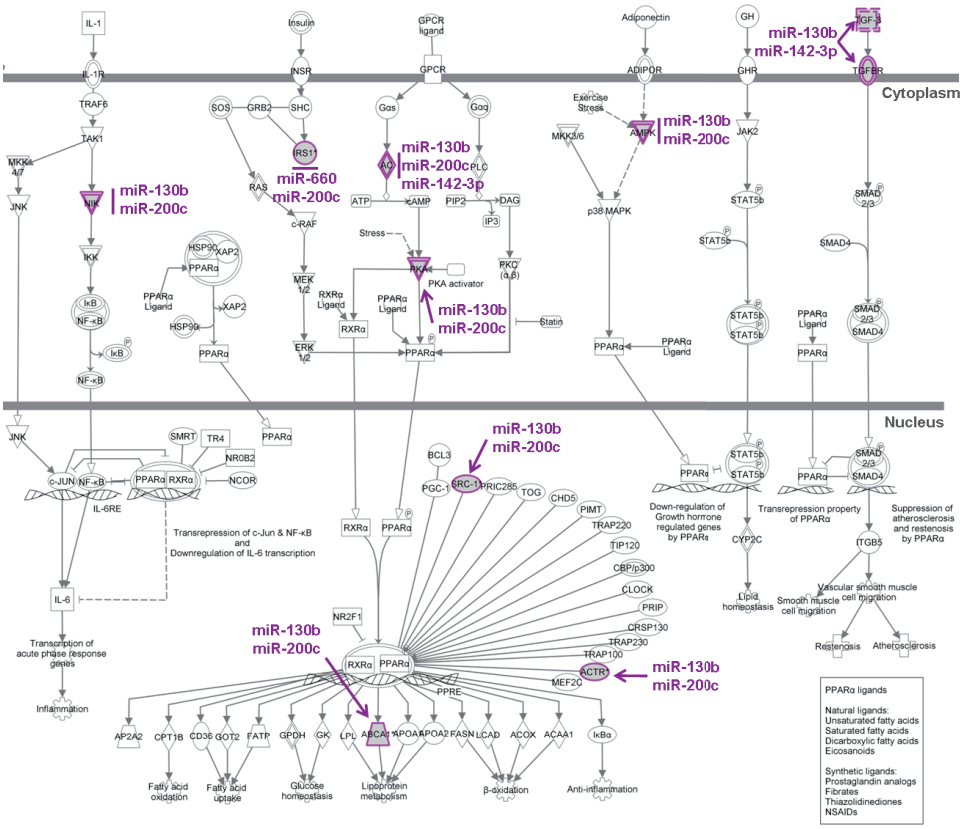


Exosomal microRNA	AUC (95% CI)	SE	Significance
9-miRNA signature (A)	**0.850** (0.736-0.963)	0.058	*P* < 0.001
5-miRNA signature (B)	**0.795** (0.660-0.930)	0.069	*P* = 0.001
Reference Line	**0.500**	-	-

Notes: 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.
Source: Own elaboration.

Figure 5

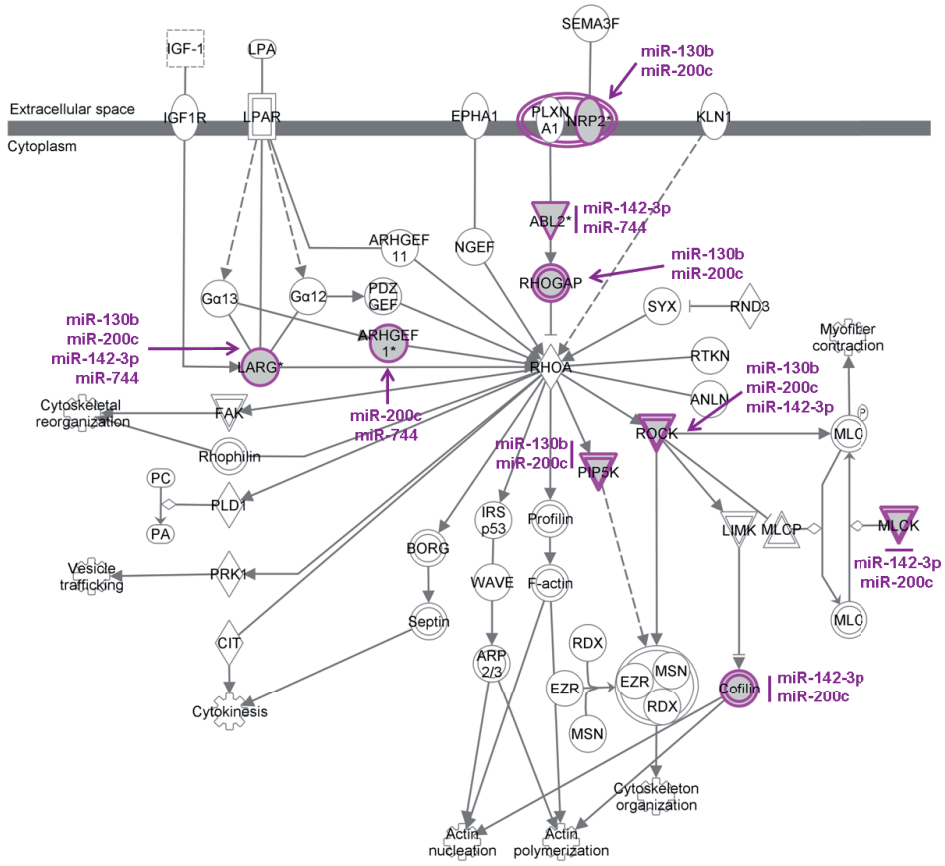
PPAR ACTIVATION SIGNALLING



Notes: 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.
Source: Own elaboration.

Figure 6

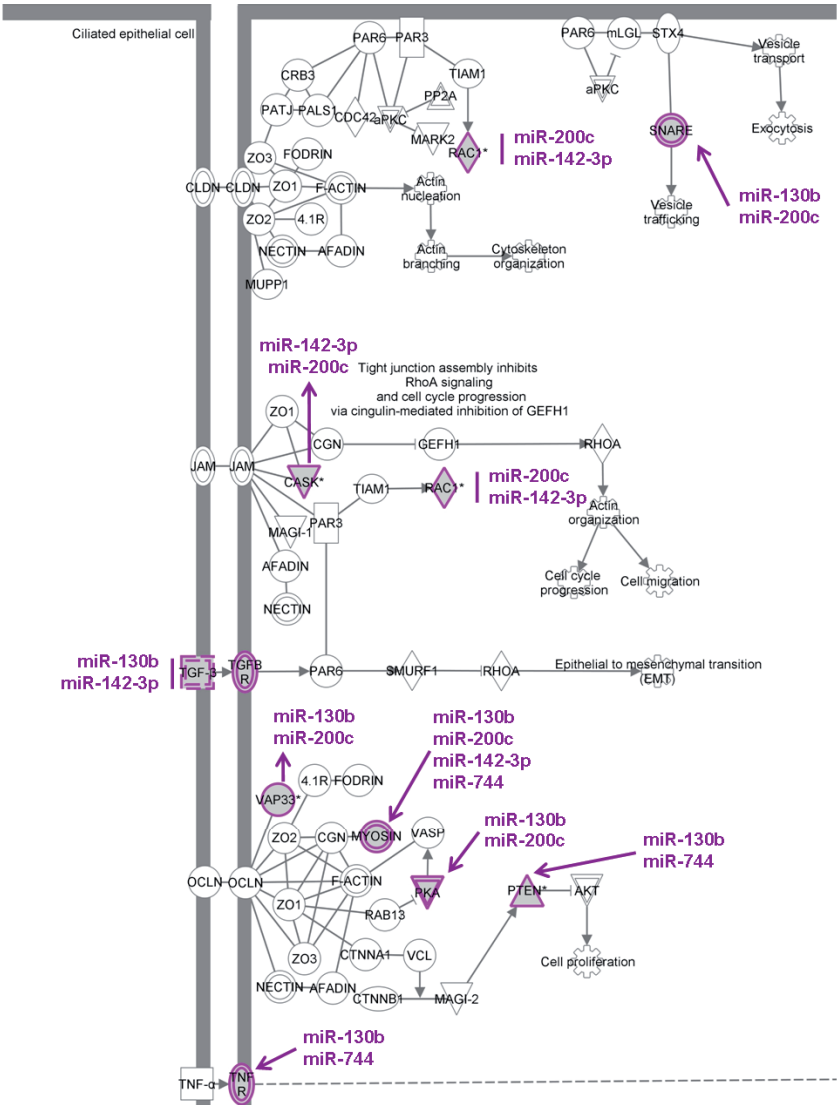
RHOA SIGNALLING



Notes: 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.
Source: Own elaboration.

Figure 7

TIGHT JUNCTION SIGNALLING



Notes: 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.
Source: Own elaboration.

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

Note: 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;
Source: Own elaboration.

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

Notes: 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.
Source: Own elaboration.

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

Note: 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.
Source: Own elaboration.

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.

5 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 (Agouni *et al.,* 2008; Naqvi *et al.,* 2010). 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 (Perez-Pujol, Marker and Key, 2007). 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 (Burger *et al.,* 2013). Recent studies have correlated AV-negative cMPs to clinical parameters in systemic diseases (Nielsen *et al.,* 2011) 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 (Mallat *et al.,* 2000).

Previous studies have shown elevated cMP levels in patients with clustering of cardiovascular risk factors (Preston *et al.,* 2003; Koga *et al.,* 2005; Nomura *et al.,* 2007) and many pathological states (Bernal-Mizrachi et al., 2004; Werner *et al.,* 2006; Bernal-Mizrachi *et al.,* 2003) such as CAD (Werner *et al.,* 2006). 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 (Dignat-George and Boulanger, 2011). Indeed, high plasma cholesterol levels directly impair flow-mediated dilatation and induce aortic stiffness, both associated with the onset and progression of atherosclerosis (Alonso *et al.,* 2001). eMPs have been proposed as potential prognostic markers for future cardiovascular events in high CV risk patients (Nozaki *et al.,* 2009). Pirro et al showed high levels of leuko-eMPs in newly-diagnosed never-LLT-treated hypercholesterolemic subjects (Pirro *et al.,* 2006). Besides, Huber *et al.,* found that those microvesicles containing oxidized phospholipids, typically in lipid-related diseases, could induce monocyte-endothelial interactions (Neumann, 1999), contributing to atherogenesis (Hartvigsen *et al.,* 2009). 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 (Hu *et al.,* 2014). 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 (Amabile *et al.,* 2014).

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 (Hoyer, *et al.,* 2012) and amplify inflammation by activation of endothelium (Wang *et al.,* 2011). It was also described that atherosclerotic plaques contain large amounts of LMPs (Leroyer *et al.,* 2007) and that plaque-MPs promote plaque progression by promoting inflammatory cell adhesion to ECs (Rautou *et al.,* 2011). 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 (Neumann *et al.,* 1999). Previously, lymphocyte-derived cMPs (ℓMPs) were shown to promote production of TNFα and IL-1β by monocytes (Scanu *et al.,* 2008) and were proposed as markers of reduced vasculoprotective properties of ECs in patients at HCVR (Martin *et al.,* 2004). 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

(Caballero *et al.,* 2012). Indeed, it has been found that aortic atherosclerosis is a marker for coronary atherosclerosis (Parthenakis *et al.,* 1996). 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 (Virmani *et al.,* 2000). 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 (Sarlon-Bartoli *et al.,* 2013). 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 (Weber and Noels, 2011). 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 (Caballero *et al.,* 2012). 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 (Del Turco *et al.,* 2014). 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 (Xu *et al.,* 2012) and other clinical studies have shown that, in fact, calcification might confer stability to the established atherosclerotic plaques (Beckman *et al.,* 2001).

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 (Graham *et al.,* 2007; Wierzbicki, Humphries and Minhas, 2008; Reiner *et al.,* 2011). Different studies indicate that only 13% of patients attained LDL-c goal on their initial lipid-lowering treatment (REALITY study (García-Ruiz *et al.,* 2004)) and only 3% of high coronary risk patients achieve lipid therapeutic targets recommended by European guidelines (DARIOS study (Marrugat *et al.,* 2011)). 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 (Liu *et al.,* 2007) and VMSC (Llorente-Cortes *et al.,* 2004) 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 (Ridker *et al.,* 2008), 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 (Sugiyama *et al.,* 2004). TF activation is one of the main triggers of thrombosis on ruptured atherosclerotic plaques (Toschi *et al.,* 1997) and it is present within the lipid-rich core components (Fernandez-Ortiz *et al.,* 1994). In addition, activated platelets contribute to thrombogenicity triggering the coagulation cascade and the subsequent formation of fibrin (Badimon, Storey and Vilahur, 2011). 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 (Butenas *et al.,* 2005), today is generally accepted that platelets carry TF (Camera *et al.,* 2010) and that platelets possess mechanisms to internalize TF-rich MPs (Escolar *et al.,* 2008) suggesting that TF in platelets could be the result of plasma transfer, expression or even be acquired by internalization of mMPs (Del Conde *et al.,* 2005; Camera *et al.,* 2012). In contrast, there is agreement on monocytes as the most important source of TF^+-cMPs (Osterud and Bjorklid, 2006). cMP-associated TF activity had been mainly attributed to mMPs (Van Der Meijden *et al.,* 2012). 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 (Owens *et al.,* 2012). 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 (Badimon, Storey and Vilahur, 2011). In FH, platelets might become activated because of the chronic hypercholesterolemia and the ensuing inflammatory processes (Chen *et al.,* 2011). 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 (Nomura *et al.,* 2001), induce PCA *(Paper 1),* promote thrombin generation (Van Der Meijden *et al.,* 2012) and interact with leukocytes (Forlow, McEver and Nollert, 2000).

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 (van der Pol *et al.,* 2012, 2013, 2014), 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 (Falk, 2013). 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 (Tan and Lip, 2005). 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 (Nomura *et al.,* 2000; Miyazaki *et al.,* 1996; Horigome *et al.,* 2002; Diehl *et al.,* 2008; Reininger *et al.,* 2006; Chen, Chen and Wang, 2010; Forestier *et al.,* 2008; Haga, Slack and Jennings, 2003; Holme *et al.,* 1997; Ikeda *et al.,* 2003; Macey, Wolf and Lawson, 2010). 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 (Forlow, McEver and Nollert, 2000). 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 FeCl3-mediated thrombosis model (Barr *et al.,* 2013). 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 (Nantakomol *et al.,* 2012; Kozuma *et al.,* 2011) such as sickle cell disease (SCD) (Nebor *et al.,* 2013; Tantawy *et al.,* 2012). ErMPs have recently been shown to amplify systemic inflammation by thrombin-dependent activation of complement (Zecher, Cumpelik and Schifferli, 2014). Due to their haemoglobin content and PS exposure, ErMPs may account for NO scavenging and PCA enhancement (Rubin *et al.,* 2012). In fact, ErMPs are able to support coagulation by decreasing prothrombin time (Rubin *et al.,* 2008) and, together with pMPs, have demonstrated to trigger thrombin generation in a FXII-dependent/TF-independent manner (Van Der Meijden *et al.,* 2012), being their coagulation activation involvement corroborated in the setting of SCD (Gerotziafas *et al.,* 2012; van Beers *et al.,* 2009). 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 (Camus *et al.,* 2012). 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 (Mallat *et al.,* 2000; van der Zee *et al.,* 2006; Biasucci *et al.,* 2012; Morel *et al.,* 2004; Stepien *et al.,* 2012) and associated to the area at risk during MI (Jeanneteau et al., 2012; Jung *et al.,* 2012). 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 (Armstrong *et al.,* 2013). 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 (Werner *et al.,* 2006) and $CD31^+/AV^+$-cMPs are an independent predictor of CV events in stable CAD patients (Sinning *et al.,* 2010). 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 (Zwicker *et al.,* 2011). In addition, human atherosclerotic plaques contain high levels of MPs expressing CD40L and bearing TF (Leroyer *et al.,* 2008). 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 (Habersberger *et al.,* 2012). 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 (Badimon *et al.,* 2002). 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 (Mallat *et al.,* 2000; Tan *et al.,* 2005; Vidal *et al.,* 2001). 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 (Alkhatatbeh *et al.,* 2011), and PECAM-1 increases in cMPs of patients with different cardiovascular outcomes (Sinning *et al.,* 2010). pMPs also carry thrombospondin-1 and CD63, an antigen found to be increased in pMP subpopulations of peripheral arterial disease and myocardial infarction patients (van der Zee *et al.*, 2006), 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 (Badimon *et al.,* 1988 and 1987). The present study, using two well-characterized flow perfusion devices to rheologically model stenotic coronary blood conditions (Badimon *et al.,* 1988 and 1987; Molins *et al.,* 2010 and 2008), 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 (Merten *et al.,* 1999), as well as to fibrinogen, von Willebrand factor and surface immobilized platelets (Keuren *et al.,* 2007). 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.,* 2008. 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 (van der Zee *et al.,* 2006). 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 (Garcia *et al.,* 2005; Sinauridze *et al.,* 2007). 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 (Benes *et al.,* 2012). 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 (Svensson *et al.,* 2009). 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 (Cselenyi and Lee, 2008; Mao et al., 2002; Niehrs, 2006) that recently has been identified on platelets and might enhance the amplification of platelet activation (Steele *et al.,* 2009; Ueland *et al.,* 2009).

The oxireductase protein disulfide isomerise A3 (PDIA3, ERp57, GRP58) has recently been shown to participate in thrombus formation (Holbrook *et al.,* 2012; Wu *et al.,* 2012). A previous study of our group showed that PDIA3 increases in the secretome of thrombin-activated platelets and NO donor could modulate its release (Vilahur *et al.,* 2007). 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 (Essex and Li, 1999).

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 (Rangrez *et al.,* 2011). Besides, miRNA-143 has been linked to atheroprotective shear stress-mediated communication between ECs and VSMCs through a microvesicle-mediated mechanism (Hergenreider *et al.,* 2012). 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 (Baigent *et al.,* 2005). In primary prevention results of clinical trials have shown less clear beneficial effects but yet reductions in CAD are evident (WOSCOP, Shepherd *et al.,* 1995, AFCAP/TexCAP, Downs *et al.,* 1998, ASCOT_LLA, Sever *et al.,* 2003) and even mortality was reduced in the JUPITER trial (Ridker *et al.,* 2008). However, the benefit on all-cause mortality has not been proved in a recent meta-analysis (Ray *et al.,* 2010). 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 (Normura *et al.,* 2011 and 2009). 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 (Lins *et al.,* 2014).

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.

REFERENCES

Agouni, a. *et al.* (2008), "Endothelial dysfunction caused by circulating microparticles from patients with metabolic syndrome," *Am J Pathol,* 173(4): 1210-1219.

Alkhatatbeh, M. J. *et al.* (2011), "The putative diabetic plasma marker, soluble CD36, is non-cleaved, non-soluble and entirely associated with microparticles," *J Thromb Haemost,* 9(4): 844-851.

Alonso, R. *et al.* (2001), "Sustained long-term improvement of arterial endothelial function in heterozygous familial hypercholesterolemia patients treated with simvastatin," *Atherosclerosis,* 157(2): 423-429.

Amabile, N. *et al.* (2014), "Association of circulating endothelial microparticles with cardiometabolic risk factors in the Framingham Heart Study," *Eur Heart J*.

Armstrong, P. W. *et al.* (2013), "Fibrinolysis or primary PCI in ST-segment elevation myocardial infarction," *N Engl J Med,* 368(15): 1379-1387.

Badimon, L. *et al.* (1987), "Characterization of a tubular flow chamber for studying platelet interaction with biologic and prosthetic materials: deposition of indium 111-labeled platelets on collagen, subendothelium, and expanded polytetrafluoroethylene," *J Lab Clin Med,* 110(6): 706-718.

Badimon, L. *et al.* (1988), "Platelet thrombus formation on collagen type I. A model of deep vessel injury. Influence of blood rheology, von Willebrand factor, and blood coagulation," *Circulation,* 78(6): 1431-1442.

Badimon, L. *et al.* (2002), "Pathogenesis of the acute coronary syndromes and therapeutic implications," *Pathophysiol Haemost Thromb,* 32(5-6): 225-231.

Badimon, L.; Storey, R. F. and Vilahur, G. (2011), "Update on lipids, inflammation and atherothrombosis," *Thromb Haemost,* 105 Suppl 1: S34-42.

Baigent, C. *et al.* (2005), "Efficacy and safety of cholesterol-lowering treatment: prospective meta-analysis of data from 90,056 participants in 14 randomised trials of statins," *Lancet,* 366(9493): 1267-1278.

Barr, J. D. *et al.* (2013), "Red blood cells mediate the onset of thrombosis in the ferric chloride murine model," *Blood,* 121(18): 3733-3741.

Benes, C. H. *et al.* (2012), "The SRC-associated protein CUB Domain-Containing Protein-1 regulates adhesion and motility," *Oncogene,* 31(5): 653-663.

Bernal-Mizrachi, L. *et al.* (2003), "High levels of circulating endothelial microparticles in patients with acute coronary syndromes," *Am Heart J,* 145(6): 962-970.

Bernal-Mizrachi, L. *et al.* (2004), "Endothelial microparticles correlate with high-risk angiographic lesions in acute coronary syndromes," *Int J Cardiol,* 97(3): 439-446.

Biasucci, L. M. *et al.* (2012), "Differences in microparticle release in patients with acute coronary syndrome and stable angina," *Circ J,* 76(9): 2174-2182.

Burger, D. *et al.* (2013), "Microparticles: biomarkers and beyond," *Clin Sci (Lond),* 124(7): 423-441.

Butenas, S. *et al.* (2005), "Tissue factor activity in whole blood," *Blood,* 105(7): 2764-2770.

Caballero, P. *et al.* (2012), "Detection of subclinical atherosclerosis in familial hypercholesterolemia using non-invasive imaging modalities," *Atherosclerosis,* 222(2): 468-472.

Camera, M. *et al.* (2010), "Tissue factor expression on platelets is a dynamic event," *Blood,* 116(23): 5076-5077.

Camera, M. *et al.* (2012), "Tissue factor and atherosclerosis: not only vessel wall-derived TF, but also platelet-associated TF," *Thromb Res,* 129(3): 279-284.

Camus, S. M. *et al.* (2012), "Erythrocyte microparticles can induce kidney vaso-occlusions in a murine model of sickle cell disease," *Blood,* 120(25): 5050-5058.

Chen, K. *et al.* (2011), "Vav guanine nucleotide exchange factors link hyperlipidemia and a prothrombotic state," *Blood,* 117(21): 5744-5750.

Chen, Y. W. ; Chen, J. K. and Wang, J. S. (2010), "Strenuous exercise promotes shear-induced thrombin generation by increasing the shedding of procoagulant microparticles from platelets," *Thromb Haemost,* 104(2): 293-301.

Cselenyi, C. S. and Lee, E. (2008), "Context-dependent activation or inhibition of Wnt-beta-catenin signaling by Kremen," *Sci Signal,* 1(8): pe10.

Del Conde, I. *et al.* (2005), "Tissue-factor-bearing microvesicles arise from lipid rafts and fuse with activated platelets to initiate coagulation," *Blood,* 106(5): 1604-1611.

Del Turco, S. *et al.* (2014), "Procoagulant activity of circulating microparticles is associated with the presence of moderate calcified plaque burden detected by multislice computed tomography," *J Geriatr Cardiol,* 11(1): 13-19.

Diehl, P. *et al.* (2008), "Increased levels of circulating microparticles in patients with severe aortic valve stenosis," *Thromb Haemost,* 99(4): 711-719.

Dignat-George, F. and Boulanger, C. M. (2011), "The many faces of endothelial microparticles," *Arterioscler Thromb Vasc Biol,* 31(1): 27-33.

Downs, J. R. *et al.* (1998), "Primary prevention of acute coronary events with lovastatin in men and women with average cholesterol levels: results of AFCAPS/TexCAPS. Air Force/Texas Coronary Atherosclerosis Prevention Study," *Jama,* 279(20): 1615-1622.

Escolar, G. *et al.* (2008), "Internalization of tissue factor by platelets," *Thromb Res,* 122 Suppl 1: S37-41.

Essex, D. W. and Li, M. (1999), "Protein disulphide isomerase mediates platelet aggregation and secretion," *Br J Haematol,* 104(3): 448-454.

Falk, E. *et al.* (2013), "Update on acute coronary syndromes: the pathologists' view," *Eur Heart J,* 34(10): 719-728.

Fernandez-Ortiz, A. *et al.* (1994), "Characterization of the relative thrombogenicity of atherosclerotic plaque components: implications for consequences of plaque rupture," *J Am Coll Cardiol,* 23(7): 1562-1569.

Forestier, M. *et al.* (2008), "Platelet microparticle suppressing antibody against GP Ibalpha acts independently of the filamin cleavage and increases protein tyrosine phosphorylation," *Blood Coagul Fibrinolysis,* 19(8): 801-806.

Forlow, S. B.; Mcever, R. P. and Nollert, M. U. (2000), "Leukocyte-leukocyte interactions mediated by platelet microparticles under flow," *Blood,* 95(4): 1317-1323.

Garcia, B. A. *et al.* (2005), "The platelet microparticle proteome," *J Proteome Res,* 4(5): 1516-1521.

Garcia Ruiz, F. J. *et al.* (2004), "Current lipid management and low cholesterol goal attainment in common daily practice in Spain. The REALITY Study," *Pharmacoeconomics,* 22 Suppl 3: 1-12.

Gerotziafas, G. T. *et al.* (2012), "The acceleration of the propagation phase of thrombin generation in patients with steady-state sickle cell disease is associated with circulating erythrocyte-derived microparticles," *Thromb Haemost,* 107(6): 1044-1052.

Graham, I. *et al.* (2007), "European guidelines on cardiovascular disease prevention in clinical practice: executive summary: Fourth Joint Task Force of the European Society of Cardiology and Other Societies on Cardiovascular Disease Prevention in Clinical Practice (Constituted by representatives of nine societies and by invited experts)," *Eur Heart J,* 28(19): 2375-2414.

Habersberger, J. *et al.* (2012), "Circulating microparticles generate and transport monomeric C-reactive protein in patients with myocardial infarction," *Cardiovasc Res,* 96(1): 64-72.

Haga, J. H.; Slack, S. M. and Jennings, L. K. (2003), "Comparison of shear stress-induced platelet microparticle formation and phosphatidylserine expression in presence of alphaIIbbeta3 antagonists," *J Cardiovasc Pharmacol,* 41(3): 363-371.

Hartvigsen, K. *et al.* (2009), "The role of innate immunity in atherogenesis," *J Lipid Res,* 50 Suppl: S388-393.

Hergenreider, E. *et al.* (2012), "Atheroprotective communication between endothelial cells and smooth muscle cells through miRNAs," *Nat Cell Biol,* 14(3): 249-256.

Holbrook, L. M. *et al.* (2012), "The platelet-surface thiol isomerase enzyme ERp57 modulates platelet function," *J Thromb Haemost,* 10(2): 278-288.

Holme, P. A. *et al.* (1997), "Shear-induced platelet activation and platelet microparticle formation at blood flow conditions as in arteries with a severe stenosis," *Arterioscler Thromb Vasc Biol,* 17(4): 646-653.

Horigome, H. *et al.* (2002), "Overproduction of platelet microparticles in cyanotic congenital heart disease with polycythemia," *J Am Coll Cardiol,* 39(6): 1072-1077.

Hoyer, F. F. *et al.* (2012), "Monocytic microparticles promote atherogenesis by modulating inflammatory cells in mice," *J Cell Mol Med,* 16(11): 2777-2788.

Hu, S. S. *et al.* (2014), "CD144(+)EMPs/CD62E(+)EMPs: A couple of new biomarkers to monitor endothelial function in hypertension with hyperlipidemia involved," *Int J Cardiol,* 175(1): 203.

Ikeda, M. *et al.* (2003), "Increased platelet aggregation and production of platelet-derived microparticles after surgery for upper gastrointestinal malignancy," *J Surg Res,* 115(2): 174-183.

Jeanneteau, J. *et al.* (2012), "Microparticle release in remote ischemic conditioning mechanism," *Am J Physiol Heart Circ Physiol,* 303(7): H871-877.

Jung, C. *et al.* (2012), "Circulating endothelial and platelet derived microparticles reflect the size of myocardium at risk in patients with ST-elevation myocardial infarction," *Atherosclerosis,* 221(1): 226-231.

Keuren, J. F. *et al.* (2007), "Microparticles adhere to collagen type I, fibrinogen, von Willebrand factor and surface immobilised platelets at physiological shear rates," *Br J Haematol,* 138(4): 527-533.

Kim, H. K. *et al.* (2008), "The role of cellular microparticles in platelet-related hemostasis measured using Platelet Function Analyzer-100," *Blood Coagul Fibrinolysis,* 19(4): 328-330.

Koga, H. *et al.* (2005), "Elevated levels of VE-cadherin-positive endothelial microparticles in patients with type 2 diabetes mellitus and coronary artery disease," *J Am Coll Cardiol,* 45(10): 1622-1630.

Kozuma, Y. *et al.* (2011), "Procoagulant properties of microparticles released from red blood cells in paroxysmal nocturnal haemoglobinuria," *Br J Haematol,* 152(5): 631-639.

Lins, L. C. *et al.* (2014), "Effects of Ezetimibe on Endothelial Progenitor Cells and Microparticles in High-Risk Patients," *Cell Biochem Biophys*.

Liu, M. L. *et al.* (2007), "Cholesterol enrichment of human monocyte/macrophages induces surface exposure of phosphatidylserine and the release of biologically-active tissue factor-positive microvesicles," *Arterioscler Thromb Vasc Biol,* 27(2): 430-435.

Llorente-Cortes, V. *et al.* (2004), "Aggregated low-density lipoprotein uptake induces membrane tissue factor procoagulant activity and microparticle release in human vascular smooth muscle cells," *Circulation,* 110(4): 452-459.

Macey, M. G.; Wolf, S. I. and Lawson, C. (2010), "Microparticle formation after exposure of blood to activated endothelium under flow," *Cytometry A,* 77(8): 761-768.

Mallat, Z. *et al.* (2000), "Elevated levels of shed membrane microparticles with procoagulant potential in the peripheral circulating blood of patients with acute coronary syndromes," *Circulation,* 101(8): 841-843.

Mao, B. *et al.* (2002), "Kremen proteins are Dickkopf receptors that regulate Wnt/beta-catenin signalling," *Nature,* 417(6889): 664-667.

Marrugat, J. *et al.* (2011), "Relative Validity of the 10-Year Cardiovascular Risk Estimate in a Population Cohort of the REGICOR Study," *Rev Esp Cardiol (Engl Ed),* 64(5): 385-394.

Martin, S. *et al.* (2004), "Shed membrane particles from T lymphocytes impair endothelial function and regulate endothelial protein expression," *Circulation,* 109(13): 1653-1659.

Merten, M. *et al.* (1999), "Platelet microparticles promote platelet interaction with subendothelial matrix in a glycoprotein IIb/IIIa-dependent mechanism," *Circulation,* 99(19): 2577-2582.

Miyazaki, Y. *et al.* (1996), "High shear stress can initiate both platelet aggregation and shedding of procoagulant containing microparticles," *Blood,* 88(9): 3456-3464.

Molins, B. *et al.* (2008), "C-reactive protein isoforms differ in their effects on thrombus growth," *Arterioscler Thromb Vasc Biol,* 28(12): 2239-2246.

Molins, B. *et al.* (2010), "Glucose-regulated protein 78 and platelet deposition: effect of rosuvastatin," *Arterioscler Thromb Vasc Biol,* 30(6): 1246-1252.

Morel, O. *et al.* (2004), "Sustained elevated amounts of circulating procoagulant membrane microparticles and soluble GPV after acute myocardial infarction in diabetes mellitus," *Thromb Haemost,* 91(2): 345-353.

Nantakomol, D. *et al.* (2012), "Red cell and platelet-derived microparticles are increased in G6PD-deficient subjects," *Eur J Haematol,* 89(5): 423-429.

Naqvi, T. Z. *et al.* (2010), "High prevalence of ultrasound detected carotid atherosclerosis in subjects with low Framingham risk score: potential implications for screening for subclinical atherosclerosis," *J Am Soc Echocardiogr,* 23(8): 809-815.

Nebor, D. *et al.* (2013), "Fetal hemoglobin and hydroxycarbamide modulate both plasma concentration and cellular origin of circulating microparticles in sickle cell anemia children," *Haematologica*.

Neumann, F. J. *et al.* (1999), "Effect of glycoprotein IIb/IIIa receptor blockade on platelet-leukocyte interaction and surface expression of the leukocyte integrin Mac-1 in acute myocardial infarction," *J Am Coll Cardiol,* 34(5): 1420-1426.

Niehrs, C. (2006), "Function and biological roles of the Dickkopf family of Wnt modulators," *Oncogene,* 25(57): 7469-7481.

Nielsen, C. T. *et al.* (2011), "Distinct features of circulating microparticles and their relationship to clinical manifestations in systemic lupus erythematosus," *Arthritis Rheum,* 63(10): 3067-3077.

Nomura, S. *et al.* (2000), "Platelet-derived microparticles in patients with arteriosclerosis obliterans: enhancement of high shear-induced microparticle generation by cytokines," *Thromb Res,* 98(4): 257-268.

Nomura, S. *et al.* (2007), "Effect of nifedipine on adiponectin in hypertensive patients with type 2 diabetes mellitus," *J Hum Hypertens,* 21(1): 38-44.

Nomura, S. *et al.* (2009), "The effects of pitavastatin, eicosapentaenoic acid and combined therapy on platelet-derived microparticles and adiponectin in hyperlipidemic, diabetic patients," *Platelets,* 20(1): 16-22.

Nomura, S. *et al.* (2011), "Effects of miglitol in platelet-derived microparticle, adiponectin, and selectin level in patients with type 2 diabetes mellitus," *Int J Gen Med,* 4: 539-545.

Nomura, S. *et al.* (2001), "High-shear-stress-induced activation of platelets and microparticles enhances expression of cell adhesion molecules in THP-1 and endothelial cells," *Atherosclerosis,* 158(2): 277-287.

Nozaki, T. *et al.* (2009), "Significance of a multiple biomarkers strategy including endothelial dysfunction to improve risk stratification for cardiovascular events in patients at high risk for coronary heart disease," *J Am Coll Cardiol,* 54(7): 601-608.

Osterud, B. and Bjorklid, E. (2006), "Sources of tissue factor," *Semin Thromb Hemost,* 32(1): 11-23.

Owens, A. P., 3RD *et al.* (2012), "Monocyte tissue factor-dependent activation of coagulation in hypercholesterolemic mice and monkeys is inhibited by simvastatin," *J Clin Invest,* 122(2): 558-568.

Parthenakis, F. *et al.* (1996), "Absence of atherosclerotic lesions in the thoracic aorta indicates absence of significant coronary artery disease," *Am J Cardiol,* 77(12): 1118-1121 SERIALS SUBS JAF.

Perez-Pujol, S.; Marker, P. H. and Key, N. S. (2007), "Platelet microparticles are heterogeneous and highly dependent on the activation mechanism: studies using a new digital flow cytometer," *Cytometry A,* 71(1): 38-45.

Pirro, M. *et al.* (2006), "Increased ratio of CD31+/CD42- microparticles to endothelial progenitors as a novel marker of atherosclerosis in hypercholesterolemia," *Arterioscler Thromb Vasc Biol,* 26(11): 2530-2535.

Preston, R. A. *et al.* (2003), "Effects of severe hypertension on endothelial and platelet microparticles," *Hypertension,* 41(2): 211-217.

Rangrez, A. Y. *et al.* (2011), "miR-143 and miR-145: molecular keys to switch the phenotype of vascular smooth muscle cells," *Circ Cardiovasc Genet,* 4(2): 197-205.

Rautou, P. E. *et al.* (2011), "Microparticles from human atherosclerotic plaques promote endothelial ICAM-1-dependent monocyte adhesion and transendothelial migration," *Circ Res,* 108(3): 335-343.

Ray, K. K. *et al.* (2010), "Statins and all-cause mortality in high-risk primary prevention: a meta-analysis of 11 randomized controlled trials involving 65,229 participants," *Arch Intern Med,* 170(12): 1024-1031.

Reiner, Z. *et al.* (2011), "ESC/EAS Guidelines for the management of dyslipidaemias: the Task Force for the management of dyslipidaemias of the European Society of Cardiology (ESC) and the European Atherosclerosis Society (EAS)," *Eur Heart J,* 32(14): 1769-1818.

Reininger, A. J. *et al.* (2006), "Mechanism of platelet adhesion to von Willebrand factor and microparticle formation under high shear stress," *Blood,* 107(9): 3537-3545.

Ridker, P. M. *et al.* (2008), "Rosuvastatin to prevent vascular events in men and women with elevated C-reactive protein," *N Engl J Med,* 359(21): 2195-2207.

Rubin, O. *et al.* (2008), "Microparticles in stored red blood cells: an approach using flow cytometry and proteomic tools," *Vox Sang,* 95(4): 288-297.

Rubin, O. *et al.* (2012), "Red blood cell microparticles: clinical relevance," *Transfus Med Hemother,* 39(5): 342-347.

Sarlon-Bartoli, G. *et al.* (2013), "Plasmatic level of leukocyte-derived microparticles is associated with unstable plaque in asymptomatic patients with high-grade carotid stenosis," *J Am Coll Cardiol,* 62(16): 1436-1441.

Scanu, A. *et al.* (2008), "Stimulated T cells generate microparticles, which mimic cellular contact activation of human monocytes: differential regulation of pro- and anti-inflammatory cytokine production by high-density lipoproteins," *J Leukoc Biol,* 83(4): 921-927.

Sever, P. S. *et al.* (2003), "Prevention of coronary and stroke events with atorvastatin in hypertensive patients who have average or lower-than-average cholesterol concentrations, in the Anglo-

Scandinavian Cardiac Outcomes Trial--Lipid Lowering Arm (ASCOT-LLA): a multicentre randomised controlled trial," *Lancet,* 361(9364): 1149-1158.

Shepherd, J. *et al.* (1995), "Prevention of coronary heart disease with pravastatin in men with hypercholesterolemia. West of Scotland Coronary Prevention Study Group," *N Engl J Med,* 333(20): 1301-1307.

Sinauridze, E. I. *et al.* (2007), "Platelet microparticle membranes have 50- to 100-fold higher specific procoagulant activity than activated platelets," *Thromb Haemost,* 97(3): 425-434.

Sinning, J. M. *et al.* (2010), "Circulating CD31+/Annexin V+ microparticles correlate with cardiovascular outcomes," *Eur Heart J,* 32(16): 2034-2041.

Steele, B. M. *et al.* (2009), "Canonical Wnt signaling negatively regulates platelet function," *Proc Natl Acad Sci U S A,* 106(47): 19836-19841.

Stepien, E. *et al.* (2012), "Number of microparticles generated during acute myocardial infarction and stable angina correlates with platelet activation," *Arch Med Res,* 43(1): 31-35.

Sugiyama, S. *et al.* (2004), "Hypochlorous acid, a macrophage product, induces endothelial apoptosis and tissue factor expression: involvement of myeloperoxidase-mediated oxidant in plaque erosion and thrombogenesis," *Arterioscler Thromb Vasc Biol,* 24(7): 1309-1314.

Svensson, L. *et al.* (2009), "Leukocyte adhesion deficiency-III is caused by mutations in KINDLIN3 affecting integrin activation," *Nat Med,* 15(3): 306-312.

Tan, K. T. and Lip, G. Y. (2005), "The potential role of platelet microparticles in atherosclerosis," *Thromb Haemost,* 94(3): 488-492.

Tan, K. T. *et al.* (2005), "Elevated platelet microparticles in stable coronary artery disease are unrelated to disease severity or to indices of inflammation," *Platelets,* 16(6): 368-371.

Tantawy, A. A. *et al.* (2012), "Circulating platelet and erythrocyte microparticles in young children and adolescents with sickle cell disease: Relation to cardiovascular complications," *Platelets*.

Toschi, V. *et al.* (1997), "Tissue factor modulates the thrombogenicity of human atherosclerotic plaques," *Circulation,* 95(3): 594-599.

Ueland, T. *et al.* (2009), "Dickkopf-1 enhances inflammatory interaction between platelets and endothelial cells and shows increased expression in atherosclerosis," *Arterioscler Thromb Vasc Biol,* 29(8): 1228-1234.

Van Beers, E. J. *et al.* (2009), "Circulating erythrocyte-derived microparticles are associated with coagulation activation in sickle cell disease," *Haematologica,* 94(11): 1513-1519.

Van der Meijden, P. E. *et al.* (2012), "Platelet- and erythrocyte-derived microparticles trigger thrombin generation via factor XIIa," *J Thromb Haemost,* 10(7): 1355-1362.

Van der Pol, E. *et al.* (2012), "Single vs. swarm detection of microparticles and exosomes by flow cytometry," *J Thromb Haemost,* 10(5): 919-930.

Van der Pol, E. *et al.* (2013), "Innovation in detection of microparticles and exosomes," *J Thromb Haemost,* 11 Suppl 1: 36-45.

Van der Pol, E. *et al.* (2014), "Particle size distribution of exosomes and microvesicles by transmission electron microscopy, flow cytometry, nanoparticle tracking analysis, and resistive pulse sensing," *J Thromb Haemost*.

Van der Zee, P. M. *et al.* (2006), "P-selectin- and CD63-exposing platelet microparticles reflect platelet activation in peripheral arterial disease and myocardial infarction," *Clin Chem,* 52(4): 657-664.

Vidal, C. *et al.* (2001), "Flow cytometry detection of platelet procoagulation activity and microparticles in patients with unstable angina treated by percutaneous coronary angioplasty and stent implantation," *Thromb Haemost,* 86(3): 784-790.

Vilahur, G. *et al.* (2007), "Protein disulphide isomerase-mediated LA419- NO release provides additional antithrombotic effects to the blockade of the ADP receptor," *Thromb Haemost,* 97(4): 650-657.

Virmani, R. *et al.* (2000), "Lessons from sudden coronary death: a comprehensive morphological classification scheme for atherosclerotic lesions," *Arterioscler Thromb Vasc Biol,* 20(5): 1262-1275.

Wang, J. G. *et al.* (2011), "Monocytic microparticles activate endothelial cells in an IL-1beta-dependent manner," *Blood,* 118(8): 2366-2374.

Weber, C. and Noels, H. (2011), "Atherosclerosis: current pathogenesis and therapeutic options," *Nat Med,* 17(11): 1410-1422.

Werner, N. *et al.* (2006), "Circulating CD31+/annexin V+ apoptotic microparticles correlate with coronary endothelial function in patients with coronary artery disease," *Arterioscler Thromb Vasc Biol,* 26(1): 112-116.

Wierzbicki, A.S.; Humphries, S.E. and Minhas, R. (2008), "Familial hypercholesterolaemia: summary of NICE guidance," *Bmj,* 337: a 1095.

Wu, Y. *et al.* (2012), "The disulfide isomerase ERp57 mediates platelet aggregation, hemostasis, and thrombosis," *Blood,* 119(7): 1737-1746.

Xu, Y. *et al.* (2012), "Prevalence, distribution, predictors, and outcomes of patients with calcified nodules in native coronary arteries: a 3-vessel intravascular ultrasound analysis from Providing Regional Observations to Study Predictors of Events in the Coronary Tree (PROSPECT)," *Circulation,* 126(5): 537-545.

Zecher, D.; Cumpelik, A. and Schifferli, J. A. (2014), "Erythrocyte-derived microvesicles amplify systemic inflammation by thrombin-dependent activation of complement," *Arterioscler Thromb Vasc Biol,* 34(2): 313-320.

Zwicker, J. I. *et al.* (2011), "Tissue factor-bearing microparticles and thrombus formation," *Arterioscler Thromb Vasc Biol,* 31(4): 728-733.

6 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.

ABOUT THE AUTHOR

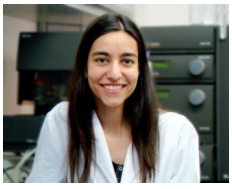
ROSA SUADES, PhD

PERSONAL INFORMATION

Date of Birth May 28, 1984, female

Institute Cardiovascular Research Center (CSIC-ICCC)
Catalan Institute of Cardiovascular Sciences
C/ Sant Antoni Ma. Claret, 167, 08025
Barcelona (Spain)

Current job Postdoctoral researcher



SCIENTIFIC EDUCATION AND ACADEMIC DEGREES

2014 Dr. phil. (*cum laude*), Universitat Pompeu Fabra (UPF), Advisor: Prof. Lina Badimon.
PhD title: *"Role of microparticles in atherothrombosis"*.
Doctoral School PhD Extraordinary Award.

2008 MSc in Pharmaceutical Sciences, Universitat Pompeu Fabra (UPF).

2007 Degree in Biology, Universitat Pompeu Fabra (UPF).

GRANTS

Predoctoral fellowship from ISCIII (Instituto de Salud Carlos III) [PFIS-FI09/00092].

Predoctoral fellowship from CIBER OBN (Obesidad y Nutrición)

AWARDS

- Best oral communication award at the Spanish Society of Cardiology (SEC) from "Riesgo Vascular y Rehabilitación" section of SEC, Zaragoza, October 27-29th 2016.
- "Enrique Fuentes Quintana" Health Science PhD Award from Funcas, 2016.
- Doctoral School PhD Extraordinary Award from Universitat Pompeu Fabra (UPF).
- Young Investigator Award at the 24th Biennal International Congress on Thrombosis from the Mediterranean League Against Thromboembolic Diseases (MLTD), Istambul, May 4-7th 2016.
- Young Investigator Award at the 23rd Biennal International Congress on Thrombosis from the Mediterranean League Against Thromboembolic Diseases (MLTD), Valencia, May 14-17th 2014.
- Young Investigator Award at the XXIV Congress of the International Society of Thrombosis and Haemostasis (ISTH), Amsterdam, July 2nd 2013.

RESEARCH INTERESTS

Extracellular vesicles, microparticles, exosomes, microRNAs, platelets, cardiovascular risk factors, hypercholesterolemia, atherothrombosis, and ischaemic heart disease.

LANGUAGES

Catalan and Spanish – native or bilingual proficiency

English – Full professional

ACADEMIC AND PROFESSIONAL EXPERIENCE

Institution: Academic Medical Centre, Clinical Chemistry Department (Amsterdam, the Netherlands).
Supervisor: Dr. Rienk Nieuwland.
Year: 2009.
Project: Circulating microvesicle characterization and quantifitication by flow cytometry.

Institution: LABORATORIOS ALMIRALL, SA. Departamento de Farmacología.
Supervisor: Dr. Jordi Gras and Dr. Jesús Llenas.
Year: 2007.
Project: Study of cardiac remodeling after myocardial infarction in rats.

Institution: Respiratory Muscle Laboratory, Royal Brompton Hospital (London, UK).
Supervisor: Dr. Michael I Polkey.
Year: 2006.
Project: Maximal Sniff Nasal Inspiratory Pressure versus Inspiratory-to-Total Lung Capacity Ratio in the Prediction of Mortality in Advanced Chronic Obstructive Pulmonary Disease.

Institution: Laboratorio de Neurofarmacología, Departamento de Ciencias Experimentales y de la Salut, Universidad Pompeu Fabra (UPF). Barcelona (Spain).
Supervisor: Dr. Rafael Maldonado and Dr. Olga Valverde.
Year: 2005.
Project: Involvement of CB1 cannabinoid receptors in emotional behaviour and depression.

Institution: Laboratorio Centre Hospitalari de Manresa, Fundació ALTHAIA (Spain).
Supervisor: Dr. Fina Franquesas and Dr. Dolors Estivill.
Year: 2005.
Project: Antifungal activity of different compounds against yeast by agar diffusion method.

Institution: Biochemistry Department of Medical University of Bydgoszcz (Poland).
Supervisor: Dr. K. Kozłowska
Year: 2004.
Project: Effect of arterial hypertension on glutatione peroxidase activity in erythrocytes and plasma from aged patients.

PARTICIPATION IN FUNDED PROJECTS

- RD12/0042/0027 - Red de Investigación Cardiovascular (RIC), Fondo Investigación Sanitaria - Redes Temáticas Investigación Cooperativa (RETICS), 03/2013 - 03/2016.
- SAF 2012-40208 - Efectos funcionales y moleculares de distintas formas de HDL en el remodelado cardiaco post-infarto: aplicación de biología de sistemas para identificar las dianas moleculares. Plan Nacional de Investigación Científica y Desarrollo tecnológico I+D+i, 01/01/2012-31/12/2015.
- CB06/003 - Fisiopatología de la obesidad y nutrición (CIBERobn), 01/01/2012-31/12/2015. Fondo de Investigación Sanitaria – Centro de Investigación Biomédica en Red – CIBER
- CNIC-08-2008 - Prognostic factors of cardiovascular mortality and morbidity in a cohort of families with genetic diagnosis of familial hypercholesterolaemia. From hypercholesterolemia to atherosclerosis in hFH patients: Characterization and functional effects on resident vascular cells of plasma lipoproteins (HDL, LDL) and circulating microparticles. CNIC, 01/12/2008-30/12/2011.
- SAF 2006-10091 - Mecanismos celulares y moleculares en arteriosclerosis coronaria e isquemia miocárdica. - Plan Nacional I+D+I – Tipo C Consolider-, 01/01/2006-30/09/2011

LIST OF PUBLICATIONS

1. Escate R, Padro T, Borrell-Pages M, SUADES R, Aledo R, Mata P, Badimon L. 2016. Macrophages of genetically characterized familial hypercholesterolaemia patients show up-regulation of LDL-receptor-related proteins. **Journal of Cellular and Molecular Medicine**; Sep 29. doi: 10.1111/jcmm.12993. [Epub ahead of print]
2. Badimon L, SUADES R, Fuentes E, Palomo I, Padro T. 2016. Role of Platelet-Derived Microvesicles As Crosstalk Mediators in Atherothrombosis and Future Pharmacology Targets: A Link between Inflammation, Atherosclerosis, and Thrombosis. **Frontiers in Pharmacology**; 7:293. eCollection 2016. *doi: 10.3389/fphar.2016.00293.*
3. Chiva-Blanch G, SUADES R, Crespo J, Peña E, Padró T, Jiménez-Xarrié E, Martí-Fàbregas J, Badimon L. 2016. Microparticle shedding from neural progenitor cells and vascular compartment cells is increased in ischemic stroke. **PLoS One**; 11(1):e0148176. doi:10.1371/journal.pone.0148176.
4. Chiva-Blanch G, SUADES R, Crespo J, Vilahur G, Arderiu G, Padró T, Corella D, Salas-Salvadó J, Arós F, Martínez-Gonzàlez MA, Ros E, Fitó M, Estruch R, Badimon L. 2016. CD3+/CD45+ and SMA-α+ circulating microparticles are increased in individuals at high cardiovascular risk who will develop a major cardiovascular event. **International Journal of Cardiology**; 208:147-9. Doi:10.1016/j.ijcard.2016.01.211.
5. Chiva-Blanch G, SUADES R, Padró T, Vilahur G, Peña E, Ybarra J, Pou JM, Badimon L. 2016. Microparticle Shedding by Erythrocytes, Monocytes and Vascular Smooth Muscular Cells Is Reduced by Aspirin in Diabetic Patients. **Revista Española de Cardiologia**; *In press.* Doi: 10.1016/j.rec.2015.12.033.
6. Chiva-Blanch G, Crespo J, SUADES R, Arderiu G, Padro T, Vilahur G, Cubedo J, Corella D, Salas-Salvadó J, Arós F, Martínez-González MA, Ros E, Fitó M, Estruch R, Badimon L. 2016. CD142+/CD61+, CD146+ and CD45+ microparticles predict cardiovascular events in high risk patients following a Mediterranean diet supplemented with nuts. **Thrombosis and Haemostasis**; 116(1).
7. SUADES R, Padró T, Crespo J, Ramaiola I, Martin-Yuste V, Sabaté M, Sans-Roselló J, Sionis A, Badimon L. 2016. Circulating microparticle signature in coronary and peripheral blood of ST elevation myocardial infarction patients in relation to pain-to-PCI elapsed time. **International Journal of Cardiology**; 202:378-387
8. SUADES R, Padró T, Alonso R, Mata P, Badimon L. 2015. High levels of TSP1+/CD142+ platelet-derived microparticles characterize young patients with high cardiovascular risk and subclinical atherosclerosis. **Thrombosis and Haemostasis**; 114(6):1310-21.
9. SUADES R, Padró T, Vilahur G, Martin-Yuste V, Sabaté M, Sans-Roselló J, Sionis A, Badimon L. 2015. Growing thrombi release increased levels of CD235a(+) microparticles and decreased levels of activated platelet-derived microparticles. Validation in ST-elevation myocardial infarction patients. **Journal of Thrombosis and Haemostasis**; 13(10):1776-86.
10. SUADES R, Padró T, Badimon L. 2015. The role of blood-borne microparticles in inflammation and haemostasis. **Seminars in Thrombosis and Haemostasis**; 41(6):590-606;2015.
11. SUADES R, Padró T, Alonso R, López-Mirando J, Mata P, Badimon L. 2014. Circulating CD45+/CD3+ lymphocyte-derived microparticles map lipid-rich atherosclerotic plaques in familial hypercholesterolaemia patients. **Thrombosis and Haemostasis**; 111(1):111-121.
12. SUADES R, Padró T, Alonso R, Mata P, Badimon L. 2013. Lipid-lowering therapy with statins reduces microparticle shedding from endothelium, platelets and inflammatory cells. **Thrombosis and Haemostasis**; 110(2):366-377.
13. SUADES R, Padró T, Vilahur G, Badimon L. 2012. Circulating and platelet-derived microparticles in human blood enhance thrombosis on atherosclerotic plaques. **Thrombosis and Haemostasis**; 108(6):1208-1219.
14. Alastair J. Moore, ROSA SUADES SOLER, Edward J. Cetti, S. Amanda Sathyapala, Nicholas S. Hopkinson, Michael Roughton, John Moxham c, Michael I. Polkey. 2010. Sniff nasal inspiratory pressure versus inspiratory-to-total lung capacity ratio in the prediction of mortality in advanced chronic obstructive pulmonary disease. **Respiratory Medicine**; 104:1319-1325.

Últimos números publicados

N.º 40. DOS ENSAYOS SOBRE FINANCIACIÓN AUTONÓMICA *(Serie ECONOMÍA Y SOCIEDAD),*
por Carlos Monasterio Escudero e Ignacio Zubiri Oria.

N.º 41. EFICIENCIA Y CONCENTRACIÓN DEL SISTEMA BANCARIO ESPAÑOL *(Serie ANÁLISIS),*
por Fernando Maravall, Silviu Glavan y Analistas Financieros Internacionales.

N.º 42. ANÁLISIS DE REFORMAS DEL IMPUESTO SOBRE LA RENTA PERSONAL A PARTIR DE MICRODATOS TRIBUTARIOS *(Serie ANÁLISIS),*
por José Félix Sanz Sanz, Juan Manuel Castañer Carrasco y Desiderio Romero Jordán.

N.º 43. COMPORTAMIENTO ESTRATÉGICO DE LA BANCA AL POR MENOR EN ESPAÑA: FUSIONES Y ESPECIALIZACIÓN GEOGRÁFICA *(Serie TESIS),*
por Cristina Bernad Morcate.

N.º 44. LA VERTIENTE CUALITATIVA DE LA MATERIALIDAD EN AUDITORÍA: MARCO TEÓRICO Y ESTUDIO EMPÍRICO PARA EL CASO ESPAÑOL *(Serie TESIS),*
por Javier Montoya del Corte.

N.º 45. LA DECISIÓN DE INTERNACIONALIZACIÓN DE LAS EMPRESAS: UN MODELO TEÓRICO CON INVERSIÓN HORIZONTAL Y VERTICAL *(Serie TESIS),*
por Jaime Turrión Sánchez.

N.º 46. FINANCIACIÓN DE LA ENSEÑANZA OBLIGATORIA: LOS BONOS ESCOLARES EN LA TEORÍA Y EN LA PRÁCTICA *(Serie ECONOMÍA Y SOCIEDAD),*
por Javier Díaz Malledo (coordinador), Clive R. Belfield, Henry M. Levin, Alejandra Mizala, Anders Böhlmark, Mikael Lindahl, Rafael Granell Pérez y María Jesús San Segundo.

N.º 47. SERVICIOS Y REGIONES EN ESPAÑA *(Serie ECONOMÍA Y SOCIEDAD),*
por Juan R. Cuadrado Roura y Andrés Maroto Sánchez.

N.º 48. LAS EMPRESAS DEL SECTOR DE LA CONSTRUCCIÓN E INMOBILIARIO EN ESPAÑA: DEL *BOOM* A LA RECESIÓN ECONÓMICA *(Serie ECONOMÍA Y SOCIEDAD),*
por Belén Gill de Albornoz (Dir.), Juan Fernández de Guevara, Begoña Giner y Luis Martínez.

N.º 49. INSTRUMENTOS PARA MEJORAR LA EQUIDAD, TRANSPARENCIA Y SOSTENIBILIDAD DE LOS SISTEMAS DE PENSIONES DE REPARTO *(Serie TESIS),*
por M.ª del Carmen Boado-Penas.

N.º 50. EL IMPUESTO DE FLUJOS DE CAJA EMPRESARIAL: UNA ALTERNATIVA AL IMPUESTO SOBRE LA RENTA DE SOCIEDADES *(Serie TESIS),*
por Lourdes Jerez Barroso.

N.º 51. LA SUBCONTRATACIÓN DE SERVICIOS DE I+D: EVIDENCIA DE EMPRESAS EUROPEAS Y DE EE.UU. *(Serie TESIS),*
por Andrea Martínez Noya.

N.º 52. IMPOSICIÓN EFECTIVA SOBRE LAS RENTAS DEL CAPITAL CORPORATIVO: MEDICIÓN E INTERPRETACIÓN. EL IMPUESTO SOBRE SOCIEDADES EN ESPAÑA Y EN LOS PAÍSES DE LA UNIÓN EUROPEA EN EL CAMBIO DE MILENIO *(Serie ANÁLISIS),*
por José Félix Sanz Sanz, Desiderio Romero Jordán y Begoña Barruso Castillo.

N.º 53. ¿ES RENTABLE EDUCARSE? MARCO CONCEPTUAL Y PRINCIPALES EXPERIENCIAS EN LOS CONTEXTOS ESPAÑOL, EUROPEO Y EN PAÍSES EMERGENTES *(Serie ECONOMÍA Y SOCIEDAD),*
por José Luis Raymond (coordinador).

N.º 54. LA DINÁMICA EXTERIOR DE LAS REGIONES ESPAÑOLAS *(Serie ECONOMÍA Y SOCIEDAD),*
por José Villaverde Castro y Adolfo Maza Fernández.

N.º 55. EFECTOS DEL *STOCK* DE CAPITAL EN LA PRODUCCIÓN Y EL EMPLEO DE LA ECONOMÍA *(Serie TESIS),*
por Carolina Cosculluela Martínez.

N.º 56. LA PROCICLICIDAD Y LA REGULACIÓN PRUDENCIAL DEL SISTEMA BANCARIO ***(Serie TESIS),***
por Mario José Deprés Polo.

N.º 57. ENSAYO SOBRE ACTIVOS INTANGIBLES Y PODER DE MERCADO DE LAS EMPRESAS. APLICACIÓN A LA BANCA ESPAÑOLA ***(Serie TESIS),***
por Alfredo Martín Oliver.

N.º 58. LOS ATRACTIVOS DE LOCALIZACIÓN PARA LAS EMPRESAS ESPAÑOLAS. EXPLOTACIÓN DE LA ENCUESTA SOBRE ATRACTIVOS DE LOCALIZACIÓN ***(Serie ECONOMÍA Y SOCIEDAD),***
por Encarnación Cereijo, David Martín, Juan Andrés Núñez, Jaime Turrión y Francisco J. Velázquez.

N.º 59. ESTUDIO ECONÓMICO DE LOS COSTES DE LA ENFERMEDAD: APLICACIÓN EMPÍRICA AL CASO DEL ALZHEIMER Y LOS CONSUMOS DE DROGAS ILEGALES ***(Serie TESIS),***
por Bruno Casal Rodríguez.

N.º 60. BUBBLES, CURRENCY SPECULATION, AND TECHNOLOGY ADOPTION ***(Serie TESIS),***
por Carlos J. Pérez.

N.º 61. DISCAPACIDAD Y MERCADO DE TRABAJO: TRES ANÁLISIS EMPÍRICOS CON LA MUESTRA CONTINUA DE VIDAS LABORALES ***(Serie TESIS),***
por Vanesa Rodríguez Álvarez.

N.º 62. EL ANÁLISIS DE LOS IMPUESTOS INDIRECTOS A PARTIR DE LA ENCUESTA DE PRESUPUESTOS FAMILIARES (*SERIE ANÁLISIS*),
por José Félix Sanz Sanz, Desiderio Romero Jordán y Juan Manuel Castañer Carrasco.

N.º 63. EUROPA, ALEMANIA Y ESPAÑA: IMÁGENES Y DEBATES EN TORNO A LA CRISIS ***(Serie ECONOMÍA Y SOCIEDAD),***
por Víctor Pérez-Díaz, Juan Carlos Rodríguez y Elisa Chuliá.

N.º 64. INTEGRACIÓN, INMIGRANTES E INTERCULTURALIDAD: MODELOS FAMILIARES Y PATRONES CULTURALES A TRAVÉS DE LA PRENSA EN ESPAÑA (2010-11) ***(Serie ECONOMÍA Y SOCIEDAD),***
por Enrique Uldemolins, Alfonso Corral, Cayetano Fernández, Miguel Ángel Motis, Antonio Prieto y María Luisa Sierra.

N.º 65. SOSTENIBILIDAD DEL SISTEMA DE PENSIONES DE REPARTO EN ESPAÑA Y MODELIZACIÓN DE LOS RENDIMIENTOS FINANCIEROS ***(Serie TESIS),***
por Clara Isabel González Martínez.

N.º 66. EVOLUCIÓN DE LAS FUNDACIONES BANCARIAS ITALIANAS: DE *HOLDING* DE SOCIEDADES BANCARIAS A UN MODELO INNOVADOR DE "BENEFICIENCIA PRIVADA" ***(Serie ECONOMÍA Y SOCIEDAD),***
por Paolo Baroli, Claudia Imperatore, Rosella Locatelli y Marco Trombetta.

N.º 67. LAS CLAVES DEL CRÉDITO BANCARIO TRAS LA CRISIS ***(Serie ECONOMÍA Y SOCIEDAD),***
por Santiago Carbó Valverde, José García Montalvo, Joaquín Maudos y Francisco Rodríguez Fernández.

N.º 68. ENTRE DESEQUILIBRIOS Y REFORMAS. ECONOMÍA POLÍTICA, SOCIEDAD Y CULTURA ENTRE DOS SIGLOS ***(Serie ECONOMÍA Y SOCIEDAD),***
por Víctor Pérez-Díaz y Juan Carlos Rodríguez.

N.º 69. REFORMA DEL MERCADO DE SERVICIOS PROFESIONALES EN ESPAÑA ***(Serie ECONOMÍA Y SOCIEDAD),***
por María Paz Espinosa, Aitor Ciarreta y Aitor Zurimendi.

N.º 71. BUILDING A EUROPEAN ENERGY MARKET: LEGISLATION, IMPLEMENTATION AND CHALLENGES ***(Serie ECONOMÍA Y SOCIEDAD),***
por Tomás Gómez y Rodrigo Escobar.

N.º 72. ESSAYS IN TRADE, INNOVATION AND PRODUCTIVITY
(Serie TESIS),
por Aránzazu Crespo Rodríguez.

N.º 73. ENDEUDAMIENTO DE ESPAÑA: ¿QUIÉN DEBE A QUIÉN?
(SERIE ECONOMÍA Y SOCIEDAD),
por Analístas Financieros Internacionales (AFI).

N.º 74. AGENTES SOCIALES, CULTURA Y TEJIDO PRODUCTIVO EN LA ESPAÑA ACTUAL
(SERIE ECONOMÍA Y SOCIEDAD),
por Víctor Pérez-Díaz, Juan Carlos Rodríguez, Joaquín Pedro López-Novo y Elisa Chuliá.

N.º 75. EVOLUCIÓN RECIENTE DEL CRÉDITO Y LAS CONDICIONES DE FINANCIACIÓN: ESPAÑA EN EL CONTEXTO EUROPEO
(SERIE ECONOMÍA Y SOCIEDAD),
por Joaquín Maudos.

N.º 76. EFICIENCIA DE LOS SISTEMAS REGIONALES DE INNOVACIÓN EN ESPAÑA
(SERIE ANÁLISIS),
por Mikel Buesa, Joost Heijs, Thomas Baumert y Cristian Gutiérrez.

N.º 77. ENCOURAGING BLOOD AND LIVING ORGAN DONATIONS
(Serie TESIS),
por María Errea y Juan M. Cabasés (director).

N.º 78. EMPLEO Y MATERNIDAD: OBSTÁCULOS Y DESAFIOS A LA CONCILIACIÓN DE LA VIDA LABORAL Y FAMILIAR *(Serie ECONOMÍA Y SOCIEDAD),*
por Margarita León Borja (coordinadora).

N.º 79. PEOPLE MANAGEMENT IN MICRO AND SMALL COMPANIES - A COMPARATIVE ANALYSIS. EMPLOYEE VOICE PRACTICES AND EMPLOYMENT RELATIONS,
(Serie ANÁLISIS),
por Sylvia Rohlfer, con la colaboración de Carlos Salvador Muñoz y Alesia Slocum.

N.º 80. LA CRISIS, ¿UNA OPORTUNIDAD PARA LA ECONOMÍA SOCIAL ESPAÑOLA
(Serie ECONOMÍA Y SOCIEDAD),
por Pierre Perard.

N.º 81. UN TRIÁNGULO EUROPEO: ELITES POLÍTICAS, BANCOS CENTRALES Y POPULISMOS
(Serie ECONOMÍA Y SOCIEDAD),
por Víctor Pérez Díaz, Juan Carlos Rodríguez y Elisa Chuliá.

N.º 82. EL MERCADO ESPAÑOL DE ELECTRICIDAD
(Serie ECONOMÍA Y SOCIEDAD),
por Aitor Ciarreta, María Paz Espinosa y Aitor Zurimendi.

N.º 83. THREE ESSAYS IN LONG-TERM ECONOMIC PERSISTENCE
(Serie TESIS),
por Felipe Valencia Caicedo

ESTUDIOS
DE LA FUNDACIÓN
SERIE TESIS

Pedidos e información:

Funcas

Caballero de Gracia, 28
28013 Madrid
Teléfono: 91 596 54 81
Fax: 91 596 57 96

publica@funcas.es
www.funcas.es

P.V.P.: Edición papel, 12€ (IVA incluido)
P.V.P.: Edición digital, 9€ (IVA incluido)

ISBN 978-84-15722-57-1