TESI DOCTORAL UPF / 2013

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Alla mia Famiglia

ACKNOWLEDGEMENTS

¡Por fin llegó este día tan esperado! ¡Aún me cuesta creerlo! Tras un largo y arduo camino, entre un montón de experimentos, resultados, desesperación por lo que no sale y alegría cuando parece que las cosas van por buen camino, se me hace difícil agradeceros a todos los que de una forma u otra habéis hecho posible que llegue hasta aquí y que haya conseguido acabar una etapa muy importante en mi vida.

En primer lugar quiero agradecer a mis directoras, Lina y Teresa que desde el primer día confiaron en mí.

Lina, muchas gracias por haberme ofrecido la oportunidad de trabajar contigo, por todo lo que me has enseñado, por haberme dado la posibilidad de aprender mucho, por tu dirección, por tus consejos y por el apoyo que me has dado. ¡Muchas gracias!

Teresa, me has llevado paso a paso hasta el final de esta etapa y por eso tengo mucho que agradecerte. En primer lugar quiero darte las gracias por haber confiado en mí, por todo lo que me has enseñado a lo largo de estos años, por tu paciencia, tus consejos, por el tiempo que me has dedicado, por tus múltiples correcciones, por los ánimos cuando la cosas no salían, por alegrarte cuando finalmente salieron y por haber conseguido que confiara más en mí misma. Además quiero darte las gracias por tu interés y por tu cercanía tanto a nivel profesional como personal. ¡Mil gracias!

Quiero agradecer también al Dr. David Andreu por haber aceptado ser mi tutor. ¡Muchas gracias!

Llegar a este día hubiera sido imposible sin la ayuda incansable de mis compañeras de laboratorio. Me siento muy afortunada por haber sido parte del 004 por todos estos años, por haber recibido vuestros consejos, ánimos y porque siempre que lo necesité me

habéis echado una mano sin pensarlo, por estar siempre pendientes de todo, por las risas y los arranques de limpieza y orden que de vez en cuando nos afectan y sobre todo por haber compartido con vosotras esta experiencia.

Ilaria…mi compatriota! Prima di tutto voglio ringraziarti per avermi fatto sentire come in casa (anche se non sono dell'oltrepó!), per poter parlare un po in italiano e soprattutto in dialetto!!!! Per avermi insegnato che l'ordine e la organizzazione sono di vitale importanza (in questo aspetto ci sto ancora lavorando!) e per essere meglio di un`agenzia di viaggi, ma soprattutto per essere sempre disponibile ad aiutare e a trovare una soluzione ai problemi, sei un esempio! Tieni duro che ci sei quasi!

Judit, ha sido un placer trabajar contigo, gracias por tus consejos y comprensión, por estar siempre disponible para cualquier cosa, por tu "soporte proteómico" y por la seguridad que transmites. Muchas gracias por interesarte siempre por cómo me van las cosas y gracias por tener todo apuntado en tu primera libreta….sin ella la rosu aún estaría entre nosotros! Eres fundamental para el laboratorio.

Loli, a ti tengo mucho que agradecerte, empezando por todos los wb y 2D (…y tranquila que aún no se han acabado!), por todas las horas pasadas haciendo "caracoles", por la colección de "pinos" y todo tipo de hortalizas que salieron intentando sacar un gel decente de las MEC. Gracias por tus ganas de trabajar y por tu dedicación (y también por tu paella y por el queso).

Rosa, muchas gracias por tu ayuda a lo largo de estos años y por tus consejos. Muchos ánimos para lo que te falta de tesis.

Quiero agradecer de forma especial al grupo de trasplantes. Oriol, muchas gracias por estar siempre pendiente de mí y preocuparte, por lo que me has enseñado, por nuestras conversaciones y sobre todo por las llamadas y los paseos nocturnos en el hospital. He aprendido mucho contigo! Maribel, muchas gracias por lo que me has enseñado, por tu disponibilidad, por tu ayuda y por todas las veces que me despertaste con un susto!!! Por organizar los congeladores y por pelearte con el equipo del agua! Ha sido divertido trabajar contigo! Raquel, gracias por informarme sobre cómo vamos con los turnos, sin tu control no me aclararía nunca. Carolina y Elena, gracias por los momentos compartidos, mucha suerte en esta etapa. Mar, gracias por invitarme siempre a tus conciertos y a las actividades extraescolares, y por las fotos del trasplante! Ingrid, gracias por tu alegría, optimismo y por animarme en esta última etapa, mucha suerte.

A los de cultivos, Esther G. muchas gracias por estar siempre disponible a echar una mano, por todos los favores que me hiciste, por tu trabajo y tu dedicación, ha sido un placer trabajar contigo. Sonia V. tengo que agradecerte, sobre todo por todas las horas que coincidimos en cultivos, por haberme echado una mano con las células siempre y por decirme: "en un plis plas acabamos" cuando estábamos sumergidas entre flascones de células! Y además, gracias por tu alegría y optimismo! Mucha suerte en todo! Nia, muchas gracias por mantener el orden en cultivos y por preocuparte cuando mis células estaban en peligro de contaminación. Patri, aunque coincidimos poco tiempo muchas gracias! Laura, muchas gracias por tu trabajo tanto en cultivos como en el cuartito de lavados, tu ayuda ha sido muy importante!

Además quiero agradecer a todos los becarios, técnicos y post-

docs de otros laboratorios con los cuales he coincidido en estos años.

Los del 005. Maite, eres un ejemplo a seguir, he tenido mucha suerte en conocerte, gracias por estar pendiente, por tu sonrisa y buen rollo. Mucha suerte en lo que te falta. Mónica P, fuiste una de las primeras personas que conocí cuando entré al centro y debuté asistiendo a una clase magistral de peinados que no se me olvidará! Muchas gracias por tu ayuda, alegría y espontaneidad! Javier, gracias por estar siempre pendiente y darme ánimos. Raquel F, muchas gracias por tu ayuda a lo largo de estos años. Blanca M. aunque coincidimos poco en el centro muchas gracias por tu interés y por todos los momentos fuera del labo, las cañas, caipis, cenas y todos tipos de celebraciones!

Blanca O. mi compañera de biblio! He tenido la suerte de coincidir contigo en esta última etapa, y sobre todo de compartir la "experiencia" de la escritura de la tesis, los momentos de crisis y las risas. Muchas gracias por estar pendiente y por echarme siempre una mano!

Sergi L. gracias por ser mi "guardaespaldas" en la biblio, por no coincidir nunca con la sensación de calor/frío, por tu ayuda con las ratas y por todo el tiempo compartido.

María Angeles, gracias por tu buen humor y por tu disponibilidad en ayudar.

Los del 111. Jose, muchas gracias por todo el tiempo en que compartimos mesa, por iluminarme sobre el LRP-1, por haber sido parte del grupo de trasplantes y haber intentado mejorarlo, por estar siempre disponible para ayudar, por todo lo que sabes sobre series, música, camisetas… y también por todas las veces que

coincidimos fuera del labo. Mucha suerte en tu nuevo trabajo! Carlota, mucha gracias por todo los momentos que hemos compartidos y por las risas. Vale, grazie per essere una buona amica, per tutte le cene a casa tua, per i dolci, la pizza e tutte le feste pazze di questi anni, per preoccuparti sempre e perché só che posso contare su di te. Laura N, muchas gracias por toda tu ayuda, por las lipos, por las fiestas, los días en la playa y las caipis. David, mucha suerte y gracias por compartir las lipos conmigo! Marta, gracias por tu alegría y por tus inagotables ganas de trabajar. Roi, gracias por darme ánimos. Paula, muchas gracias por toda tu ayuda durante los primeros años, por enseñarme lo de los trasplantes, por tu interés y por tu alegría. Judith S. gracias por todas las veces que coincidimos dentro y fuera del labo. Sandra, muchas gracias por toda tu ayuda y tus consejos, por ayudarme a solucionar los problemas y enseñarme todos los trucos para trabajar con RNA.

También me gustaría agradecer a las personas del 110 con la que he coincidido a lo largo de estos años: Javi F, María de Burgos, Anna, Ricardo, Olivier, Maurizio, Silvia, Judith, Beatriz, Saray, María Galán, Pepe y Cristina. ¡Muchas gracias! Javi Crespo, muchas gracias por estar siempre al día de todo lo que pasa en el centro, por tu buen humor y por intentar hablar en italiano!

Rafael, gracias por confiar en mis conocimientos sobre los western, mucha suerte en esta etapa. Chema, gracias por haberme iniciado en el mundo de las PCR. Sonia E, suerte con la tesis.

Quiero agradecer a todos los técnicos: Montse, Olaya, Mari, Sonia H, Mónica A, Ona, Ana, Pablo, Josep y Álvaro, por vuestra ayuda.

A los de Jerónimo, María gracias por darlo todo en las clases del teatro, Eli por el tiempo en la biblio, Marta Vilalta por preguntarme siempre que tal me iba todo y Juli por tu optimismo y por estar siempre pendiente, mucha suerte en tu nueva aventura.

María Granada, muchas gracias por tu alegría, por las risas y tus historias y por los futuros aperitivos en Milán. Elena gracias por tus fiestas en Blanes, por tu sentido del humor y por estar pendiente. Erick, eres pura alegría, ha sido un placer conocerte, mucha suerte en la vuelta.

Además quiero agradecer de forma muy especial a Esther Peña, por todas las horas pasadas en confocal, por todo lo que me has enseñado, por ayudarme a entender los resultados, por estar siempre disponible cuando lo he necesitado, por animarme y entenderme, miles de gracias! Maisa, muchas gracias por tu ayuda, por haberme enseñado mucho, por estar pendiente y preocuparte, por todas las muestras pasadas por el MALDI, por la rosu y por las fiestas de cumple de Alexia. Laura Casaní gracias por ayudarme con las ratas y las dietas. Rosa Aledo, muchas gracias por estar siempre hasta las tantas en la biblio, por el pijama party (fecha aún por concretar), por saludarme "a la monja" y por darme galletas y todo tipo de alimentos y sobre todo por tu ayuda! Vicenta, muchas gracias por todas las veces que me dejaste los anticuerpos del LRP-1, por estar siempre disponible para ayudarme, por los consejos y por estar pendiente. Gemma V, gracias por toda tu ayuda, y por las risas mirando las fotos de Córdoba. Gemma A. muchas gracias por tu ayuda y sobre todo por todo el tiempo que coincidimos esperando el ascensor! María Borrell, gracias por tu disponibilidad y por tus ganas de ayudar.

Quiero agradecer también al Dr. Joan Carles Escolà y a David del grupo de bioquímica del Institut de Recerca del Hospital de Sant Pau por haberme ayudado con la bioquímica de las ratas, por vuestra disponibilidad y amabilidad y por el tiempo que me dedicasteis. ¡Muchas gracias!

Además quiero dar las gracias a otras personas que me ayudaron a lo largo de estos años: los gerentes Angel, Jaume y Joaquim. A Silvia y Maria Jose por ayudarme cada vez que lo he necesitado y por estar siempre pendientes. A María, por todos los pedidos y el material de oficina. Nuria, Montse, Meritxell, Pepi y Patri, gracias por todas vuestras gestiones.

Además quería agradecer a Roger de mantenimiento, al señor que limpia los cristales que siempre me saluda, a Filo y a las dos Carmen por no haberme dado con el mocho todas las veces que pisé el suelo mojado, por preocuparos y por lo dulces y los rollitos de queso de la Filo! Muchas gracias!

Y gracias por todo a los que habéis formado parte del ICCC en estos años!

Ovviamente voglio ringraziare tutte le persone esterne al laboratorio che mi hanno aiutato!

Rocky, grazie per essere come un fratello, perché só che sempre potró contare su di te!...senza il tuo aiuto sarebbe stato imposibile! Ivan, sei una persona stupenda. Gracias por todo, por tu lado artístico, por tu cariño y por tu amistad! Ariel e Lauretta, siete dei veri amici, anche nella distanza so che posso sempre contare su di voi, grazie mille, siete come una famiglia.

Sandra e Nando, non so come ringraziarvi per tutto quello che avete fatto e state facendo per me, per essere un punto di contatto

con i miei genitori, per tutti pranzi e le cene (e per le foto in tempo reale!) e soprattutto perché so di poter contare sul vostro appoggio, grazie mille! Anna, grazie per tutte le conversazioni e i preziosi consigli sul futuro.

Emma e Dome, grazie per avermi sempre trattato come parte della famiglia e per il vostro interesse.

Gracias a Norma, Carlos, María Elena, Carlos, Ana y la Abuelita por haberme hecho sentir parte de vuestra familia y por estar pendientes. Muchas gracias!

Alla nonna Maria e al nonno Luigi, grazie per il vostro aiuto, per tutto quello che avete fatto e state facendo.

Alla zia Anna e allo zio Ignazio, grazie per il vostro interesse e il vostro aiuto. Al mio cugi Andrea, grazie per chiedermi sempre come va e cercarmi ogni volta che vengo a casa!

A mia mamma e mio papá, per avermi fatto diventare quello che sono oggi, per il vostro appoggio, la vostra pazienza e per aver sempre creduto in me.

Rodri, sin tu ayuda hubiera sido imposible, gracias por el apoyo, los ánimos y por aguantar mis manías y mis momentos de crisis. Gracias por todos los momentos juntos, y por entenderme tan bien!

SUMMARY

High levels of circulating low-density lipoproteins (LDL) are one of the major cardiovascular risk factors. Hypercholesterolemia induces endothelial dysfunction and chronic intimal inflammatory cell accumulation, hallmarks of the initiation of atherosclerosis. Additionally, growing human atherosclerotic plaques show proliferation and migration of vascular smooth muscle cells (VSMC) towards the intima producing remodeling of the vascular wall. However, those plaques that are most prone to rupture show a progressive loss of VSMC becoming soft and vulnerable and these lipid-rich high risk plaques cause clinical episodes resulting in morbid or fatal ischemic events. The mechanisms involved in the transformation of a plaque into a vulnerable VSMC-depleted atheroma have not been completely elucidated. Lipid-rich-VSMC have an impaired vascular repair function due to changes in cytoskeleton proteins. However, the effects of LDL on VSMC function during plaque remodeling and vascular repair are not fully understood. Thus, the aim of this thesis was to investigate early changes directly induced by LDL on VSMC phenotype and function and to identify the molecular mechanisms involved.

This thesis demonstrates that the cardiovascular risk of hypercholesterolemia involves the interaction of LDL with VSMC and the regulation at a molecular level of different pathways that converge in the cell's migratory capacity. Migratory function of lipid-loaded VSMC can be restored by inhibition of 3-hydroxy-methylglutaryl coenzyme A (HMG-CoA) through a Rho kinase and myosin light chain phosphatase dependent mechanism. In addition, the studies performed in this thesis show that LDL affect VSMC adhesion, migration and cytoskeleton dynamics through the abrogation of the urokinase-plasminogen activator (uPA)/uPA receptor (uPAR) system function and by modulation of HSP27 phosphorylation and subcellular localization.

RESUMEN

El nivel elevado de lipoproteínas de baja densidad (LDL), uno de los principales factores de riesgo cardiovascular, conllevan a una disfunción endotelial y acumulación crónica de células inflamatorias en la íntima arterial en la etapa inicial de desarrollo de la arterosclerosis. Además, la progresión de las placas arterioscleróticas se caracteriza por un proceso de remodelado vascular consecuencia de la proliferación y migración de células musculares lisas vasculares (CML) en la íntima. Sin embargo, las placas ateroscleróticas con mayor susceptibilidad a la ruptura presentan una pérdida progresiva de CML, siendo estas placas ricas en lípidos y altamente vulnerables las que provocan eventos isquémicos mórbidos o fatales. Hoy día desconocemos todavía los mecanismos involucrados en la transformación de las placas en ateromas vulnerables. Las CML ricas en lípidos presentan alteraciones en su capacidad de reparación vascular debido a alteraciones en proteínas del citoesqueleto. Sin embargo, los efectos de las LDL en la función de las CML durante el remodelado de las placas y reparación vascular se desconocen en gran medida. Por ello, el objetivo de esta tesis ha sido investigar los cambios iniciales inducidos directamente por las LDL en el fenotipo y la función de las CML e identificar los mecanismos moleculares involucrados.

Esta tesis demuestra que el riesgo cardiovascular de la hipercolesterolemia implica la interacción entre LDL y CML y la regulación a nivel molecular de diferentes vías de señalización que convergen en la migración celular. La capacidad de migración de CML cargadas de lípidos puede restituirse mediante la inhibición de la 3-hidroxi-3-metilglutaril coenzima-A (HMG-CoA), a través de un mecanismo dependiente de la quinasa Rho. Además, los estudios realizados en esta tesis demuestran que las LDL afectan la adhesión, migración y dinámica de formación del citoesqueleto de las CML a través de la alteración de la función del sistema del activador del plasminogeno tipo uroquinasa (uPA)/uPA receptor (uPAR) y mediante la modulación de la fosforilación y localización subcelular de la HSP27.

PREFACE

Cardiovascular disease remains one of the major causes of mortality and morbidity in the world. Atherosclerosis is considered the leading underlying cause of the appearance of cardiovascular episodes including coronary syndromes, stroke and peripheral artery disease.

Atherosclerosis is a diffuse pathological process that involves structural changes in the intima and media of arterial vessels mainly driven by cholesterol accumulation, endothelial dysfunction, inflammatory cells infiltration, and vascular smooth muscle cells migration. Atherosclerosis is a systemic disease that starts early in life, asymptomatically progressing through adulthood, until clinically manifested. Initial fatty streak lesions evolve into fibrous plaques, some of which are prone to rupture, leading to subsequent atherothrombotic episodes. Predominant factors that affect atherosclerotic plaques stability are their cell composition and the ratio of extracellular matrix to lipid content. Indeed, unstable plaques contain a large lipid core, rich in cholesterol esters and with little collagen, and a small number of vascular smooth muscle cells.

Previous studies at the Institut Català de Ciències Cardiovasculars (ICCC) in Barcelona have shown that high levels of low density lipoproteins impair vascular smooth muscle cells functions, such as actin cytoskeleton organization, adhesion and migration, processes that are closely related with the atherosclerotic plaque cell content. However, the molecular mechanisms that drive these effects of low density lipoproteins on vascular smooth muscle cells function still remain poorly understood.

The work presented in this thesis has been conducted at ICCC in Barcelona, and is focused on the identification of mechanisms and molecular players that are either directly involved or participate in the atherogenic effects of low density lipoproteins on vascular smooth muscle cell function, which may contribute to the development of new therapeutic targets to control plaque stability.

INDEX OF CONTENTS

INDEX OF FIGURES

INDEX OF TABLES

ABBREVIATIONS

ADF	Actin-depolymerizing factor
agLDL	Aggregated low density lipoproteins
Akt	Protein kinase B
AngII	Angiotensin II
Apo	Apolipoprotein
apoER2	Apolipoprotein E receptor 2
Arp	Actin-related protein
AT1	Angiotensin II receptor type 1
ATF	Amino terminal fragment
BCA	Bicinchoninic acid
bFGF	Basic fibroblast growth factor
BMI	Body mass index
BSA	Bovine serum albumin
cDNA	Complementary DNA
CE	Cholesteryl ester
CM	Chylomicrons
CREA	Creatinine
CRP	C-reactive protein
CVD	Cardiovascular disease
DAF	Decay-accelerating factor
DALY	Disability Adjusted Life Years
DNA	Deoxyribonucleic acid
EC	Endothelial cells
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme Linked ImmunoSorbent Assay
eNOS	Endothelial nitric oxide synthase
EPC	Endothelial progenitor cell
ERK	Extracellular signal-regulated kinase
FA	Focal adhesion
FAK	Focal adhesion kinase
FAK-p	Phosphorylated focal adhesion kinase

FBS	Fetal bovin serum
GAP	GTPase activating protein
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
gC1qR	Binding protein for the globular head domains of complement component C1q
GEF	Guanine exchange factor
GFD	Growth factor-like domain
GGPP	Geranyl-geraniol pyrophosphate
GOT	Glutamic oxaloacetic transaminase
gp130	Glycoprotein 130
GPI	Glycosyl-phosphatidylinositol
HB-EGF	Heparin-binding epidermal growth factor
HDL	High density lipoproteins
HDL-C	HDL cholesterol
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HMWK	High molecular weight kinin-free kininogen
HSP	Heat shock protein
ICAM	Intercellular adhesion molecule
IDL	Intermediate density lipoproteins
IFN	Interferon
IGF-1R	Insulin-like growth factor 1 receptor
IL	Interleukin
ILK	Integrin-linked kinase
JAK	Janus kinases
KBr	Potassium bromide
LCAT	Lecithin/cholesterol acyltransferase
LDL	Low density lipoproteins
LDL-C	LDL cholesterol
LDL-R	LDL receptor
LFA	Lymphocyte function-associated antigen
LOX-1	Lectin-like ox-LDL receptor
LPL	Lipoprotein lipase
LRP	Low density lipoprotein receptor-related protein

MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight
MAPK	Ras-mitogen-activated protein kinase
MAPKAP kinase 2	MAPK-activated protein kinase 2
MAPKAP kinase 2-p	Phosphorylated MAPKAP kinase 2
MCP	Monocyte chemoattractant protein
M-CSF	Macrophage colony-stimulating factor
MEGF7	Epidermal growth factor-like protein 7
MHC	Major histocompatibility complex
ML9	Inhibitor of myosin light chain kinase
MLC	Myosin light chain
MLCK	MLC kinase
MLCP	MLC phosphatase
MMPs	Matrix metalloproteases
MRLC	Myosin regulatory light chain
MRLC-p	Phosphorylated MRLC
mRNA	Messenger ribonucleic acid
MTOC	Microtubule-organizing center
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
nLDL	Native LDL
NO	Nitric oxide
oxLDL	Oxidized LDL
PAF-AH	Platelet-activating factor acetylhydrolase
PAI	Plasminogen activator inhibitor
PAK	p21-activated kinase
PAK-p	Phosphorylated PAK
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDGFR	PDGF receptor
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol 4,5-biphosphate

PPARα	Peroxisome proliferator-activated receptor
RAGE	Receptor for advanced glycation end products
RAP	Receptor associated protein
RBD	Rho-binding domain
RLCs	Regulatory light chains
ROCK	Rho kinase
RVSMC	Rat vascular smooth muscle cells
SAA4	Serum amyloid A4
sc-uPA	Single-chain uPA
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis
SHP-2	Src homology 2-containing phosphotyrosine phosphatase
siRNA	Small interfering RNA
SR	Scavenger receptor
SREBP	Sterol regulatory element binding proteins
STAT	Signal Transducer and Activator of Transcription
TBARS	Thiobarbituric acid reactive substances
TFPI	Tissue factor pathway inhibitor
TIMPs	Tissue inhibitor of metalloproteinases
TNF	Tumor necrosis factor
tPA	Tissue plasminogen activator
uPA	Urokinase plasminogen activator
uPAR	uPA receptor
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VLDL	Very low density lipoproteins
VLDL-C	VLDL cholesterol
VLDL-R	VLDL receptor
VN	Vitronectin
VSMC	Vascular smooth muscle cells
vWF	Von Willebrand factor
WASP	Wiskott–Aldrich Syndrome Protein

WAVE	Wiskott-Aldrich syndrome protein family member 2
WHO	World Health Organization
Y27632	Inhibitor of Rho-associated protein kinase

I. INTRODUCTION

1. Cardiovascular disease

According to the World Health Organization (WHO), cardiovascular disease (CVD) is the leading cause of death and disability in the world despite currently available drug therapies.

CVD is responsible for over 17.3 million deaths per year worldwide (World Health Organization, 2011), being atherosclerosis its major cause. Indeed, this represents 30% of all-cause mortality (32% in woman and 27% in men) and 48% of that by non-communicable diseases **(Figure 1)**.

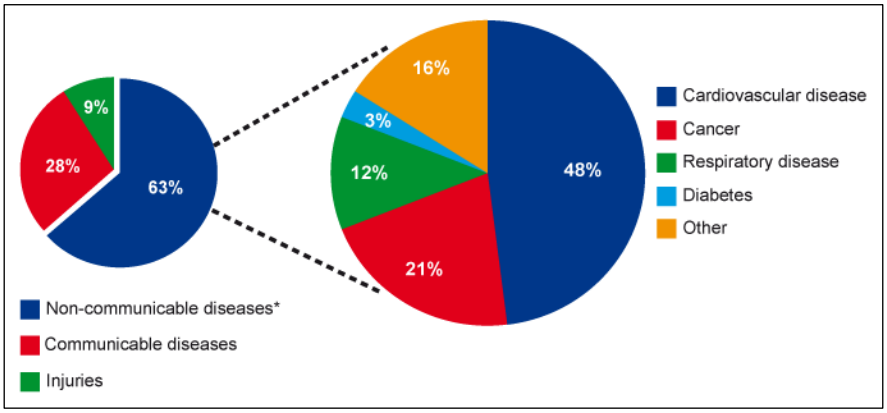

Figure 1. Deaths by non-communicable diseases worldwide. Adapted from WHO (World Health Organization, 2011) *Include non-communicable, maternal, perinatal and nutritional conditions.

Moreover, CVD is responsible for 151 million Disability Adjusted Life Years (DALY), accounting for 10% of the total DALY estimated on a global scale for the year 2008 (World Health Organization, 2011).

In addition, it has been estimated that the number of deaths caused by CVD will increase to reach 23.4 million people by the year 2030 (World Health Organization, 2008).

In Spain, according to the report published by the Ministry of Health, Social Policies and Equality, CVD is also the main cause of death accounting for 29% of all-cause mortality. This is slightly different from that observed in Catalonia, where it represents a 26.4% (Ministerio de Sanidad Servicios Sociales e Igualdad, 2008) **(Table 1)**.

Causes of death	SPAIN		CATALONIA	
	Number of deaths	% of all deaths	Number of deaths	% of all deaths
CVD*	112,008	29.0	15,888	26.4
Cancer	100,675	26.1	15,976	26.6
Respiratory diseases	14,875	3.9	2,600	4.3
Injuries	10,924	2.8	2,007	3.3
Diabetes	10,153	2.6	1,721	2.9
TOTAL	**388,324**	**100%**	**60,110**	**100%**

Table 1. Leading causes of deaths in Spain and Catalonia in 2008. Adapted from the Ministry of Health, Social Policy and Equality report (Ministerio de Sanidad Servicios Sociales e Igualdad, 2008). *Include cerebrovascular and cardiovascular diseases.

Risk Factors

CVD is considered a dynamic, progressive and multifactorial disease which develops as a consequence of interactions between genetic and environmental factors. Indeed, large prospective cohort studies such as the Framingham Heart Study have detected several conditions that favor the appearance of cardiovascular events (Wilson, P. W. et al., 1998) and that allow to stratify patients by estimating their probability of developing cardiovascular disease within a specified amount of time. These conditions are called cardiovascular risk factors and while some of them can be controlled, treated or modified (e.g. hypercholesterolemia, hypertension, obesity, etc.), others such as age, gender and family history cannot be controlled (World Health Organization, 2011) **(Figure 2)**.

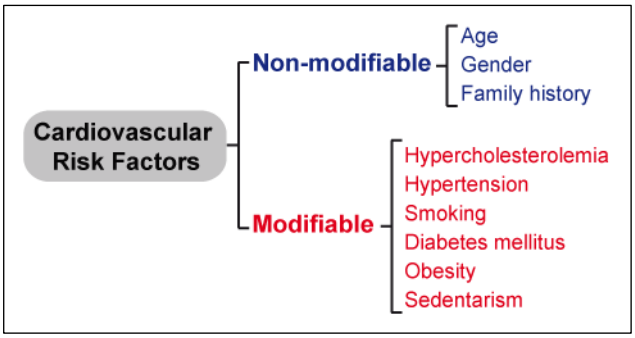

Figure 2. Major cardiovascular risk factors.

Among the main modifiable risk factors we find:

- **Hypercholesterolemia:** several studies, including INTERHEART (Yusuf, Salim et al., 2004), have reported a strong association between the advent of cardiovascular events and hypercholesterolemia, especially high levels of low density lipoprotein cholesterol (LDL-C), indicating that it can be considered as a major cause of vascular damage and remodeling (Badimon, L. et al., 2009) **(Figure 3)**. Indeed, high levels of total cholesterol (>200mg/dl), low density lipoprotein cholesterol (>130mg/dl) and triglycerides (>150mg/dl) increase the risk of heart disease and stroke, causing approximately 2.6 million deaths (4.5% of total deaths) (World Health Organization, 2009).

- **Hypertension:** considered as one of the most important causes of premature death. Blood pressure levels have been shown to be positively and progressively related to the risk of stroke and coronary heart disease (Lawes, C. M. et al., 2008).

- **Smoking:** a large number of epidemiological, clinical and laboratory studies demonstrated that smoking causes harmful effects on the heart and blood vessels, where it contributes to endothelial dysfunction, an

early key event in atherogenesis (Leone, A., 2003). Smoking is estimated to cause nearly 9% of all CVD.

- **Sedentarism:** physical inactivity is associated with the progression of CVD. Regular exercise training can decrease arterial wall thickness (a marker of pre-clinical atherosclerosis) in healthy asymptomatic subjects as well as in subjects with cardiovascular risk factors (Thijssen, D. H. et al., 2012). In addition, physical activity increases nitric oxide (NO) bioavailability improving endothelial function (Walther, C. et al., 2004).

- **Obesity**: is a growing health problem worldwide. It is strongly related to other major cardiovascular risk factors such as hypertension, high blood cholesterol and triglycerides levels and insulin resistance. Risk of cardiovascular disease and type 2 diabetes increases steadily with an increasing body mass index (BMI, a measure of weight relative to height).

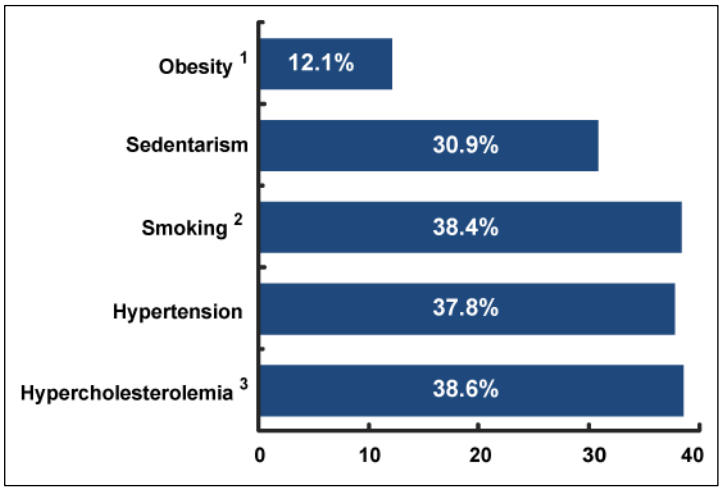

Figure 3. Prevalence of major cardiovascular risk factors worldwide. Adapted from WHO (World Health Organization, 2011). [1] People with BMI>30; [2] daily smokers of any tobacco product, [3] total cholesterol levels ≥193mg/dl.

2. Atherosclerosis

Atherosclerosis, the major cause of CVD, is a systemic disease that begins early in life and progresses asymptomatically through adulthood, until clinical manifestations such as myocardial infarction, stroke, and gangrene occur. Atherosclerosis is characterized by the progressive accumulation of lipids and other blood-borne components within the wall of medium and large-sized arteries (Viles-Gonzalez, J. F. et al., 2004).

2.1. Anatomy of arterial wall

All the arterial vessels, with the exception of the capillaries, are composed of three layers **(Figure 4)**:

- **Intima:** is the innermost layer and is in direct 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:** is the middle layer of the vascular wall. It 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:** is the external layer of the vascular wall. Its thickness varies considerably depending on the type and location of the vessel. This layer consists of elastic fibers, collagen, fibroblast, nerves and small blood vessels (*vasa vasorum*).

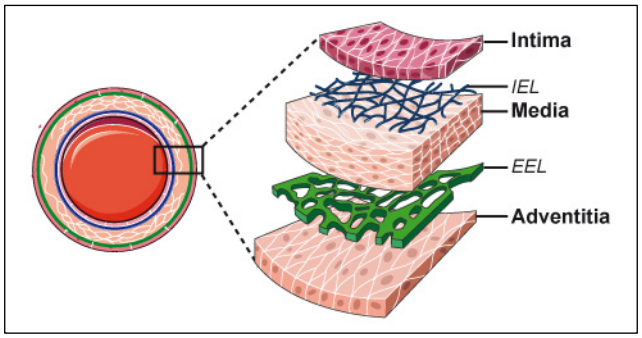

Figure 4. Arterial wall structure. *IEL*: internal elastic lamina; *EEL*: external elastic lamina.

2.2. Patophysiology of atherosclerosis

Our understanding of the etiology of atherosclerosis has evolved throughout the years. In 1852, Rotikansky postulated the *"Thrombogenic hypothesis"* suggesting that fibrin deposition with secondary lipid accumulation resulted in the intimal thickening. In 1856, Virchow proposed the *"Lipid hypothesis"*, which postulates that increased transudation of plasma lipids caused an accumulation of lipids within the arterial wall and that this was caused by the combination of increased lipid deposition with reduced lipid removal.

One hundred years later, these two theories were linked by Ross (1993) in his *"Response to injury hypothesis"*, where he established that atherosclerosis is a chronic inflammatory disease and that endothelial dysfunction induced by various risk factors (hyperlipemia, hypertension, cigarettes smoking, etc.) alters its permeability facilitating the initiation and progression of atherosclerotic lesions.

Thus, atherosclerotic lesions result from a complex interplay between circulating factors and the various cell types present in the vessel wall. This process is triggered by the chronic and repeated exposure to several systemic and local injurious stimuli, such as increased plasma lipid levels

(Badimon, L. et al., 2006), and can result in morbid or fatal ischemic events with different clinical manifestations depending on the affected area (i.e. coronary artery disease, cerebrovascular disease, and peripheral artery disease) (Faxon, D. P. et al., 2004) **(Figure 5)**.

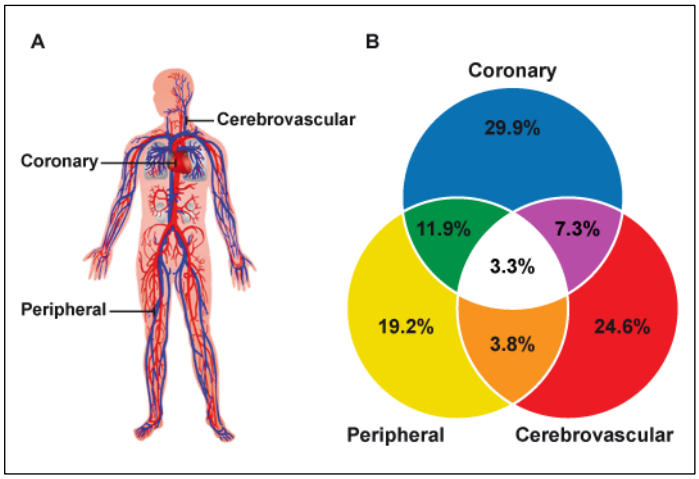

Figure 5. Distribution of atherosclerotic lesions. (A) Main territories affected by atherosclerotic lesions. **(B)** Percentage of atherosclerotic events in different territories. Data obtained from CAPRIE trial (Coccheri, S., 1998).

Initial lesion

The earliest changes that precede the formation of atherosclerotic lesions take place in the endothelium. EC are highly specialized cells able to modulate their functional stage in response to different stimuli and modify its functional status to contribute to the homeostasis of the vascular wall (Michiels, C., 2003). Early lesions are called *fatty streaks* and consist in subendothelial accumulation of low density lipoproteins (LDL), infiltration of immune cells, such as macrophages, T cells and mast cells **(Figure 6)**.

Lipid accumulation is increased when the levels of circulating LDL are raised. LDL diffuses through EC junctions and its retention in the vessel

wall involves interaction between LDL component apolipoprotein B (apoB) and matrix proteoglycans (Boren, J. et al., 1998). In addition to LDL, other apoB-containing lipoproteins can also accumulate in the intima and promote atherosclerosis.

Lipoproteins retained in the subendothelial space become modified displaying characteristics different from native LDL that make them even more atherogenic. Indeed, trapped LDL undergo aggregation and oxidation and these modifications contribute to inflammation. Oxidized-LDL stimulate endothelial cells to produce adhesion molecules and chemotactic proteins, such as the monocyte chemoattractant protein-1 (MCP-1) and the macrophage colony-stimulating factor (M-CSF), resulting in the recruitment of monocytes and T-lymphocytes into the vessel wall. It has been reported that adhesion molecules such as ICAM-1, VCAM-1, P-selectin, E-selectin (Dong, Z. M. et al., 1998; Nakashima, Y. et al., 1998; Collins, R. G. et al., 2000) are important for adhesion of leukocytes and monocytes to endothelium, promoting its migration into subendothelial space. The presence of molecules such as M-CSF contributes to monocyte differentiation to macrophages, increasing their expression of scavenger receptors that will allow them to uptake greater amounts of lipids, transforming into foam cells (Kunjathoor, V. V. et al., 2002).

As activated macrophages, T-cells and EC continue to release growth-regulatory molecules and cytokines they contribute to the progression of atherosclerosis.

Progression of lesion

Lesion progression involves the migration of VSMC from the media layer to the intima layer and its proliferation in response to mediators such as

platelet-derived growth factor (PDGF).

Once in the intima layer, VSMC produce extracellular matrix (ECM) macromolecules such as collagen, elastin and proteoglycans and form a fibrous cap that covers the plaque (Katsuda, S. et al., 2003). VSMC and ECM accumulation promote a fibrous plaque development resulting in the progressive narrowing of the vessel lumen. VSMC that migrated into the intima are able to further proliferate and internalize modified LDL, such as aggregated LDL (agLDL) by expressing the low density lipoprotein receptor-related protein 1 (LRP-1) (Llorente-Cortes, V. et al., 2000) or oxidized LDL (oxLDL) through the lectin-like ox-LDL receptor (LOX-1) mediated mechanism (Aoyama, T. et al., 2000) thus contributing to foam cell formation. With time, foam cells (VSMC and macrophages) undergo necrosis and release lipids that accumulate extracellulalry. The inefficient clearance of dead cells can promote the accumulation of cellular debris and extracellular lipids forming a lipid-rich pool called the necrotic core of the plaque (Ross, R., 1999; Virmani, R. et al., 2000) **(Figure 6)**.

Advanced lesions

As atherosclerotic plaques continue to grow, the arterial wall thickness to compensate possible lumen reduction. However, when plaque occupies approximately 40% of the arterial area, compensation ceases and any additional growth results in a narrowing of the arterial lumen (stenosis) that can lead to distal ischemic events.

However, the main complications of atherosclerosis are those caused by the formation of an occlusive thrombus as a consequence of plaque rupture or erosion (Badimon, L. et al., 2009), when procoagulant material from the plaque's necrotic core is exposed to coagulation proteins present

in the blood (Lusis, A. J., 2000). Thrombi formed can then interrupt blood flow locally or embolize and lodge in distal arteries.

Plaque vulnerability depends mainly on its composition. Stable lesions are characterized by low lipid content and a thick and uniform fibrous cap. On the other hand, plaques susceptible to rupture (vulnerable plaques) are characterized by a thin and collagen-poor fibrous cap with few VSMC and abundant macrophages. High content of extra- and intra-cellular lipid deposits are also associated with a high-risk of vulnerability to rupture (Badimon, L. et al., 2009).

Activated macrophages, T-cells, and mast cells present at lesion sites produce several types of molecules (i.e. inflammatory cytokines, proteases, coagulation factors, radicals, and vasoactive molecules) that can contribute to destabilize the plaque by affecting the formation of a stable fibrous cap. Indeed, collagen synthesis inhibition due to pro-inflammatory cytokines such as interferon-γ (IFN-γ) as well as degradation of ECM components by the proteolytic activity of matrix metalloproteases (MMPs) and cysteine proteases are key events in the destabilization of plaques. Besides, it can lead to hemorrhage from the *vasa vasorum* resulting in thrombus formation (Lusis, A. J., 2000; Libby, P., 2002). In addition, the combination of pro-inflammatory cytokines, such as interleukin-1β (IL-1β), tissue necrosis factor-α (TNF-α), and IFN-γ increase VSMC apoptosis promoting plaque rupture (Rudijanto, A., 2007) **(Figure 6)**.

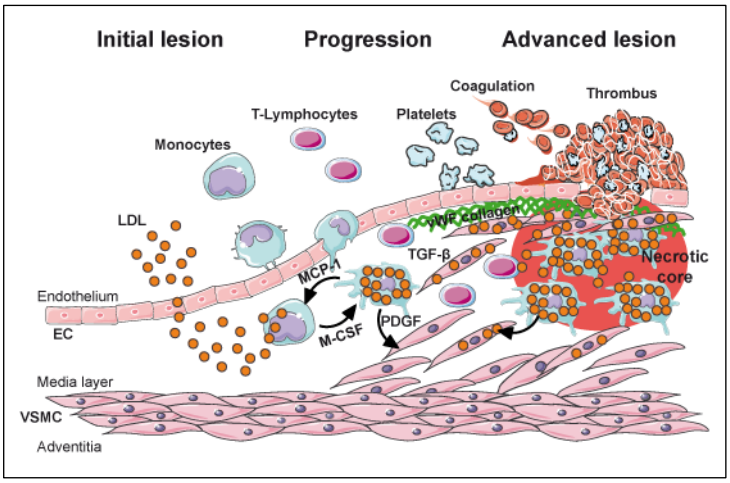

Figure 6. Stages of atherosclerotic plaque formation. EC, endothelial cells; VSMC, vascular smooth muscle cells, LDL, low density lipoproteins; M-CSF, macrophage colony-stimulating factor; MCP-I, monocyte chemotactic protein-1; PDGF, platelet-derived growth factor; TGF-β, tranforming growth factor beta; vWF, von Willebrand factor. Adapted from (Badimon, L. et al., 2009).

3. Lipoproteins

Lipoproteins are high molecular weight complexes of lipids and proteins that circulate in the blood plasma. Their physiologic function is to transport lipids to cells for energy, growth requirements or storage and their metabolism is closely interrelated with the initiation and progression of atherosclerosis.

In general, lipoproteins are composed of a core of cholesteryl esters and triglycerides and a surface layer of unesterified cholesterol and phospholipids, in which one or more proteins are embedded. These proteins are called apolipoproteins (apo) and contain amphipatic regions which enable them to bind lipids and at the same time be transported in plasma (Badimon, J. J. et al., 1993) **(Figure 7)**.

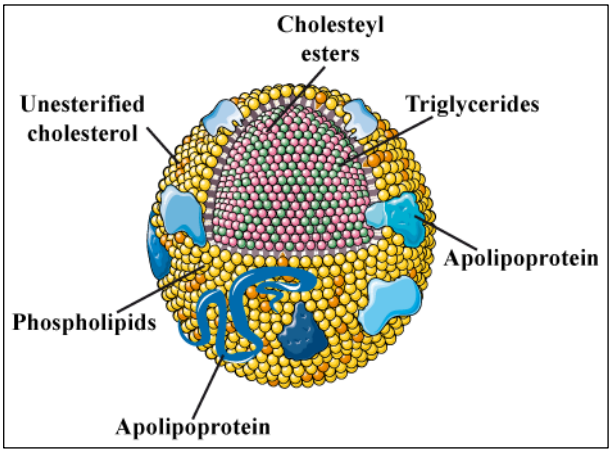

Figure 7. Lipoprotein structure.

Lipoproteins are in a state of constant flux, changing their composition and physical structure as peripheral tissues take up various components before the remnants return to the liver.

There are many different types of lipoproteins present in human blood with the same general structure, but with very different ratios between lipid and protein content, as well as different protein types. For this reason, the lipoproteins are usually classified according to their density. Lipoproteins can be grouped in five different families: chylomicrons (CM), very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL) and high density lipoproteins (HDL) **(Table 2)**.

	CM	VLDL	IDL	LDL	HDL
Density (g/ml)	0.93	0.93-1.006	1.006-1.019	1.019-1.063	1.063-1.21
Diameter (nm)	75-1200	30-80	25-35	18-25	5-12
Composition (% of mass)					
Triglycerides	86	55	23	6	4
Cholesteryl esters	3	12	29	42	14
Unesterified cholesterol	2	7	9	8	4
Phospholipids	7	18	19	22	34
Proteins	2	8	19	22	45

Table 2. Physical properties and composition of plasma lipoproteins. *percentage of mass. Adapted from (Kane, J. P., 1996).

3.1. Chylomicrons and very low density lipoproteins

CM and VLDL are triglyceride-rich lipoproteins. CM are secreted in the intestine and carry exogenous triglycerides into the plasma, whereas VLDL originate in the liver and transport endogenous triglycerides.

CM mainly consist of various members of the apolipoprotein family: apoB-48, apoA-I, apoA-II, apoA-IV, apoC and apoE, whereas, VLDL contain apoB-100, apoE, apoC-I, apoC-II and apoC-III.

These circulating particles undergo lipolysis by lipoprotein lipase (LPL), liberating free fatty acids. As a result, these lipoproteins decrease their size and the cores become relatively enriched in cholesterol esters, forming remnant lipoproteins.

Remnant triglyceride-rich lipoproteins contribute to atherosclerosis development via indirect mechanisms, particularly those involving its delipidation and retention in the arterial wall, where they can be internalized by macrophages and VSMC (Ginsberg, H. N., 2002; Tomkin, G. H. et al., 2012).

3.2. Low density lipoproteins

LDL originate from the catabolism of CM and VLDL. This type of lipoproteins carries the majority of plasma cholesterol and delivers it to peripheral tissues. LDL are considered as the main atherogenic lipoproteins. Indeed, most lipids deposited in the atherosclerotic lesions are derived from plasma LDL (Badimon, J. J. et al., 1993).

ApoB-100 accounts for more than 95% of the total protein mass of LDL (Segrest, J. P. et al., 2001). However, minor amounts of other apolipoproteins associated to LDL have been reported. Several of these proteins, such as apoE, apoC-III, and platelet-activating factor acetylhydrolase (PAF-AH), have important roles in LDL metabolism and modulate its atherogenicity despite their low concentration (Bancells, C. et al., 2010). In addition, in recent years proteomics have allowed to detect other minor proteins in LDL such as apoC-II, apoA-I, apoA-IV, apoM, apoJ, serum amyloid A4 (SAA4), calgranulin A, lysozyme C, apoD, apoH, α1-antitrypsin, orosomucoid-1, paraoxonase-1, retinol binding protein, and prenylcysteine lyase-1(Davidsson, P. et al., 2005; Karlsson,

H. et al., 2005; Stahlman, M. et al., 2008; Banfi, C. et al., 2009; Sun, H. Y. et al., 2010).

ApoB-100, present in VLDL and LDL, is a large protein of 515 KDa that contains different functional domains such as an LDL-receptor binding site, lysine and arginin-rich segments that interact with ECM proteins and several binding sites for glycosaminoglycans (Segrest, J. P. et al., 2001) that promote their retention in the vessel wall.

The entry and retention of LDL in the subendothelial layer mainly depend on its sustained plasma levels. However, other possible determinants as lipoprotein size, cholesterol enrichment and endothelial permeability may also affect LDL entry and retention (Tabas, I. et al., 2007).

In the arterial intima, the organized tight network of the ECM promotes lipoprotein binding. The major components of vascular ECM are collagen, elastin, and proteoglycans, which are considered as the most-important lipoprotein-retaining molecules in the subendothelial space (Khalil, M. F. et al., 2004).

Proteoglycans are abundant in atherosclerotic lesions, and they associate with several specific regions of the apoB-100 fraction of LDL. LDL retained in the subendothelium can become modified displaying characteristics different from those of native LDL that make them even more atherogenic (Badimon, L. et al., 2012).

3.2.1 LDL modifications

In the arterial intima, there are several proteolytic and lipolytic enzymes and oxidants capable of modifying LDL. Indeed, initial retention of LDL in the ECM by proteoglycans of arterial wall appears to be a prerequisite for LDL modification. The most commonly found LDL modifications are aggregation and oxidation (Badimon, L. et al., 2012).

Chondroitin sulphate proteoglycans, such as versican, are the main structural proteoglycans of the ECM and are considered important atherogenic elements given that they can strongly interact with and retain circulating LDL (Pentikainen, M. O. et al., 1997; Tirziu, D. et al., 1999).

Aggregated LDL (agLDL). LDL aggregation is due to the modification of the surface structure of LDL particles that can result in loss of particle stability. This, again, affects interactions between particles and can lead to their aggregation. Aggregation of LDL brings the surfaces of different LDL particles into contact and if particle modifications are sufficiently extensive, energetic stabilization will result in the subsequent fusion of the attached particles (Oorni, K. et al., 2000) thus increasing their atherogenicity.

agLDL are potent inducers of massive intracellular cholesteryl ester (CE) accumulation in VSMC and in macrophages leading to foam cell formation, a major characteristic of the early atherosclerotic lesion.

In vitro studies have shown that unmodified LDL (native LDL) are taken up very slowly by macrophages (Nievelstein, P. F. et al., 1991) and VSMC, however, when LDL were induced to self-aggregate either by vortexing or by incubation with versican (Llorente-Cortes, V. et al., 2002a), agLDL are rapidly taken up by macrophages (Buton, X. et al., 1999) and VSMC (Llorente-Cortes, V. et al., 2005).

agLDL are internalized by VSMC through LDL receptor-related protein 1 (LRP-1) (Llorente-Cortes, V. et al., 2005) which in turn is overexpressed by the presence of agLDL. Indeed, in situ hybridization analysis has shown that, in hypercholesterolemic animals, vascular wall LRP-1 mRNA is increased (Llorente-Cortes, V. et al., 2002b), indicating that hypercholesterolemia might favor LDL capture by VSMC through its

capacity to regulate the amount of LRP-1.

On the other hand, there is considerable controversy regarding the mechanism involved in agLDL uptake by macrophages. While some authors suggest a phagocytic process (Khoo, J. C. et al., 1988), others, using experimental designs that simulate *in vivo* interaction between macrophages and subendothelial LDL particles, have defined a new internalization mechanism known as "pathocytosis," which consists of capturing large amounts of agLDL by means of vesicular membrane formations (Kruth, H. S., 2002). More recently, however, it has been described that agLDL can be internalized by macrophages through LRP-1 in a process regulated by sterol regulatory element binding proteins transcription factors, SREBP1 and SREBP2, which in turn control LRP-1 expression (Llorente-Cortes, V. et al., 2007).

Oxidized LDL (oxLDL). This LDL modification is due to the activity of certain enzymes present in the arterial wall such as 15-lipoxigenase, myeloperoxidase and heme-oxydase as well as by the presence of NO synthase and NADPH oxidase that can produce free radicals capable of oxidizing LDL (Oorni, K. et al., 2000). oxLDL are known to enhance the expression of pro-inflammatory genes leading to monocytes recruitment into the vessel wall and dysfunction of the vascular endothelium (Li, D. et al., 2000). The biological effects of oxLDL are mediated by receptors. A number of scavenger receptors for oxLDL present on VSMC, monocytes and macrophages have been identified, such as type 1 scavenger receptor (SR-A1) and type 2 (SR-A2), type B scavenger receptor CD36 and CD68. Whereas, lectin-like oxLDL receptor 1 (LOX-1) was identified as a specific receptor for oxLDL uptake by EC (Sawamura, T. et al., 1997). The internalization of oxLDL produces formation of foam cells and activation of EC with subsequent increase in subendothelial permeability.

3.2.2. LDL receptors

Two families of lipoprotein receptors are considered responsible for LDL uptake by cells of the vascular wall: scavenger receptors family and LDL receptors family.

Scavenger receptors family

The scavenger receptors family is composed of at least eight different subclasses (Krieger, M. et al., 1994; Rigotti, A., 2000; Badimon, L. et al., 2006), which bear little sequence homology. Scavenger receptors recognize a wide variety of ligands including oxLDL, apoptotic cells and pathogens. Although the pathophysiological roles played by these receptors are still unproven, data from murine models of atherosclerosis have demonstrated a significant role of two receptors, SR-A and CD36 in atherosclerotic foam cell formation and vascular lesion development (Rigotti, A., 2000). SR-A isoforms are largely expressed in macrophages but can also be detected in EC and VSMC (Svensson, L. et al., 2002) and bind oxLDL at the cell surface. On the other hand, CD36 is expressed in monocytes, macrophages, platelets, EC, adipocytes and VSMC and is one of the major receptors of oxLDL playing an important role in foam cell formation from monocytes-macrophages in atherosclerotic lesions (Matsumoto, K. et al., 2000). Finally, LOX-1 is a type-E scavenger receptor, expressed by EC, macrophages and VSMC that also recognizes oxLDL (Murase, T. et al., 2000).

LDL receptor family

The first member of the LDL receptor family, the LDL receptor (LDL-R), is involved in the cellular binding, internalization and degradation of plasma native LDL (Goldstein, J. L. et al., 2009). Other members of this

family are the Low Density Lipoprotein-related Proteins (LRP): LRP-1, LRP-1b, LRP-2, epidermal growth factor-like protein 7 (MEGF7), apolipoprotein E receptor 2 (apoER2), very low density lipoprotein receptor (VLDL-R), LRP-5 and LRP-6 (Badimon, L. et al., 2006).

LRP-1 is a member of the LDL receptor family, which contains several structurally homologous regions that are composed of modular structures. Like other members of the LDL receptor family, LRP-1 has: cysteine-rich complement-type repeats, epidermal growth factor (EGF) repeats, β-propeller (YWTD) domains, a transmembrane domain and a cytoplasmic domain (NPxY motifs). The cytoplasmic domain acts as a docking site for the endocytosis machinery and for cytoplasmic adaptor and scaffolding proteins involved in signaling events.

The extracellular complement-type, or ligand-binding repeats, are found in four clusters (I, II, III and IV) and are responsible for recognizing ligands (Herz, J. et al., 2001; Lillis, A. P. et al., 2008) **(Figure 8)**.

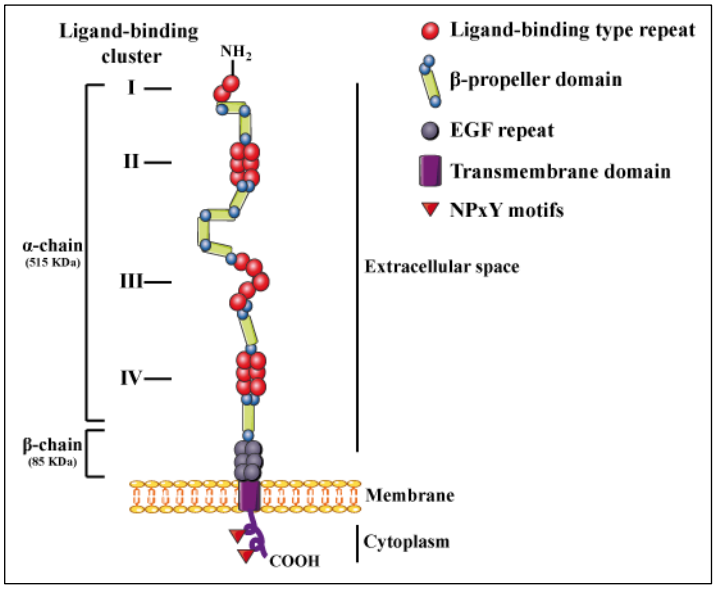

Figure 8. Structural domains of LRP-1 molecule. Adapted from (Herz, J. et al., 2001).

LRP-1 is a large endocytic receptor widely expressed in several tissues. Originally identified as an endocytic receptor for apoE-enriched lipoproteins and activated forms of α2-macroglobulin, LRP-1 is known to recognize at least of 30 distinct ligands including lipoproteins, proteases, proteinase inhibitor complexes, matrix proteins, bacterial toxins, viruses, intracellular proteins and growth factors (Lillis, A. P. et al., 2008) **(Table 3)**. Furthermore, LRP-1 is implicated in several signal transduction pathways involved in vascular wall integrity (Boucher, P. et al., 2003), cell migration (Okada, S. S. et al., 1996; Wijnberg, M. J. et al., 1997) and modulation of the integrity of the blood-brain barrier.

Besides, LRP-1 is also considered to play a role in the binding and internalization of agLDL in VSMC, since in the absence of LRP-1, VSMC are unable to accumulate cholesterol (Llorente-Cortes, V. et al., 2000; Llorente-Cortes, V. et al., 2002a). As LRP-1 is highly expressed in atherosclerotic plaques and subendothelial LDL retention and aggregation are key events in atherogenesis, the uptake of lipoprotein ligands through LRP-1 could have a critical role in VSMC-lipid deposition and VSMC-functions in atherosclerotic plaques. Indeed, VSMC derived from advanced atherosclerotic plaques showed high expression of LRP-1 and higher intracellular lipid deposition (Llorente-Cortes, V. et al., 2005). In addition, lipid-loaded cells are characterized by low adhesion and migration capacities and impairment in cytoskeleton dynamics (Padro, T. et al., 2008).

LRP-1 ligands	Main Functions
ApoE Lipoprotein lipase Hepatic lipase	Lipoprotein metabolism and transport
tPA	Fibrinolysis; signaling function in brain
uPA, uPA/uPAR	Cell migration, wound healing
Factor IX, VIIIa, VIIa	Blood coagulation
MMP-13, -9	Angiogenesis, metastasis
Pregnancy Zone Protein α2-Macroglobulin	Proteinase inhibitors
Complement C3	Infection
PAI-1	Regulates tPA/uPA activity
C1 inhibitor	Regulates C1r/C1s activity
Antithrombin II TFPI Heparin cofactor II	Regulate blood clotting
α1-antitrypsin	Regulates neutrophil elastase
Thrombospondin-1	TGF-β activation, matrix cell interaction
Thrombospondin-2	Collagen assembly, matrix cell interaction
Lactoferrin	Antibacterial
RAP	Chaperone
HSP-96	Chaperone
HIV-Tat protein	Transcriptional activation

Table 3. Main ligands binding the extracellular domain of LRP1. tPA, tissue-type plasminogen activator; uPA urokinase plasminogen activator; uPAR, urokinase plasminogen activator receptor, MMP, matrix metalloproteinases; PAI-1, plasminogen ativator inhibitor 1; TFPI, tissue factor pathway inhibitor; RAP, receptor associated protein; HSP-96, heat shock protein-96. Adapted from (Herz, J. et al., 2001).

3.3. High Density Lipoproteins

HDL are composed of lipids (phospholipids, cholesterol, and triglycerides) and apolipoproteins. The main structural apolipoproteins of HDL are apoA-I and apoA-II and other minor proteins found in smaller amounts include apoC proteins, apoE, apoA-IV, apoA-V, apoD, apoJ and apoL (Rader, D. J., 2005; Gordon, S. et al., 2010). ApoA-I is produced mainly by the liver, and released into the plasma. Circulating apoA-I interacts with serum phospholipids and forms nascent pre-β-HDL. Once

pre-β-HDL are generated, they trigger cholesterol efflux from macrophages and fibroblasts in the subendothelial space. Externalized cholesterol is then absorbed by pre-β-HDL and subsequently esterified by lecithin/cholesterol acyltransferase (LCAT). HDL particles are enriched with cholesteryl esters and become larger, resulting in HDL3 and HDL2. These cholesteryl esters are then delivered back to the liver via LDL-R, converted to bile salts, and eliminated through the gastrointestinal tract (Ohashi, R. et al., 2005).

Multiple epidemiological studies have provided robust evidence of an inverse correlation between HDL plasma levels and cardiovascular risk (Badimon, J. J. et al., 2010). The Framingham study confirmed that low HDL cholesterol concentration predicted future cardiovascular events, and was, along with the total cholesterol/HDL ratio, the only independent predictor of CVD (Wilson, P. W. et al., 1998). More recent studies have confirmed this relationship, and found that patients with HDL <35 mg/dL have an incidence of cardiovascular events eight times higher than individuals with HDL >65 mg/dL (Assmann, G. et al., 1996; Goldbourt, U. et al., 1997).

It is currently believed that most of the atheroprotective effects of HDL stem from their capacity to remove cholesterol from the vasculature and to deliver it to the liver for disposal in a process commonly referred to as reverse cholesterol transport. This prevents atherosclerosis progression and offers the possibility to effectively cause regression of established atherosclerotic lesions (Badimon, L. et al., 2012). However, recently, other features of HDL have been suggested to contribute to their overall anti-atherothrombotic effects, including anti-inflammatory, immunomodulatory, antioxidant, antithrombotic, and endothelial cell repair effects (Choi, B. G. et al., 2006; Ibanez, B. et al., 2007; Badimon, J. J. et al., 2010).

4. Statins in atherosclerosis

Coronary risk rises progressively with an increase in cholesterol levels, particularly when cholesterol levels rise above 200 mg/dl. There is substantial evidence that lowering total and LDL-C levels reduces the incidence of coronary heart disease.

Several pharmacological strategies have been used in order to reduce plasma cholesterol levels, including statins (Mills, E. J. et al., 2011), nicotinic acid (Ganji, S. H. et al., 2003), and fibrates (Tokuno, A. et al., 2007) among others. Statins, considered as effective and safe drugs, are the most commonly used in the clinic.

Statins interfere with the conversion of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) to the cholesterol precursor mevalonate by HMG-CoA reductase, an early and rate-limiting step in cholesterol biosynthesis **(Figure 9)**.

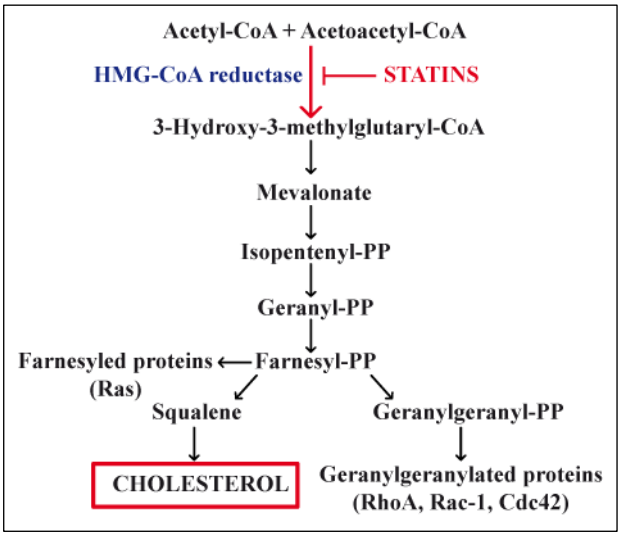

Figure 9. Statins and cholesterol biosynthesis.

Statins competitively inhibit HMG-CoA reductase by binding to the enzyme and sterically inhibiting substrate binding. However, the degree of inhibition exhibited by different statins may differ depending on the strength of their binding to the enzyme.

Besides inhibiting HMG-CoA reductase, statins are also characterized by their ability to produce large reductions in LDL cholesterol and non-HDL cholesterol (Ballantyne, C. M. et al., 2008; Mihaylova, B. et al., 2012).

In fact, reducing cholesterol synthesis with statin therapy causes a reduction in intracellular cholesterol concentrations and subsequent upregulation of hepatocyte LDL receptors, increasing uptake and degradation of LDL and VLDL particles by the cells. In addition, HMG-CoA reductase inhibition may lead to an increase in HDL cholesterol by producing a reduction of downstream farnesyl-PP production, inducing upregulation of peroxisome proliferator-activated receptor alpha (PPARα) and consequently increasing apoA-I (the major apolipoprotein in HDL). Other mechanism for increasing HDL cholesterol is a reduction cholesteryl esters' transfer from HDL to VLDL and LDL particles via inhibition of cholesteryl esters transfer protein (Lamon-Fava, S., 2013).

Despite that all statins share many features, they also exhibit differences in their pharmacologic attributes, such as relative lipophilicity, bioavailability and elimination half-time that may contribute to differences in the clinical utility and effectiveness in modifying lipid risk factors in coronary disease**.**

Pleiotropic effects of statins

Beside the well-known ability of statins to lower cholesterol, it is now clear that their clinical benefits are substantially higher than expected and several clinical trials have indicated that such effects are independent of

cholesterol reduction (Nordmann, A. J. et al., 2012). These cholesterol-independent actions have been named "pleiotropic effects" and include: improvement of endothelial function, stabilization of atherosclerotic plaque, anti-oxidant and anti-inflammatory effects, decreased platelet activation, inhibition of cardiac hypertrophy, reduction of cytokine-mediated VSMC proliferation, and among others (Davignon, J., 2004; Liao, J. K. et al., 2005; Mihos, C. G. et al., 2010) **(Figure 10)**.

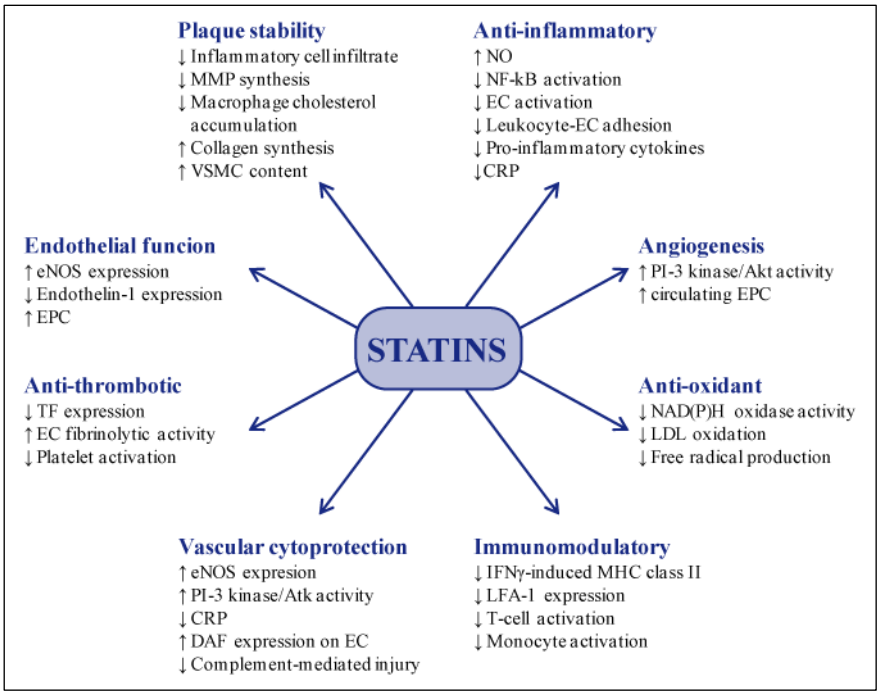

Figure 10. Pleiotropic effects of statins. eNOS, endothelial nitric oxide synthase; EC, endothelial cells; MMP, matrix metalloproteases; TF, tissue factor; NO, nitric oxide; NF-kB, nuclear factor kappa-light-chain-enhancer of activated B cells; CRP, C-reactive protein; IFN-γ, interferon gamma; MHC class II, major histocompatibility complex class II; LFA-1, lymphocyte function-associated antigen; DAF, decay-accelerating factor; PI-3 kinase, phosphoinositide 3-kinase; Akt, protein kinase B; EPC, endothelial progenitor cell.

As described previously, endothelial dysfunction is one of the earliest manifestations of atherosclerosis. An important characteristic of endothelial dysfunction is the impaired synthesis, release, and activity of

endothelial NO. Indeed, NO, a fundamental mediator of vascular homeostasis, has been shown to mediate vasodilatation and inhibition of several features of the atherogenic process including platelet aggregation, vascular smooth muscle proliferation, and endothelial-leukocyte interactions (Liao, J. K. et al., 2005). The molecular basis of the association between statins and endothelial function relates to the interaction with NO production pathways at the cellular level. Indeed, statins improve endothelial function by upregulating the expression and activity of eNOS as well as through their antioxidant effect. eNOS expression is upregulated via two different pathways. The first one is by activating protein kinase Akt, which is an important regulator of a number of cellular processes (Kureishi, Y. et al., 2000). The second pathway is the inhibition of geranylgeranylation of the small G-protein Rho (Laufs, U. et al., 1999).

Another mechanism through which statins may affect the endothelium is through their antioxidant effects. Statins attenuate angiotensin II (Ang II) induced free radical production in vascular smooth muscle cells by inhibiting Rac1-mediated NAD(P)H oxidase activity and downregulating angiotensin AT1-receptor expression (Wassmann, S. et al., 2001).

In addition, inhibition of the formation of mevalonate, and thus of isoprenoids, leads to the inhibition of critical proteins in the inflammatory cascade (in particular Ras, Rho and Rab), which act via NF-kB; thus, statins also exhibit a powerful anti-inflammatory effect (Antonopoulos, A. S. et al., 2012).

Moreover, statins have beneficial effects on thrombosis. Indeed, it has been observed that statins produce a significant reduction of platelet factors for thrombin formation, such as CD40 ligand-mediators in hypercholesterolemic patients treated with statins (Sanguigni, V. et al., 2005). A number of studies have demonstrated that lipid lowering by

statins may contribute to plaque stability by reducing plaque size or by modifying the physicochemical properties of the lipid core (Koh, K. K., 2000; Fukumoto, Y. et al., 2001). However, changes in plaque size by lipid lowering tend to occur over extended time and are quite minimal as assessed by angiography. Rather, the clinical benefits from lipid lowering are probably due to a decrease in macrophage accumulation in atherosclerotic lesions and by inhibition of MMP production by activated macrophages (Aikawa, M. et al., 2001). Thus, the plaque stabilizing properties of statins, therefore, are mediated through a combined reduction in lipids, macrophages, and MMPs (Crisby, M. et al., 2001).

5. Vascular smooth muscle cells: vascular remodeling and atherosclerosis

VSMC are the only cellular type present in the media layer and are highly specialized cells whose principal function is the regulation of blood vessel tone.

VSMC exist in a diverse range of phenotypes (Gittenberger-de Groot, A. C. et al., 1999). Indeed, during normal vascular development, dedifferentiated VSMC (synthetic VSMC) exhibit a high rate of proliferation, migration, and production of ECM components such as collagen, elastin, and proteoglycans that form the vessel wall. Whereas, in mature blood vessels, VSMC exhibit a contractile or differentiated phenotype characterized by a reduction of ECM protein secretion, an increase in intracellular myofilament formation and the expression of contractile markers specific to smooth muscle, such as smooth muscle myosin heavy chain, smooth muscle α-actin, h-caldesmon, and calponin (Owens, G. K., 1995). This transition, from synthetic to a contractile state, is required for VSMC to perform contraction and dilatation of the blood vessel wall (Rudijanto, A., 2007).

The high plasticity of VSMC allows them to modulate their phenotype and reprogramme their expression pattern in response to environmental stimuli (Lacolley, P. et al., 2012). Therefore, under pathological conditions, such as when vascular injury occurs, differentiated VSMC can undergo a phenotypic switch from their contractile state to a synthetic, proliferative, and migratory state, promoting the migration of VSMC from the media to the intima layer (Stegemann, J. P. et al., 2005). This change in VSMC phenotype also occurs after exposure to multiple growth factors and cytokines released by different cell types, including EC, platelets and inflammatory cells.

Regarding the role of VSMC in atherosclerosis, today there are still important gaps in our knowledge of the underlying mechanisms that contribute to the development and progression of atherosclerotic lesions and end-stage clinical events, including plaque instability and rupture. On early stages, VSMC may contribute to the development of the atherosclerotic plaque through the production of pro-inflammatory mediators and the synthesis of ECM molecules that increase lipoprotein retention.

VSMC represent 90-95% of the cells present in the initial lesions and are also important to maintain the stability of the plaque through the formation of a firm fibrous cap, reviewed in (Badimon, L. et al., 2009). Indeed, in lipid-loaded lesions in which the fibrous cap is thin and weak, there is evidence of loss of VSMC, decreasing its proportion to 50% and leading to a plaque prone to rupture (Dzau, V. J. et al., 2002) **(Figure 11)**. In fact, most common acute coronary syndromes, (e.g. unstable angina, myocardial infarction, sudden death, and cerebrovascular syndromes) are associated with vulnerable plaques. These data reflect the importance of identifying and elucidating the mechanisms that lead to the loss of VSMC in advanced lesions, as this might help to define new therapeutic strategies.

Recent studies have shown that atherogenic concentrations of LDL significantly reduce the migratory capacity of human VSMC and that LDL particles affect the expression and phenotypic profile of different proteins associated with the cytoskeleton of VSMC (Padro, T. et al., 2008). In addition, the internalization of LDL by VSMC has been shown to induce a decrease in matrix metalloproteinase-9 (MMP-9) activity and also potentiate the inhibitory effect of LDL on VSMC migration thereby contributing to the instability and vulnerability of plaques in advanced stages (Otero-Vinas, M. et al., 2007). However, up-to-date the overall

mechanisms that lead to loss of VSMC in advanced lesions remain unclear.

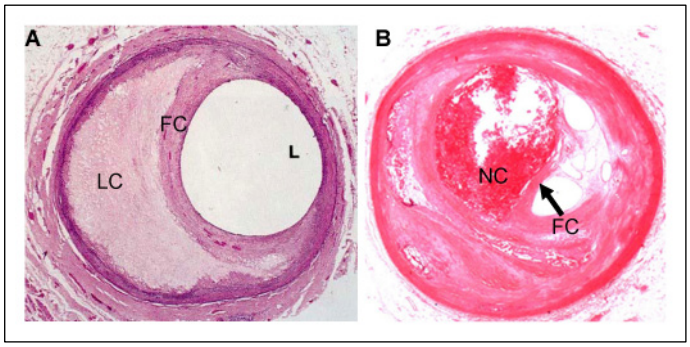

Figure 11. Atherosclerotic lesions in human coronary arteries. (A) Histology of a stable atherosclerotic plaque with a firm fibrous cap (FC) and a large lipid-rich core (LC); L, lumen. Adapted from (Sambola, A. et al., 2003). **(B)** Histology of a vulnerable atherosclerotic plaque characterized by a thin fibrous cap (FC) and a large lipid necrotic core (NC). Adapted from (Badimon, L. et al., 2009)**.**

5.1. VSMC migration

Cell migration is a complex process that requires tight control of several mechanisms, including dynamic reorganization of the actin cytoskeleton and adhesion to the ECM in response to promigratory stimuli. After initial injury, different cell types, including EC, platelets, and inflammatory cells release mitogenic and chemotactic factors that stimulate VSMC migration. The promigratory stimuli shown in **Table 4** then activate signal transduction cascades that trigger the remodeling of the cytoskeleton, change the adhesiveness of the cell to the ECM and activate motor proteins (Gerthoffer, W. T., 2007; Rudijanto, A., 2007).

Cytokines	Growth factors	Others
IL-1	bFGF	Thrombin
IL-4	HB-EGF	uPA
IL-6	IGF-1	Angiotensin II
TNF-α	PDGF	Endothelin-1
IFN-γ	VEGF	

Table 4. VSMC promigratory stimuli present in atherosclerotic lesion. bFGF, basic fibroblast growth factor; HB-EGF, heparin-binding epidermal growth factor; IGF, insulin-like growth factor; IL-1,4,6, interleukins; PDGF, platelet-derived growth factor; TNF-α, tissue necrotic factor-alpha; IFN-γ, interferon-gamma; uPA, urokinase plasminogen activator; VEGF, vascular endothelial growth factor.

Cell migration consists of different steps, mainly: cell polarization, membrane protrusion, focal adhesions' formation and stabilization, contraction, focal adhesion disassembly and rear retraction (Ridley, A. J. et al., 2003) **(Figure 12)**.

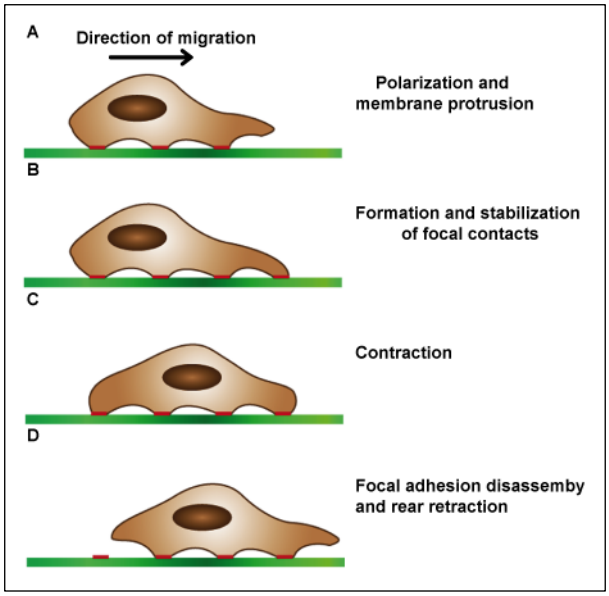

Figure 12. Steps of VSMC migration.

Cell polarization

Cells initiate migration by polarizing and extending protrusions of the cell membrane towards the cue. Cell polarity in response to extracellular stimuli appears to be mediated by a set of interlinked positive feedback loops involving Rho family GTPases, phosphoinositide 3-kinase (PI3K), integrins, microtubules and vesicular transport. In particular, Cdc42 (a member of the Rho family of GTPases) is considered as the main regulator of this process. Indeed, Cdc42 is activated at the leading edge of migrating cells by its downstream target, the p21-activated kinase-1 (PAK1) through a positive feedback loop and by integrins (Li, Z. et al., 2003). Activated Cdc42 then influences cell polarity by localizing the microtubule-organizing center (MTOC) and the Golgi apparatus in front of the nucleus and oriented towards the leading edge (Etienne-Manneville, S. et al., 2002).

Membrane protrusion

Once polarized, cells extend protrusions comprised of large and broad lamellipodia, spike-like filopodia or both. These protrusions are driven by the polymerization of actin filaments in the direction of migration (Higgs, H. N. et al., 1999; Pollard, T. D. et al., 2003) and are stabilized by adhesion to the ECM or adjacent cells via transmembrane receptors linked to the actin cytoskeleton.

Focal adhesion

To anchor the protrusions, the cell's front interacts with the ECM forming nascent adhesions (or focal complexes) that are connected to the intracellular lamellipodial actin network. Transient focal contacts between the cell membrane and the ECM are necessary for migration to proceed

beyond the initial stages and provide the traction for propulsion of the cell over or through the ECM.

Nascent adhesions can either disassemble or, in response to actomyosin force, mature into larger structures called focal adhesions (FA) that assemble contractile actomyosin filaments (also called stress fibers) (Ridley, A. J. et al., 2003). Adhesion disassembly is observed both at the leading edge, where it accompanies the formation of new protrusions, and at the cell rear, where it promotes traction.

Turnover of FA in protrusions is regulated by the focal adhesion kinase (FAK) and Src tyrosine kinases (Webb, D. J. et al., 2004). Indeed, its activation accompanies the formation of an adhesion signaling complex that in turn mediates the localized activation of Rac and extracellular signal-regulated kinase (ERK) contributing to the turnover of adhesion at the leading edge (Ridley, A. J. et al., 2003). On the other hand, adhesive release at the cell rear can involve weakening or severing of the integrin-ECM or integrin-cytoskeletal linkages and is driven, at least in part, through contractile forces.

More than 150 components have been identified as adhesion-associated, including integral membrane proteins such as integrins and syndecans, proteins linked to actin (e.g. vinculin, talin, α-actinin), signaling and adaptor proteins such as Src tyrosine kinases, FAK, paxillin, and integrin-linked kinase (ILK) (Turner, C. E., 2000; Zamir, E. et al., 2001; Legate, K. R. et al., 2006; Romer, L. H. et al., 2006; Zaidel-Bar, R. et al., 2010). Focal contacts also contain proteins that transiently associate with FA and regulate migration, such as PAK, Rho family proteins (Ridley, A. J., 1994), calcium-dependent protease calpain 2 and tyrosine phosphatase SHP-2 (Beckerle, M. C. et al., 1987).

Contraction of actin stress fibers

The last major phase of cell migration is the contraction of the cell body. Actin stress fibers are connected at both ends to FA and transmit the contractile force through activation of myosin II, which moves antiparallel actin filaments past each other. The mechanism regulating myosin activation is detailed below.

5.2. Cell cytoskeleton and VSMC migration

The cytoskeleton is an active network of cytosolic filaments composed by three types of protein fibers: microtubules, intermediate filaments and microfilaments (also known as actin filaments) **(Figure 13)**. The cytoskeleton ensures cell rigidity and anchors subcellular organelles in place and its constant reorganization allows internal movement including chromosome displacement and membrane deformation, thus enabling endocytosis and cell migration, reviewed in (Blain, E. J., 2009; Wettstein, G. et al., 2012).

- Microtubules (~25 nm diameter) are responsible for structural strength and cell shape. They allow organelles to move within cells and they act like rails on which kinesin and dynein, motor proteins, can pull organelles (Bryantseva, S. A. et al., 2012).
- Intermediate filaments (~10 nm diameter) are ubiquitous cytoskeletal elements and are among the most insoluble and most resilient solid structures of eukaryotic cells (Goldman, R. D. et al., 2008). The key function of intermediate filaments is to support the cell membrane, serving as a structural scaffold to maintain cell shape. They are fixed to the membrane through transmembrane proteins such as cadherins, a protein family involved in the formation of cell–cell tight junctions.

- Actin filaments (~7 nm diameter) play a role in many different cellular processes including maintenance of cell shape and mechanical strength, membrane trafficking, cell division, and migration. The main characteristics of these filaments are detailed below.

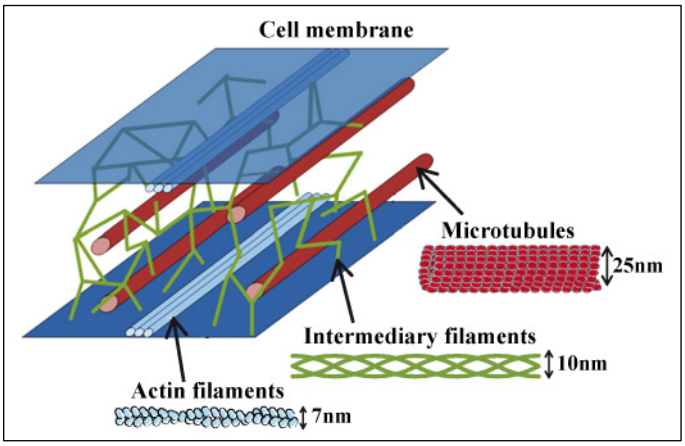

Figure 13. Schematic representation of the intracellular organization of the three main components of the cytoskeleton. Microtubules with a diameter of ~25nm; intermediary filaments with a diameter of ~10nm and actin filaments with a diameter of ~7nm. Adapted from (Wettstein, G. et al., 2012).

Actin cytoskeleton

The actin cytoskeleton is a dynamic structure that plays a fundamental role in different physical processes in eukaryotic cells. Actin-dependent cellular mechanisms are typically associated with membrane dynamics and the coordinated polymerization of actin filaments provides the force for these processes. Probably, the most thoroughly characterized of such mechanisms is cell migration.

During cell migration, precisely coordinated polymerization of actin filaments induces the formation of membrane protrusions and the consequent advancement of the leading edge of the cell. Moreover, actin

filaments together with myosin filaments form contractile structures in cells.

Actin is a globular protein with a molecular weight of 42KDa that contains a nucleotide (either ATP or ADP) associated with the cleft located between the two lobes of the protein. Under physiological conditions, actin monomers (G-actin) assemble into polarized helical filaments (F-actin) that have two structurally and biochemically distinct ends called “barbed end” and “pointed end”.

Under steady-state conditions, the net addition of subunits to actin filaments occurs at the barbed end. Irreversible hydrolysis of bound ATP destabilizes the filament and results in net dissociation of actin subunits at the pointed end. This ATP-dependent polymerization of actin filaments at their barbed ends and depolymerization at the pointed end results in “filament treadmilling” which plays a central role in the actin-based migration process in cells **(Figure 14)**.

The structure and dynamics of the actin cytoskeleton in cells are regulated by a vast number of actin-binding proteins regulated by Ca2+, phosphatidylinositol 4,5-biphosphate (PIP2) and Rho family proteins (particularly Rac, Cdc42 and RhoG). These include proteins that promote nucleation of new actin filaments (including Arp2/3 complex, WASP/WAVE families and formins), proteins that enhance actin filament severing and depolymerization (including ADF/cofilins and gelsolin) (Ono, S., 2007; Chesarone, M. A. et al., 2009) and proteins that regulate actin filament polymerization either by interacting with actin monomers (including profilin, twinfilin and β-thymosins) or with filament barbed ends such as capping proteins that play crucial roles in cell actin dynamics (Paavilainen, V. O. et al., 2004; Cooper, J. A. et al., 2008; Le Clainche, C. et al., 2008; Saarikangas, J. et al., 2010) **(Figure 14)**.

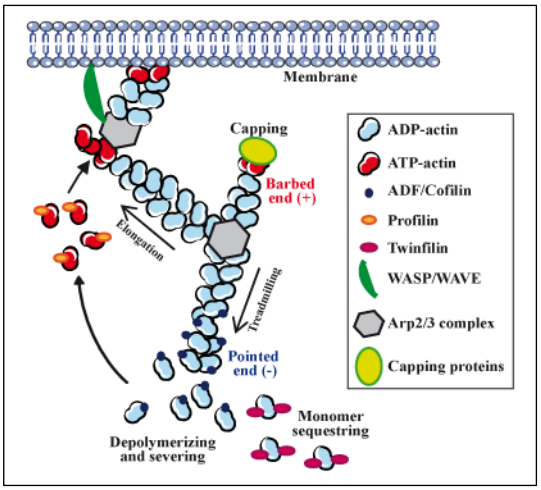

Figure 14. Regulation of actin dynamics in cell motility. Adapted from (Saarikangas, J. et al., 2010).

As previously mentioned, the actin-depolymerizing factor (ADF, also called cofilin) and capping proteins are key regulatory factors for actin treadmilling.

ADF/cofilin proteins are small (molecular mass 15–20 KDa) and abundant proteins that interact with both monomeric and filamentous actin, preferring ADP-actin. ADF/cofilins promote rapid actin dynamics by depolymerizing and severing actin filaments. In addition to promoting actin filament disassembly in cells, ADF/cofilins may also contribute to stimulus-responsive actin filament assembly by creating new polymerization-competent filament barbed ends through their severing activity (Saarikangas, J. et al., 2010). The activity of ADF/cofilin is regulated by phosphorylation, pH, PIP2 and interactions with other proteins (Saarikangas, J. et al., 2010).

On the other hand, **capping proteins** interact with barbed ends of actin filaments with high affinity. By blocking the majority of the barbed ends, these proteins increase the concentration of G-actin. Capping proteins are

required for efficient motility of many cells, indeed by blocking a large fraction of the barbed ends, capping proteins funnel the flux of pointed-end depolymerization inducing a faster growth of non-capped filaments (Pantaloni, D. et al., 2001) and promoting the formation of lamellipodium protrusions, an important step in the migration process (Le Clainche, C. et al., 2008).

The activities of actin-binding proteins are controlled through various signaling pathways that ensure proper spatial and temporal regulation of actin dynamics in cells. The most thoroughly characterized regulators of actin-binding proteins are members of the Rho-family small GTPases such as RhoA that induces the formation of contractile stress fibers, Rac1 that drives the formation of lamellipodial actin filament networks at the leading edge of migrating cells, and Cdc42 that induces the formation of thin actin-rich filopodial protrusions at the cell periphery (Spiering, D. et al., 2011). The role and regulation of Rho-family small GTPases is detailed below.

In addition to small GTPases, membrane phosphoinositides also play a key role in regulating the dynamics of the actin cytoskeleton. Phosphoinositides regulate the activities of Rho GTPases, but they can also directly associate with actin-binding proteins to regulate their interactions with actin (Di Paolo, G. et al., 2006).

Contraction of actin stress fibers is mediated by myosin II, which moves antiparallel actin filaments and thereby provides the force to rearrange the actin cytoskeleton (Aratyn-Schaus, Y. et al., 2011). Myosin II is comprised of two heavy chains, two regulatory light chains (RLCs) and two essential light chains and has three isoforms (myosin IIA, myosin IIB, and myosin IIC), which are specified by the different heavy chains that they contain. Myosin IIA and IIB are present in most cells, whereas myosin IIC is not widely expressed (Vicente-Manzanares, M. et al.,

2009). Myosin II activity (ATP hydrolysis and actin filament formation) is regulated by the reversible phosphorylation of Thr18 and Ser19 of the regulatory light chain of the myosin II molecule driving the actomyosin assembly. This phosphorylation is controlled by the protein myosin light chain kinase (MLCK) and by the protein myosin light chain phosphatase (MLCP), that are in turn regulated by Rho GTPases (Hirano, K. et al., 2004; Watanabe, T. et al., 2007) **(Figure 15)**.

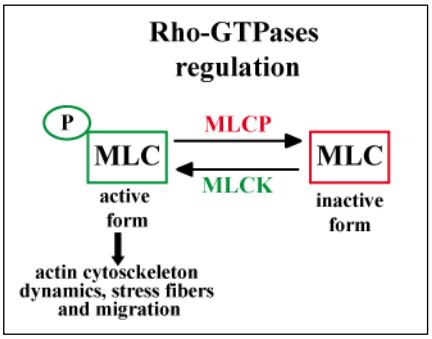

Figure 15. Regulation of myosin regulatory light chain activity. MLC-P, phosphorylated myosin light chain; MLC, non-phosphorylated myosin light chain; MLCP, myosin light chain phosphatase; MLCK, myosin light chain kinase.

5.3. Rho family GTPases and actin cytoskeleton

Rho family GTPases are members of the Ras superfamily of monomeric 20-30 KDa GTP-binding proteins and are identified as the master regulators of the actin cytoskeleton. However, Rho GTPases also control a remarkable diversity of cell functions implicated in the pathogenesis of atherosclerosis including cell contraction, migration, proliferation, and apoptosis (Jaffe, A. B. et al., 2005).

At least twenty members of the Rho family of GTPases have been identified in humans, being RhoA/B, Rac1/2 and Cdc42 the most extensively characterized (Guilluy, C. et al., 2011).

Each of these GTPases acts as a molecular switch, cycling between an active state (GTP-bound, induced by guanine exchange factor, GEF) and an inactive state (GDP-bound, induced by GTPase activating protein, GAP). In their GTP-bound form, Rho GTPases are able to interact with effectors or target molecules to initiate downstream responses, while an intrinsic GTPase activity returns the proteins to their GDP-bound state to complete the cycle and terminate signal transduction (Bar-Sagi, D. et al., 2000).

Rho GTPases exhibit distinct functions in cell migration. Indeed, several studies performed in migrating cells have linked Cdc42 to cell polarization, Rac1 to the protrusion of the leading edge and RhoA to focal adhesion complex formation and organization and cell contraction (Burridge, K. et al., 2004; Saarikangas, J. et al., 2010; Yan, Xiumin et al., 2010). However, recently, active RhoA has also been reported to accumulate at the front of migrating cells, particularly in lamellipodia and filopodial, where it is involved in the regulation of actin-myosin mediated protrusion and retraction events (Heasman, S. J. et al., 2010; Wu, C. et al., 2011).

RhoA in its active form (RhoA–GTP) binds the Rho-binding domain (RBD) of Rho kinases 1 and 2 (ROCK1 and ROCK2 respectively) disrupting the interaction between the catalytic N-terminal and the inhibitory C-terminal regions of the enzyme and stimulating the phosphotransferase activity of ROCK (Loirand, G. et al., 2006). ROCK is characterized by the ability to mediate actin cytoskeleton dynamics by increasing phosphorylation of MLC (Totsukawa, G. et al., 2000). As previously reported, phosphorylation and dephosphorylation of MLC are

key events in the regulation of VSMC migration and are mediated by MLCK and MLCP, respectively. ROCKs enhance MLC phosphorylation through the inhibition of the myosin-binding subunit of MLCP (MBS) or by direct phosphorylation of MLC (Noma, K. et al., 2006).

Additionally, MLC phosphorylation can also be regulated by the activation of MLCK through Ca2+-calmodulin or by MLCK inactivation mediated by PAK, a downstream effector of Cdc42 and Rac1. Indeed, upon GTPase binding, PAK undergoes a conformational change that leads to its activation and autophosphorylation at several sites (Parrini, M. C. et al., 2002). Phosphorylated PAK (PAK-P) inhibits MLCK activity and consequently inhibits MLC phosphorylation (Sanders, L. C. et al., 1999) **(Figure 16)**.

Growing evidence from animal and clinical studies highlights the importance of Rho GTPases in the pathogenesis of cardiovascular disease. Indeed, many of the pleiotropic effects of statins may be due to their ability to block isoprenoid synthesis. In particular, inhibition of geranylgeranyl pyrophosphate (GGPP) synthesis leads to inhibition of Rho and its downstream target, ROCK (Zhou, Q. et al., 2009) **(Figure 9 in chapter 4)**. In addition, increased ROCK activity is associated with endothelial dysfunction, whereas, inhibition of ROCK by statins or selective ROCK inhibitors leads to eNOS up-regulation, decreased vascular inflammation, and reduced atherosclerotic plaque formation (Zhou, Q. et al., 2009).

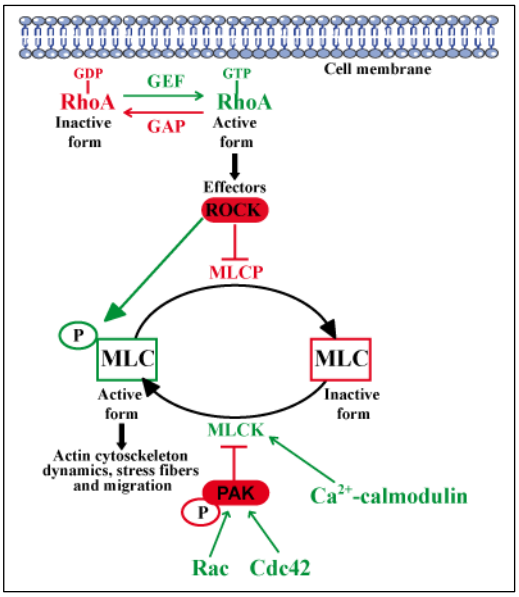

Figure 16. Rho GTPases activation/deactivation cycle and regulation of myosin light chain phosphorylation. GEF, guanine exchange factor; GAP, GTPase activating protein; ROCK, Rho kinase; MLC, myosin light chain, MLC-P, phosphorylated MLC; MLCP myosin phosphatase; MLCK, MLC kinase; PAK, p21-activated kinase.

5.4. Small heat shock proteins: HSP27

Heat shock proteins (HSPs) are a highly conserved group of proteins that are constitutively expressed and function as molecular chaperones, aiding in protein folding and preventing the accumulation of misfolded proteins. Several studies have related HSPs with the pathogenesis of atherosclerosis (Kilic, A. et al., 2012; Xu, Q. et al., 2012). Indeed, HSPs expression is modulated by different stimuli involved in all steps of atherogenesis including oxidative stress, proteolytic aggression, or inflammation (Madrigal-Matute, J. et al., 2011). However, recent studies, have also related HSPs with the regulation of processes that promote VSMC migration such as rearrangement of actin fibers and the stabilization of FA

(Murakami, S. et al., 2001; Grifoni, S. C. et al., 2008; Chen, H. F. et al., 2009).

HSPs comprise several classes of functionally related families of heat-responsive cell stress proteins expressed in response to stressful environmental conditions that include heat, free radicals, toxins and ischemia (Ferns, G. et al., 2006). HSPs are commonly divided into seven families based on their size and molecular structure: HSP10 (10KDa), small HSPs (15–30 kDa), HSP40 (40KDa), HSP60 (60KDa), HSP70 (70KDa), HSP90 (90KDa), HSP100 (100KDa) (Ghayour-Mobarhan, M. et al., 2012).

The small heat shock proteins (sHSPs) family contains ten different members including HSP27, also called HSPB1, with a molecular weight of approximately 27 KDa.

Emerging data has implicated HSP27 in the pathogenesis of atherosclerosis. Indeed, a study of human atherosclerotic plaques revealed an increase in the expression of HSP27 in normal-appearing vessels adjacent to the plaque, with decreased levels in plaque itself (Park, H. K. et al., 2006). Another study found that HSP27 was significantly decreased in atherosclerotic plaques and that circulating levels of soluble HSP27 were also significantly decreased in patients with carotid stenosis compared with healthy controls (Martin-Ventura, J. L. et al., 2004). Moreover, proteomic analyses of stable versus unstable human atherosclerotic plaques found reduced levels of HSP27 in unstable lesions attributing HSP27 a role in plaque stability (Lepedda, A. J. et al., 2009).

Structurally, HSP27 contains a C-terminal domain called α-crystallin that is conserved among all members of the small HSPs family and a N-terminal motif WDPF (Acunzo, J. et al., 2012) **(Figure 17)**.

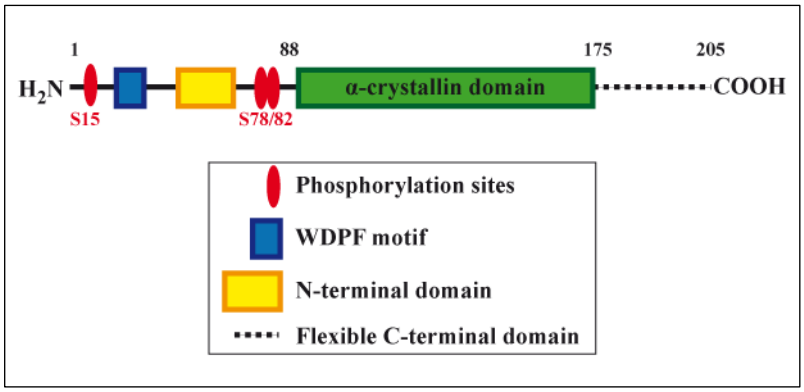

Figure 17. Structural domains of HSP27. This schematic representation indicates the N-terminal domain, the flexible C-terminal domain, the WDPF motif and the three serines (S) susceptible of phosphorylation. Adapted from (Acunzo, J. et al., 2012).

In addition to its chaperoning functions, HSP27 also appears to be involved in a diverse range of cellular functions. HSP27 promotes cell survival through effects on the apoptotic pathway and plays an important role in cytoskeleton dynamics, cell differentiation and embryogenesis (Ghayour-Mobarhan, M. et al., 2012).

sHSPs properties are often modulated by post-translational modifications such as phosphorylation, deamidation, oxidation, glycation and isomerization (Garrido, C. et al., 2012). The phosphorylation status represents an important factor for its chaperone activity and is produced in response to heat shock, differentiating agents, mitogens, inflammatory cytokines such as TNF-α, and IL-1β and in response to hydrogen peroxide and other oxidants (Vertii, A. et al., 2006). HSP27 is phosphorylated in serine 15 (S15), S78 and S82 by the MAPKAP kinase 2/3 via the activation of the p38 MAPK pathway (Guay, J. et al., 1997) **(Figure 17)**. Following phosphorylation, HSP27 reorganizes itself into smaller oligomers, often dimers and tetramers and can interact with other proteins (Vidyasagar, A. et al., 2012).

In non-stress conditions, the cytoskeleton and principally actin fibers are involved in several cellular processes such as motility and pinocytosis. When stress appears, its first observable effects are the disruption of the cytoskeleton and the disaggregation of actin fibers. The stabilization of actin fibers and major cytoskeleton fibers becomes critical for cell survival (Garrido, C. et al., 2012). It has been demonstrated that HSP27 is able to cap the barbed end of actin filaments, thus preventing the fixation of new actin monomers, thus being considered as an inhibitor of actin polymerization. This inhibitory activity of HSP27 is, however, only effective when HSP27 is in its non-phosphorylated monomer state (Wettstein, G. et al., 2012). Indeed, small phosphorylated dimers/tetramers bind F-actin preventing filament degeneration and promote polymerization (Guay, J. et al., 1997); on the contrary, non-phosphorylated HSP27 acts as an actin capping protein limiting filaments growth (Ferns, G. et al., 2006) **(Figure 18)**.

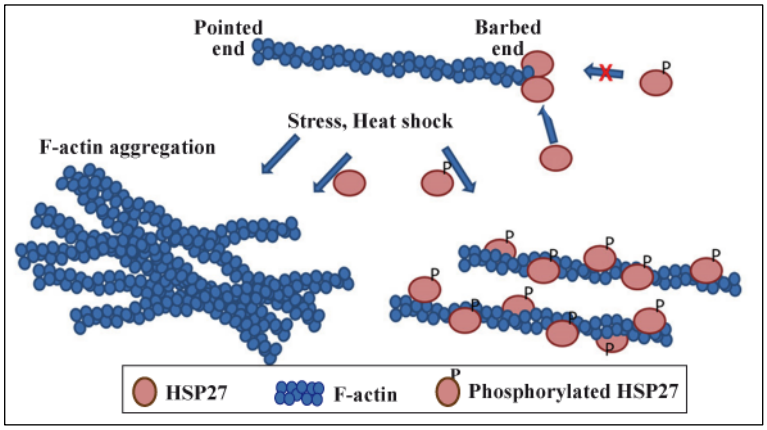

Figure 18. Schematic representation of the interaction between HSP27 and actin cytoskeleton. HSP27 in its monomeric form (non-phosphorylated) can cap actin fibers and inhibits polymerization. In its phosphorylated form HSP27 can prevent F-actin aggregation. Neither HSP27 phosphorylated monomers nor HSP27 oligomers are able to act as capping proteins. Adapted from (Wettstein, G. et al., 2012).

5.5. Regulation of VSMC migration during vascular remodeling

As already mentioned, VSMC migration is considered as one of the main processes involved in the vascular remodeling that takes place during the development and complication of atherosclerotic plaques. Vascular remodeling occurs in all stages of the atherosclerotic process and involves the modification of the ECM composition and structure as well as an imbalance between synthesis and degradation of ECM components.

Up-to-date, many factors present in atherosclerotic lesions have been suggested to alter VSMC behavior. Inflammatory cytokines and growth factors have been shown to stimulate migration of VSMC to lesion sites. In contrast, other factors such as LDL particles have been proven to exert inhibitory effects, alter the expression and the phenotypic profile of different cytoskeleton-related proteins involved in VSMC migration (Padro, T. et al., 2008).

Among promigratory stimuli, proteolytic enzymes such as MMPs (i.e. MMP-2 and MMP-9) (Johnson, C. et al., 2004), and serine proteases including the urokinase plasminogen activator (uPA), participate directly in the ECM-degradative events necessary to drive VSMC migration and thus contribute to atherosclerotic lesion progression. Therefore, the deregulation of these enzymes or their inhibitors [tissue inhibitor of metalloproteinases (TIMPs) and plasminogen activator inhibitor-1 (PAI-1)] may play a key role in atherosclerotic lesion development.

6. The urokinase-type plasminogen activator system: vascular remodeling and atherosclerosis

The plasminogen activator system is an important participant in diverse physiologic processes including fibrinolysis, tissue growth and remodeling, wound healing, angiogenesis, and embryogenesis (Nicholl, S. M. et al., 2006). However it also plays a key role in pathologic processes such as cancer, chronic inflammation, and blood vessel diseases.

The urokinase-type plasminogen activator (uPA) is an important component of the extracellular protease system since it specifically converts the zymogen plasminogen into plasmin, a serine protease with wide substrate specificity. Upon the generation of intravascular thrombi mainly constituted of polymeric fibrin, plasminogen activators are triggered to recover the blood flow, and plasmin directly degrades fibrin leading to thrombus dissolving.

On the cell surface, plasmin activates a number of MMPs that degrade ECM proteins and components of the basal membrane, such as collagen, fibronectin, and laminin (Lijnen, H. R., 2001; Alfano, D. et al., 2005).

Thus, the combined effects of plasminogen activators, plasmin and MMPs promote cell movement due to the destruction of cell-cell contacts and of the ECM and also due to the activation or release of latent or matrix-bound growth factors possessing chemotactic properties (Deryugina, E. I. et al., 2012).

On the cell surface, uPA binds with high affinity its specific receptor protein, uPA receptor (uPAR). This binding promotes local proteolysis in the direction of cell migration. However, the uPA and uPAR working as a complex or independently of each other can also activate intracellular signaling that regulates different cell functions, including cell adhesion, migration and proliferation (Alfano, D. et al., 2005; Fuhrman, B., 2012).

Altogether these characteristics allow considering the system of plasminogen activators as a separate group of regulators of cell movement and communication which provide proteolytic and signaling functions **(Figure 19)**.

Several evidences relate the uPA system with atherosclerosis progression, plaque instability, restenosis, and aneurysm formation (Parfyonova, Y. V. et al., 2002; Nicholl, S. M. et al., 2006). Indeed, observational studies have shown that uPA and uPAR are present in human atherosclerotic lesions (Padro, T. et al., 1995; Kienast, J. et al., 1998; Steins, M. B. et al., 2004).

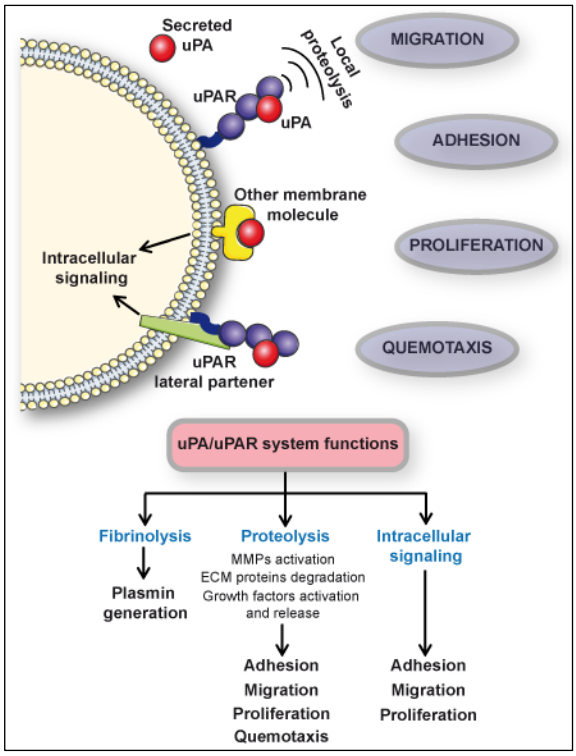

Figure 19. uPA/uPAR system functions. uPA, urokinase plasminogen activator; uPAR, uPA urokinase plasminogen activator receptor; MMPs, matrix metalloproteases; ECM, extracellular matrix.

6.1. The uPA/uPAR system in the pathogenesis of atherosclerosis

uPA is a crucial protein for neointimal growth and vascular remodeling. The exact role of uPA dependently or independently of the uPAR in the development of the atherosclerotic plaque is not fully clarified yet, but it is now clear that all cells present in the atherosclerotic arterial wall (EC, VSMC and macrophages) can express and secrete uPA (Lupu, F. et al., 1995; Padro, T. et al., 1995; Kienast, J. et al., 1998) and can express surface uPAR (Shen, G. X., 1998). A number of recent studies in humans and in animal models have suggested that uPA plays a role in the initiation and development of atherosclerosis, but only a few studies were directed to elucidate its possible role in the pathogenesis of atherosclerosis.

Evidences appear to support the notion that uPA over-expression contributes to progression and complications of atherosclerosis (Falkenberg, M. et al., 2002). Gyongyosi et al. have demonstrated that plasma levels of uPA are elevated in patients with unstable angina suggesting that the uPA system is associated with signs of plaque instability (Gyongyosi, M. et al., 2004). On the contrary, Dellas et al. using a double knockout apoE$^{-/-}$uPA$^{-/-}$ mice showed that uPA is essential for maintaining cellularity and collagen content, thus stabilizing the plaques (Dellas, C. et al., 2007). In addition, determination of uPAR and uPAR-bound uPA levels in segments of human coronary and aortic vessels with different degrees of atherosclerotic lesions revealed that the uPAR content increased progressively with the severity of atherosclerosis (Steins, M. B. et al., 2004) suggesting that overexpression of uPAR in advanced atherosclerotic lesions contributes to lesion development. Therefore, up-to-date there is no consensus on the contribution of membrane-bound uPA and uPAR to the development of atherosclerosis.

uPA/uPAR and VSMC in vascular remodeling

Neointimal formation and inward vessel remodeling, known also as negative vascular remodeling, are a fundamental mechanisms responsible for lumen narrowing in atherosclerosis. On the contrary, outward, or positive vascular remodeling, is a compensatory change in shape and size of the vessel wall area to accommodate the plaque and maintain constant flow and lumen size (Korshunov, V. A. et al., 2007).

uPA expression has been shown to increase in the media and developing neointimal after balloon catheter injury, throughout increasing VSMC migration, proliferation and adhesion (Clowes, A. W. et al., 1990). In addition, uPA was found to stimulate reactive oxygen species generation in VSMC via regulation and activation of cellular oxidases, and this effect is essential for uPA-mediated VSMC proliferation (Menshikov, M. et al., 2006). Furthermore, uPA was recently shown to promote inward arterial remodeling by regulating oxidative stress and inflammation after arterial injury (Plekhanova, O. et al., 2009). Interaction of VSMC with monocytes recruited to the arterial wall at injured sites resulting in VSMC migration is central to the development of vascular intimal thickening. Indeed, uPA expressed by monocytes has been shown to be a potent chemotactic factor for VSMC in a co-culture model, suggesting that monocyte uPA acts as an activator of VSMC migration, contributing and accelerating vascular remodeling (Kusch, A. et al., 2002)**.**

6.2. Urokinase Plasminogen Activator

uPA is a serine protease synthesized by vascular resident and infiltrating cells as EC, VSMC, fibroblasts, monocytes and macrophages (Parfyonova, Y. V. et al., 2002).

The uPA is a 53 kDa multidomain glycoprotein of 411 residues, synthesized and secreted as a single-chain zymogen (pro-uPA or sc-uPA). Once secreted, sc-uPA is exposed to the action of proteases which may generate enzymatically active or inactive forms of uPA (Ulisse, S. et al., 2009).

The uPA molecule consists of 3 structural domains: the N-terminal domain homologous to the epidermal growth factor-like domain (amino acids 9-45), the kringle domain (amino acids 46-143) and the C-terminal proteolytic domain (amino acids 144-411), reviewed in (Stepanova, V. V. et al., 2002) **(Figure 20)**. The growth factor-like domain (GFD) is responsible for the interaction of uPA with its receptor uPAR (Appella, E. et al., 1987). The kringle domain contains a sequence for the interaction with its specific inhibitor, plasminogen activator inhibitor-1 (PAI-1) (Mimuro, J. et al., 1992), and the protease domain includes the active site of the enzyme represented by a specific fragment with its catalytic triad His204, Asp255, and Ser356.

As mentioned above, uPA is a multifunctional protein that has been implicated in several physiological and pathological processes, including cell proliferation, migration, tissue regeneration, inflammatory responses, and tumor growth/metastases. All these complex processes involve intracellular signal transduction and regulation of gene transcription in addition to proteolysis.

Several studies have showed that uPA-induced signal transduction occurs via both uPAR-dependent and uPAR-independent pathways (Blasi, F. et al., 2002; Stepanova, V. et al., 2008). Indeed, it has been demonstrated that uPA can bind directly other proteins including LRP/α2-macroglobulin (Kounnas, M. Z. et al., 1993) or maspin, a member of the serpin family proteins (Al-Ayyoubi, M. et al., 2007). However, the most reported uPA-

induced signaling involved in cell migration, adhesion and proliferation are associated with the interaction with its receptor uPAR.

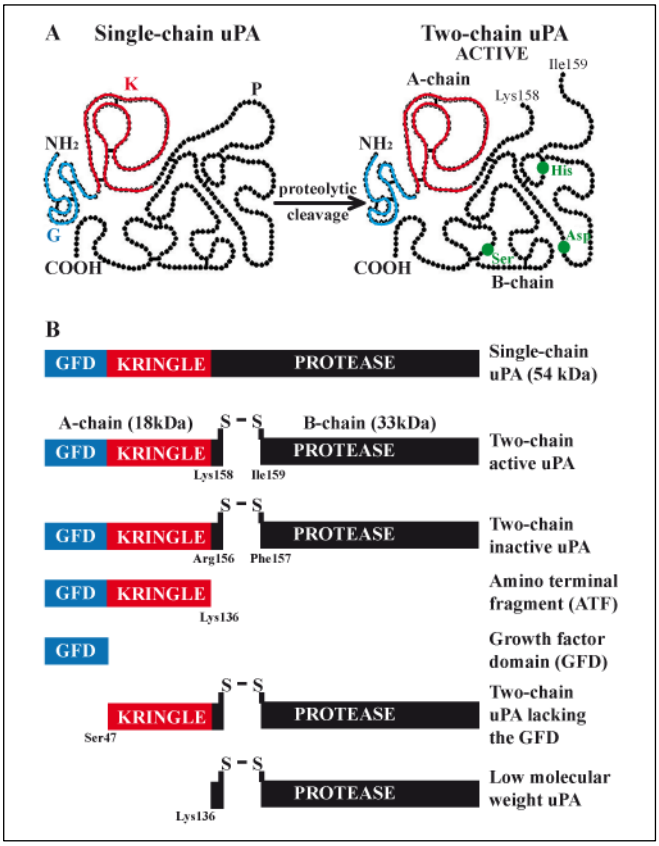

Figure 20. Structure and processing of uPA. (A) Structure of single chain and two-chain uPA form. G, growth factor like domain (GFD); K, kringle domain; P, protease domain. **(B)** Different fragments generated upon proteolytic cleavage. A-chain, light chain; B-chain, heavy chain; ATF, amino terminal fragment. Adapted from (Stepanova, V. V. et al., 2002).

6.3. Urokinase Plasminogen Activator Receptor

uPAR (CD87) is a glycoprotein of 55-60 kDa with 5 potential N-linked glycosylation sites and it is synthesized as a single polypeptide chain of 313 amino acid residues containing a 21 residue signaling peptide. uPAR lacks a transmembrane domain and during its maturation process, post-translational events lead to the cleavage of 30 carboxyterminal residues

and to the attachment of a glycosyl-phosphatidylinositol (GPI) tail to Gly283 that anchors the receptor to the cell surface (GPI-uPAR) (Ploug, M. et al., 1991).

The receptor is formed by three similarly sized (about 90 residues each) homologous domains: the amino-terminal D1 domain, the linker D2 domain and the carboxy-terminal D3 domain (Blasi, F. et al., 2002). These three extracellular domains of uPAR form a globular-like structure providing a large external surface that is suited for lateral interactions, and constitute a central pocket which is the binding site of its physiological ligand uPA (Barinka, C. et al., 2006). Indeed, uPA and uPAR interact through the N-amino terminal GFD domain of uPA and the large hydrophobic binding pocket involving residues from all three domains of uPAR (Kjaergaard, M. et al., 2008) **(Figure 21)**.

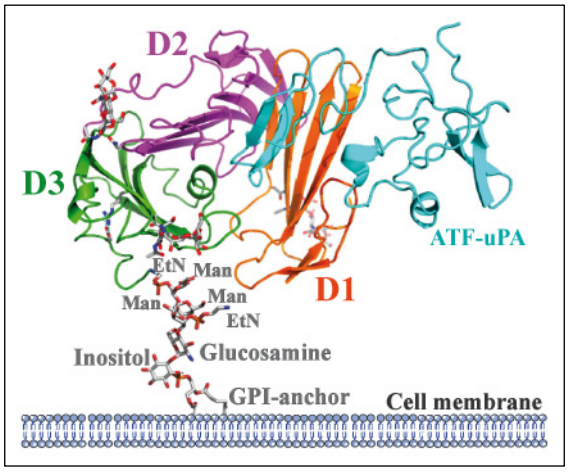

Figure 21. Structure of uPAR forming a complex with the ATF domain of uPA. The individual domains in uPAR are color coded as follow: D1, orange; D2 magenta; D3, green and in cyan its natural ligand was represented (uPA, ATF domain). In addition, the GPI-membrane anchor is shown. Man, mannose; Etn, ethanolamine. Adapted from (Xu, X. et al., 2012).

6.4. Cell surface associated uPA/uPAR system

6.4.1. uPAR binding ligands

As a receptor for uPA, uPAR may be considered as "constitutively active" as high-affinity binding occurs without the need of any additional co-factors. However, the interaction requires the intact three-domain structure of uPAR, explaining why cleavage of uPAR in the linker region connecting D1 and D2 is an irreversible inhibitory event. In addition to uPAR cleavage, the affinity of the interaction is moderately dependent upon expression levels and on the type and degree of uPAR glycosylation (Blasi, F. et al., 2010).

The uPAR was originally identified as the membrane receptor of uPA, involving uPAR in the plasminogen activator cascade and in the regulation of pericellular proteolysis by degradation of ECM proteins (Ellis, V. et al., 1992). Later on, vitronectin was shown to be another major ligand indicating a role of uPAR in cell adhesion. Other unrelated ligands have been subsequently reported including coagulation factor XII (a zymogen, precursor of the serine protease factor XIIa, involved in coagulation cascade) and high molecular weight kinin-free kininogen (HMWK, pro-inflammatory plasma protein), expanding the functions of uPAR (Eden, G. et al., 2011).

6.4.2. uPAR co-receptors

Recent studies have indicated that uPAR acts not only as a proteinase receptor. Indeed, uPAR also induces intracellular signaling events involved mainly in the regulation of the actin cytoskeleton and promoting cell migration, adhesion and proliferation. uPAR's molecular properties and structure are largely responsible for conferring this versatile role

(Blasi, F. et al., 2002). Since, uPAR lacks a transmembrane domain, uPAR-dependent signaling requires lateral interactions with other proteins of the plasma membrane. As shown in **Table 5**, a wide variety of uPAR lateral partners have been reported in the literature.

Family	Partners	Functions
Integrins	α3β1; α4β1; α5β1; α9β1; αvβ3; αvβ5; αMβ2; αLβ2; αXβ2	Regulate cell adhesion and migration Modulate uPAR expression, localization and activity Mediate uPAR-induced signaling
Receptor tyrosine kinases	EGF; PDGFR; IGF-1R	Promote cell proliferation, and migration Mediate uPAR-induced signaling
Endocytic receptors	LRP-1; LRP-1B; VLDL-R; LR11; IGF II-R; Endo180	Regulate uPAR activity Promote uPAR endocytosis
Scaffolding/ structural proteins	Caveolin-1	Regulate uPAR activity
Protein tyrosine phosphatases	SHP2	Regulate uPAR expression
Cytokine receptors	gp130	Mediate uPAR-induced signaling
Complement receptors	gC1qR	Forms a complex with uPAR involved in the binding of factor-XII and HMWK
Intermediate filaments	Keratin 1	Forms a complex with uPAR involved in the binding of factor-XII and HMWK
Pattern recognition receptors	RAGE	Cell signaling; vascular permeability
Selectins	L-selectin	Ca^{2+} mobilization
Serine proteases	Fibroblast activation protein-α	ECM degradation
Heat shock proteins	MRJ	Promotes cell adhesion on vitronectin

Table 5. The lateral partners of uPAR. PDGFR (platelet derived growth factor receptor), IGF-1R (insulin-like growth factor 1 receptor), LR11 (lipoprotein receptor 11), gp130 (glycoprotein 130), gC1qR (binding protein for the globular

head domains of complement component C1q), RAGE (receptor for advanced glycation end products), MRJ (DNAJ homolog subfamily B member). Adapted from Eden, G. et al. (Eden, G. et al., 2011).

As a result of these interactions, uPAR might activate several intracellular signaling pathways including the Ras-mitogen-activated protein kinase (MAPK) pathway, and intracellular molecules such as FAK, Src, Rho family small GTPase Rac, Janus kinases (JAK)-signal transducer, activator of transcription (STAT), and PI3K-Akt **(Figure 22)**.

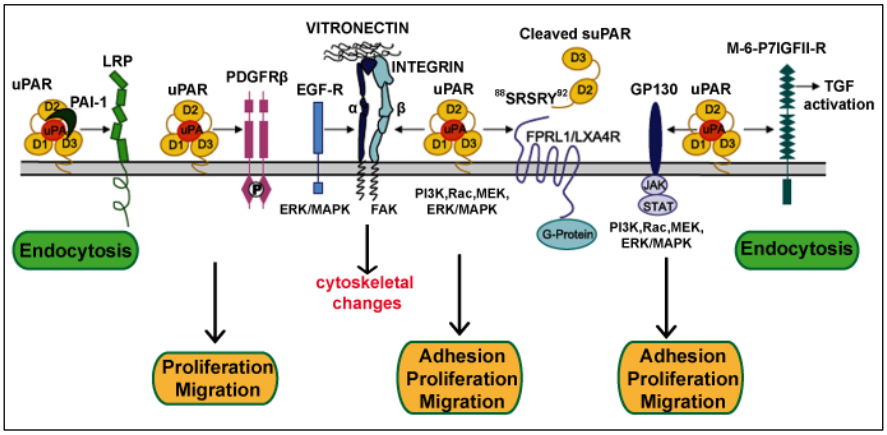

Figure 22. Possible interactions between uPAR and its most common lateral partners leading to signaling events. Image adapted from (Binder, B. R. et al., 2007).

Up to now, the mechanisms that regulate uPAR's interaction with one particular lateral partner rather than other are far for being completely understood.

All major ligands of uPAR may influence its lateral interactions. Thus, it has been suggested that binding of uPA to uPAR promotes a change in uPAR conformation converting the "uPAR receptor" into "uPAR ligand", which leads to the exposure of the SRSRY motif responsible of the

interaction of uPAR with G protein-coupled receptors (GPCRs) (Resnati, M. et al., 2002) **(Figure 23)**. In addition, uPAR binding with vitronectin (VN) might increase the association of uPAR with integrins, and the binding with factor XII the formation of uPAR-integrin-EGFR complex (LaRusch, G. A. et al., 2010).

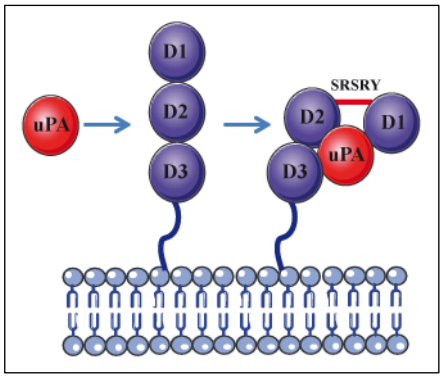

Figure 23. Suggested uPAR conformational change in response to uPA binding. The binding of uPA to uPAR induces a conformational change in uPAR that leads to the exposure of the SRSRY motif located in the linker-1 between domain D1 and D2. This change promotes the interaction between uPA/uPAR complex and GPCRs.

6.4.3. Regulation of uPA/uPAR on cell surface

The regulation of uPA and uPAR on the cell surface can occur through different mechanisms including uPAR cleavage and uPA/uPAR complex endocytosis.

uPAR cleavage in the D1-D2 linker by proteases, such as uPA (Hoyer-Hansen, G. et al., 1992), plasmin (Tjwa, M. et al., 2009) and MMPs (Andolfo, A. et al., 2002), creates a soluble D1 fragment and a D2-D3 fragment that can be membrane associated or shed (Hoyer-Hansen, G. et al., 1992). Cleaved uPAR cannot bind uPA or VN and the uPAR-integrin interaction is abrogated, suggesting that cleavage inhibits uPAR signaling. However, studies have shown that cleaved uPAR might signal through

pathways independently of uPA, VN and integrins (Resnati, M. et al., 2002).

On the other hand, cell surface expression of GPI-anchored uPAR can be regulated by endocytosis and recycling. This process is mediated by its interaction with LRP and other LDL receptor family members (Webb, D. J. et al., 1999; Czekay, R. P. et al., 2001). Up-to-date, LRP–mediated endocytosis is the best characterized uPA/uPAR endocytosis mechanism. When uPA binds uPAR, it rapidly reacts with the plasminogen activator inhibitor-1 (PAI-1) forming a complex that is internalized by LRP. During endocytosis, uPAR remains associated with the uPA-PAI-1 complex and is also internalized. This process leads to clathrin-dependent endocytosis, lysosomal degradation of uPA and PAI-1 and recycling of uPAR and LRP to the plasma membrane (Nykjaer, A. et al., 1997; Czekay, R. P. et al., 2001) **(Figure 24)**.

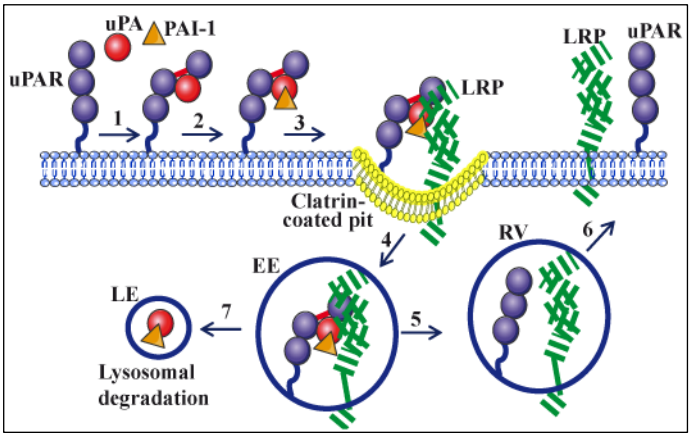

Figure 24. Endocytosis, degradation and recycling of uPAR/uPA/PAI-1 complex through LRP. 1) Active uPA binds to uPAR. **2)** PAI-1 binds and inhibits uPA. **3)** Binding of PAI-1also triggers the binding with LRP and the formation of a quaternary complex that is rapidly distributed into clathrin-coated pits of the plasma membrane. **4)** Endocytosis and trafficking to the early endosome (EE). **5)** Sorting of LRP and uPAR into recycling vesicles (RV). **6)** recycling of LRP and uPAR back to plasma membrane. **7)** uPA:PAI-1complex is delivered to the late endosome (LE) or lysosome for degradation.

Endocytosis and recycling have a complex role in uPAR signaling. Some studies report that endocytosis can reduce the amount of cell surface uPAR available for signaling, thereby preventing uPAR-mediated Rac, ERK1 and ERK2 activation and inhibiting cell migration and chemotaxis (Webb, D. J. et al., 2000; Ma, Z. et al., 2002). Conversely, other studies show that uPAR endocytosis and recycling can enhance cell migration through the localization of uPAR into nascent adhesion complexes, in which it can signal through integrins or other transmembrane partner (Prager, G. W. et al., 2004).

6.5. uPA and uPAR signaling

uPA may activate a number of signaling pathways in different cell types. The signaling effects of uPA are suggested to be mediated by uPAR, or other membrane uPA-binding proteins (Binder, B. R. et al., 2007). To this respect it has been reported that uPA-induced cell migration is associated with the activation of Src and Janus kinases (Dumler, I. et al., 1998) and that uPAR can interact with several members of the tyrosine- and serine-protein kinases such FAK, ERK/mitogen-activated protein kinase (MAPK) and hematopoietic cell kinase (Hck) (Blasi, F. et al., 2002; Tkachuk, V. A. et al., 2009). Such intracellular signal transduction pathways can mediate the expression of MMPs in the vessel wall induced by uPA, which might be important for vascular remodeling. In addition, Kiyan et al. have been demonstrated that PDGF receptor may also be involved in uPA-induced signaling in VSMC, thereby participating in the regulation of cell migration (Kiyan, J. et al., 2005).

Furthermore, uPAR can activate other signaling pathways involved in cell migration and adhesion through its interaction with VN and with several members of integrin family (Degryse, B. et al., 2001).

6.6. uPA and uPAR in cell adhesion and migration

Several studies indicate that uPA may induce migration through both proteolysis-dependent and proteolysis-independent mechanisms (Tkachuk, V. A. et al., 2009; Blasi, F. et al., 2010).

In migrating cells, the coordinated expression of uPA and uPAR exists at cell-substrate and cell-cell contact sites where uPA/uPAR complexes concentrate plasmin production that provides ECM proteolysis, weakened cell-cell contact, and increased cell motility. The proteolytic mechanism includes uPA-induced plasmin generation at focal adhesion sites, which result in ECM degradation and thus facilitate the detachment of the cell's trailing edge (Tkachuk, V. A. et al., 2009).

On the other hand, uPA stimulates cell migration via non proteolytic mechanisms by enhancing adhesion at the leading edge of the cell, stimulating binding of uPAR to VN, modulating uPAR/integrin interaction, and initiating signal transduction cascades that result in cytoskeleton reorganization and cell migration. As previously mentioned, the uPA and uPAR migratory effect can also be regulated by their endocytosis that ensures uPAR recycling on the leading edge that accelerates a new cycle of adhesion and cytoskeleton reorganization that are required for cell movement (Prager, G. W. et al., 2004)

7. Concluding remarks and unresolved issues

Atherosclerosis is a systemic disease that starts early in life and progresses asymptomatically through adulthood when clinical manifestations usually occur. Initial fatty streak lesions evolve into complicated plaques, some of which are vulnerable to rupture or erosion and cause atherothrombotic events that can result in morbid or fatal ischemic events.

Advances in the understanding of the atherothrombotic process have shown that plaque composition, rather than the degree of stenosis, is the major pathophysiologic determinant of acute ischemic events. Indeed, human arteriotomy specimens and necropsies have revealed that the major intrinsic features that characterize vulnerable plaques are a large lipid core (rich in cholesterol esters and with little collagen) and a small number of VSMC (Stary, H. C. et al., 1992; Fuster, V. et al., 2005). However, the causes that lead this lack of VSMC in advanced lesions are not fully understood.

Previous studies have related high levels of LDL, particularly modified LDL, with a reduced number of VSMC, indicating that circulating lipoproteins may exert cellular effects not exclusively related to their lipid transport function. Indeed, our group has demonstrated that aggregated LDL induce intracellular cholesteryl esters accumulation in VSMC and that lipid loaded-VSMC show defects in actin cytoskeleton organization and cell migration due to changes in the phenotypic profile of MRLC, a protein that exerts a key role in VSMC cytoskeleton dynamics and migration (Padro, T. et al., 2008). However, the mechanisms that mediate these effects of LDL on the actin cytoskeleton and migration kinetics of VSMC, as well as in the vascular remodeling still remain to be elucidated.

On the other hand, proteolytic enzymes have been shown to be involved in the complication of atherosclerotic plaques. Previous studies have

demonstrated that LDL affects the expression and activity of MMP-9 in VSMC (Otero-Vinas, M. et al., 2007). This evidence suggests that LDL might affect other proteolytic enzymes present in lesion sites further increasing plaque instability. Therefore, additional studies are needed to determine the effects of LDL on these enzymes and to investigate the mechanisms involved.

Finally, several studies have suggested that LDL-cholesterol lowering with statins promotes plaque-stabilization through an increase in VSMC and collagen content (Crisby, M. et al., 2001; Suzuki, H. et al., 2003). However, further investigation is required in order to unravel the mechanisms behind this effect.

II. HYPOTHESIS AND AIMS OF THE STUDY

A reduced number of VSMC and high lipid content are major features of unstable atherosclerotic plaques prone to rupture. Previous studies from our group have provided evidence that native and modified LDL particles induce changes in VSMC cytoskeleton-dynamics and migration-kinetics. However, the mechanisms through which LDL affect VSMC functions are not completely understood. Therefore, this doctoral thesis seeks to thoroughly investigate the molecular mechanisms and mediators involved in the detrimental effects of atherogenic levels of LDL on VSMC motility in order to identify potential therapeutic targets to promote plaque stabilization. The study is based on the **hypothesis** that **"atherogenic levels of LDL alter proteins directly involved in the regulation of VSMC migration".**

The specific objectives proposed to prove this hypothesis were:

- **Investigate the mechanisms by which atherogenic LDL affect VSMC migration.** This includes the analysis of:
  - Mechanisms by which LDL alter VSMC actin cytoskeleton dynamics
  - Effects of atherogenic LDL levels on uPA/uPAR-mediated VSMC adhesion and migration
  - The relevance of LRP-1 in the UPA/UPAR-mediated migration of VSMC exposed to atherogenic LDL
- **Analyze the effects of LDL-C lowering treatments over VSMC migration and the molecular mechanisms involved**

III. MATERIALS AND METHODS

1. Experimental design

This study was performed mainly in primary human VSMC cultures exposed to atherogenic concentrations of native LDL or LDL modified by aggregation. Lipid effects were analyzed in cell functional studies. Gene expression, protein levels, protein subcellular distribution as well as differential proteome profile were analyzed. The *in vivo* studies were performed in hyperlipidemic rat model. The effects of high cholesterol levels were analyzed on VSMC outgrowth from aortic explants. **Figure 25** shows a flow-diagram of experimental design used in this thesis.

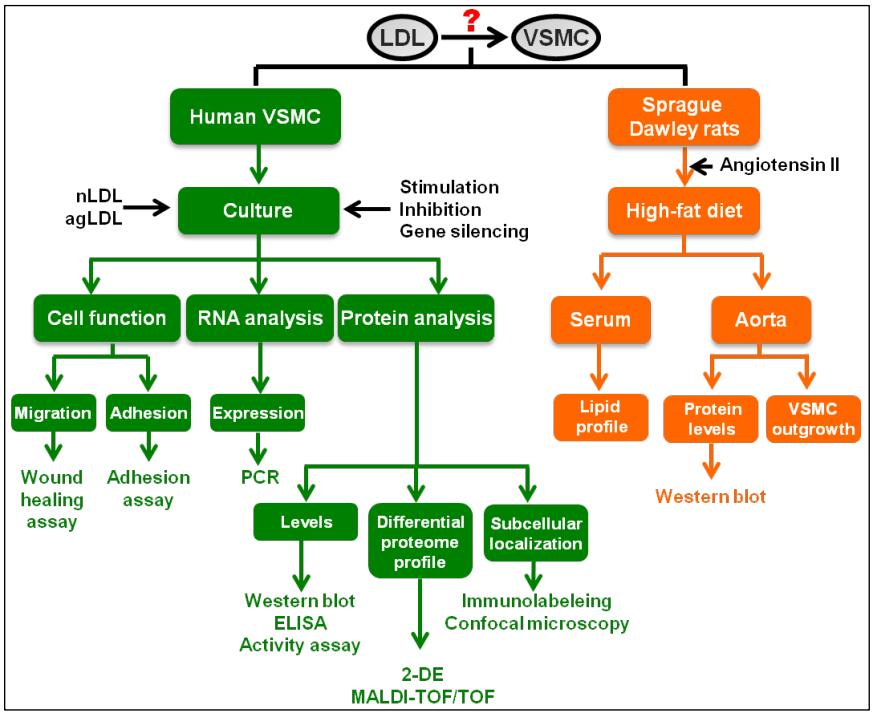

Figure 25. Schematic experimental design. LDL, low density lipoproteins; VSMC, vascular smooth muscle cells; nLDL, native LDL; agLDL, aggregated LDL; PCR, real time polymerase chain reaction assay; ELISA, enzyme linked immonosorbent assay; 2-DE, two dimensional electrophoresis; MALDI-TOF/TOF, matrix-assisted laser desorption/ionization time-of-flight.

2. Cell culture

2.1. Vascular smooth muscle cells

2.1.1. Human coronary vascular smooth muscle cells

Primary cultures of human coronary vascular smooth muscle cells (VSMC) were obtained by an explant technique from human non-atherosclerotic coronary arteries of hearts obtained from heart transplantation surgery performed at the Hospital de la Santa Creu i Sant Pau (Barcelona, Spain).

Human coronary arteries were immediately excised from the extracted heart and transported in tissue transport medium **(Table 6)** to the cell culture facility. In order to prepare artery-explants, coronaries were cleaned removing the surrounding fat tissue, opened longitudinally, and the endothelium was gently scraped off. Then, the adventitia layer was discarded and the media layer containing VSMC was cut into approximately 1.5 mm^2 segments.

Coronary explants were then placed in tissue culture dishes with M199 complete medium **(Table 6)** and incubated at 37°C in a humidified atmosphere of 5% CO_2 to obtain VSMC outgrowth. Culture medium was replaced every 48 hours and VSMC derived from explants were frozen and cryopreserved in liquid nitrogen at passage three **(Figure 26)**.

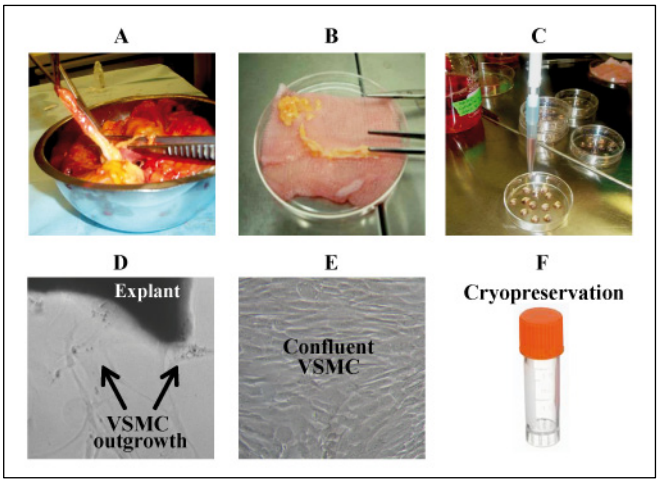

Figure 26. Schematic representation of human coronary VSMC isolation from transplanted hearts. Human coronary arteries are excised from the removed heart **(A).** After coronary excision, surrounding fat tissue is removed, the artery is opened longitudinally and the endothelium and adventitia are discarded **(B).** Remaining tissue (media layer) is then cut into approximately 1.5mm^2 explants, placed in tissue culture dishes (15 explants per dish) and cultured in M199 complete medium **(C)**. After approximately one week, VSMC start to migrate from explants **(D)** and proliferate covering the surface of the culture dish **(E)**. VSMC are expanded and cryopreserved at passage three **(F)**.

Coronary VSMC were characterized morphologically using light microscopy. At subconfluence, VSMC showed a spindle-shaped appearance and confluent VSMC cultures showed the characteristic "hill-and-valley" growth pattern **(Figure 27)**. Additionally, cells were characterized by immunological staining of cytoskeleton proteins specific to VSMC such as human α-smooth muscle actin (clone 1A4; Abcam, ab7817). To exclude any endothelial or fibroblast cells contamination, immunostaining for von Willebrand factor (clone F8/86; Abcam, ab778), and fibroblast surface protein (clone 1B10; Abcam; ab11333) were performed.

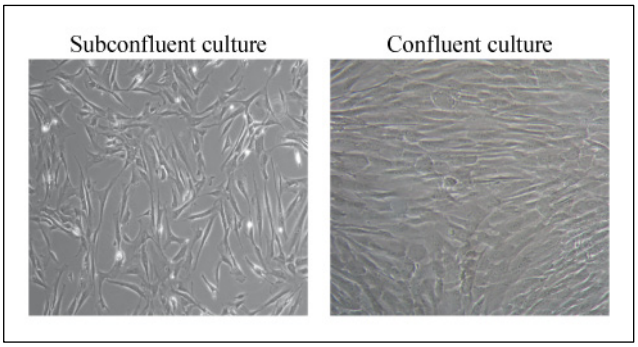

Figure 27. Light microscopy images of cultured VSMC. Subconfluent and confluent VSMC culture. Images were obtained using an inverted microscope (10X lens).

For experimental studies, VSMC were cultured in M199 complete medium **Table 6** and used between passages four and eight.

These cells were used in Papers 1 and 2.

2.1.2. Human umbilical vein vascular smooth muscle cells

Primary cultures of human VSMC derived from umbilical vein (pool of 10 donors, Technoclone) were used in experiments focused on studying uPA/uPAR system due to its high expression of these molecules.

These cells were also cultured in M199 complete medium as previously described for coronary VSMC (**Table 6)**. Cell treatments used in this thesis were checked in VSMC derived from human coronary arteries and in VSMC derived from human umbilical vein and cell response was similarly in both cell cultures.

These cells were used in Papers 3 and 4.

2.2. Cell culture media

The different cell culture media used throughout this study are shown in **Table 6**.

Medium	Composition	Paper
Tissue transport medium	M199 100U/mL Penicillin 100µg/mL Streptomycin 15µM Hepes	1, 2
M199 complete medium	M199 20% Fetal bovine serum (FBS) 2% Human serum 100U/mL Penicillin 100µg/mL Streptomycin 2µM L-Glutamine	1, 2, 3, 4
M199 migration medium	M199 10% Fetal bovine serum (FBS) 2% Human serum 100U/mL Penicillin 100µg/mL Streptomycin 2µM L-Glutamine	1, 2, 4
M199 adhesion medium	M199 5% Fetal bovine serum (FBS) 2% Human serum 100U/mL Penicillin 100µg/mL Streptomycin 2µM L-Glutamine	2, 3
M199 growth-arrest medium	M199 100U/mL Penicillin 100µg/mL Streptomycin 2µM L-Glutamine	1, 2, 3, 4

Table 6. Summary of culture media used. M199 (Gibco); Penicillin/Streptomycin (Fisher bioblock); L-Glutamine (Fisher bioblock); FBS (Biological Industries).

2.3. Cell treatments

2.3.1. Low density lipoproteins

LDL isolation

Human LDL were isolated from pooled sera of normocholesterolemic volunteers (Banco de Sangre y Tejidos, Barcelona), based on their specific density, by sequential ultracentrifugation (Havel, R. J. et al., 1955) in density gradients with potassium bromide (KBr) followed by dialyzation. LDL preparations were maintained protected from light at 4ºC until used (always within 7 days of preparation).

LDL characterization

The nLDL fraction was quantified by determining both protein and total cholesterol concentrations. Additional analyses were performed to determine purity as well as to exclude oxidation.

LDL protein concentrations were determined using the bicinchoninic acid (BCA) method (Pierce) and total cholesterol concentration using a commercial kit (Kit RefLab.Brand) based on the method by Allain et al. (Allain, C. C. et al., 1974). Purity of LDL was assessed by agarose gel electrophoresis (Paragon Lipoprotein Electrophoresis kit, Beckman Coulter) and lipid peroxidation was indirectly determined by measuring thiobarbituric-acid-reactive-substances (TBARS; Thiobarbituric Acid Reactive Substances, Janssen Chimica). TBARS values were in all cases <1.2mmol malonaldehyde/mg of LDL protein. In addition, an oxidized LDL ELISA (Mercodia) was performed.

LDL aggregation

agLDL were prepared by vortexing nLDL (1mg/mL in Phosphate Buffered Saline, PBS) for 4 minutes (Guyton, J. R. et al., 1991) as previously described in our group (Llorente-Cortes, V. et al., 2002a). Aggregates (LDL in fused form, precipitable fraction) were then separated from non-aggregated LDL (non-precipitable fraction) by centrifugation at 10000xg for 10 minutes at room temperature. Precipitated agLDL were suspended in PBS at a final concentration of 1mg/ml (Khoo, J. C. et al., 1988; Llorente-Cortes, V. et al., 1999).

agLDL obtained in this manner have previously been proven to be similar in size and structure to fused-LDL produced by the interaction between versican (an ECM proteoglycan) and LDL and those described in atherosclerotic lesions) (Llorente-Cortes, V. et al., 2002a) **(Figure 28)**.

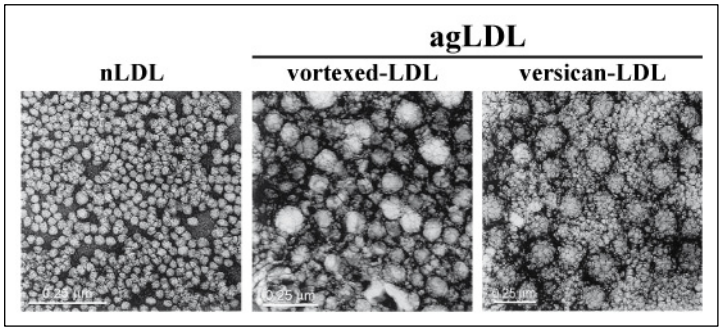

Figure 28. Electron microscopy of LDL. nLDL (native LDL; unmodified LDL), agLDL (aggregated LDL);LDL modified by vortexing (vortexed-LDL) and LDL modified by versican (versican-LDL). Adapted from (Llorente-Cortes, V. et al., 2002a).

LDL treatment

To study the effects of LDL on VSMC function, cells were exposed to atherogenic concentrations of nLDL or agLDL (100μg/mL) for up to 18

hours. VSMC lipid internalization in cells incubated with LDL was regularly assessed by staining with Sudan III-Sudan IV Herxheimer solution **(Figure 29)**. Briefly, cells were seeded on bottom glass chamber slides, treated with/without 100µg/mL nLDL or agLDL for 18 hours and then fixed with 4% paraformaldehyde in PBS. Lipids were stained with filtered Herxheimer solution (0.5gr Sudan III, 0.5gr Sudan IV, 50mL Alcohol 70º and 50 mL Acetone) and nuclei with Mayer Hematoxylin.

nLDL were used in Papers 1 and 2, whereas, agLDL were used in Papers 1, 2, 3 and 4.

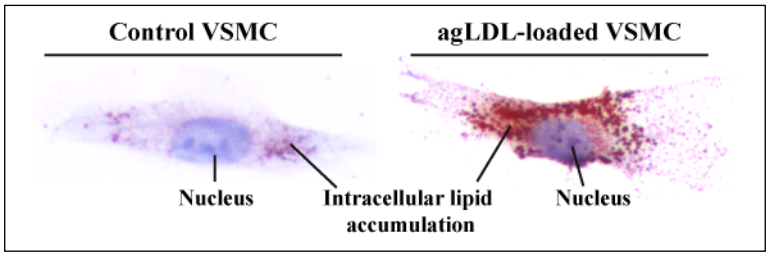

Figure 29. Intracellular lipid staining. Control VSMC and VSMC exposed to 100µg/ml agLDL for 18 hours (agLDL-loaded VSMC). Lipids were stained with Herxheimer solution (Sudan III and Sudan IV), red signal. Nuclei were stained with Mayer Hematoxylin, blue signal.

2.3.2. Induction/Inhibition experiments

A set of inhibitors, inducers, and specific antibodies were used in proof-of concept studies. VSMC treatments and conditions used in these different studies are provided in **Table 7**.

Treatment	Origin	Concentration	Duration	Paper
STATINS				
Rosuvastatin	-	2.5; 5; 10; 40µM	20h*	2, 4
Simvastatin	-	2.5µM	20h*	2
INHIBITORS				1
Okadaic Acid	-	20nM	20h/2h	1
Fostriecin	-	200nM	30min/2h*	
Y27632	-	10µM	30min/2h*#	2
ML9	-	50µM	30min*	2
RAP	-	200nM	30min*	4
ANTIBODIES				
anti-UPA	Goat-pol	40µg/mL	30min*	4
anti-UPA ATF	Mouse-mon	40µg/mL	30min*#	3, 4
anti-UPAR	Mouse-mon	5µg/mL	30min*#	3
OTHERS				
GGPP	-	10µM	20h	2
Exogenous UPA	Hu-urine	2µg/mL	30min*#	3, 4
Angiotensin II	-	1µM	20h*	3,4

Table 7. Summary of VSMC treatments and incubation times. *In wound healing and adhesion assays, incubation times refer to time periods prior to performing the scratch in the cell monolayer or cell harvesting and seeding in adhesion assays. #Treatments added at time 0 during wound healing assay or just after cell seeding during adhesion assays. Y27632, inhibitor of Rho-associated protein kinase (p160 ROCK); ML9, inhibitor of myosin light chain kinase (MLCK); RAP, receptor associated protein; UPA, urokinase plasminogen activator; UPAR, urokinase plasminogen activator receptor; GGPP, geranyl-geraniol pyrophosphate. mon, monoclonal; pol, polyclonal; Hu, human.

2.3.3. Gene silencing

In order to obtain uPA or HSP27 deficient VSMC, uPA expression was silenced with a commercial siRNA Silencer® siRNA ID No: s10611 (Ambion) (*used in Papers 3 and 4*) and HSP27 with Silencer® siRNA ID No: s194538 (Ambion) (*used in Paper 1*) using the Human AoSMC Nucleofector kit (Amaxa), according to the manufacturer's instructions. siRNA random Silencer® Select Negative Control siRNA (Ambion) was used as control.

Briefly, cells ($6x10^5$) were electroporated in the presence of 600nM siRNA against uPA, HSP27 or random siRNA. Suppression of gene expression was confirmed by real time- PCR and western blot at 24, 36 and 48 hours after cell nucleofection.

3. Cell function assays

3.1. Wound healing assay

This assay was performed in human coronary and umbilical VSMC cultured in 6-well plates at 90% confluence.

Briefly, double-sided scrape-wounds were produced on the cell monolayer using a sterile micropipette tip. Scrapped cells were washed with PBS and the culture maintained in M199 migration medium **(Table 6)** during wound healing follow-up. Cell migration and wound repair were controlled under a 10x lens using an inverted microscope (Leica DMIRE2) attached to a video SPOT camera (Leica-DFC350FX). Images of the initial denuded area were taken at 2 hours intervals over the 10 hours period following the scratch (four different fields along the linear scratch, covering more than the 95% of the wound). During migration, cells were maintained at 37°C in a humidified atmosphere of 5% CO_2. Alternatively, 4 hours after performing the scratch, cells were fixed for immunolabeling and confocal microcopy analysis as described below. A flow-chart of wound healing assays performed in this study is shown in **(Figure 30)**.

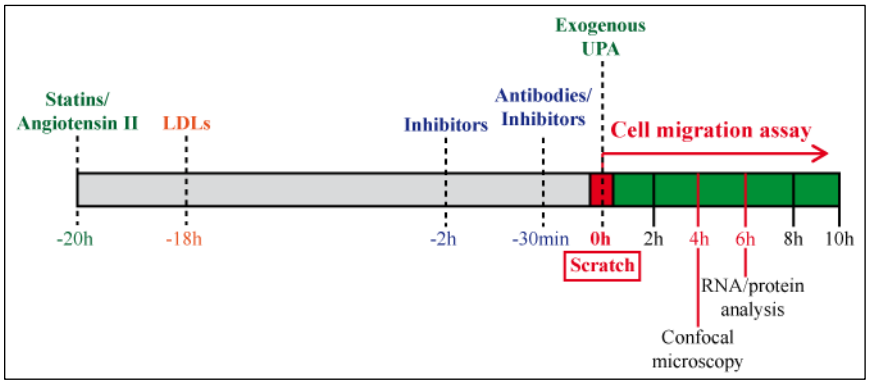

Figure 30. Flow-chart of the wound healing assay.

Quantification of cell migration

To quantify cell migration, photomicrographs along the initial cell denuded area were taken at 2 hours interval over a 10 hours period (4 defined fields covering >95% of the wound) **(Figure 31A)**. The cell-free area of each field was quantitatively determined using Image-J software (National Institutes of Health, NIH) **(Figure 31B)** and the average cell-free area was determined for each time interval. Wound coverage was expressed as a percentage of the initial cell-free area and 3 independent experiments were performed for each condition.

Wound healing assays were performed in Papers 1, 2 and 4.

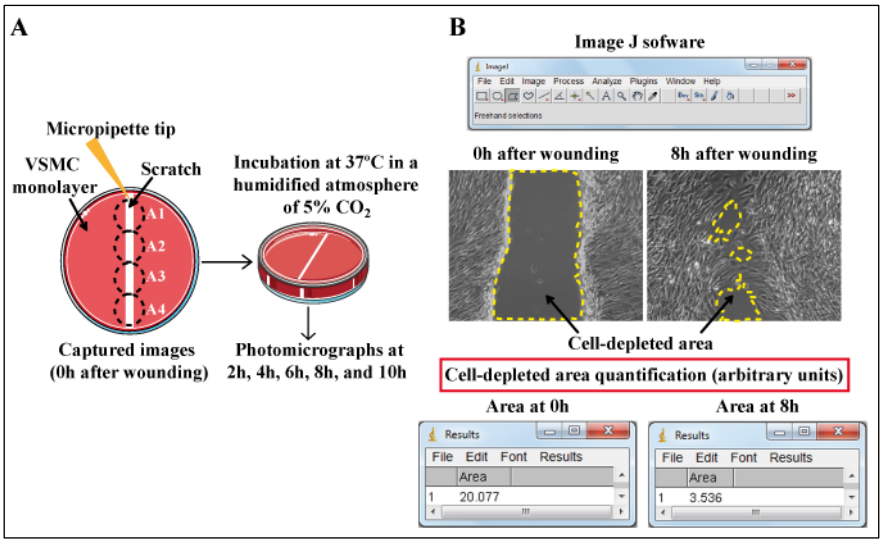

Figure 31. Quantification of cell migration. (A) Schematic representation of a wound healing assay; A, area. **(B)** Cell migration quantification using ImageJ software. The same area along the linear scratch was measured at different time periods after wounding. Cell migration was calculated as percentage of the cell-free area measured at 0 hours and considered as 0% of cell migration.

3.2. Cell adhesion assay

Subconfluent VSMC cultures, were incubated with specific treatments for the time periods and conditions described in **Table 7** and **Figure 32**, harvested with trypsin-EDTA (Gibco) and suspended in M199 adhesion medium **(Table 6)**. Cells ($1x10^5$) were seeded in FBS-coated 6-well plates in the presence of each specific treatment and maintained at 37°C in a humidified atmosphere of 5% CO_2 during adhesion assay. At different time periods after seeding (30 minutes, 1 hour and 3 hours), attached cells were harvested and counted using a Neubauer-chamber. Alternatively, at different time periods cells were fixed with 4% paraformaldehyde for immunolabeling and confocal microscopy analysis *(Papers1 and 2)* or harvested for gene and protein expression analyses as described below. *This assay was used in Papers 1, 2 and 3.*

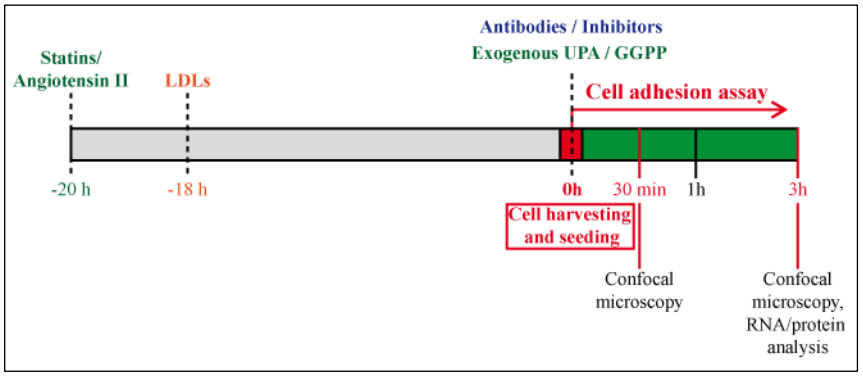

Figure 32. Flow-chart of the cell adhesion assay.

3.3. VSMC outgrowth assay from aortic explants

Aortas were dissected from rats after *in vivo* studies and explants were cleaned as previously described for human coronary arteries (*Materials and Methods section 2.2.1.*).

From each aorta, twenty explants from the thoracic aorta (size 1x1mm) were placed in tissue culture dishes with the luminal side down and maintained in M199 medium supplemented with 10% FBS at 37°C in a 95% air-5% CO_2 humidified atmosphere. Medium was replaced every 48 hours and the number of explants showing rat vascular smooth muscle cells (RVSMC) outgrowth was recorded daily over a period of 6 days. *This assay was used in Paper 4.*

4. RNA analysis

Briefly, total RNA was extracted from migrating VSMC or growth-arrested VSMC using the RNeasy Mini Kit (Qiagen; *used in Paper 4*) or Ultraspec RNA Isolation System reagent (Biotecx Laboratories, inc.; *used*

in Paper 3) according to the manufacturer's instructions. RNA concentration was assessed using Nanodrop Spectrophotometer (Thermo Scientific). cDNA was synthesized from 1μg of RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). uPA, uPAR and LRP-1 mRNA gene expression was analyzed by real-time PCR using TaqMan fluorescent real-time PCR primers and probes (Applied Biosystems): uPA (Hs01547054_m1), uPAR (Hs00182181_m1) and LRP-1 (Hs00233899_m1). Human GAPDH (4326317E) was used as a standardization control for the $2^{-\Delta\Delta Ct}$ method.

5. Protein analysis

5.1. Total protein extraction

Total VSMC lysates were obtained from migrating cells *(Paper 4)*, cells during attachment and spreading (*Paper 3*) or growth-arrested cells (*Papers 1, 2, 3, and 4*). Cells were washed with ice-cold PBS and collected in RIPA buffer (50mM Tris HCl pH 8; 150mM NaCl; 1% NP-40; 0.5% sodium Deoxycholate; 0.1% SDS) supplemented with a protease inhibitor cocktail (Complete, EDTA-free, Roche, 11873580001). Cell suspensions were transferred into microcentrifuge tubes, incubated for 30 minutes on ice and passed through a 20G gauge needle to facilitate cell breaking up. Residual cell fragments were discarded by centrifugation (10 minutes at 10000 rpm at 4ºC) and supernatants were stored at -80ºC for further analysis.

5.2. Membrane and cytosol protein extraction

For quantitative analysis of specific proteins at cell membrane and in the cytosol compartment, protein extraction was performed at subcellular level.

Briefly, frozen cell pellets were sequentially extracted based on differential protein solubility. In a first step, samples were homogenized in 40mM tris-base buffer (tris-buffer), incubated for 30 minutes at room temperature in the presence of 1x NucleaseMix (GE-Healthcare) and centrifuged at 16000xg (20 minutes). Supernatants containing the hydrophilic cellular proteins (cytosolic proteins) were collected and stored at -80ºC. Remnant protein pellets were washed in tris-buffer and further extracted with a urea/thiourea-chaps buffer (7M urea; 2M thiourea; 4% CHAPS; 40mM tris-base) for 15 minutes at room temperature obtaining a urea-detergent soluble fraction (hydrophobic cellular proteins, including cytoskeleton and integral-membrane proteins). *This extraction protocol was used in Papers 1 and 2.*

5.3. Tissue protein extraction

For rat aortic tissue protein extraction, tripure isolation reagent (Roche) was used after tissue homogenization following manufacturer´s instructions. *Used in Paper 4.*

5.4. Protein extract quantification

Sample protein concentrations were determined with the BCA Protein Assay Kit (ThermoScientific) based on the biuret reaction where Cu^{2+} is reduced to Cu^{1+} by proteins in an alkaline medium. Addition of BCA leads to a purple-colored end product that is detected colometrically at

562nm on a plate reader (Spectra Max 250, Molecular Device). *Used in Papers 3 and 4.* Alternatively, protein quantification of samples prepared with reagent that are incompatibles with BCA assay were performed using 2D-Quant Kit (GE-Healthcare) as indicated by the manufacturer. *Used in Papers 1, 2 and 3.*

5.5. Assays for protein levels quantification

5.5.1. Western blot

Protein extracts were separated in a one dimensional Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE), as originally described by Laemmli (Laemmli, 1970). Briefly, 25μg 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 10-12% running gel). Separated proteins were transferred to nitrocellulose membranes using a wet electroblotting or 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 then blocked to avoid unspecific binding of antibodies. Primary antibodies used in the different studies are listed in **Table 8**. Specific secondary antibodies against different animal species were used conjugated with horseradish peroxidase. Detection of proteins bands was achieved by chemiluminescence using a peroxidase enzymatic reaction (Supersignal, Pierce) and images were obtained with a ChemiDocTM XRS system. Bands intensities were quantified using Image Lab software (Bio-Rad). For normalization of results, β-actin (*Papers 1, 2, 3 and 4*) or tubulin (*Paper 4*) were used as loading controls.

Primary antibody	Reference	Host species	Dilution	Paper
FAK-p	Abcam/ab4803	Rabbit polyclonal	1/1000	3
HSP27-pSer15	Stressgen/SPA-525	Rabbit polyclonal	1/1000	1
HSP27	Abcam/ab2790	Mouse monoclonal	1/1000	1
HSP27-pSer78	Stressgen/SPA-523	Rabbit policlonal	1/1000	1
HSP27-pSer82	Stressgen/SPA-524	Rabbit polyclonal	1/1000	1
MAPK Phospho-p38	Cell Signaling/4631	Rabbit monoclonal	1/1000	1
MAPKAP Kinase2	Abcam /ab131531	Rabbit polyclonal	1/1000	1
MAPKAP Kinase2-p	Abcam/ab136490	Mouse monoclonal	1/1000	1
MRLC	Abcam/ab11082	Mouse monoclonal	1/500	2, 3
MRLC-p	Abcam/ab2480	Rabbit polyclonal	1/500	2, 3
PAK1-p	Abcam/ab40795	Rabbit monoclonal	1/10000	2
p38	Abcam/ab7952	Rabbit polyclonal	1/1000	2
RhoA	SC/418	Mouse monoclonal	1/500	2
ROCK-p160	SC/87349	Rabbit polyclonal	1/500	2
UPA	AD/ 398	Goat polyclonal	1/200	3, 4
UPA	Abcam/ab133563	Rabbit monoclonal	1/1000	4
UPAR	AD/ 3937	Mouse monoclonal	1/500	3, 4
β-actin	Abcam/ab8226	Mouse monoclonal	1/5000	1, 2, 3, 4
β-actin	Abcam/ab8227	Rabbit polyclonal	1/5000	1, 2, 3, 4
β-tubulin	Abcam/ab6046	Rabbit policlonal	1/500	4

Table 8. Primary antibodies and working conditions used for western blot analysis. AD, American Diagnostica; SC, SantaCruz.

5.5.2. Enzyme Linked ImmunoSorbent Assay (ELISA)

A specific quantitative sandwich enzyme immunoassay (ELISA, Assaypro, EU1001-1) was used to quantify levels of secreted uPA in cell adhesion studies, 3 hours after seeding. Cell culture medium was concentrated using 3KDa cut-off filters before performing the assay. Values were expressed as ng UPA secreted by $1x10^7$ cells. *This assay was used in Paper 3.*

5.5.3. Protein activity assay

Levels of active RhoA were measured in the cytosolic fraction as well as in total cell lysates using a RhoA activation assay kit (Cytoskeleton, BK036) according to manufacturer's instructions. Briefly, VSMC cultured in growth-arrest medium **(Table 6)** for 24 hour and exposed to specific treatments, were harvested either with 40mM Tris-base (cytosol extract) or with the lysis buffer (total cell lysates) provided by the RhoA activation assay kit.

Immediately after extraction, protein concentration was determined (Precision Red Advanced Protein Assay Reagent, ADV02, Cytoskeleton) and equal amounts of protein were incubated with GST-Rhotekin sepharose beads to pull down active RhoA. The complete pulled down sample, containing only active Rho family members, was then resuspended in Laemmli loading buffer and analyzed by western blot.
This assay was used in Paper 2.

5.6. Protein subcellular localization. Immunolabeling and confocal microscopy

Subcellular distribution of proteins and protein colocalization were analyzed by immunolabeling and confocal microscopy. Immunolabeling was performed during cell adhesion and migration or in cultures of growth-arrested cells.

For confocal microscopy, VSMC were seeded on glass bottom culture dishes and experiments were performed as previously described in *Materials and Methods sections 3.1.* and *3.2.*.

Tween-20/PBS permeabilized cells were used in studies of total protein localization (cytosolic and membrane distribution) and non-permeabilized cells in studies of cell surface/membrane protein localization.

Cells were fixed with paraformaldehyde 4% in PBS (v/v) for 15 minutes at room temperature, washed with PBS and permeabilized with 0.5% Tween-20/PBS for 5 minutes at room temperature, whenever it was experiment required. Thereafter, cells were blocked with 1% bovine serum albumin (BSA)/PBS for 1 hour at room temperature, incubated for 30 minutes with Signal Enhancer (Invitrogen), and then for 1 hour with the specific primary antibodies **(Table 9)**. After washing the primary antibody, cells were incubated with fluorophore-conjugated secondary antibodies (AlexaFluor-488, AlexaFluor-633 or AlexaFluor-647; dilution 1/100, 1 hour). Hoechst dye (Molecular Probes, dilution: 1/500, blue fluorescent stain specific for DNA) was used for nuclei staining and AlexaFluor-633 or -488 Phalloidin for labeling F-actin (fluorescent F-actin stain, Molecular Probes, dilution 1/20; for permeabilized conditions). Afterwards, cells were washed and mounted for confocal microscopy using ProLong Gold Antifade Reagent (Invitrogen) to maintain fluorescence signal. In each experiment, negative controls were included. Samples were always stored protected from light at 4ºC until

analysis by confocal microscopy. Cell labeling was examined in an inverted fluorescence confocal microscope (Leica TCS SP2-AOBS). Fluorescent images were acquired in a scan format of 1024x1024 pixels at intervals of 0.1µM (20 slides) and were processed with the TCS-AOBS software (Leica). Labeling intensity was obtained as maximal intensity projections or complete xyz-stack series (20 slides, 0.1µm/slide). Labeling intensity was calculated in a minimum of 3 independent experiments using LAS AF Leica Software. A minimum of 5 cells/group were used in cell adhesion assays (*Papers 2 and 3*) and an area 12-15µm wide along the migrating edge in wound healing assays. Values are given as Intensity/μm^2 or as % of colocalization after subtraction of background signal.

In some experiments, protein subcellular distribution was analyzed by three-dimensional projections with rotation of +90º, +65º, +45º, +25º and 0º. Z-values are represented by a color scale, being blue Z-minimum (cell bottom) and pink Z-maximum (cell top). These projections were performed using LAS AF Leica Software (*Paper 3*).

Primary antibody	Reference	Dilution	Secondary antibody	Paper
RhoA	SC-418	1/50	Anti-mouse AlexaFuor-488	2
pMRLC	Abcam/ab2480	1/50	Anti-rabbit AlexaFuor-488	2
HSP27	Abcam/ab2790	1/50	Anti-mouse AlexaFuor-488	1
pSer82-HSP27	Stressgene/SPA-524	1/50	Anti-rabbit AlexaFuor-488	1
Gelsolin	Abcam/ab11081	1/50	Anti-mouse AlexaFuor-647	1
uPA	AD/398	1/50	Anti-goat AlexaFuor-488/-633	3, 4
uPAR	AD/3937	1/50	Anti-mouse AlexaFluor-488/-633	3, 4
pFAK	Abcam/ab4803	1/50	Anti-rabbit AlexaFluor-488	3
LRP-1	Fitzgerald/10R-L107A	1/30	Anti-mouse AlexaFluor-488	4

Table 9. Primary antibodies and fluorophore-conjugated secondary antibodies used for immunolabeling and confocal microscopy. AD, American Diagnostica; SC, SantaCruz.

5.7. Proteomic analysis

Analysis of differential protein expression patterns and protein identification was performed by a proteomic approach using two-dimensional electrophoresis (2-DE; *used in Papers 1, 2 and 3*), or one-dimensional electrophoresis (1-DE; *used in Paper 3*), and mass-spectrometry identification. Sample contaminants (salts, nucleic acids, lipids) were removed using a commercial kit (2D-CleanUp Kit, GE-Healthcare).

For 1-DE experiments, proteins extracts were separated in 10% SDS-PAGE gel, as described above (*Materials and Methods section 5.5.1.*). 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).

For 2-DE analysis, protein extracts (120µg to 300µg) were loaded on 17cm dry strips (pH 3-10 linear range, GE Healthcare) and proteins were separated by electrofocussing according to their isoelectric point (pI). Second dimension was resolved in 12% SDS-PAGE gels. Gels were developed by fluorescent Flamingo staining using a Typhoon 9400 scanner (GE-HealthCare, Uppsala, Sweden). Analysis of differences in protein patterns was performed with the PD-Quest 8.0 software (BioRad).

Protein bands (1DE) or spots of interest (2DE) were excised from gels and analyzed by MALDI-TOF/TOF mass spectrometry for protein identification.

In *Papers 1 and 2*, protein identification was performed by peptide-mass fingerprinting using an Ettan MALDI-TOF Pro (matrix-assisted laser desorption/ionization time-of-flight mass spectrometer, GEHealthcare) operating in delayed extraction/reflector mode. The peptide masses were searched against the National Center for Biotechnology Information non redundant mammalian database using ProFound and confirmed using a Mascot 2.3 search from Matrix Science, selecting the Swiss-Prot database. Identification was accepted with a score higher than 56 (Padro, T. et al., 2008). Whereas, *in Paper 3*, proteins were identified after in-gel tryptic digestion and extraction of peptides from the gel pieces using AutoFlex III Smartbeam MALDI-TOF/TOF spectrometer (Bruker Daltonics). In order to perform this analysis, samples were placed in Prespotted AnchorChip plates (Bruker Daltonics) surrounding the calibrants provided on the plates. Spectra were acquired using flexControl software on reflector mode. Each sample was processed with flexAnalysis software (version 3.0, Bruker Daltonics) considering a signal-to-noise ratio over 3, applying statistical calibration and eliminating background peaks. For

identification, spectra were analyzed with the BioTools software (version 3.2, Bruker Daltonics) and proteins were identified using the MASCOT search on the Swiss-Prot. Identification was accepted with a score higher than 56 (Cubedo, J. et al., 2013).

6. *In vivo* experiments

6.1. Animal model

In vivo experiments were performed in female seven-week old Sprague-Dawley rats (Harlan) fed with a western type high-fat diet (Cocoa Butter Diet and Purina Mouse Chow, Harlan, TD.88051) or a regular diet (Teklad Global 2019 Extruded Rodent Diet, Harlan, 2019) for 14 days (N=10 animals in each group).

Animals had free access to tap water and chow. On day 1, rats were implanted with subcutaneous osmotic mini-pumps (model 1002, Alzet). During the 2 week-period, 50% of the animals in each diet-group (hyperlipemic and normolipemic) were infused with 200ng/Kg per minute of angII (Sigma, A9525) and 50% saline solution, as previously described (Sendra, J. et al., 2008).

On day 14, animals were anesthetized with a mix of Ketamine (75mg/Kg) and Medetomidine (0.5mg/Kg) and Buprenorphine (0.1mg/Kg) was used as an analgesic. Blood was obtained by intracardiac puncture and animals were euthanized by intracardiac injection of a pentobarbital overdose (400mg/Kg). Aortas were immediately dissected and the aortic VSMC outgrowth or protein expression was analyzed as described above (*Materials and Methods section 3.3. and 5.5.1.* respectively).

All procedures fulfilled the criteria established by the “Guide for the Care and Use of Laboratory Animals” published by the United States National

Institutes of Health (NIH Publication No.85-23, revised 1996) and were approved by the Internal Animal Committee Review Board. *This animal model was used in Paper 4.*

6.2. Lipid profile analysis

Plasma lipid profiles of all animals were analyzed. Total cholesterol and triglycerides levels were directly determined from plasma by enzymatic reaction using commercial kits adapted to an BM/HITACHI 911 autoanalyzer (Roche Diagnostics, Rotkreuz, Switzerland) (Escola-Gil, J. C. et al., 2011). To isolate the HDL-Cholesterol (HDL-C) fraction from total plasma, apoB-containing apolipoproteins were selectively precipitated using a commercial phosphotungstic-$MgCl_2$ (PT) method (Boehringer Mannheim) and samples were centrifuged for 10 minutes at 10000 rpm. Supernatants containing the HDL-C fraction were isolated and HDL-C levels were determined by enzymatic reaction using the same commercial kit previously used for total cholesterol. Finally, the VLDL-cholesterol (VLDL-C) fraction was isolated from total plasma by sequential ultracentrifugation (16 hours at 36000 rpm) in a 1.006 density solution. The obtained floating band was isolated and VLDL-C levels were determined with the same kit previously described for total and HDL-C. LDL-Cholesterol (LDL-C) levels were calculated from total, HDL, and VLDL cholesterol levels according to the formula:

$$\text{Total cholesterol} = \text{VLDL-C} + \text{LDL-C} + \text{HDL-C}$$

Glucose, glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT) and serum creatinine (CREA) were determined using a CLIMA MC-15 analyzer (RAL).

These analyses were used in Paper 4.

7. Statistical analysis

Results are presented as mean±standard error over the mean (SEM) or as median (interquartile range, IQR) and the number of experiments is shown in every case. Statistical differences between treatments or groups were analyzed using by parametric tests as t-student, one-way analysis of variance (ANOVA) or two-way ANOVA, followed by the Fisher's PLSD test for group differences or by non-parametric tests (Kruskal Wallis, Mann-Whitney or Chi-Square), as indicated. A p-value ≤ 0.05 was considered significant. Statistical analyses were performed using statistical software StatView 5.0.1.

8. Additional methods

Additional methods detailed in the papers (*Results section*):

- Monocyte isolation and macrophage differentiation *(Paper 1)*
- Size exclusion chromatography *(Paper 1)*
- Immunohistochemistry of coronary arteries *(Paper 1)*
- Cell proliferation assay *(Paper 2)*
- Co-immunoprecipitation *(Paper 3)*

IV. RESULTS

1. Paper 1

"Low-Density Lipoproteins Induce Heat Shock Protein 27 Dephosphorylation, Oligomerization, and Subcellular Relocalization in Human Vascular Smooth Muscle Cells".

Maísa García-Arguinzonis, Teresa Padro´, Roberta Lugano, Vicenta Llorente-Cortes, Lina Badimon

Arterioscler Thromb Vasc Biol. 2010 Jun;30(6):1212-9.

The *objective* of this study was to investigate whether exposure of VSMC to LDL induces changes on the proteomic profile of the heat shock protein (HSP) family, molecular chaperones involved in atherosclerosis. The *main results* revealed that LDL modifies the proteomic profile of HSP27 (HSPB1). In addition, a significant HSP27 dephosphorylation was detected after exposure of cells to native LDL (nLDL) and aggregated-LDL (agLDL) for 24 hours. Dephosphorylation of HSP27 was not related to changes in p38MAPK phosphorylation. Both nLDL and agLDL induced relocalization of unphosphorylated HSP27 to the tip of actin stress fibers and focal adhesion structures in VSMC. During cell adhesion, phospho-HSP27 was located at the cell surface contact region in LDL-treated cells, whereas it remained cytosolic in control cells. Moreover, immunohistochemistry studies showed that phosphorylated HSP27 is rarely found in lipid-rich areas of atherosclerotic plaques in human coronary arteries. *In conclusion*, our results indicate that in VSMC, LDL modulate HSP27 phosphorylation and subcellular localization, affecting actin polymerization and cytoskeleton dynamics.

Arterioscler Thromb Vasc Biol. 2010 Jun; 30(6):1212-9. doi: 10.1161/ATVBAHA.109.198440. Epub 2010 Apr 8.

Low-density lipoproteins induce heat shock protein 27 dephosphorylation, oligomerization, and subcellular relocalization in human vascular smooth muscle cells.

Maísa García-Arguinzonis, Teresa Padró, Roberta Lugano, Vicenta Llorente-Cortes, Lina Badimon

2. Paper 2

"LDL-Induced Impairment of Human Vascular Smooth Muscle Cells Repair Function Is Reversed by HMG-CoA Reductase Inhibition"

Teresa Padró, Roberta Lugano, Maisa García-Arguinzonis, Lina Badimon

PLoS One. 2012 Jun;7(6):e38935.

The *objective* of this study was to investigate whether HMG-CoA reductase inhibition with rosuvastatin can reverse the effects induced by atherogenic concentrations of LDL either in the native (nLDL) form or modified by aggregation (agLDL) on human VSMC motility. The *main results* revealed that treatment of human coronary VSMC with rosuvastatin significantly prevented (and reversed) the inhibitory effect of nLDL and agLDL in the repair of the cell depleted areas. In addition, rosuvastatin significantly abolished the agLDL-induced dephosphorylation of myosin regulatory light chain as demonstrated by 2DE-electrophoresis and mass spectrometry. Besides, confocal microscopy showed that rosuvastatin enhances actin-cytoskeleton reorganization during lipid-loaded-VSMC attachment and spreading. The effects of rosuvastatin on actin-cytoskeleton dynamics and cell migration were dependent on ROCK-signaling. Furthermore, rosuvastatin caused a significant increase in RhoA-GTP in the cytosol of VSMC. *In conclusion* our study demonstrated that inhibition of HMG-CoA reductase restores the migratory capacity and repair function of VSMC that is impaired by native and aggregated LDL. This mechanism may contribute to the stabilization of lipid-rich atherosclerotic plaques afforded by statins.

PLoS One. 2012 Jun;7(6):e38935. doi: 10.1371/journal.pone.0038935. Epub 2012 Jun 12.

LDL-Induced Impairment of Human Vascular Smooth Muscle Cells Repair Function Is Reversed by HMG-CoA Reductase Inhibition.

Teresa Padró, Roberta Lugano, Maisa García-Arguinzonis, Lina Badimon

3. Paper 3

"Aggregated low-density lipoprotein induce impairment of the cytoskeleton dynamics through urokinase-type plasminogen activator/urokinase-type plasminogen activator receptor in human vascular smooth muscle cell"

Roberta Lugano, Esther Peña, Lina Badimon, Teresa Padro

Journal of Thrombosis and Haemostasis. 2012 Oct;10(10): 2158–2167.

The *objective* of this study was to investigate whether mechanisms underlying inhibition of cytoskeleton dynamics in lipid-loaded VSMC occurs through a UPA-mediated process. The *main results* revealed that agLDL induced UPA subcellular delocalization and significantly decreased UPA levels during attachment of VSMC. UPA (enhanced endogenous-expression or exogenous added) acting as a urokinase-type plasminogen activator receptor (UPAR)-ligand restored actin-cytoskeleton organization and adhesion capacity of lipid-loaded cells to control levels. UPAR co-immunoprecipitated with the unphosphorylated form of myosin regulatory light chain (MRLC) in lipid-loaded cells. The detrimental effects of agLDL on MRLC phosphorylation were reversed by high levels of UPA. The UPA effects on VSMC exposed to agLDL involved FAK phosphorylation. *In conclusion***,** the detrimental effects of atherogenic LDL on VSMC are mediated by a decrease and delocalization of the UPA–UPAR interaction that result in an impairment of cytoskeleton dynamics and adhesion capacity affecting cell phenotype and function.

J Thromb Haemost. 2012 Oct;10(10):2158-67. doi: 10.1111/j.1538-7836.2012.04896.x.

Aggregated low-density lipoprotein induce impairment of the cytoskeleton dynamics through urokinase-type plasminogen activator/urokinase-type plasminogen activator receptor in human vascular smooth muscle cell.

Roberta Lugano, Esther Peña, Lina Badimon, Teresa Padro

4. Paper 4

"UPA promotes lipid-loaded vascular smooth muscle cell migration through LRP-1"

Roberta Lugano, Esther Peña, Laura Casani, Lina Badimon, Teresa Padró

Cardiovasc Res (2013). In press.

The *objective* of this study was to investigate whether UPA-ligand binding is involved in the detrimental effects of lipid loading in VSMC migration. The *main results* revealed that rats fed a high-fat diet had 10fold higher cholesterol-LDL plasma levels, >60% decrease in aortic UPA-protein expression and VSMC showed impaired out-growth from aortic-explants. Angiotensin-II infusion significantly increased aortic UPA-expression and accelerated VSMC migration. In addition, using an in vitro model of wound repair, we showed that agLDL inhibits UPA-mediated VSMC migration. UPA-silencing reduced migration in control cells to levels observed in lipid-loaded-VSMC. UPA-silencing did not affect migration in lipid-loaded VSMC. UPA-expression was significantly decreased in agLDL-exposed VSMC. agLDL also induced changes in the subcellular localization of UPA, with a reduction in colocalization with UPAR strongly evident at the front edge of agLDL-treated migrating cells. Rescue experiments showed that UPA acting as UPAR-ligand restored migration capacity of agLDL-VSMC to control levels. The effects of UPA/UPAR on migration of lipid-loaded cells occurred through the binding to LRP-1. In *conclusion,* UPA-ligand binding regulates VSMC migration, a process that is interfered by LDL. Thus tissue infiltrated LDL, through the abrogation of UPA function reduces VSMC-regulated vascular repair.

Cardiovasc Res. (2013). In press.

UPA promotes lipid-loaded vascular smooth muscle cell migration through LRP-1.

Roberta Lugano, Esther Peña, Laura Casani, Lina Badimon, Teresa Padró

V. DISCUSSION

Atherosclerosis is the leading underlying cause of clinical cardiovascular events including coronary syndromes, stroke and peripheral artery disease. Despite currently available drug therapies, atherosclerosis represents one of the leading causes of death and disability in the world (World Health Organization, 2011).

Accumulation of lipids within the arterial wall is associated with intimal disorganization and thickening, and often, in advanced atherosclerotic lesions, with a loss of VSMC that can contribute to plaque rupture and thrombosis (Stary, H. C. et al., 1994; Libby, P., 2001). Previous studies have demonstrated that LDL infiltrated in the vessel wall became aggregated and retained in the intima by proteoglycans (Camejo, G. et al., 1998; Oorni, K. et al., 2000; Badimon, L. et al., 2006). agLDL are internalized by VSMC through the scavenger receptor LRP-1 (Llorente-Cortes, V. et al., 2000) and impair cell functions as cytoskeleton dynamics, cell migration and vascular repair after injury (Padro, T. et al., 2008). The composition of the atherosclerotic plaque, rather than the degree of arterial stenosis, has been shown to be the main determinant of acute manifestations of the disease. Indeed, today we know that intrinsic features characterizing unstable plaques include: a large necrotic lipid core occupying more than 40% of the total plaque volume, a thin fibrous cap, and a reduced VSMC and collagen content. Whereas VSMC account for 90-95% of the cell component in initial lesions, this proportion decreases to 50% in advanced atherosclerotic plaques, reviewed in (Badimon, L. et al., 2009). However, the causes underlying this reduction of VSMC in unstable plaques are poorly understood. Previous studies have related high levels of LDL, particularly modified LDL, with a reduced number of VSMC, indicating that circulating lipoproteins may exert cellular effects not exclusively related to their lipid transport function.

This thesis was aimed to address early changes induced by atherogenic LDL levels in human VSMC phenotype and function, features that have been found to be highly modified during atherosclerotic vascular remodeling.

VSMC produce most of the main components of the ECM such as proteoglycans, collagen, and elastin, as well as a large number of proteins responsible for the equilibrium between synthesis and degradation of the ECM during the atherogenic development, reviewed in (Badimon, L. et al., 2009). VSMC migration is considered as one of the main processes involved in vascular remodeling and thus may modulate VSMC content in the atherosclerotic plaque. Therefore, a better understanding of the effects of LDL on VSMC phenotype and function may represent a key issue to understand vascular remodelling during atherosclerosis. Indeed, the detrimental effects of LDL, particularly agLDL, on VSMC migration, adhesion and cytoskeleton reorganization can contribute to reduce the number of VSMC at the lesion site promoting atherosclerotic plaque vulnerability.

Atherogenic levels of native LDL and agLDL have been related with changes in cytoskeleton proteins associated to VSMC migration (Padro, T. et al., 2008). Indeed, it has been demonstrated that LDL induce dephosphorylation of MRLC and produce striking changes in the subcellular localization of its phosphorylated form (pMRLC) (Padro, T. et al., 2008). MRLC is a cytoskeleton protein involved in VSMC migration kinetics as pMRLC activates myosin II through its ATPase motor domain, a critical regulator of cytokinesis, and consequently promotes cell migration (Hirano, K. et al., 2004; Watanabe, T. et al., 2007). HMG-CoA reductase inhibitors, as statins, are widely used cholesterol-lowering drugs that have been shown to reduce clinical cardiovascular disease presentation (Baigent, C. et al., 2005; Brugts, J. J. et al., 2009). Among

them, rosuvastatin has shown efficacy in reducing primary presentation and inducing regression or halt of disease burden (Nissen, S. E. et al., 2006; Crouse, J. R., 3rd et al., 2007; Ridker, P. M. et al., 2008). Several studies have shown that statins favourably alter the fibromuscular composition of plaques by increasing collagen content and SMC-α-actin in both hyperlipidaemic ApoE$^{-/-}$ mice (Schafer, K. et al., 2005; Gronros, J. et al., 2008) and human carotid plaques (Crisby, M. et al., 2001).

A substudy of this thesis was addressed to investigate whether HMG-CoA reductase inhibition with rosuvastatin can reverse the effects induced by atherogenic concentrations of LDL, either in their native form or modified by aggregation, on VSMC motility and cytoskeleton dynamics (P*aper 2*). Based on an *in vitro* VSMC culture approach and using a wound repair model, we have demonstrated that rosuvastatin prevents, in a dose-dependent manner, agLDL-induced dephosphorylation of MRLC. Furthermore, rosuvastatin abolished the impairment of VSMC migration induced by the presence of atherogenic LDL.

HMG-CoA reductase inhibitors block the synthesis of farnesyl pyrophosphate (FPPP) and geranylgeranyl pyrophosphate (GGPP) isoprenoids and thereby disrupt posttranslational isoprenylation and intracellular translocation from cytoplasm to membrane of isoprenylated proteins involved in cell signaling, such as the members of the Rho family (Seasholtz, T. M. et al., 2004). Rho GTPases are key regulators of cytoskeleton dynamics in a wide variety of events related to cell functions, including cell migration (Etienne-Manneville, S. et al., 2002). The results obtained in this thesis further revealed that rosuvastatin leads to a significant increase in functional RhoA-GTP in the cytosol of VSMC and to a significant decrease in the amount of RhoA partitioning in the membrane compartment. This novel finding is consistent with those of previous studies in monocytes, HEL cells and endothelial cells showing

that statins promote Rho-GTP binding and activation (Cordle, A. et al., 2005; Turner, S. J. et al., 2008). This result suggest that anchoring of active RhoA in the cell membrane is not required for the increase in cell motility induced by rosuvastatin in lipid-loaded VSMC. The functional relevance of this finding is reinforced by studies showing that unprenylated G-proteins are also biologically active (Allal, C. et al., 2000; Turner, S. J. et al., 2008; Kou, R. et al., 2009).

MRLC-phosphorylation is mainly influenced by the opposing activities of MLC-kinase (MLCK) and MLC-phosphatase (MLC-P), which in turn are regulated by p21-activated protein kinase (PAK) and Rho-dependent kinase (ROCK) (Amano, M. et al., 1996; Sanders, L. C. et al., 1999). RhoA in its active form (RhoA–GTP) binds ROCK stimulating the phosphotransferase activity of ROCK (Loirand, G. et al., 2006), which in turn mediates actin cytoskeleton dynamics by increasing phosphorylation of MLC (Totsukawa, G. et al., 2000) mainly through the inhibition of the MLCP or by direct phosphorylation of MLC (Noma, K. et al., 2006). Additionally, MLC phosphorylation can also be regulated by the activation of MLCK through Ca^{2+}-calmodulin or by MLCK inactivation mediated by PAK, a downstream effector of Cdc42 and Rac1. Phosphorylated PAK (pPAK) inhibits MLCK activity and consequently inhibits MLC phosphorylation (Sanders, L. C. et al., 1999). In LDL-loaded VSMC, ROCK inhibition completely reverted rosuvastatin induced MRLC-phosphorylation, whereas MLCK inhibition did not induce a significant effect and rosuvastatin did not revert the increase in pPAK induced by agLDL, thus emphasizing the relevance of the ROCK signaling pathway in MRLC-phosphorylation. Taken together, these results indicate that rosuvastatin induces MRLC phosphorylation, enhances cytoskeleton organization and promotes cell adhesion and

migration of LDL-loaded-VSMC through a mechanism directly involving the activation of the RhoA/ROCK downstream pathway.

There are evidences showing that the phosphorylation of MRLC in a ROCK-dependent manner can be induced by the amino terminal-fragment of the urokinase plasminogen activator (uPA) (Kiian, I. et al., 2003). uPA is a serine protease that besides its proteolytic function is also involved in signal transduction pathways of several physiological processes such as cell proliferation, adhesion and migration (Blasi, F. et al., 2002). Indeed, uPA and its receptor uPAR have also been shown to be active participants in vascular remodeling and cardiovascular disease progression (Parfyonova, Y. et al., 2004; Parfenova, E. V. et al., 2009; Farris, S. D. et al., 2011). In fact, uPA and uPAR are strongly expressed in human atherosclerotic lesions (Padro, T. et al., 1995; Steins, M. B. et al., 2004; Farris, S. D. et al., 2011), although the overall effect of uPA on plaque composition and stability remains controversial. Throughout this thesis, we have demonstrated that some of the inhibitory effects of agLDL on VSMC cytoskeleton reorganization, actin fiber formation, cell adhesion and migration are mediated by alterations in the uPA-uPAR system and that the effect of rosuvastatin on lipid-loaded VSMC migration is mediated by uPA (*Paper 3 and 4*).

Lipid-loaded VSMC expressed low levels of uPA even when located at the border of the wound and confocal microscopy showed changes in its subcellular localization during cell adhesion and spreading, as well as during cell migration. In fact, at early stages of cell adhesion, uPA labeling was mainly located at the cell border and in the filopodial extensions of control VSMC. In contrast, lipid-loaded cells showed a weak uPA labeling, particularly evident at the cell surface where uPA acts as an uPAR ligand. Similarly, in migrating cells, uPA was strongly polarized at the leading edge in control cells, whereas agLDL-loaded cells

showed a reduced uPA signal at the front of migration. In addition, this study has shown that angiotensin II (angII) increases endogenous uPA levels in LDL-loaded VSMC and shifts their pattern of uPA subcellular distribution in the interface with the extracellular matrix during adhesion and at the leading edge during VSMC migration. The observation that an increase of endogenous uPA or the addition of exogenous uPA was able to restore adhesion and migration of VSMC exposed to agLDL, provides insights into the relevance of uPA on the atherogenic effects of LDL on VSMC functions. These findings are in line with previous reports in mice supporting the notion that uPA is essential to maintain cellularity and stability of atherosclerotic lesions (Dellas, C. et al., 2007).

uPA is active both as a serine protease and a signaling ligand in both uPAR-dependent and uPAR-independent pathways (Padro, T. et al., 1995; Stepanova, V. et al., 2008). However, this study demonstrated that the effect of UPA on cell adhesion and migration occurs via uPAR binding. The presence of agLDL significantly reduces uPA/uPAR colocalization at the leading edge of migrating cells and as consequence impairs cell motility (*Paper 4*). Moreover, blockade of the uPA-uPAR interaction with specific antibodies clearly inhibited the effects of angII or exogenous uPA on LDL-VSMC adhesion and migration. Additional data that linked uPA to the detrimental effects of LDL have been provided using a high fat diet rat model, showing that high levels of plasma LDL-C impair rat VSMC capacity to migrate from aortic explants and decrease uPA levels. I*n vivo* studies also showed that aorta uPA up-regulation by angII was linked to an accelerated VSMC outgrowth from aortic explants of apparently normal arterial tissue even in hyperlipidaemic animals, further supporting the relevance of uPA for VSMC migration (*Paper 4*).

To better understand the mechanisms by which LDL affect VSMC motility, regulation of cytoskeleton dynamics in VSMC was further

investigated. During cell migration, precise coordinated polymerization of actin filaments induces the formation of membrane protrusions and the consequent advancement of the leading edge of the cell. Actin cytoskeleton dynamics are regulated by a vast number of actin-binding proteins that mediate assembly, stability, and organization of actin filaments (Le Clainche, C. et al., 2008). Confocal microscopy analysis of lipid-loaded VSMC at early stages of cell adhesion and spreading showed a deregulation of cytoskeleton reorganization and actin fiber formation which was related to an impaired cell adhesion compared with control VSMC (*Papers 2 and 3*). Besides the effect of LDL on actin-fibers formation through regulation of MRLC phosphorylation, this thesis has also shown that atherogenic LDL modulate actin cytoskeleton reorganization through the dephosphorylation of the small heat shock protein HSP27 (*Paper 1*). HSP27 has been implicated in a wide variety of process, both physiological and pathological, including atherosclerosis (Ghayour-Mobarhan, M. et al., 2012). The use of a proteomic approach together with confocal microscopy studies provided evidence that atherogenic LDL significantly affect the phosphorylation pattern of this small HSP as well as its subcellular localization. Many protective roles of HSP27, including the regulation of cell motility and the coordination of actin dynamics, are modulated by its phosphorylated forms (Guay, J. et al., 1997). Phosphorylated HSP27 (pHSP27) organized in small oligomers interacts with F-actin protecting actin filaments against breakage by actin-severing proteins and promotes its subsequent reorganization. In contrast, non-phosphorylated HSP27 acts as an actin-capping protein blocking actin fiber polymerization (Wettstein, G. et al., 2012). In addition, confocal microscopy studies provided evidence that LDL induce translocation of non-phosphorylated HSP27 towards the cell borders and more specifically towards the lamellipodia in human VSMC. This relocalization of non-phosphorylated HSP27 at the borders of lipid-loaded VSMC showed an

increased colocalization with gelsolin, a protein that enhances actin filament severing and depolymerization, strengthening the relevance of non-phosphorylated HSP27 as an actin-capping protein in the presence of LDL.

In agreement with the *in vitro* studies, inmunohistochemical analysis of advanced human atherosclerotic lesions showed a weak or undetectable pHSP27 staining, particularly in LDL-rich-areas (high content of apoB),. On the contrary, clear positive signals for total HSP27 were usually observed in smooth muscle cell–rich areas of the neointima. These results are in line with previous studies that demonstrate increased levels of pHSP27 in patients with cardiac allograft vasculopathy (De Souza, A. I. et al., 2005), strongly supporting the findings of this thesis, because graft-related accelerated atherosclerotic lesions are restenotic with little participation of lipids.

The regulation of pHSP27 is mediated by a well-know signaling pathway involving MAPK (Landry, J. et al., 1995). However, LDL-related dephosphorylation of HSP27 is not due to the switch-off of MAPK but to a counterregulation by phosphatase PPA2, because inhibition of PPA2 blocked the effect of LDL on pHSP27. Thus, LDL modulation of pHSP27 seems to be a new mechanism by which vessel wall-infiltrated LDL may affect VSMC function and vascular remodeling.

Detrimental effects of LDL on cytoskeleton organization were reverted by increasing uPA levels either by promoting endogenous expression or by the addition of exogenous uPA (*Paper 3*). This observation was supported by previous studies demonstrating that uPA induces cytoskeleton rearrangements in migrating VSMC (Kusch, A. et al., 2000) and by the analysis of uPAR interacting proteins assessed by protein identification using mass spectrometry of uPAR co-immunoprecipitates in the present study. This analysis provided evidence of an interaction of uPAR with

cytoskeleton related proteins, particularly with the phosphorylated and unphosphorylated forms of MRLC in control and agLDL VSMC, respectively. Furthermore, this thesis demonstrated that uPA restores MRLC phosphorylation in agLDL-VSMC to levels similar to those of control cells. This process may contribute to the improvement of cytoskeleton dynamics, cell adhesion and migration induced by uPA in VSMC exposed to atherogenic LDL. These new findings in lipid-loaded VSMC are in line with a previous study demonstrating that the amino terminal-fragment of the uPA induced MRLC phosphorylation in a ROCK and PI3K-dependent manner (Kiian, I. et al., 2003). Indeed, here, we found that angII did not enhance MRLC phosphorylation levels, actin fiber organization and cell attachment in lipid-loaded VSMC when uPA gene expression was silenced.

Results of this thesis also showed that agLDL significantly decrease focal adhesion kinase (FAK) phosphorylation in VSMC during cell adhesion and that the addition of exogenous uPA reverts this effect (*Paper 3*). FAK is a non-receptor tyrosine kinase involved in cytoskeleton remodeling and formation and in the disassembly of cell adhesion structures (Hanks, S. K. et al., 2003). Our results suggest that uPA effects on cytoskeleton reorganization of lipid-loaded VSMC can be mediated by the FAK-induced signaling pathway enhancing MRLC phosphorylation and promoting actin fiber formation and adhesion. Indeed, it has previously been demonstrated that FAK activation is linked to RhoA/ROCK signaling (Gerthoffer, W. T. et al., 2001; Seo, C. H. et al., 2011), a mechanism that induces MRLC phosphorylation through the inactivation of MLCP (Ridley, A. J., 2001). These results are in agreement with our previous observation that MRLC phosphorylation is mediated by RhoA/ROCK pathway in LDL-loaded VSMC in presence of an HMG-CoA reductase inhibitor (*Paper 2*).

uPAR lacks an intracellular domain; therefore, the response to uPA acting as uPAR binding ligand requires the formation of multi-protein complexes with other adaptor proteins. Indeed, several uPAR transmembrane adaptors have been identified (Binder, B. R. et al., 2007; Eden, G. et al., 2011). In this study we have demonstrated that uPA-uPAR signaling in lipid-loaded VSMC requires the binding of LRP-1, an endocytic and cell-signaling receptor for diverse ligands with a potential role in cell migration (Lillis, A. P. et al., 2005; Dedieu, S. et al., 2008) and in agLDL intracellular accumulation in VSMC (Llorente-Cortes, V. et al., 2000) .

Wound-healing assays have demonstrated that the blockade of the LRP-1 binding site by RAP significantly abolishes the effects of exogenous uPA or angII on VSMC migration. These evidences emphasize that uPA/uPAR/LRP-1 association is required for the observed uPA-induced increase of lipid-loaded VSMC migration. Although the effects of LRP-1 on uPA internalization and signaling have previously been examined in different systems (Gonias, S. L. et al., 2011), this is the first study to show a linked contribution of LRP-1 and uPA to lipid-loaded VSMC migration. Indeed, this study has demonstrated that the presence of exogenous uPA facilitates cell-surface localization of LRP-1 in focal clusters at the front edge of migrating LDL-VSMC, a distribution pattern characteristic of migrating control cells.

In summary, the results obtained from the different substudies included in this thesis provide new notions about the mechanisms by which vessel wall-infiltrated LDL impair VSMC functions. Actin cytoskeleton dynamics, cell adhesion and migration, cell processes with a key role in vascular remodeling during atherosclerosis are affected by LDL infiltrated in the vessel wall. These detrimental effects of LDL on cell adhesion and migration provide new clues for a better understanding of the evolution of lipid-rich plaques to unstable lesions with a high risk of rupture and

clinical outcomes. To this respect, the results of this thesis have contributed to highlight novel molecular targets with potential relevance for new therapeutic approaches to prevent vascular remodeling during atherosclerosis.

VI. CONCLUSIONS

From all results presented in this thesis we can establish that:

- HMG-CoA reductase inhibitors restore the migratory capacity of lipid-loaded VSMC though RhoA/ROCK mediated mechanism.
- The detrimental effects of LDL in VSMC adhesion and migration are related to the uPA/uPAR system downregulation.
- Atherogenic concentration of LDL affects actin polymerization and cytoskeleton dynamics through changes in HSP27 phosphorylation and subcellular localization.
- uPA reverts the detrimental effects of aggregated LDL in VSMC adhesion and migration.
- uPA effects on lipid-loaded VSMC are mediated by uPAR binding.
- LRP-1 acts as uPA/uPAR transmembrane adaptor for uPA signal transduction in LDL-VSMC.

In summary, the main concluding remarks are:

1. **The atherogenic levels of LDL impair VSMC adhesion and migration through mechanisms that involve cytoskeleton-related proteins**.
2. **Tissue infiltrated LDL through the abrogation of uPA function reduces VSMC-mediated vascular repair**.
3. **Statins may contribute to the stabilization of lipid-rich atherosclerotic plaques through a mechanism that restore the migratory capacity and repair function of lipid-loaded VSMC.**

These results contribute to elucidate the emerging role of LDL in VSMC processes involved in the loss of cellularity at the atherosclerotic lesion sites, a characteristic feature of vulnerable plaque and identify potential therapeutic targets to promote plaque stabilization.

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