THROMBUS COMPOSITION IN ACUTE CORONARY SYNDROME

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SUMMARY

Atherothrombosis and, specifically intracoronary thrombosis is a major cause of acute coronary syndromes (ACS) and consequently of morbidity and mortality throughout the world. While management of acute ST-elevation myocardial infarction (STEMI) has dramatically improved over the last years, there is still a need to find thrombosis-related biomarkers for an early identification of ischemic processes and a better stratification of patients that have suffered an ACS. In fact, the ischemia time, defined as the time from the onset of symptoms to reperfusion, has been recently suggested as the "New Gold Standard for STEMI-Care". This thesis mainly focuses on the protein composition of the occluding coronary thrombus, occurring both in the native coronary arteries and in the commonly implanted coronary stents. The study based on the proteomic analysis of coronary thrombi, obtained after percutaneous coronary intervention (PCI), has provided consistent evidence of the dynamic composition of the coronary thrombi in relation with the time of ischemia, and has resulted in the identification of novel biomarkers of potential use to be translated to the clinical practice. Furthermore, the comparison of native and in-stent-thrombosis has allowed the identification of proteins that might serve as interesting therapeutic targets to prevent thrombosis in patients who undergo PCI with stent-implantation.

RESUMEN

La enfermedad aterotrombotica y concretamente la trombosis intracoronaria es la mayor causa de los síndromes coronarios agudos (SCA), y consecuentemente de morbilidad y mortalidad en el mundo. El manejo de los pacientes con infarto agudo de miocardio con elevación del segmento ST ha mejorado considerablemente en los últimos años, a pesar de esto sigue siendo necesario encontrar biomarcadores para la detección temprana de los procesos isquémicos y que permitan una estratificación más eficiente de los pacientes que han sufrido un evento isquémico agudo. De hecho, el tiempo de isquemia, definido como el tiempo entre el inicio del dolor y la revascularización, ha sido recientemente definido como el parámetro fundamental en el tratamiento de los pacientes con STEMI. Este trabajo de tesis está enfocado a elucidar la composición proteica de los trombos coronarios oclusivos que se forman tanto en las arterias coronarias nativas como en aquellas con stent. El estudio se basa en el análisis proteómico de trombos coronarios en relación al tiempo de isquemia, con la finalidad de encontrar nuevos biomarcadores para trasladar a la práctica clínica. Además, la comparación entre trombos nativos y trombos desarrollados sobre el stent permite la identificación de proteínas diferenciales que podrían ser futuras dianas terapéuticas para prevenir la formación del trombo en pacientes sometidos a angioplastia coronaria transluminal percutánea (ACTP) con implantación de un stent coronario.

PREFACE

Cardiovascular disease remains one of the major causes of mortality and morbidity in the world. Acute coronary syndromes (ACS) most often result from atherothrombosis, a disease characterized by atherosclerotic lesion disruption with superposed thrombus formation that critically decrease coronary blood flow. ACS are life-threatening conditions, which comprise a group of clinical symptoms associated with acute myocardial ischaemia with or without infarction. There are three types of ACS, ST-segment elevated myocardial infarction (STEMI), non-STsegment elevated myocardial infarction (NSTEMI), and unstable angina. Particularly, STEMI is a leading cause of mortality in developed countries. Therefore, STEMI-patients require rapid initiation of therapy aimed at achieving reperfusion and cardiovascular protective medications. Today, diagnosis of STEMI-patients is made by electrocardiography and the use of plasma biomarkers that result from the necrotic cardiac tissue due to the ischemia period. The total ischaemic time between symptom onset and provision of reperfusion therapy is a major determinant of the clinical outcomes. Indeed, increasing body of evidences support the view that the ischemic time rather than medical contact to treatment time should be the new gold standard for STEMI care. For this reason, there is an increasing need for the identification of new biomarkers for an early detection of atherothrombotic processes and ischemic events turning in an STEMI-ACS.

The introduction of intracoronary stents greatly increased the safety and applicability of percutaneous coronary interventions. However, stent thrombosis (ST), the most feared complication after stent implantation, has emerged as an important entity to be prevented because of its clinical impact owing to a high risk of myocardial infarction and death.

The work presented in this thesis has been conducted in Professor Badimon's research group, at the "Institut Català de Cieències

Cardiovasculars" (ICCC) in Barcelona, a group with a high translational research experience, specifically focusing on the understanding of mechanisms involved in the initiation, progression and complication of atherosclerosis and ischemic diseases. To this respect, the work of this thesis has been mainly focused on the characterization of the evolving composition of human coronary thrombus obtained from STEMI patients and the identification of thrombus proteins that change in relation both pain-to-PCI elapsed time and in-stent thrombosis, with potential relevance in clinical outcome.

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ABBREVIATIONS

AAA	Abdominal aortic aneurysm
ACN	Acetonitrile
ACS	Acute coronary syndromes
ADP	Adenosine diphosphate
Apo	Apolipoprotein
AMI	Acute myocardial infarction
Anti-HT	Antihipertensive
Arp	Actin-related protein
ASA	Acetylsalicylic acid
ATP	Adenosine triphosphate
AU	Arbitrary units
AUC	Area under the curve
A2MG	Alpha-2-macroglobulin
BMI	Body mass index
BMS	Bare-metal stent
BSA	Bovine serum albumin
CABG	Coronary artery bypass grafting
CAD	Coronary artery disease
cDNA	Complementary DNA
CD40L	CD40 ligand
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CHD	Coronary artey disease
CK-MB	Creatin-Kinase isoform B
COX	Cyclooxygenase
CPK	Creatine phosphokinase
CRP	C-reactive protein
CT	Thrombus on native coronary artery
CV	Coefficient of variation
CVD	Cardiovascular disease
Cx	Circumflex artery
DALY	Disability Adjusted Life Years
DE	Distal embolization

DES	Drug-eluting stents
DIGE	Difference gel electrophoresis
DTT	Dithiothreitol
ECs	Endothelial cells
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked ImmunoSorbent Assay
ESC	European society of cardiology
ESI	Electrospray ionization
eNOS	Endothelial nitric oxide synthase
FFAu	Unbound free fatty acids
FITC	Fluorescein isothiocyanate
FMC	First medical contact
GOT	Glutamic-oxaloacetic transaminase
GP	Glycoprotein
GPT	Glutamic-pyruvic transaminase
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HDL	High density lipoproteins
HDL-C	High density lipoproteins cholesterol
HIV	Human immunodeficiency virus
HMW	High molecular weight
HMWK	High molecular weight kininogen
HRG	Histidine-rich glycoprotein
hs-CRP	High sensitivity CRP
Hsp	Heat shock protein
H_2O_2	Hydrogen peroxide
ICAM-1	Intracellular cell adhesion molecule-1
IEF	Isoelectric focusing
IFN-γ	Interferon- γ
ILT	Intraluminal thrombus
IL-1β	Interleukin-1β

IMA	Ischaemia modified albumin
IPA	Ingenuity pathway analysis
IQR	Interquartile range
IST	In stent thrombosis
IT	Ion trap
IU	International unit
i.v.	Intravenous
IVUS	Intravascular ultrasound
LAD	Left anterior descending artery
LC	Liquid chromatography
LDL	Low density lipoproteins
LDL-C	Low density lipoprotein cholesterol
LMW	Low molecular weight
LVEF	Left ventricular ejection fraction
MACE	Major adverse cardiac events
MALDI	Matrix-assisted laser desorption/ionization
MCP-1	Monocyte chemotactic protein-1
M-CSF	Macrophage colony-stimulating factor
MDD	Minumum detectable dose
MI	Myocardial infarction
MMPs	Matrix metalloproteases
MPO	Myeloperoxidase
MPs	Microparticles
MRI	Magnetic resonance imaging
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
m/z	Mass-to-charge ratio
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
NOS	Nitric oxide synthase
NOX	Nicotinamide adenine dinucleotide phosphate oxidase
NSTEMI	Non-ST segment elevated myocardial infarction

OCT	Optimal cutting temperature
OPUS	Orbofiban in Patients with Unstable coronary Syndromes
oxLDL	Oxidized LDL
$O_2^{\cdot -}$	superoxide
PAI-1	Plasminogen activator inhibitor-1
PAPP-A	Pregnancy-associated plasma protein-A
PARs	Platelet protease-activated receptors
PBS	Phosphate buffered saline
PCI	Percutaneous coronary intervention
PDGF	Platelet –derived growth factor
Pfn-1	Profilin-1
PFP	Platelets free plasma
pI	Isoelectric point
PMF	Peptide mass fingerprint
PMNs	Polymorphonuclear neutrophils
pPCI	Primary percutaneous coronary intervention
PRDX-1	Peroxiredoxin-1
PRDX-2	Peroxiredoxin-2
PRP	Platelets rich plasma
PSGL-1	P-selectin glycoprotein ligand 1
PVDF	Polyvinylidene difluoride
Q	Quadrupole
RBCs	Red blood cells , Erithrocytes
RCA	Right coronary artery
ROC	Receiver-operating characteristic
ROS	Reactive oxygen species
SAP	Serum amyloid P component
sCD40L	Soluble CD40 ligand
SD	Standard deviation
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis
SES	sirolimus-eluting stent

SM	Smooth muscle
SMC	Smooth muscle cells
SOD	Superoxide dismutase
STEMI	ST-segment elevated myocardial infarction
TAFI	Thrombin activable fibrinolysis inhibitor
TF	Tissue factor
TG	Triglyceride
TGF-β	Transforming growth factor beta
TIMI	Thrombolysis in Myocardial Infarction
TNF-α	Tissue necrosis factor- α
TnT	Troponin T
tPA	Tissue plasminogen activator
TOF	Time-of-flight
Total-C	Total cholesterol
TRAP	Thrombin receptor activating peptide
TXA2	Thromboxane A2
UA	Unstable angina
uPA	Urokinase plasminogen activator
VCAM-1	Vascular cell adhesion molecule-1
VSMC	Vascular smooth muscle cells
vWF	Von Willebrand factor
WB	Whole blood
2-DE	Two-dimensional electrophoresis
2-D PAGE	Two-dimensional polyacrylamide gel electrophoresis

I. INTRODUCTION

1. Cardiovascular disease

Cardiovascular disease (CVD) is currently the most common cause of death globally, (Murray et al., 2012). According to the Global Burden of Disease Study the 29.6% of all deaths worldwide (15 616.1 million deaths) were caused by CVD in 2010, (Nichols et al., 2014) which represents more than all communicable, maternal, neonatal and nutritional disorders combined, and doubles the number of deaths caused by cancers (Lozano et al., 2012). 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, 2013).

CVD is also the leading cause of death in Europe **(Figure 1)**. Despite the recent decrease in mortality rates in many countries, CVD is still responsible for over 4 million deaths per year, close to half of all deaths in Europe. Death rates from coronary heart disease (CHD) are generally higher in Central and Eastern Europe than in Northern, Southern and Western Europe. The proportion of all deaths that are attributable to CVD is substantially greater among women (51%) than men (42%) (Nichols et al 2014).

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 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).

Of the total cost of CVD in the European Union, around 54% is due to health care costs, 24% due to productivity losses and 22% due to the informal care of people with CVD.

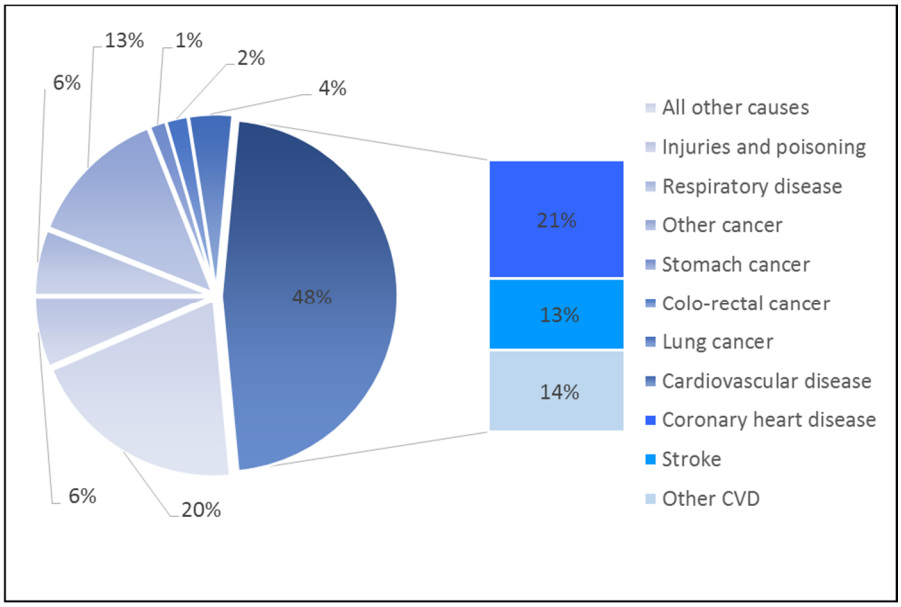

Figure 1. Proportion of all death due to major causes in Europe. Adapted from (Nichols et al 2014) *No data are available for Andorra

2. Acute coronary syndrome

Acute coronary syndromes (ACS) are life-threatening conditions which comprise a group of clinical symptoms associated with acute myocardial ischaemia with or without infarction (Badimon et al., 2002). These conditions are usually the result of a reduction in blood flow associated with a coronary artery becoming narrowed or blocked through atherosclerosis and atherothrombosis. Common pathophysiology of ACS is plaque disruption or erosion. There are three types of ACS, ST-segment elevated myocardial infarction (STEMI), non-STsegment elevated myocardial infarction (NSTEMI) and unstable angina all of which are diagnosed using electrocardiograms and biomarkers.

Particularly, STEMI is a leading cause of mortality in developed countries. The objective of treatment for a patient with ACS is to restore the blood flow to the heart (revascularization) For patients with the clinical presentation of STEMI within 12h of symptom onset and with persistent ST-segment elevation, the European society of cardiology (ESC) Guidelines 2012 recommend that early mechanical (Percutaneous coronary intervention, PCI) or pharmacological reperfusion should be performed as early as possible (Authors/Task Force et al., 2014; Task Force on the management of et al., 2012).

Retrospective, a study on nearly 100,000 patients with STEMI in the US has revealed that, the improvements in door-to-balloon times (from a median 83 minutes in 2005–2006 to 67 minutes in 2008–2009), apparently is not significantly translated into a reduction in in-hospital mortality (Menees et al., 2013), highlighting the fact that door-to-balloon time constitutes a fraction of the total myocardial ischaemia time that begins at the time of the onset of the symptoms and ends after successful reperfusion. In the STEMI guidelines, the concept of “door-to balloon time” was replaced with “first medical contact/FMC-to device time”

(Aggarwal and Menon, 2013). The total ischaemic time, between symptom onset and provision of reperfusion therapy is probably the most important factor to improve clinical outcomes. For this reason, there is an increasing need for the identification of new biomarkers for an early detection of atherothrombotic events such as acute myocardial infarction.

The introduction of intracoronary stents greatly increased the safety and applicability of PCI. However, in-stent thrombosis (IST), the most feared complication after stent implantation, has emerged as an important entity to prevent because of its clinical impact owing to a high risk of myocardial infarction and death (Burzotta et al., 2009; Claessen et al., 2014; De Luca et al., 2013).

3. Atherothrombosis

Atherothrombosis is a systemic arterial disease, originally involving mostly the intima of large- and medium-sized systemic arteries such as carotid, aorta, coronary, and peripheral arteries. Atherothrombosis occurs when a thrombus forms superposed on a disrupted or surface-eroded atherosclerotic plaque. The most common event triggering atherothrombosis is the rupture of unstable plaques (55-65%) (Sakakura et al., 2013) leading to direct contact between the intraluminal blood and the lipid rich core (Badimon et al., 2002; Fuster et al., 2005). In addition, eroded plaques, that present small or absent lipid core, may also lead to occlusive thrombosis (30-35%) (Fuster et al., 2005; Sakakura et al., 2013). Atherothrombosis is clinically manifested as coronary heart disease (myocardial infarction and angina), peripheral arterial disease and cerebral ischaemia (Leys, 2001; Viles-Gonzalez et al., 2004) **(Figure 2)**. Atherosclerosis, mechanism underlying atherothrombosis, is a systemic disease that starts early in childhood and progresses asymptomatically through the adult life until dramatically changes its course when complicated by thrombosis. Atherosclerosis is characterized by the progressive accumulation of lipids, cells and other blood components within the wall of medium and large-sized arteries (Viles-Gonzalez et al., 2004). This process can be summarized in different phases, from early lesions to plaque rupture. In the early phases endothelial dysfunction, cholesterol transport, innate and adaptive immune response and calcification mechanisms. , play an important role. For the advanced phases, remodeling, vasa vasorum neovascularization, and mechanisms of plaque rupture are key steps.

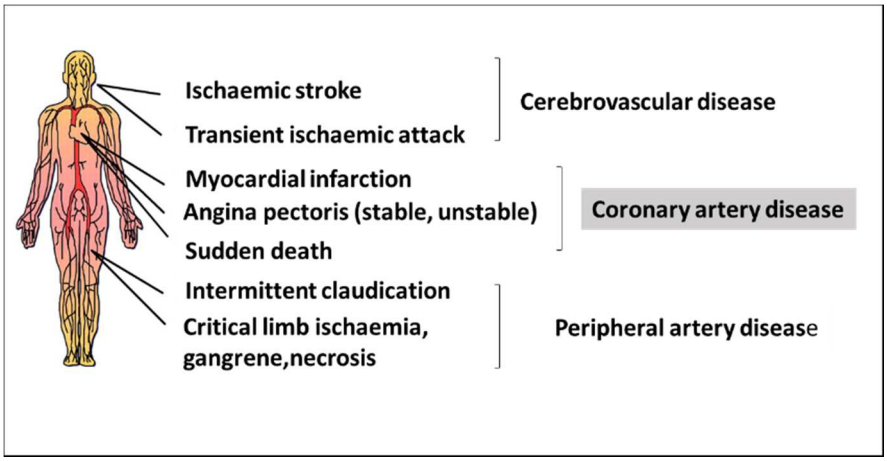

Figure 2. Clinical manifestation of atherothrombosis Adapted from Vile-Gonzalez et al (2004)

3.1. Pathophysiology of atherothrombosis

3.1.1 Initiation and progression of atherosclerotic lesion

The earliest changes that precede the formation of atherosclerotic lesions take place in the vascular endothelium. Endothelial cells (ECs) modulate their functional capacity in response to different stimuli and modify its functional status to contribute to the homeostasis of the vascular wall (Michiels, 2003).

Under physiological conditions, endothelial cells are resistant to adhesion and aggregation of inflammatory cells and platelets, promote fibrinolysis and control vascular tone. These antiatherogenic properties are mainly driven by the endothelial nitric oxide synthase (eNOS) enzyme (Loscalzo, 2001). Nitric oxide (NO) synthesis and release prevents platelet activation, induces vasodilatation and blocks the expression of nuclear-factor kappa B (NF-κB) -regulated genes. NF-κB comprises a family of redox-sensitive transcription factors recognized as critical regulators of the expression of many proinflammatory genes, such as those encoding

for adhesion molecules [vascular cell adhesion molecule-1 (VCAM-1), intercellular cell adhesion molecule-1 (ICAM- 1) and E-selectin], chemokines, cytokines, and enzymes producing inflammatory mediators, such as cyclooxygenase (COX)-2. Under pathological conditions, such as risk factors and mechanical injury, the endothelium becomes dysfunctional which is characterized by decreased NO synthesis, thus facilitating vessel wall entry and oxidation of circulating lipoproteins, monocyte internalization, smooth cell proliferation and extracellular matrix deposition, finally leading to a prothrombotic state within the vessel lumen (Ignarro and Napoli, 2004; Voetsch et al., 2004).

Early lesions are called *fatty streaks* and consist in subendothelial accumulation of low density lipoproteins (LDL) and infiltration of immune cells, such as macrophages, T cells and mast cells **(Figure 3)**

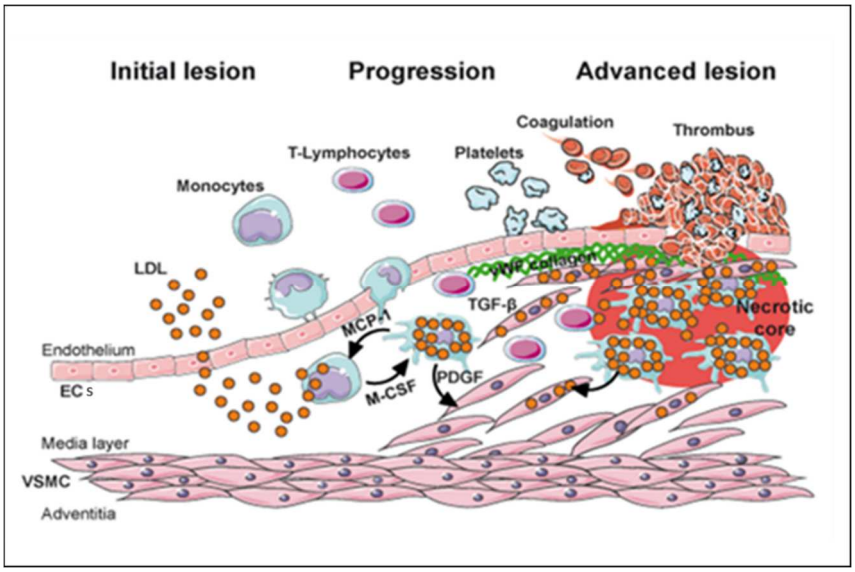

Figure 3. Atherosclerotic plaque formation. ECs, endothelial cells; VSMC, vascular smooth muscle cells, LDL, low density lipoproteins; M-CSF, macrophage colony-stimulating factor; MCP-I, monocyte chemotactic protein-1; PDGF, platelet-derived growth factor; TGF-β, tranforming growth factor beta; vWF, von Willebrand factor. Adapted from Badimon, L. et al., (2009).

Intravascular lipid accumulation is increased when the levels of circulating LDL are elevated. LDL penetrate through the arterial endothelium into the intima, there the interaction with proteoglycans favours their retention in the vessel wall (Boren et al., 1998). Once in the subendothelial space, LDL suffer modifications (oxidation, aggregation, glycation) and become more atherogenic, triggering a cascade of pro-inflammatory processes, which contribute to 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 (Collins et al., 2000; Dong et al., 1998; Nakashima et al., 1998) are important for adhesion of leukocytes and monocytes to endothelium, promoting their migration into the subendothelial space. Furthermore the presence of molecules such as macrophage colony-stimulating factor (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 et al., 2002). Activated macrophages, T-cells and ECs continue releasing growth-regulatory molecules and cytokines that contribute to the progression of atherosclerosis.

The progression of the lesion involves the migration of vascular smooth muscle cells (VSMC) from the media layer to the intima layer and their proliferation in response to mediators such as platelet-derived growth factor (PDGF). VSMC produce extracellular matrix (ECM) macromolecules such as collagen, elastin and proteoglycans and form a fibrous cap that covers the plaque (Katsuda and Kaji, 2003). VSMC phenotype and ECM composition are key determinants for plaque progression. Moreover VSMC and macrophages within the atherosclerotic intima internalize modified LDL evolving into foam cells that will contribute to the evolution of the plaque and the formation of the necrotic

core, a key process in the progression of the atherosclerotic plaques and their evolution to unstable plaques with high risk of rupture (Ross, 1999; Virmani et al., 2000) **(Figure 4)**.

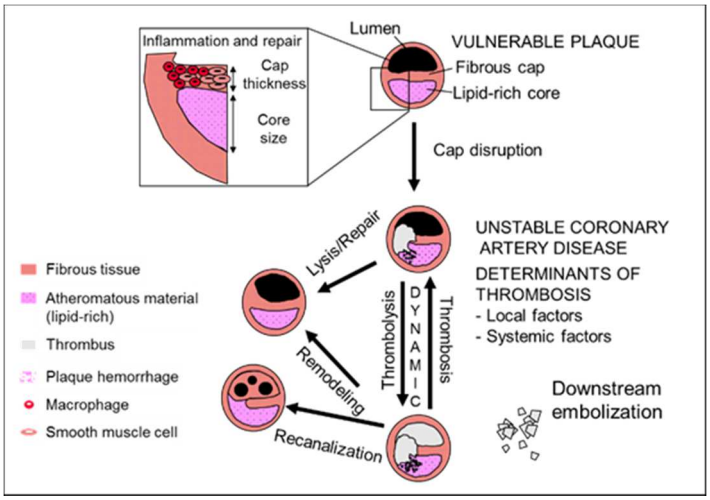

Figure 4. Determinants of plaque vulnerability. Adapted from Fuster et al (2005).

3.1.2 Complication of advanced lesion

The main complications of atherosclerosis are those caused by the formation of an occlusive thrombus as a consequence of plaque rupture or erosion (Badimon et al., 2009), when the sub-endothelial space containing tissue factor, collagen, and von Wilembrand factor (vWF) or the plaque's necrotic core are exposed to blood components (Lusis, 2000). Thrombi formed can interrupt blood flow locally or embolize and lodge in distal arteries (Libby et al., 2011).

Mayor components of atherothrombotic plaques are 1) connective tissue extracellular matrix, including collagen, proteoglycans, and fibronectin elastic fibers; 2) crystalline cholesterol, cholesteryl esters, and

phospholipids; 3) cells such as monocyte-derived macrophages, T-lymphocytes, and smooth-muscle cells; and 4) thrombotic material with platelets and fibrin deposition. These above described components vary in proportion among different plaques, thus giving rise to a heterogeneity or spectrum of lesions with different pathophysiological patterns. Plaques susceptible to erosion are those with loss and/or dysfunction of the luminal endothelial cells. There is usually no additional defect or gap in the plaque, which is often rich in smooth muscle cells and proteoglycans. Plaques susceptible to rupture (vulnerable plaques) **(Figure 4)** 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 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, free 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 (Libby, 2002; Lusis, 2000). In addition, the combination of pro-inflammatory cytokines, such as interleukin-1β (IL-1β), tissue necrosis factor-α (TNF-α), and IFN-γ induce apoptosis of VSMC promoting plaque rupture (Rudijanto, A., 2007) **(Figure 3)** Platelets also play a fundamental role in both the formation of the atheromatous plaque and the thrombotic process resulting in the clinical presentation of acute atherothrombotic events, following plaque rupture.

3.2. Risk factors

Atherothrombosis is considered a dynamic, progressive and multifactorial disease, which develops as a consequence of the interaction between genetic and environmental factors. These conditions are called 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 modified (World Health Organization, 2011,) **(Figure 5)**.

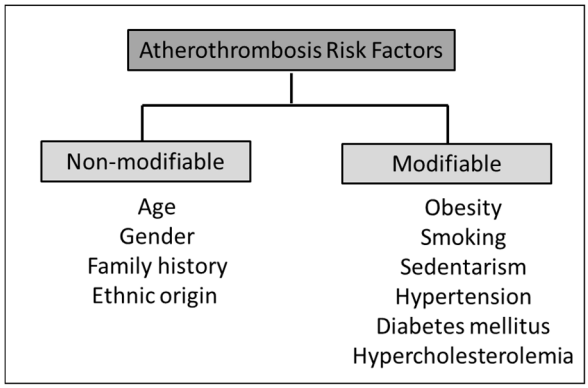

Figure 5. Major risk factors for atherothrombosis.

Among the main modifiable risk factors, we find:

Hypercholesterolemia: several studies, including INTERHEART (Yusuf 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 et al., 2009). 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: is 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 et al., 2008).

Smoking: a large number of epidemiological, clinical and laboratory studies have 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, 2003). Smoking is estimated to cause nearly 9% of all CVD (World Health Organization, 2009).

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) (Kumanyika et al., 2008).

4. Arterial Thrombosis

Arterial thrombi that develop at site of plaque rupture or erosion are dynamic and evolve in stage. When the thin fibrous cap is disrupted, collagen and tissue factor (TF) become exposed to flowing blood, which triggers the accumulation and activation of platelets and converts fibrinogen to fibrin, thereby initiating thrombus formation. Thrombus in acute atherothrombotic events can be either partially or completely occlusive. The former is primarily composed of platelet aggregates, and the latter of platelet aggregates and a fibrin-rich clot that is generated by the coagulation cascade. The presence of those thrombi may block blood flow and reduce oxygen supply (ischaemia) in the affected arteries, resulting in the clinical manifestations of atherothrombotic diseases (Yunoki et al., 2013).

In rare cases, ACS may have a non-atherosclerotic aetiology such as arteritis, trauma, dissection, thromboembolism, congenital anomaly, cocaine abuse or complication of cardiac catheterization (Fuster et al., 2005).

4.1. Cellular components of the thrombi

Although fibrin forms the core matrix of thrombi, their structure also depends on the cellular elements embedded in the network. Platelets are essential in the initial stages of thrombus formation. They adhere and aggregate at sites of blood vessel wall injury and then serve as a surface for coagulation reactions, which determine the final structure of the fibrin clot. Leukocytes form mixed aggregates with platelets and thus influence the structure of thrombi. After activation they secrete different proteases (elastase, cathepsin G, and matrix metalloproteinases) that further enhance von Willebrand factor-dependent platelet adhesion (Wohner, 2008).

4.1.1 Platelets

The first step in thrombus formation involves platelet adhesion, activation and aggregation **(Figure 6)**. Platelets roll, adhere, and spread on the subendothelial collagen matrix to form an activated platelet monolayer. During the rolling phase, adhesion is mediated by interaction between the glycoprotein (GP) Ib/V/IX receptor complexes on the platelet surface with vWF and between the GP VI and GP Ia proteins with collagen at sites of vascular injury (Savage et al., 1996). These interactions lead to the adhesion of flowing platelets to the extracellular matrix, thereby enabling the binding to the collagen receptor. Interaction between vWF and GP Ib/V/IX is required for the initial adhesion of platelets to the subendothelium under conditions of high shear such as in arterioles of the normal circulation or large arteries with pathological lumen restrictions. Binding of platelet GPVI to collagen plays a central role in platelet activation through multiple intra cytoplasmic pathways (i.e., elevation of intracellular Ca2+, phosphoinositide metabolism, phosphorylation of cytoplasmic and nuclear proteins). Platelets become activated after the initial tethering, which allows their irreversible adhesion to the surface and the binding of plasma vWF and fibrinogen to the integrin αIIbβ3 (Ruggeri et al., 1999; Savage et al., 2001) **(Figure 6)**.

The increase in the concentration of free calcium results in a number of structural and functional changes of the platelet. Morphologically, the platelet changes dramatically from a disc to a spiny sphere. The granules in the platelet are centralized and their contents are discharged into the lumen of the open canicular system, from which they are released to the exterior. Long membrane projections brought about by a shape-change reaction allow platelets to interact to form aggregates. Shape change is mediated by the platelet cytoskeleton, composed by an organized network of microtubules and actin filaments and a number of associated proteins,

linked to a variety of platelet signaling molecules (Hartwig, 1992). Thus platelet shape change results in extensive reorganization of the cytoskeleton network, polymerization of actin, and myosin light chain phosphorylation; these responses vary in a time- and stimulus-dependent manner (Daniel et al., 1984; Mannucci and Sharp, 1967). Secretion of dense and alpha granules, containing several soluble agonists, especially thromboxane A2 (TXA2), adenosine diphosphate (ADP), and serotonin, which cooperatively promote further activation, recruitment of additional platelets from the circulation and amplification of the signal for thrombus formation (Badimon et al., 2009).

In addition to the synthesis and release of soluble agonists, platelets provide a catalytic surface for the assembly of coagulation complexes necessary for thrombin generation. Thrombin is among the most potent stimulators of platelets through proteolytic cleavage and activation of platelet protease-activated receptors (PARs), specifically PAR1 and PAR4 on human platelets (Coughlin, 1999; Sambrano et al., 2001).

A final step is the formation of platelet aggregates. This event is mediated by the binding of adhesive proteins to the ligand receptive form of the GPIIb/IIIa receptor. The receptive form of GPIIb/IIIa also binds fibrinogen molecules, which form bridges between adjacent platelets and facilitate platelet aggregation. Other adhesive GPs, including fibronectin, vWF, and vitronectin, also bind to these receptors. As a result of these multiple reactions, platelets become aggregated into a hemostatic plug. P-selectin induces formation of platelet monocyte aggregates, which can stimulate the release of inflammatory mediators. Platelets interact with neutrophils through P-selectins and β2 and β3 integrins. In resting platelets, selectins are stored in the membrane of α-granules and upon activation they are redistributed to the platelet surface and initiate adhesion to leukocytes. Besides capturing neutrophils, platelets also secrete neutrophil and endothelial activators inducing production of

inflammatory cytokines and release of neutrophil granules including proteases (serine proteases and matrix metalloproteases) (Wohner, 2008).

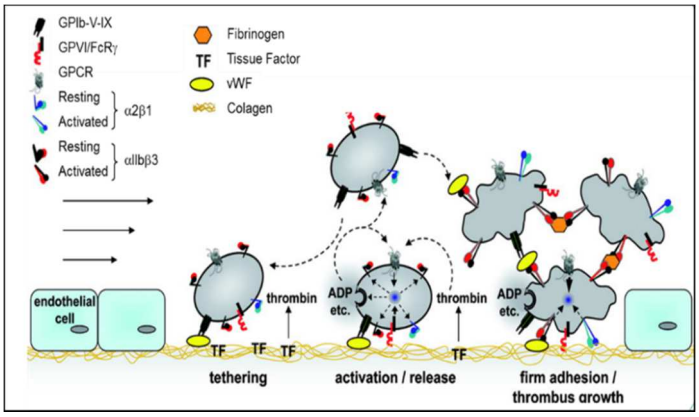

Figure 6. Platelet adhesion and aggregation Adapted from Varga-Szabo et al. (2008)

4.1.2. Leucocytes

Blood circulating, leukocytes are in a resting, low-adhesive state, Stimulatory signals mediated by selectins which are exposed on the activated endothelial surface, promote the rolling of leukocytes followed by their adhesion to allow transendothelial migration.

Specifically β2-integrins are the most important players in this process. The major counter-receptors for the β2-integrin isotypes are the intercellular adhesion molecules (ICAMs). vWf recruits leukocytes by mediating both rolling and stable adhesion of the cells. Independently of their interaction with platelets, leukocytes can directly bind to vWf through P-selectin glycoprotein ligand 1 (PSGL-1). Circulating neutrophils are captured by the selectins presented by activated endothelium, which trigger the fusion of secretory vesicle membranes with the plasma membrane and the up-regulation of neutrophil β2-

integrins and chemotactic receptors (Pendu et al., 2006; Zarbock et al., 2007).

4.1.3. Red blood cells

Erythrocytes (RBCs) predispose to thrombosis by altering blood viscosity. A high hematocrit increases the residence time of circulating platelets and coagulation factors near the activated endothelium, thereby increasing their collision with the vessel wall (Goldsmith et al., 1995). RBCs also have a chemical signaling role in hemostasis. They promote platelet aggregation and degranulation by releasing ATP and ADP. RBCs contribute to the activation of the coagulation cascade by losing their phospholipid asymmetry and serve as a procoagulant surface (Zwaal and Schroit, 1997).

Moreover, RBCs influence the structure of the fibrin network. Thus, fibrin has larger pores when it is formed in the presence of. Therefore, RBCs may determine the physical properties of fibrin, affecting profoundly the course of its dissolution (Weisel and Litvinov, 2008).

4.2. Blood coagulation

Activation of the coagulation cascade occurs promptly upon vascular injury. Proteins of the coagulation cascade generally circulate in plasma as inactive zymogens that are converted to active coagulation factors on the surface of activated platelets. A fibrin monolayer is the first circulation-related response to atherosclerotic plaque damage, suggesting a rapid onset of TF-dependent thrombin formation close to the damaged vessel wall (Celi et al., 2003; Mailhac et al., 1994; Meyer et al., 1994; Toschi et al., 1997). TF expression, mainly by endothelial cells and macrophages, increases in conditions of inflammation or injury, occurring during plaque destabilization and rupture. TF interacts with factor VIIa, forming an

activating complex which binds and activates factor X and factor IX amplifying the coagulation pathway (Badimon et al., 1999; Toschi et al., 1997). The last phase of the coagulation cascade is fibrin formation by thrombin. Thrombin interacts with fibrinogen, forming fibrin and inducing activation of factors V, VIII and XII that amplify the coagulation cascade, finally leading to clot formation and cross-linking with factor XIIIa. Thrombin formed during the initial phases of the coagulation process is significantly increased by positive feedback and platelet activation.

The implication of the extrinsic coagulation pathway in atherosclerosis has been demonstrated by a large number of studies but nowadays there is an emerging body of evidence suggesting the involvement of intrinsic coagulation proteins in the etiology of cardiovascular disease (Siegerink et al., 2012)FXII modulate fibrin clot structure, independently of thrombin generation (Konings et al., 2011) **(Figure7).**

4.3. Spontaneous fibrinolysis

The fibrinolytic system is involved in the lysis of clots and also acts to restrict thrombus propagation beyond the site of injury, as a counterregulatory mechanism of the coagulation cascade. The efficacy of fibrinolysis is demonstrated by the spontaneous reperfusion that occurs in about 30% of patients with myocardial infarction (DeWood et al., 1983) (Rentrop et al., 1989; Stone et al., 2001)Indeed spontaneous lysis of the thrombus does occur not only in unstable angina but also in acute myocardial infarction (AMI) (Fuster et al., 2005).

Fibrinolysis is activated simultaneously with fibrin formation. Plasminogen is released from the liver to the circulation and converted to plasmin (active form) by tissue plasminogen activator (tPA) and urokinase (uPA) (Abbate et al., 2012). Plasmin has great affinity for fibrin and, when incorporated into the clot, degrades fibrin and thereby promotes

proteolysis of the thrombotic substrate. However, in stable thrombi, cross-linking of fibrin masks tPA-binding sites, thus protecting fibrin from degradation. Moreover, tPA and uPA are inhibited by plasminogen activator inhibitor-1 (PAI-1) and activated by phospholipid membrane components released at sites of vascular injury. In addition, increased levels of circulating α2-antiplasmin block plasmin, leading to enhanced thrombus resistance to proteolysis. Thrombin activatable fibrinolysis inhibitor (TAFI) also contributes to the overall reduction in fibrinolysis. Thrombin may activate TAFI but the rate of activation is increased by the thrombin– thrombomodulin complex. Once activated, TAFI downregulates fibrinolysis by the removal of C-terminal lysines from fibrin. As a consequence the binding of plasminogen and t-PA to the fibrin clot is inhibited (Foley et al., 2013) **(Figure7)**.

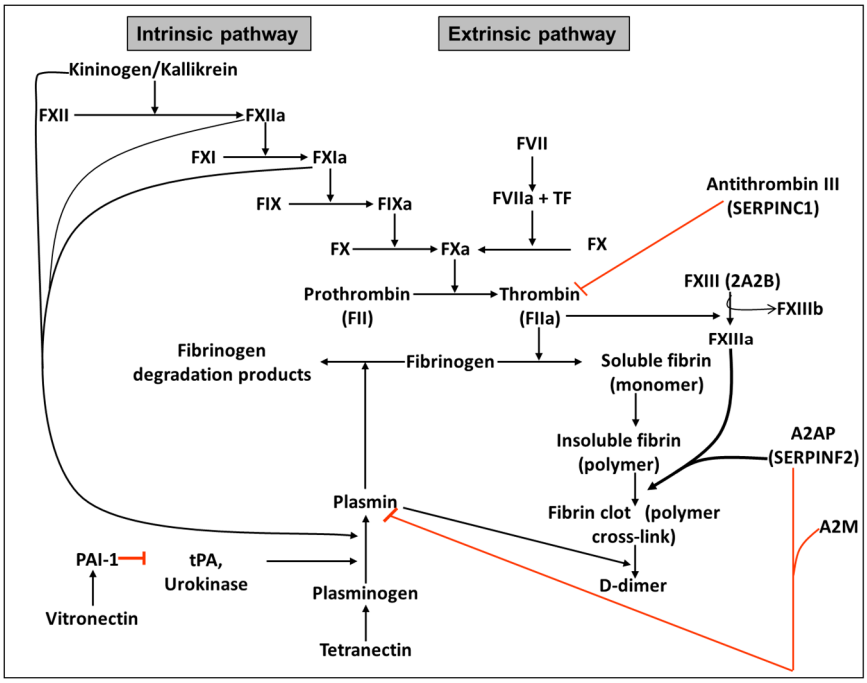

Figure 7. Coagulation and fibrinolytic system

5. Thrombus composition in native coronary arteries

Coronary thrombosis is a dynamic process that, in addition to the atherosclerotic vessel wall, includes plasma proteins and blood cells with a direct influence on its morphology, evolution and tentative resolution. Thrombosis has been largely studied in model systems, *in vitro* or *ex vivo*, in peripheral vessel and microcirculation or *post-mortem* (Kramer et al., 2010; Sartori et al., 2011; Shenkman et al., 2012; Vilahur et al., 2004; Virmani et al., 2006). Nowadays aspiration of coronary thrombus at PCI allows studying *in vivo* retrieved samples of ongoing thrombosis in humans. Up to now, available information is mainly based on histopathological and immunohistochemical analysis and only in the recent years other techniques as scanning electron microscope and proteomics tools have been applied (Alonso-Orgaz et al., 2014; Silvain et al., 2011).

The most accepted classification of coronary thrombus based on their composition, have been done using histopathological techniques which have allowed to determine the thrombus age (Henriques de Gouveia et al., 2002; Murakami et al., 1998; Rittersma et al., 2005). Thrombi composed by alternating layers of platelets mixed with fibrin and intact granulocytes and erythrocytes are classified as early thrombi (<1 day). The lytic thrombus (1 to 5 days) present a homogenization of structural elements and degraded inflammatory cells. The organized thrombus (<5 days) is composed by a basal in-growth of smooth muscle cells (SMC) and or EC with or without accumulated proteoglycan matrix. Moreover, the presence of activated TF has been detected in coronary thrombi of STEMI patients (Palmerini et al., 2013).

5.1. Coronary thrombus composition and ischaemia time

Up to now, thrombus age, considered as the time frame between the initiations of thrombus formation and the lumen occlusion, has been related to thrombus composition as mentioned above. However in the recent years the time of ischaemia defined as the time from the symptom onset to clinical intervention (PCI) has been considered as a more relevant parameter in the study of the thrombus composition. Recent studies, based on electron microscopy (Silvain et al., 2011), have shown a dynamic evolution both in fibrin and cell composition of the thrombi during STEMI. These results suggest that the time of ischaemia is a key determinant in the type of interaction between the thrombus and the vessel and, hence, on the progression of the underlying vascular disease. Studies based on immunohistological analysis have also supported the influence of the ischaemia elapsed time in the platelet content of the coronary thrombus showing platelets as the dominant cell component of thrombi in the early phase of STEMI (Nagata et al., 2004)

However the dynamic evolution of intracoronary thrombus growth and composition in ACS is still poorly known. Up to now, there is a lack of studies analyzing the protein pattern of coronary thrombi in relation to their dynamic evolution.

It is extremely important to elucidate the evolution of thrombus composition during the ischaemia to improve patients' prognosis because in this time-frame the clinical manifestation an the consequent myocardial damage occurs.

5.2. Coronary thrombus composition and clinical outcome

Several studies have investigated the relation between thrombus composition and patients' clinical outcome. Erythrocyte component in coronary thrombi has been linked with the presence of distal embolization (DE) during PCI(Fokkema et al., 2009) and erythrocyte-rich thrombi are frequently observed in patients with impaired myocardial reperfusion (Yunoki et al 2012). High neutrophil density in the aspirated thrombi has been associated with impaired coronary microcirculation and left ventricular dysfunction (Arakawa et al., 2009). Furthermore the presence of CD34- positive primitive cells in intracoronary thrombi positively correlates with restenosis (Iwata et al., 2010). In addition the presence of organized thrombus, which showed ingrowth of smooth muscle cells, is an independent predictor of long term mortality (Kramer et al., 2008).

Recently, Barba and coworkers, using magnetic resonance imaging (MRI) have reported a correlation between the characteristics of the thrombi and the myocardial perfusion status as measured with ST segment resolution in the ECG in STEMI patients after PCI (Barba et al., 2011).

Although association between coronary thrombus component and clinical outcomes has been investigated, no exhaustive studies have been performed on the relation between protein composition of the coronary thrombus and patient's clinical outcome.

6. Coronary stent thrombosis

In stent thrombosis (IST) is a rare but usually catastrophic event, leading to acute vessel closure, frequently associated with ST-elevation MI or sudden cardiac death (Daemen et al., 2007; Holmes et al., 2010).

Thrombosis associated to coronary stenting is dependent on several factors related to patient, lesion, and procedural, and post-procedural intervention.

Pathophysiological mechanisms leading to IST are: 1) activation of the extrinsic pathway of the coagulation cascade induced by exposure of blood to prothrombotic subendothelial constituents, 2) the characteristics of the stent struts, and/or polymer material leading to 3) persistent slow coronary blood flow and low shear stress; 4) inadequate pharmacological suppression of platelet activation; and 5) the presence of a systemic prothrombotic state (Claessen et al., 2014). Mainly mechanical mechanisms have been proposed to explain the high risk of IST in STEMI. Stent strut penetration of an underlying necrotic core may augment inflammation and fibrin deposition and inhibit neointimal growth, resulting in uncovered stent struts (Costantini et al., 2004).

Acute, subacute, late and very late IST have been defined as IST occurring within 24 hours, between 24 h and 30 days, between 30 days and one year, and longer than one year from pPCI, respectively (Cutlip et al., 2007). Very late drug-eluting stents (DES) thrombosis is associated with histopathological signs of inflammation and intravascular ultrasound (IVUS) evidence of vessel remodeling. Compared with other causes of myocardial infarction (MI), eosinophilic infiltrates are more common in thrombi harvested from very late DES thrombosis, particularly in sirolimus-eluting stent (SES), and correlate with the extent of stent malapposition (Cook and Windecker, 2009).

Few studies, based mostly on histochemical analysis (Nishihira et al., 2010), have compared native with stent coronary thrombi suggesting no differences between both groups. Up to now, however, there is a lack of consistent information on differences in composition between stent-induced and in native coronary arteries formed thrombus.

6.1. Predictors of stent thrombosis

Predictors of IST have been studied in registries and post hoc analyses from clinical trials, (van Werkum et al., 2009) and may be categorized into those related to the (1) **stent** (hypersensivity to drug coating or polymer, incomplete endothelialisation, stent design); (2) **patient** (diabetes mellitus, high C-reactive protein (CRP) levels, renal failure, specific genetic background); (3) **lesion characteristics** (lesion length, implantation of multiple stents, small vessel diameter); and (4) **procedural intervention** (inadequate stent expansion, incomplete stent apposition, stent deployment in necrotic core) (Holmes et al., 2010).
Despite the numerous possible risk factors, the most common and consistent predictors of stent thrombosis are early antiplatelet therapy discontinuation, extent of coronary disease, and stent number/length (D'Ascenzo et al., 2013).

6.2. Oxidative stress after coronary artery intervention

Oxidative stress, characterized by an imbalance between the generation of reactive oxygen species (ROS) and the capacity of the intrinsic antioxidant defense system, has been implicated in the different stages of the pathogenesis of cardiovascular diseases. ROS are produced by various biological systems, including uncoupling of nitric oxide synthase (NOS), nicotinamide adenine dinucleotide phosphate oxidase (NOX),

mitochondrial uncoupled respiration, xanthine oxidase, and cyclooxygenase (Bedard and Krause, 2007; Liu and Huang, 2008; Turrens, 2003).

Coronary stent interventions are associated with increased vascular levels of reactive oxygen species in conjunction with altered endothelial and smooth muscle cell function (Juni et al., 2013; Kochiadakis et al., 2010).

After bare-metal stent (BMS) deployment, there is recruitment of inflammatory cells, such as neutrophils. These cells produce ROS, contributing to the vascular pathological condition (Ohtani et al., 2006). Moreover, BMS implantation increases expression of NOX subunits leading to additional superoxide ($O_2^{\cdot -}$) production. This pathway is considered to be the main ROS-generating pathway during coronary stent implantation. $O_2^{\cdot -}$ anions induce VSMC and fibroblast proliferation and migration, leading to neointimal growth. The proliferating neointimal cells produce ROS, leading to more accumulation of the radicals that may react with NO, resulting in decreased NO bioavailability with subsequent endothelial dysfunction (Chaabane et al., 2013; Kochiadakis et al., 2010).

Furthermore, DES are a double-edged sword. On one hand, they prevent IST by elution of an antiproliferative drug that inhibits VSMC proliferation and migration (Chaabane et al., 2013). On the other they contribute to vascular abnormalities by induction of ROS in the vessel wall. Specifically NOX and mitochondria play a fundamental role in the vascular ROS generation after DES deployment, leading to a reduced NO bioavailability and endothelial dysfunction (Jabs et al., 2008). The endothelial dysfunction ultimately leads to the development of in-stent thrombosis.

7. Biomarkers in acute coronary syndromes

The National Institutes of Health define a biomarker as “a characteristic that is objectively measured and evaluated as an indicator of normal biological process and pathogenic process”.

In the past, diagnosis of ACS in the emergency department was mainly based on clinical symptoms and electrocardiographic findings, and markers of tissue damage and necrosis were used to support clinical suspicion.

New biomarkers are necessary for a better diagnostic and management of patients that present chest pain. Over the last few years, the role of markers has taken up increasingly more space in non-life-threatening conditions.

That is the reason why in the recent years, a large body of research has focused on the search for biomarkers for early detection of ACS (Kossaify et al., 2013; Mueller, 2014; Tousoulis et al., 2012).

Biomarkers can be classified as **risk stratification biomarkers** (screening for subclinical disease), **diagnostic biomarkers** (recognizing overt disease), and **prognostic biomarkers** (predicting future disease course, including recurrence and response to therapy, and monitoring efficacy of therapy) (Aldous, 2013; Gerszten et al., 2011) **(Figure 8)**.

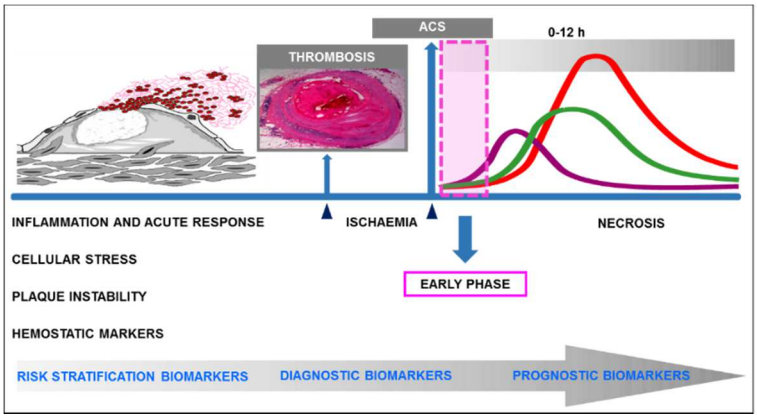

Figure 8. Biomarkers in ACS

Necrosis

Until now the only group of accepted biomarkers for the diagnosis of ACS are cardiac troponins (Daubert and Jeremias, 2010).The troponin T and I subunits are expressed only in cardiac muscle, which allows these biomarkers to be highly specific for myocardial damage. After myocardial cell death, cardiac troponin levels remain detectable for days (4–7 days for subunit I and more than 10–14 days for subunit T). However, detection of troponin in the blood could be delayed in myocardial injury, given that cellular necrosis typically requires 2–4 hours to take place after an ischemic event (Aldous et al., 2012). Despite the development of new high-sensitivity troponin assays to reduce the threshold for early diagnosis of myocardial infarction (Keller et al., 2009; Melanson et al., 2007; Reichlin et al., 2009), troponin elevation has been detected in several other condition different from ACS presentation (Gupta and Alagona, 2008). Therefore, the use of high-sensitive troponins for the early detection of MI has shown controversial results due to its lack of specificity (Patil et al., 2011).

Another necrosis marker is Creatin-Kinase isoform B (CK-MB), an enzyme that catalyzes the conversion of creatine to phosphocreatine consuming ATP and releasing energy for muscle contraction. For many years, CK-MB activity was used in the diagnosis of MI because of its early release pattern (2–4 hours after cardiac injury). Given the prolonged half-life of CK-MB, current guidelines recommend CK-MB mass dosage in combination with other more specific markers (such as cardiac troponin and myoglobin) in the evaluation of patients with suspected reinfarction soon after acute MI or cardiac surgery (Hamm et al., 2011; Taylor, 2012) **(Table 1)**.

Ischaemia

In order to improve the patient clinical outcome, it is important to make a diagnosis of myocardial ischaemia in advance, or in the absence, of the occurrence of irreversible damage (Morrow et al., 2003). As the explicit goal, only a marker that precedes necrosis and permits the prevention of its consequences can meet clinical needs (Morrow et al., 2003).

A marker of cardiac ischaemia could also be valuable in distinguishing acute MI from non-ischaemic causes of myocardial necrosis that lead to the increase in cardiac troponins.

The increase in free fatty acids unbound to albumin (FFAu) in the blood with acute myocardial ischaemia has been evaluated for the early identification of cardiac ischaemia (Apple et al., 2002). FFAu elevations occur early before the raising of traditional markers of cardiac necrosis (Kleinfeld et al., 1996) (Kleinfeld et al 2002; Adams et al 2002). Moreover FFAu has shown more that 90% of sensitivity in the diagnosis of myocardial ischemia at admission (Kleinfeld et al., 1996; Panteghini, 2004) **(Table 1)**.

Albumin is a plasma protein produced by hepatocytes, and is the most important protein regulating blood pressure and transporting of several molecules and ions. It has been shown that, during acute myocardial ischemia, the N-terminal site of serum albumin is altered by free radicals, reducing its cobalt binding capacity. This reduction is the basis of the albumin cobalt-binding test for laboratory determination of ischaemia-modified albumin (Bar-Or et al., 2001; Bar-Or et al., 2000; Panteghini, 2004). However, increases in IMA can also be observed during ischaemia related to the injury of organs other than myocardium. In addition, a deletion defect of the N-terminus of albumin has been documented in a

non-ischaemic individual that was responsible for reduced cobalt binding and, consequently, for false-positive test results. Thus, the specificity of the measurement of IMA for myocardial ischaemia requires additional investigation (Bhagavan et al., 2003) **(Table 1)**.

Hemostatic markers

The most important hemostatic biomarker is D –Dimer, a specific product of degradation of cross-linked fibrin. It appears as a result of the balance between the ongoing process of thrombus formation and the dissolution that occurs at the site of active plaques in ACS preceding myocardial cell damage and the release of protein content (Hunt et al., 1985). It is detectable early and remains elevated for days after an ischemic event. The role of D-dimer in ACS is not totally clear and there are controversial results. Gurfinkel et al. described normal levels of D-dimer in patients with MI (Gurfinkel et al., 1995). In contrast there are different authors that have reported significantly higher plasma D-dimer concentrations in patients with ischemia compared with non-ischemic patients (Bayes-Genis et al., 2000; Orak et al., 2010). Besides, Lee et al proposed D-dimer not only as a diagnostic biomarker but also as a useful clinical tool for predicting complications of MI (Lee et al., 1997) **(Table 1)**.

Inflammatory markers

Inflammation plays a key role in the development and progression of atherosclerotic lesion (Libby, 2002). Starting from this concept, many inflammatory markers have been proposed as ACS markers over time. Among them, C-reactive protein (CRP), myeloperoxidase (MPO), soluble CD40 ligand (sCD40L), and white blood cells (WBC) count have been widely studied (Hatmi et al., 2010; Tousoulis et al., 2012) **(Table 1)**.

CRP is an acute-phase protein of the innate immune system. The use of high sensitivity CRP (hs-CRP) assays have demonstrated a strong correlation as an independent risk factor for future cardiac events (Heeschen et al., 2000; Koenig, 2013; Lindahl et al., 2000; Liuzzo et al., 1994; Morrow et al., 1998). Specifically, CRP levels (> 10mg/L) are consistent with an increased risk of a cardiovascular event and may reflect a silent inflammatory process in patients without evidence of other causes of inflammation. (Riker et al 2004) Different studies have suggested that hs-CRP predicts new coronary events in patients with ACS and unstable angina, and risk of restenosis after revascularization procedures, independently of troponin T (TnT)(Heeschen et al., 2000; Lindahl et al., 2000; Morrow et al., 1998).
Furthermore, high levels of hs-CRP have been described as a poor prognostic factor of long-term cardiovascular outcomes in STEMI patients with a long ischaemia time (> 6 hours) (Kim et al., 2013) **(Table 1)**.

CD40 ligand (CD40L) is a cellular cytokine expressed by activated platelets, stimulated lymphocytes, endothelial cells, smooth muscle cells, and macrophages. It is released into the peripheral circulation as sCD40L after being cleaved by proteases. CD40L has a potential role as a proinflammatory and procoagulant mediator. Therefore it has been investigated as a prognostic biomarker of atherothrombotic risk. However, there is still poor evidence for its use in clinical practice as a diagnostic biomarker and controversial evidence for its use as a prognostic biomarker (Jefferis et al., 2011; Plaikner et al., 2009) **(Table 1)**.

MPO is a lysosomal enzyme, released from neutrophilic granules, monocytes, and some subtypes of tissue macrophages. Patients with ACS have a reduction in intracellular neutrophil MPO across both the left and

right coronary beds, suggesting that widespread neutrophil activation may underlie ACS. In a prospective study patients with ACS and elevated MPO levels had an increase in the risk of death or MI at 72 hours, (Baldus et al., 2003). In this study MPO levels were not related to TnT, CRP, or sCD40L, suggesting that MPO provides independent prognostic information distinct from other established biomarkers. The most important use of MPO may be early risk stratification of patients with non-ST-elevation MI (NSTEMI)(Armstrong et al., 2006). Prolonged ischaemia induces a greater inflammatory response than short periods of ischemia with an increase of MPO (Tanaka et al., 2005) **(Table 1)**.

Another, even simpler and universally available marker of inflammation is the WBC count. Increases in WBC count have been associated with the development of coronary artery disease (CAD), with an increased event rate in stable CAD, and a higher 30 day mortality in the setting of AMI (Cannon et al., 2001; Mueller et al., 2003) **(Table 1)**.

However, inflammatory markers are not specific for ACS. Thus any coexisting chronic disease could modify the basal body inflammatory level, resulting in very low specificity (Yayan, 2013). Because of these characteristics, all these markers of inflammation showed no added value in early stratification of MI. Nevertheless, because of their sensitivity these markers have been proposed as prognostic and evolution-monitoring markers (Rashidinejad et al., 2012).

Plaque instability

Pregnancy-associated plasma protein-A (PAPP-A) is a zinc-binding metalloproteinase that is produced in the placenta, and also by vascular

endothelial cells and fibroblasts. In ACS patients, PAPP-A has a good correlation with risk stratification and adverse cardiac events, with a release pattern between 2 and 30 hours after cell damage. An extensive meta-analysis by Long et al suggested that higher levels of PAPP-A could indicate a moderate increase in the long-term risk of adverse cardiovascular outcomes, supporting its further investigation as a valuable prognostic predictor in ACS (Long et al., 2013) **(Table 1)**.

Monocyte chemotactic protein-1 (MCP-1) is a chemokine that activates mononuclear phagocytes by promoting leukocyte-endothelium binding and migration to sites of inflammation. In the OPUS (Orbofiban in Patients with Unstable coronary Syndromes) TIMI 16 trial, MCP-1 levels were associated with an increased risk of death or MI after 10 months, even after adjustment for traditional risk factors. Measurement of MCP-1 in the coronary sinus blood of patients with unstable angina has demonstrated an association between MCP-1 levels and the extent of coronary atherosclerosis as assessed by coronary angiogram.(Amstrong 2006) A direct linear correlation was found between ischemia time and MCP-1 production (Tanaka et al., 2005) **(Table 1)**.

Cellular stress

MI leads to an oxidative stress situation that causes protein alteration both at functional and structural level (Westfall and Solaro, 1992). Heat shock proteins (Hsp) are molecular chaperones with a main function in intracellular repair processes (Gething and Sambrook, 1992; Lindquist and Craig, 1988). Hsp proteins have a dual functional role in MI. Thus, Hsp levels change in response to stress situations and for this reason they can be considered as markers of MI. Moreover, because of their chaperone activity Hsp have a potential value as a therapeutic tool

Different authors have described an increase of Hsp70 in response to ischaemia state (Benjamin et al., 1990; Knowlton et al., 1991). Moreover, decreased Hsp27 plasma levels have been reported in patients with atherosclerosis, which supports the view of Hsp as a marker of this pathological condition (Martin-Ventura et al., 2004). In addition, several studies have related increased Hsp levels with a cardioprotective role (Donnelly et al., 1992; Marber et al., 1993; Marber et al., 1994) **(Table 1)**.

Thus, the variety of biomarkers for ischaemia and myocardial necrosis are very useful for recognition of MI and unstable angina (UA), but have limited diagnostic value in the early stage of these syndromes. Besides, there is an increasing need for the identification of new biomarkers in order to obtain tools for an early detection and prognosis of atherothrombotic events such as AMI.

Group	Marker	Advantage	Disadvantage
Necrosis	Troponins (T,I)	High sensitivity/ specificity	Irreversible injury
	CK-MB	Standardized measurement	Low sensitivity/ specificity
	Myoglobin	High sensitivity	Unspecific
Ischaemia	FFAu	Early detection / high sensitivity	Preliminary results
	IMA	Early detection	Unspecific/ Truncated forms
Inflammation/ Acute response	CRP	Prognostic and diagnostic value	Lack of threshold
	WBC	Simple and accesible	Lack of data in clinical practice
	sCD40L	Prognostic and diagnostic value	Unspecific
	MPO	Post-event risk prediction	Overlapping values
Stress	Hsp 70	Cytoprotective role	Needs validation
	Hsp 27	Disease evolution	Needs validation
Coagulation	D-Dimer	Role in coagulation/fibrinolysis	Contradictory results
Plaque instability	MCP-1	Role in plaque instability	Overlapping values
	Coline	High sensitivity/ specificity	Lack of data in clinical practice
	PAPP-A	Early detection	Lack of data in clinical practice

Table 1 Advantage and disadvantage of biomarkers in ACS. Adapted from Vasan (2006)

8. Proteomic approach for the discovery of new biomarkers

The "omics" tools are becoming very useful in the discovery of new biomarkers; among them, proteomics is playing a significant role in the development of new tools in cardiovascular diagnosis and prognosis (Arab et al., 2006). Proteomics differs from "genomics" in both complexity and dynamic variability. Thus, the genome is stable and gives information about the potential of an organism, whereas the proteome is constantly changing according to the moment-to-moment interactions between the genome and the environment. It is important to study proteins, because they reflect what is happening in the organism (Tunon et al., 2010). Humans have 30,000 to 40,000 genes (Venter et al., 2001), however, a single gene might yield different proteins, due to alternative splicing of transcripts and protein post-translational changes. Proteomic analysis provides a unique opportunity to understand the pathophysiology of disease in a non-biased manner. Therefore, while genomic studies allow risk prediction, proteomics permits the development of candidate biomarkers for the diagnosis, staging, and tracking of disease (Arab et al., 2006).

Proteomics is defined as the systematic analysis of proteins for identification, quantification, post-translational modifications, subcellular localization, protein-protein interactions, and enzymatic activity (Aebersold, 2003). The advantage of protein markers over conventional physiological variables is the improvement in both specificity and sensitivity of disease detection and monitoring, with a concurrent increase in the information available to the clinician. Proteins are biomarkers but also molecules directly involved in the pathogenic mechanism of the

disease, thus providing relevant information about its severity, therapy responsiveness, and prognosis.

Potential markers identified by proteomic approaches must be validated prior to their routine clinical application. The progression of a potential biomarker toward routine clinical use involves three stages: identification, validation, and application (Edwards et al., 2008).

Proteomic approaches combine protein separation techniques, both gel and gel-free based methodologies, and protein identification technologies using mass spectrometry with bottom up or top down approaches (Arab et al., 2006; Gianazza et al., 2014; Tunon et al., 2010) **(Figure 9A)**.

8.1. Bi-dimensional electrophoresis (2-DE)

The main gel-based methodology for protein separation is two-dimensional gel electrophoresis (2-DE) (Gorg et al., 1999; Smith, 2009). In the first dimension, proteins are separated according to their isoelectric point (pI) within an immobilized pH gradient, a process called isoelectric focusing (IEF). In the second dimension, sodium dodecyl sulfate polyacrylamide gel electrophoresis is used to further separate the proteins according to their molecular mass. The use of these two orthogonal physico-chemical properties of separation leads to a high resolving power of the technique by allowing the mapping of hundreds of proteins simultaneously (Schoenhoff et al., 2009). After staining, (usually with Coomassie Blue, colloidal silver, or fluorescent dyes) gels are digitized and analyzed by dedicated software in order to qualitatively and quantitatively determine the proteins with different expression profiles between the compared groups. Spots of interest can then be easily excised from the gel in order to be characterized by MS (Moxon et al., 2009). Importantly, 2-DE not only allows the separation of proteins from

complex biological samples, (Schoenhoff et al., 2009) but it also enables the visualization of post translational modifications and isoforms of the same protein as a shift in their isoelectric point or molecular mass (Seo and Lee, 2004).

Another gel-based approach is called difference gel electrophoresis (DIGE). DIGE is performed by labelling up to three protein samples with specific fluorescent dyes (such as Cy2, Cy3, and Cy5) prior to be run on the same 2-DE gel. Different samples can be then co-separated in the same 2-DE gel and co-detected by using the overlay of their fluorescent patterns. An internal standard is used for normalization (Lilley and Friedman, 2004) **(Figure 9A)**.

8.2. Mass Spectrometry (MS) identification

Mass Spectrometry (MS) represents a powerful tool in the development of the proteomics field. MS permits the measurement of the mass-to-charge ratio (m/z) of gas-phase ions formed from molecules and provides a measure of the abundance of each specie (Jungblut and Thiede, 1997; Yates, 2000).

Following separation by 2-DE, proteins of interest are excised from the gel and digested by trypsin (bottom up approach). The obtained peptides are then subjected to analysis by MS. Another approach is top-down proteomics where no proteolytic cleavage were performed before ionization.

A mass spectrometer consists of three components: an **ion source**, a **mass analyzer**, and a **detector**. The detector measures the value of an indicator quantity and thus provides data for calculating the abundance of each ion present in the sample. As a result, mass spectrum is obtained, which is a plot of the ion signal as a function of the ratio m/z (Thomas et al., 2011).

Commonly used ion sources with solid and liquid biological samples respectively are matrix-assisted laser desorption/ ionization-MALDI and electrospray ionization-ESI (Gianazza et al., 2014; Zhou and Veenstra, 2008) . MALDI represents a good choice to analyze high molecular weight compounds as proteins. In MALDI, ionization is achieved by mixing the sample with organic compounds that crystallize to form a matrix. ESI allows the analysis of a larger range of molecules respect to MALDI (Griffiths and Wang, 2009). This spectrometer vaporizes the sample directly from the liquid phase by electrospray ionization or nebulizer with an electrical field to disperse the sample (Griffiths et al., 2001). For this technique, a gel-free liquid chromatography (LC) separation step is usually employed before detection.

After ionization, ions are filtered in the mass analyzer depending to their m/z before detection (Mann et al., 2001). There are several mass analyzers, among them, the time-of-flight (TOF), the quadrupole (Q) and the quadrupole ion trap (IT) (Gianazza et al., 2014) are the most commonly used.

In the TOF analyzer, the peptides are accelerated in an electrical field and are sent to a flight tube, at the end of which the detector is located. The particles have all the same charge and their velocities depend only on their masses. Small molecules fly faster than large ones. The resolution can be improved using a reflectron that corrects the kinetic energy distribution in the direction of the ion toward the detector. **(Figure 9 A-B).**

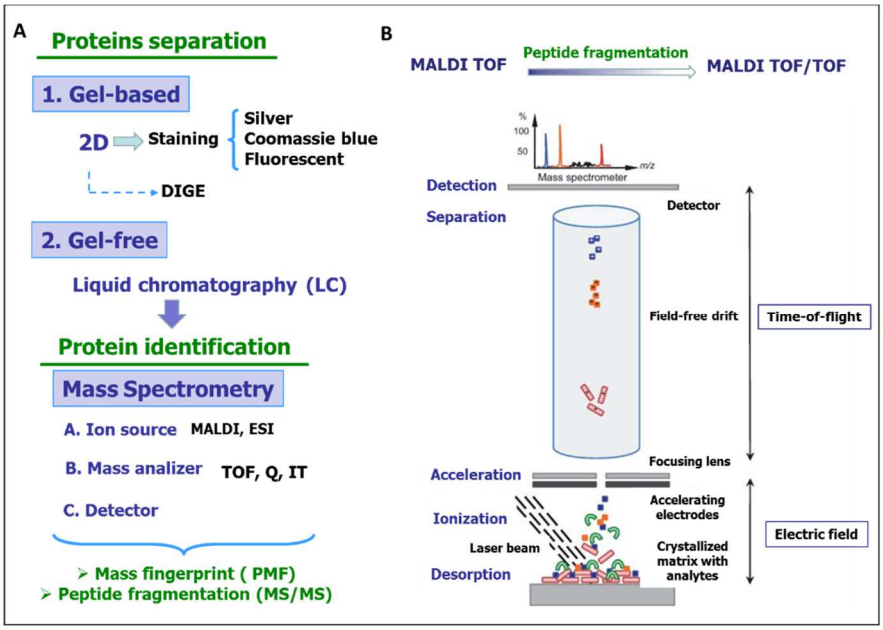

Figure 9. Schematic representation of principal proteomic approches (A) and of MALDI TOF/TOF (B)

In order to improve protein identification together with the spectra resulting from the ratio m/z, the peptide mass fingerprint (PMF) technique has been developed. This technique requires the proteolytic digestion of the proteins after separation by 2-DE by specific endo or exopeptidases **(Table 2)** (Courchesne et al., 1997; Staudenmann et al., 1998). The most commonly used enzyme is trypsin. This serine protease cleaves in a very reproducible manner peptide chains at the carboxyl side of the amino acids lysine or arginine. The measured peptides masses are compared with the theoretical mass obtained with the *in silico* digestion of the proteins in databases (Mascot, ProFound) (Griffiths and Wang, 2009; Peng and Gygi, 2001).

The presence of a protein mixture can significantly complicate the analysis and potentially compromise the results. Typical for the PMF

based protein identification is the requirement of an isolated protein. Mixtures of proteins typically require the additional use of tandem MS (MS/MS) based protein identification to achieve sufficient specificity of identification. A peptide can then be selected and broken up in a collision chamber. The resulting fragments are sent to the detector, and their masses are obtained. The sequence of the peptide is determined by the analysis of the fragmentation spectrum. These sequences are then used for database searching. Fragmentation spectra are therefore highly informative and can be powerful tools for characterizing post-translational modifications and for *de novo* sequencing of unknown proteins (Tunon et al., 2010).

Shotgun proteomics does not quantify actual protein level but relies on peptides to estimate protein abundance. Reliable quantification is more readily obtained for high abundant components for which multiple peptides are detected and accurately quantified. For low abundant proteins, reliable quantification can be impossible, especially in complex biological samples. Thus, although the gel-free approach is more sensitive in identifying low abundant proteins, it is not necessarily better for quantifying proteins in very complex biological samples or tissues (Didangelos et al., 2009).

Enzyme	Class	Cleavage Sites and Digestion Conditions
Trypsin	Serine Protease	Carboxyl side of arginine (R) and lysine (K) Ammonium bicarbonate buffers (pH 8.0). 1:20 to 1:100 (w/w) enzyme to protein ratio Digestion at 37°C for 2 hours to overnight
Endoproteinase Glu-C	Serine Endoproteinase	Carboxyl side of glutamate (G). Ammonium bicarbonate buffers (pH 7.8) Carboxyl side of glutamine (G) and asparagine (N) Phosphate buffers (pH 4.0) 1:20 to 1:100 (w/w) enzyme to protein ratio Digestion at 37°C for 2 hours to overnight
Pepsin	Digestive Protease	Carboxyl side of tyrosine (Y), tryptophan (W), phenylalanine (F), and leucine (L) Acidic conditions (pH 2-4) 1:50 (w/w) enzyme to protein ratio Digestion at 37°C for 6 hours to overnight
Chymotrypsin	Digestive Protease	Carboxyl side of tyrosine (Y), phenylalanine (F), and tryptophan (W) Ammonium bicarbonate buffers (pH 8) 1:20 to 1:100 (w/w) enzyme to protein ratio Digestion at 37°C for 2 hours to overnight
Endoproteinase Arg-C	Sulfhydryl Proteinase	Carboxyl side of arginine (R) residues Ammonium bicarbionate buffers (pH 8) 1:10 to 1:20 (w/w) enzyme to protein ratio Digestion at 37°C overnight

Table 2 Proteases used for protein digestion in mass spectrometry.

9. Background in cardiovascular proteomics

Cardiovascular proteomic studies have two main aims. Firstly the association of proteins that might be potential therapeutic targets for interventions to a specific function or disease. Secondly, the discovery of novel proteins which are altered in abundance or suffer specific modifications in response to disease states and consequently could be considered as potential biomarkers (Petricoin et al., 2002).

Proteomic technologies are increasingly being applied to identify biomarkers and therapeutic targets associated with thrombus formation and the subsequent presentation of a clinical manifestation. These studies range from the global profiling of whole plasma to more focused analyses of blood and vascular components including platelets, leukocytes, erythrocytes, and their microparticles (MPs), as well as atherosclerotic lesions (Howes et al., 2008).

9.1. Vascular proteomics

Few studies have used proteomic approaches to investigate atherosclerotic lesions. The main obstacle in these studies is the heterogeneous composition of atherosclerotic plaques (Mayr et al., 2006; Stary, 1994; Stary et al., 1994). The first attempts to analyze human atherosclerotic lesions by 2-DE were undertaken almost 30 years ago. Stastny *et al.* (Stastny et al., 1986) compared the protein composition of human fibrofatty lesions with lesion-free segments of the human aortic intima. This study suggested that changes in protein composition might occur in the human aortic intima during the initial stages of atherogenesis.

In addition, the 2-DE profile of coronary arteries of both coronary artery disease patients and healthy controls has been analyzed. Derived from this study an association between excessive iron storage and a high risk of

CAD has been reported (You et al., 2003). More recently, de la Cuesta and colleagues have described 13 differential proteins in the intimal layer of human atherosclerotic coronaries using a DIGE approach. These proteins were implicated in VSMC migration, coagulation, apoptosis, and heat shock response (de la Cuesta et al., 2011).

An alternative approach in vascular proteomics is the comparison of the secretome of cultured normal and pathological arteries (de la Cuesta et al., 2012; Martin-Ventura et al., 2004).

To overcome limitations of vessel wall heterogeneity, proteomic studies of laser capture microdissected section in human coronary atherosclerotic areas have been carried out. Based on this methodology, Bagnato and coworkers have described the first large scale proteomic map of human coronary atherosclerotic plaques, which includes a total of 806 proteins mainly involved in vascular remodeling and atherogenesis (Bagnato et al., 2007).

However, differential proteomics between diseased and normal vascular tissues remain a challenge, as a consequence mainly of the cellular heterogenicity of the atherosclerotic lesions.

The most feasible alternative is to use culture of specific cell types. To this respect studies based on differential proteomic patterns in cultured endothelial cells (EC) (Bruneel et al., 2003; Prokopi et al., 2009; Scheurer et al., 2004) and smooth muscle cells (SMC) (Dupont et al., 2005; Garcia-Arguinzonis et al., 2010; Mayr et al., 2004) in response to cardiovascular risk factors have been largely performed in the recent years.

A mayor limitation in these studies is the lack of paracrine interaction between different cells types a key process in atherosclerosis.

9.2. Plasma/serum proteomics

The human plasma/serum proteome holds the promise of a revolution in disease diagnosis and therapeutic monitoring. Plasma/serum is not only the primary clinical specimen but also represents the largest and deepest version of the human proteome present in any sample. Plasma/serum has an extraordinary dynamic range with more than 10 orders of magnitude of concentration between the abundance of albumin and the rarest proteins now measured in the clinical practice (Anderson et al., 2004).

Several studies have demonstrated changes in the plasma/serum levels of biomarkers related to inflammation, platelet activation, coagulation, myocyte necrosis, and plaque rupture in patients with ACS (Badimon et al., 2012; Donahue et al., 2006).

Plasma/serum is a very rich source for biomarker discovery. However, because of its great complexity it must be sub-fractionated prior to proteomic analysis. Therefore analysis of plasma/serum samples by 2-DE requires removal of the high abundance proteins as a previous step to obtain consistent results. However, there are studies that have used undepleted plasma samples for proteomics studies. Indeed, plasma of healthy individuals and those undergoing UA and/or MI have been compared using 2-DE/MALDI-MS (Mateos-Caceres et al., 2004). This study reported differential expression levels of specific α1-antitrypsin, apolipoprotein A1 and fibrinogen γ-chain isoforms between the analyzed groups; whereas other work (Marshall et al., 2003)based on MALDI-TOF spectral analyses of plasma peptides revealed that fibrinopeptide A and complement C3f peptides have a characteristic spectral pattern in MI. Other comparative study of plasma (albumin- and IgG-depleted) profiles between subjects with and without CAD has identified several differential proteins, including fibrinogen γ-chain, collagen α3 and complement C1 and C5a (Donahue et al., 2006). In more recent studies using 2-DE and

mass-spectrometry (MALDI-TOF/TOF), Cubedo et al. analyzed the serum proteome of patients with AMI, and their results revealed significant changes in the ApoJ proteomic profile, due to a differential glycosylation pattern, within the first 6 hours after the onset of the event (Cubedo et al., 2011). Expanding this findings Cubedo and coworkers have demonstrated a coordinated decrease in immune response inflammation-related proteins, and an increase in serum amyloid P component (SAP) related to the activation of the classical complement pathway, in the late post-AMI (Cubedo et al., 2013).

9.3. Thrombus proteomics

Thrombus is a complex sample to analyze because of its heterogeneous composition. For this reason, most of the proteomic studies are conducted on isolated components of the thrombus or using the secretome of thrombus samples.

Thus, proteomic studies have been largely applied to platelets (Lopez-Farre et al., 2011; Pena et al., 2011; Senzel et al., 2009), and in particularly on activated platelets in comparison with resting ones. Global proteomic analyses of thrombin receptor activating peptide (TRAP) stimulated platelets have identified 62 differentially regulated proteins following platelet activation (Garcia et al., 2004). More focused studies have been carried out on the tyrosine phosphoproteomes of platelets activated with collagen-related peptide and thrombin (Garcia et al., 2006). In order to point out proteins potentially relevant in thrombus formation, proteomic approaches have also been used to study other blood cells such as leucocytes, both mononuclear (Rosengren et al., 2005)and granulocytes (Lominadze et al., 2005; Yoon et al., 2005) In particular the proteomic study on polymorphonuclear neutrophils (PMNs) isolated from patients with abdominal aortic aneurysm (AAA) has revealed a decreased catalase

expression in PMNs highlighting the important role of the oxidative stress in AAA evolution and intraluminal thrombus (ILT) formation (Ramos-Mozo et al., 2011). Red blood cells are also directly involved in thrombus formation (Yunoki et al., 2012) (Yunoki et al 2012). Global red blood cells proteome profiling has been performed using LC-MS/MS, providing the identification of 181 proteins, including several members of the globin family (Goodman et al., 2007; Kakhniashvili et al., 2004).

An alternative approach is the analysis of the thrombus secretome. These studies, mainly performed in ILT revealed an association between peroxiredoxin-1 (PRDX-1) (Martinez-Pinna et al., 2011), thombospondin-1 and clusterin (Moxon et al., 2011) identified in the thrombus conditioned medium and the pathogenesis of AAA.

Recently, Alonso-Orgaz and coworkers (Alonso-Orgaz et al., 2014) have applied several proteomic approaches in an attempt to obtain a global view of the thrombus proteomic profile. Although they identified 708 proteins, they did not investigate the clinical relevance of the identified proteins in the thrombus formation process

10. Concluding remarks and unresolved issues

Atherothrombosis is a systemic multifactorial disease that occurs when a thrombus forms superposed on a disrupted or surface-eroded atherosclerotic plaque leading to the clinical presentation of acute coronary syndromes. Atherothrombosis is a dynamic process that, in addition to the atherosclerotic vessel wall, involves plasma proteins and blood cells with a direct influence on its morphology, evolution, and tentative resolution. The mechanisms of thrombus formation on disrupted and eroded atherosclerotic plaques have been the subject of substantial investigation. At the site of plaque disruption, platelets deposit and form aggregates that anchor the newly formed stable fibrin networks. In addition to platelet deposition in the injured area, the clotting mechanism is activated by the exposure of the de-endothelialized vascular surface. The implication of the tissue factor (TF)-dependent extrinsic coagulation pathway in atherosclerosis has been widely studied. Nowadays there is also an emerging body of evidence suggesting the involvement of intrinsic coagulation proteins in the etiology of cardiovascular disease.

Nevertheless, the composition of the occluding thrombus, its changes in function of the elapsed time of ischaemia and its interaction and cross-talk with the other cells is not fully known. Therefore, profiling intracoronary thrombus cellular and soluble proteins may be a strategy to identify novel factors in thrombosis.

Even though stent implantation has largely improved the clinical outcome of ACS patients, concerns still remain regarding the risk of IST and the high mortality rate associated. The cellular thrombus composition has been associated with redox imbalance in diseased arterial tissues and oxidative stress has been linked to an impaired myocardial reperfusion in STEMI patients. However, until now, there are no consistent studies comparing the proteomic composition of in-stent thrombus with those

formed in native coronary arteries, making unclear the potential molecular pathways linking the high rate of mortality in IST and thrombus composition.

II. HYPOTHESIS AND AIMS

Plasma proteins and blood cells, in addition to vessel wall components, influence thrombus morphology and its eventual resolution. Recent evidences suggest that thrombus-age is an independent predictor of long-term mortality in STEMI patients and that the mortality due to in-stent thrombosis is significantly higher compared with mortality due to a native thrombus. Nevertheless, the composition of the occluding thrombus, its changes with time of evolution and its interaction and cross-talk with other blood cell types and with the triggering substrate is not fully known. Therefore, profiling intracoronary thrombus proteins may be a strategy to identify novel factors in thrombosis.

The **hypothesis** of this study is that

"Coronary thrombus formation is a dynamic process integrating both cellular and protein components on various proportions that would contribute to its stability and characteristics. The thrombus composition would change in function of the time of ischemia or time of evolution, rendering different clinical conditions and myocardial function damage."

The specific **objectives** proposed to prove this hypothesis were:

1. Characterize the evolving cellular and protein composition of human coronary thrombus obtained from patients with STEMI in relation to pain-to-PCI elapsed time.
2. Identify serum/plasma proteins with a potential relevance as biomarkers of occlusive thrombus-age and patients' prognosis.
3. Characterize the composition of thrombi growing intra stents.
4. Investigate the coordinated changes in coagulation-related proteins and the fibrinogen distribution profile in evolving coronary thrombi obtained from patients with STEMI.

III. MATERIALS AND METHODS

1. Study design

Dynamic changes in structure and composition of coronary thrombi as a function of the ischaemia time were investigated at protein and cellular level. To this aim occlusive coronary thrombi of different age, defined by pain to PCI-elapsed time, were obtained by aspiration from patients with ST segment elevation myocardial infarction and characterized by a differential proteomic approach and advanced cellular microscopy. Protein markers of the occlusive thrombus age have been also investigated in the peripheral circulation to determine their potential value as biomarkers of the onset of pain to PCI elapsed time in STEMI. Additionally, the differential protein signature of in-stent thrombus in comparison to acute occlusive thrombi in native coronary arteries was investigated (**Figure 10).**

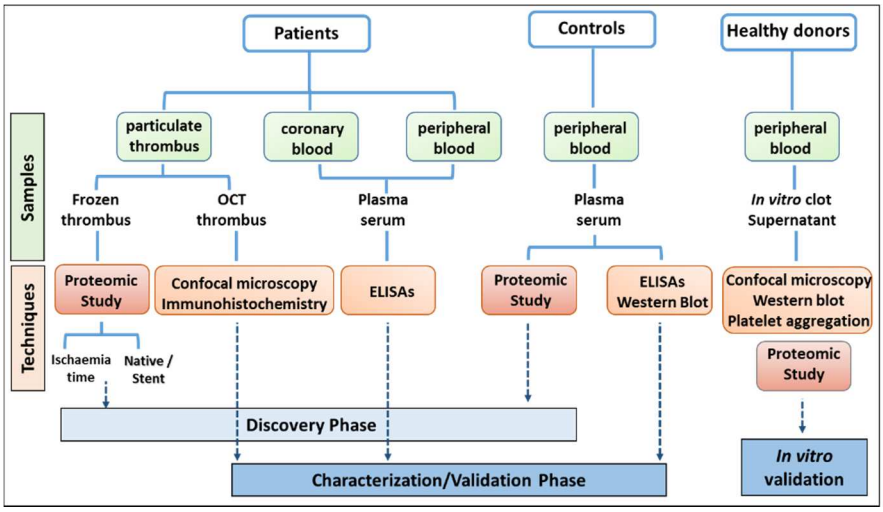

Figure 10. Schematic experimental design.

2. Study population

The present thesis includes studies performed in STEMI patients with occlusive thrombosis in native coronary arteries or after stenting. A written informed consent was obtained from all participants prior to the studies. All study protocols were approved by the corresponding Ethical Committee of the hospital in charge of the clinical study and were conducted according to good clinical practice and to the Declaration of Helsinki for studies using human subjects.

STEMI patients undergoing PCI and thrombus aspiration were intervened at the Cardiology Department of Hospital Sant Pau (Barcelona) (Group 1); a second group were treated at the Cardiology Department of Hospital Puerta de Hierro (Madrid) (Group 2). In addition, a group of STEMI-patients with an acute new-onset myocardial infarction arriving to the emergency room of Hospital Sant Pau (Group 3) were included. Control groups of healthy donors were included for comparative purposes.

Group 1: STEMI-patients admitted at the Cardiology Department of Hospital Sant Pau

A group of 86 STEMI patients undergoing PCI was included. Percutaneous coronary intervention was performed according to guidelines.

A loading dose of clopidogrel of 600 mg and maintenance of 75 mg/day were administered. Aspirin was administered as a 300 mg loading dose followed by 100 mg /day. All the patients received full dose of i.v. heparin before PCI (1 mg/kg) or 0.7 mg/kg in those patients who received abciximab. Dosages of GPIIb/ IIIa were a bolus of 0.25 mg/kg i.v. and 0.125 mg/kg/min infusion (maximum 10 mg/min) for 12 hours.

To investigate the effect of onset-of-pain-to-PCI time on the composition of STEMI thrombus, two groups were selected: <3 hours onset-of-pain-to-PCI (T3 group, n = 33) and >6 hours onset-of-pain-to-PCI (T6 group, n = 32). The range time between 3 and 6 hours was excluded in the initial stage of the study in order to clearly differentiate between early (<3h) and late (>6h) phases of ischemia. Clinical characteristics of STEMI patients are provided in **Table 3.** In the validation phase STEMI patients in the range between 3 and 6 hours (T3-6 group, n = 21) and a control group of 28 subjects without any cardiovascular event before recruitment were included.

	T3 (n = 33)	T6 (n = 32)
Past medical History of (%)		
PCI	9	6
CABG	3	3
Peripheral vasculopathy	0	0
Time delay in minutes (median [IQR])		
Symptom onset to medical contact	24 [15-44]	272 [100-455]
Hospital admission to PCI	78[45-103]	310 [265-744]
Symptom onset to PCI	110 [94-150]	615 [449-825]
Clinical presentation		
Killip (mean±SD)	1.3±0.8	1.4±0.9
TIMI flow grade (mean±SD)	0.3±0.9	0.7±1
Infarct-related artery (%)		
LAD	64	66
RCA	67	66
Cx	24	22
Antitrombotic treatment (%)		
ASA	100	91
Clopidogrel	52	68
Heparin	100	100
GpIIb/IIIa inhibitor	33	27
Reperfusion (%)		
TIMI flow grade 3 before PCI	6	13
TIMI flow grade 3 after PCI	88	80

ASA = acetylsalicylic acid; CABG = coronary artery bypass grafting; Cx = circumflex artery; LAD = left anterior descending artery; PCI = percutaneous coronary intervention; RCA = right coronary artery; TIMI = thrombolysis in Myocardial Infarction**.**

Table 3. Clinical characteristic of STEMI-patients (Group 1)

Group 2: STEMI-patients admitted at the Cardiology Department of Hospital Puerta de hierro

The study population comprised a group of 19 patients admitted with ST-elevation myocardial infarction undergoing PCI associated with thromboaspiration followed by stent implantation. Nine patients were intervened by PCI because of in-stent thrombus (IST) and ten because of thrombus formation on native coronary arteries (CT).

Both groups were matched for age, gender, cardiovascular risk factors and biochemical parameters **(Table 4)**. Similarly, pharmacological treatments have been also taken into account when possible. Loading dose of clopidogrel and aspirin was 600 and 300 mg respectively. Patients included after 2011 received a loading dose of 60 mg of prasugrel and maintenance of 10mg/day. All the patients received heparin before PCI (5000 IU) and 1000 IU every 30 minutes during the procedure. Dosages of GPIIb/IIIa were a bolus of 180 µg/kg i.v. and 2 µg/kg /min infusion for 12 h.

	CT (n = 10)	IST (n = 9)	*p*-value
Age	66±15	61±10	ns
Male (%)	80	78	ns
Risk factors (%)			
Active smoker	30	33	ns
Diabetes	10	11	ns
Hypertension	50	56	ns
Dyslipemia	60	56	ns
Ischemia time			
Symptom onset to PCI (min)	255±137	334±180	ns
Biomarkers on admission			
Troponin I (µg/L)	72±45	218±204	ns
CPK (U/L)	1798±1117	3716±45	ns
CRP (mg/L)	12±83	78±109	p=0.05
Previous treatment (%)			
Statin	50	100	p=0.01
Anti-HT	70	100	ns
GPIIb/IIIa antagonist (%)	90	89	ns
Reperfusion assessment (%)			
TIMI flow grade 3 before PCI	0	0	ns
TIMI flow grade 3 after PCI	80	89	ns

Anti-HT=antihypertensive; CPK = creatine phosphokinase; CRP= C-reactive protein; PCI = percutaneous coronary intervention; TIMI = thrombolysis in Myocardial Infarction (0= no perfusion, 1= penetration without perfusion, 2= partial reperfusion, 3= complete reperfusion).

Table 4. Background description and clinical characteristics of STEMI-patients (Group 2)

Group 3: STEMI-patients admitted at the Emergency room of Hospital Sant Pau

The study was performed in a group of patients (n=38) with an acute new-onset myocardial infarction (AMI) arriving to the Emergency Room and a control group of 60 healthy individuals who attended for a routine health check. Characteristics of AMI and control groups are outlined in **Table 5**. AMI patients were admitted with chest pain and suspected of ACS at the Hospital Sant Pau. Exclusion criteria were a previous documented or suspected myocardial infarction and antithrombotic treatment because of the AMI onset before arriving to the emergency room and time of the first blood collection. None of the patients included in the study were under anti-inflammatory treatment. Patients included in the study did not present further co-morbidities such as cancer, chronic infections (HIV, HCV and HBV), autoimmune diseases or thyroid hormones disorders.

	AMI-patient (n=38)	Controls (n=60)
Age	61 ± 2	62 ± 8
Females / Males (N)	10 / 28	15 / 45
Total-C (mg/dL)	213 ± 51	216 ± 42
HDL-C (mg/dL)	46 ± 11	49 ± 14
LDL-C (mg/dL)	135 ± 46	142 ± 37
TG (mg/dL)	184 ± 74	129 ± 84
Creatinine (µmol/L)	112 ± 38	81 ± 19
GOT (U/L)	76 ± 54	21 ± 5
GPT (U/L)	39 ± 17	22 ± 11
CRP-peak (mg/L) *	69 ± 52	-
Troponin-T (µg/L)	0	-
peak levels at 12 ± 1h*	7.9 ± 6.4	-
Necrosis (g)	19.4 ± 14.9	-
Killip class (%)		
I	87	-
II	10	-
III	-	-
IV	3	-
LVEF (%)	56 ± 12	-

GOT = Glutamic-oxaloacetic transaminase; GPT = Glutamic-pyruvic transaminase; LVEF = Left ventricular ejection fraction; * Highest values after admission.

Table 5. Clinical characteristics of AMI-patients (Group 3) and control subjects.

3. Sample collection

3.1. Thrombi samples

Thrombi were collected during Percutaneous Coronary Intervention (PCI). All thrombi were routinely macroscopically estimated in appearance for size and color. Thrombi were collected in one or more segments. Aspirated thrombi were immediately washed to eliminate attached red blood cells and weighted. Samples were snap-frozen and stored at -80ºC until analysis, or fixed in 4% paraformaldehyde, embedded in optimal cutting temperature (OCT) compound and stored at -80ºC for immunohistochemical and confocal analysis (**Figure 11**).

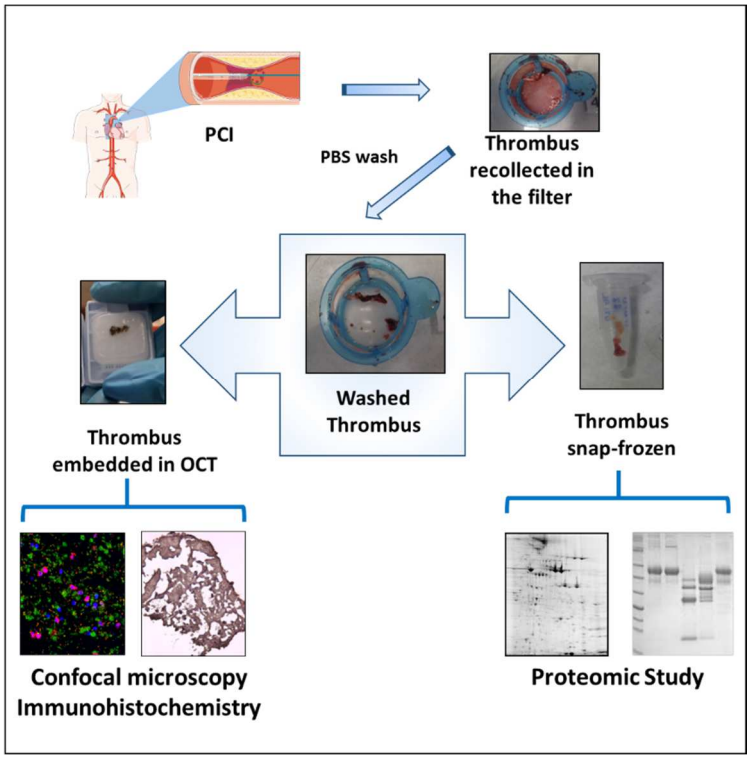

Figure 11. Thrombus sample collection

3.2. Blood from patients and healthy donors

Plasma

Peripheral and coronary blood were collected in EDTA (Ethylenediamine tetraacetic acid) tubes at the time of PCI, from cubital vein and directly from coronary artery through the thrombectomy device respectively.

Hematological parameters were measured using medonic analysis system (Medonic CA 530). In addition, blood was obtained from healthy donors in 3.8% sodium citrate or EDTA. Blood was centrifuged 10 minutes at 150g to obtained platelet rich plasma and 15 minutes at 16100g for obtaining platelet free plasma. Plasma was aliquoted and stored at -80ºC.

Serum

Blood samples, withdrawn from cubital vein from AMI-patients and healthy donors were collected to prepare serum. Blood was centrifuged 30 minutes at 1811g, aliquoted, and stored at -80ºC. Serum specimens from hemolysis blood samples were excluded.

4. Sample preparation

4.1. Total protein extraction

Frozen thrombi (coronary and *in vitro* generated) were extracted with urea/thiourea detergent buffer (7 mol/L urea; 2mol/L thiourea; 2% chaps) and sonicated. Thrombi lysate suspensions were centrifuge 10 minutes at 16000 g and supernatants were snap-frozen in liquid nitrogen and stored at -80ºC until proteomic analysis was performed.

4.2. Plasma/serum protein depletion

The six most abundant proteins (albumin, IgGs, IgAs, transferrin, α1 anti-trypsin and haptoglobin) were depleted from serum and plasma samples used for proteomic studies in order to increase resolution and detection power. To this aim a specific affinity cartridge with high binding capacity (Multiple Affinity Removal Spin Cartridge, Agilent Technologies) was used. Briefly, the targeted high-abundant proteins were simultaneously captured by immobilized antibodies when crude biological samples were passed through the cartridge. Selective immunodepletion provides an enriched pool of low abundant proteins for downstream proteomic analysis.

After protein depletion, the low-abundance proteins in the flow-through fraction were concentrated using spin concentrators with a 5,000 Da molecular weight cut-off (Spin Concentrators for Proteins, Agilent Technologies)

4.3. Sample Clean-up

Sample clean-up procedure consists in the elimination of compounds (ionic detergents, salts, nucleic acids and lipids) that can interfere with isoelectrofocusing (IEF) and western blot. After precipitation, the proteins were washed and resuspended in the IEF/2-D compatible rehydration buffer for IEF or Laemmli sample buffer, for SDS-PAGE.

In this study, the clean-up procedure has been only used for preparation of *in vitro* clot extracts for 2-DE analysis and in coronary thrombi samples prepared for western blot analysis. Clean-up was not applied to coronary thrombi samples prepared for 2-DE analysis because of a sample amount limitation. Clean-up was performed using the ReadyPrep 2-D clean-up Kit (BioRad).

4.4. Protein quantification

Protein concentration was measured with the 2-D Quant Kit (GE Healthcare).

This procedure is based on the quantitative precipitation of proteins. Therefore, the assay is compatible with the reagents used in the preparation of samples for 2-D electrophoresis, including detergents, reductants, and ampholytes. Precipitated proteins are resuspended in a copper-containing solution and unbound copper is measured with a colorimetric agent. The color density is inversely related to the protein concentration.

5. Proteomic analysis

5.1. Two dimensional electrophoresis

Rehydration step: Dry strip gels were rehydratated using IPGbox (GE Healthcare) by the immersion into the rehydration solution (7M urea, 2M thiourea, 2% chaps, 1.6% DTT, 0.2% ampholytes and 0.02% bromphenol blue) containing the sample overnight prior to the first dimension separation **(Figure 12)**.

First dimension separation: Proteins are first separated on the basis of their pI using the IEF technique. For analytical and preparative gels, 100 µg and 300 µg of protein were respectively loaded in 17-cm dry strips (pH 3-10 or 4-7 Bio-Rad Laboratories, Hercules, CA,) **(Figure 12)**.

Equilibration: A conditioning step is applied to proteins separated by IEF prior to the second-dimension. This process reduces disulfide bonds using DTT (50mM Tris-HCl pH8.8, 6M urea, 2% SDS, 30% glycerol and 2% DTT) and alkylates the resultant sulfhydryl groups of the cysteine residues using iodoacetamide (50mM Tris-HCl pH8.8, 6M urea, 2% SDS, 30% glycerol and 2,5% iodoacetamide) **(Figure 12)**.

Second dimension separation: Second dimension was resolved in 12% SDS-PAGE gels. The protein fraction loaded on a 2-D PAGE gel must be in a low ionic strength denaturing buffer that maintains the native charges of proteins and keeps them in a soluble state. Electrophoresis was performed using an Ettan Daltsix System (GE Healthcare, Uppsala, Sweden). Gels were developed by fluorescent Flamingo staining (Bio-Rad Laboratories, Hercules, CA,) and image digitalized using a fluorescence scanner (Typhoon 9400, GE-Healthcare, Uppsala, Sweden) **(Figure 12)**.

5.2. Differential protein analysis

Analysis for differences in protein patterns was performed with the PD-Quest 8.0 (Bio-Rad Laboratories) using a single master that included all gels. To each spot was assigned a relative value that corresponded to the single spot volume compared to the volume of all spots in the gel following background subtraction and normalization between gels **(Figure 12)**.

6. Protein identification by mass spectrometry

Protein identification was performed by mass-spectrometry (MALDI TOF-TOF). As a previous step, selected spots were excised from gels using an Ettan Spot Picker (GE Healthcare). Therefore the spots were processed as follows:

- washed with 500ul of Ambic 25mM
- dried by adding Ambic 25mM/50%ACN and 100%ACN
- subjected to SpeedVac to complete dryness
- digested using trypsin (Promega) 0.05-0.1 mg/mL
- generated peptides were cleaned up and concentrated using ZipTipU-C18 filters (Millipore)
- peptides were mixed (1:1) with matrix (α-cyano-4-hydroxycinnamic acid 5mg/mL)
- samples were applied to Prespotted AnchorChip plates (Bruker Daltonics, Bremen, Germany) surrounding the calibrants provided on the plates.

Samples were analyzed by matrix – assisted laser desorption/ionization time-of-flight using an AutoFlex III Smartbeam MALDI-TOF/TOF (Bruker Daltonics, Bremen, Germany). Spectra were acquired with flexControl on reflector mode (mass range 850-4000 m/z, reflector 1: 21.06 kV; reflector 2: 9.77kV; ion source 1 voltage: 19 kV; ion source 2: 16.5kV; detection gain 2.37x) with an average of 3500 added shots at a frequency of 200 Hz. Each sample was processed with flexAnalysis (version 3.0, Bruker Daltonics, Bremen, Germany) considering a signal-to-noise ratio over 3, applying statistical calibration and eliminating background peaks.

For identification, peaks between 850 and 1000 (m/z) were excluded, since they mainly refer to matrix peaks. Spectra were analyzed by the BioTools software (version 3.2, Bruker Daltonics, Bremen, Germany) and proteins were identified using the MASCOT search on Swiss-Prot 57.15 database [Taxonomy: Homo Sapiens, Mass Tolerance 50 to 100, up to 2 trypsin miss cleavage, Global Modification: Carbamidomethyl (C), Variable Modification: Oxidation (M)]. Identification was carried out by peptide mass fingerprinting (PMF) and confirmed by peptide fragmentation (TOF/TOF). Identification was accepted with a score higher than 56 **(Figure 12)**.

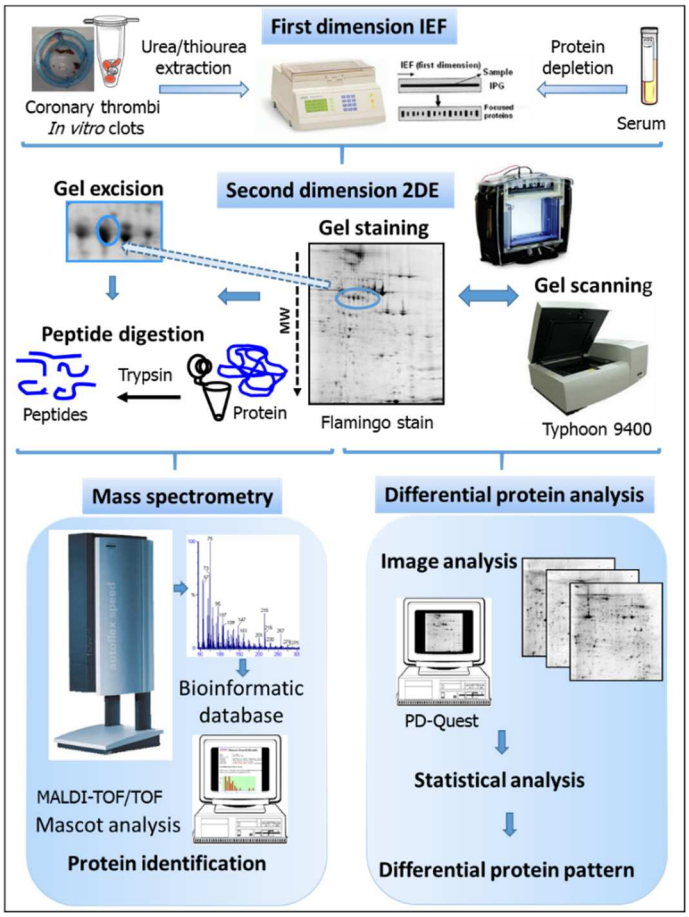

Figure 12. Schematic view of proteomic methodology

7. Assays for protein quantification and characterization

7.1. Western blot

Protein extracts were separated in 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 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). Once separated proteins were electrotransferred in semidry conditions (Semidry transfer system, or transblot BioRad), detected with specific primary antibodies (**Table 6**) and visualized either with fluorescence or chemiluminescence.

Fluorescence Protein were electrotransferred to PVDF (polyvinylidene difluoride) membranes. Detection was performed using the appropriated primary antibody combined with the Dye Double Western Blot kit (Invitrogen). Band fluorescence was determined with Typhoon 9400 (GE Healthcare) and band quantification was performed using ImageQuant TL software (GE Healthcare). Protein load was normalized using total protein fluorescent signal.

Chemiluminescence Proteins were transferred to nitrocellulose membranes. 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 using bovine serum albumin (BSA, 5%) to avoid unspecific binding of antibodies. Specific secondary antibodies against different animal species were used conjugated with horseradish peroxidase. Detection of protein bands was

achieved by chemiluminescence using a peroxidase enzymatic reaction (Supersignal, Pierce) and images were obtained with a ChemiDocTM XRS system. Band intensities were quantified using Image Lab software (Bio-Rad). Protein load was normalized using total protein Ponceau signal.

Primary antibody	Reference	Dilution	Secondary antibody	Paper
Pfn-1	0022-01, Inmunoglobe	1/500	Anti-rabbit	1
PRDX2	Ab50862, Abcam	1/2000	Anti-muose	2
SOD	574596, Calbiochem	1/1000	Anti-sheep	2
CD61	MAB20237, Merck - Millipore	1/1000	Anti-mouse	2
CD31	M0823,Dako	1/1000	Anti-mouse	2
SM actin	M0851,Dako	1/5000	Anti-mouse	2

Table 6. Antibodies and working conditions used for western blot analysis.

7.2. Enzyme Linked ImmunoSorbent Assay (ELISA)

Plasma and serum levels of specific proteins were quantitatively determined by ELISA.

Plasma profilin-1(Pfn-1), was determined in coronary and peripheral plasma samples by a double antibody sandwich enzyme-linked immunosorbent assay (Uscn Life Science, Inc. Missouri City, TX) with a lower limit of detection of 30 pg/mL. The intra-assay and the inter-assay precision are respectively CV<10% and CV<12%.

Plasma P-selectin was determined in peripheral plasma samples by a double antibody sandwich enzyme-linked immunosorbent assay (R&D

Systems, Inc., Minneapolis, MN). The minumum detectable dose (MDD) of human P-Selectin is typically less than 0.5 ng/mL. The intra-assay and the inter-assay precision are respectively CV<6 % and CV<10%.

The human high sensitivity C-Reactive Protein (hsCRP) (Millipore, Billerica, Ma) kit is a double polyclonal antibody sandwich enzyme immunoassay. The intra-assay and the inter-assay variation are respectively CV < 10 % and CV < 12% %. Sensitivity: 0.20 ng/mL.

Systemic total high molecular weight kininogen (HMWK) and FXI concentrations were determined in serum by competitive and non-competitive sandwich enzymelinked immunosorbent assay (AssayPro, St. Charles, MO; USA), respectively, using immobilised polyclonal antibodies, as described by the providers. The detection limits of the assays were 0.12 g/ml for HMWK and 1.5 ng/ml for FXI. The intra-assay and the inter-assay variation are respectively CV < 5% and CV < 8% % for HMWK and CV<6%-CV<8% for FXI **(Table 7)**.

Protein	Reference	Detection limits	Intra/inter assay variability
Pfn-1	SEC233hu, Uscn	30 pg/ml	CV<10%-CV<12%
P-selectin	BBE6, R&D	0.5 ng/ml	CV<6%-CV<10%
hsCRP	CYT298, Merck-Millipore	0.20 ng/mL	CV<10%-CV<12%
HMWK	EK1001-1, AssayPro	0.12 g/ml	CV<5%-CV<8%
FXI	EF1001-1, AssayPro	1.5 ng/ml	CV<6%-CV<8%

Table 7. ELISA kits used in the study

7.3. Immunohistochemistry

Immunohistochemical analysis has been used for identification of cellular components and fibrin content in occlusive coronary thrombi. Serial sections (5 μm thick) of OCT embedded thrombi were placed on poly-L-lysine coated slides and treated with hydrogen peroxide (H_2O_2) for inhibition of endogenous peroxidase activity and with horse or goat serum to block non-specific bindings. Therefore, slides were incubated for 2h with primary antibodies and detected with avidin-biotin immunoperoxidase technique. Antibodies used in immunohistochemical analysis are listed in **Table 8**. The chromogen used was 3.3'-diaminobenzidine. Thrombus morphology was analyzed with hematoxylin and eosin stain. The images were captured by Nikon Eclipse 80i microscope and digitized by Retiga 1300i Fast camera at 400x. Positive areas for each antigen were calculated from an average of 5-fields/sample with ImageJ 1.37v software. The values were given as a percentage of the total thrombus area (content (%) = [positive stained area/total thrombus area] x100).

Primary antibody	Reference	Dilution	Secondary antibody	Paper
macrophages	MCA1478, Serotec	1/200	biotinylated anti-mouse	1
macrophages	M1919, Sigma	1/200	biotinylated anti-mouse	1
CD61	M075, Dako	1/400	biotinylated anti-mouse	1
CD3	A0452 Dako	1/50	biotinylated anti-rabbit	1
fibrinogen	A0080, Dako	1/200	biotinylated anti-rabbit	1
neutrophil	ab6872, Abcam	1/50	biotinylated anti-rabbit	1
CD105	AF1097, R&D	1/50	biotinylated anti-goat	1
CD34	NCL_END, Novocastra	1/50	biotinylated anti-mouse	1

Table 8. Antibodies and working conditions used for immunohistochemistry analysis.

7.4. Confocal microscopy

Confocal microscopy was mainly used for studies of colocalization of proteins and specific cell component in the thrombi as well as for determining the structure of fibrin fiber. Briefly, thrombi and total blood *in vitro* clots were fixed with 4 % paraformaldehyde at room temperature. Serial thrombus sections (5 µm thick) were immobilized on poly-L lysine-coated coverslides in a humidified chamber and allowed to adhere for 2 h. Samples were then washed with PBS, permeabilized, when required, with 0.5 % Tween for 5 minutes, incubated for 30 minutes with blocking buffer (1% bovine serum albumin in PBS), and incubated with the primary antibodies. Coverslides were washed and incubated with the appropriate secondary antibody and Hoechst (for nuclei labelling). Immunostained coverslides were washed and covered with Prolong Gold antifade reagent

(Molecular Probes, Leiden, Netherlands). Images were recorded on a Leica inverted fluorescence confocal microscope (Leica TCS SP2-AOBS, Wetzlar, Germany) with HCX PL APO 63x/1.2W CORR/0.175 objective. Fluorescent images were acquired in a scan format of 1024 x 1024 pixels in a spatial data set (x y z) and were processed with the Leica Standard Software TCS-AOBS. Negative controls with no primary antibody labelling that were run with each set of experiments showed no fluorescence. Antibodies used in confocal microscopy analysis are listed in **Table 9**.

Three dimensional rotation projections were created from stack series of selected thrombi (0.2 μm distances). The maxim projection showing the z-value by colour was created and thereafter a 90º rotation animation was performed creating a projection every 2º.

Image analysis: Measurements of the length of the fibers were performed on two-dimensional reconstructions of the optical sections and each micrograph was processed with the same algorithm using the NIH Image software (public domain software by Dr Wayne Rasband, National Institutes of Health, v1.46e). After normalization of the scale for the grey level a morphological filter was applied. The results of the length of the fibers were expressed as mm. Negative controls with no primary antibody labeling run with each set of experiments showed no fluorescence.

Primary antibody	Reference	Dilution	Secondary antibody	Paper
fibrinogen-fibrin	350, American Diagnostica	1/50	Anti-mouse AlexaFuor-488	1
CD61-FITC	VI-PL2, BD Biosciences	1/50	Anti-rabbit AlexaFuor-488	1
CD105	AF1097, R&D	1/50	Anti-goat AlexaFuor-633	1
Pfn-1	0022-01, Inmunoglobe	1/50	Anti-rabbit AlexaFuor-633	1

Table 9. Primary antibodies and fluorophore-conjugated secondary antibodies used for confocal microscopy.

8. *In vitro* experiments

To perform *in vitro* studies blood was obtained from healthy donors in 3.8% sodium citrate or EDTA.

8.1. *In vitro* clot formation

Citrate whole blood (WB), platelets rich plasma (PRP) and platelets free plasma (PFP) were used to generate *in vitro* clots after addition of 25mM Cl_2Ca at 37ºC for 30 minutes. Absence of leukocytes in PRP samples was confirmed by flow cytometry (additional methods) and cell count (Hematology analyzer, Medonic CA 530). *In vitro* generated clots were washed with PBS and stored at -80ºC **(Figure 13).**

8.2. Platelet aggregation

Platelet aggregation was assessed on the Multiplate analyzer (Dynabyte® , Munich, Germany), using adenosine diphosphate [ADP:10 **µ**M], collagen [2 **µ**g/mL] and thrombin receptor-activating peptide [TRAP-6: 64 **µ**M] in citrate -anticoagulated whole blood (0.3 ml per test).

Measurements were performed between 0.75 and 1.5 hours after venipuncture (time window given by the manufacturer: 30 – 240 minutes). Aggregation was continuously recorded over 5 minutes in two independent measuring units (four silver-coated conductive copper wires) per test. The increase of impedance due to the attachment of platelets to the electrodes was detected and transformed separately to arbitrary aggregation units [U] plotted against time [AU * min]. Aggregation measured was quantified as the area under the curve [AUC]. This was calculated from the mean values from the two curves (internal control: accepted differences between curves: < 20 %). The method reproducibility was 6.5 %. After determination of aggregation time for the different agonist using Multiplate analyzer,

experiments were reproduced by stopping the reaction 10 seconds and 4 minutes after the agonist addition. After inducing platelet aggregation, samples were centrifuged to obtain platelet free plasma and stored at -80ºC **(Figure 13)**.

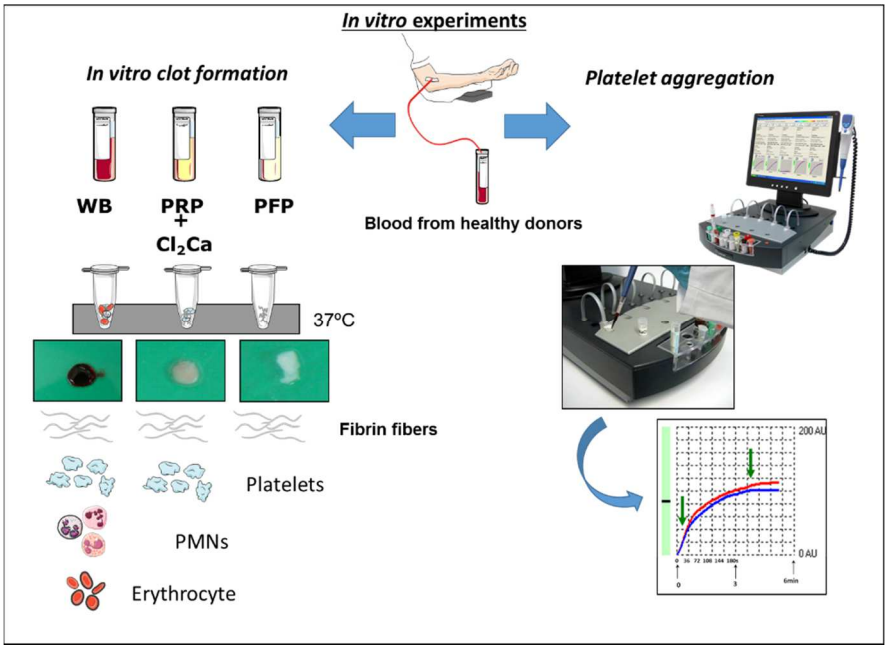

Figure 13. Schematic view of *in vitro* experiments: clot formation and platelet aggregation.

9. Bioinformatic analysis (Ingenuity pathway analysis)

The statistically significant neural network and canonical pathway in which the identified proteins were involved were generated through the use of IPA (Ingenuity System, www.ingenuity.com).

Functional analysis of a network: The functional analysis of a network identifies the biological functions and/or diseases that are most significant to the molecules in the network. Thus, the network molecules associated with biological functions and/or diseases in the Ingenuity Knowledge Base were considered for the analysis. Right-tailed Fisher's exact test was used to calculate a p-value determining the probability that each biological function and/or disease assigned to that network is due to chance alone.

Canonical pathway analysis: Canonical pathway analysis was used to identify the pathways from the IPA library that were most significant to the data set. The significance of the association between the data set and the canonical pathway was measured in 2 ways: 1) ratio of the number of molecules from the data set that maps to the pathway divided by the total number of molecules that maps to the canonical pathway is displayed. 2) Fisher's exact test was used to calculate a p-value determining the probability that the association between the genes in the data set and the canonical pathway is explained by chance alone.

10. Statistical analysis

In the different studies, data were usually expressed as median and inte quartile range (IQR) except when indicated. A test for normality was performed using the Shapiro–Wilks test. Group differences were determined by non parametrical and parametrical test (Mann–Whitney test or t-test) for non normal and normal data, respectively. Categorical variables were compared using the $\chi 2$ test or Fisher exact test. Bivariate correlations between variables were determined by Spearman or Pearson correlation coefficients.

Wilcoxon test was used for repeated measurements. To determine the correlation between two or more variables and a response variable a bivariate analysis (correlation or t-test) was performed followed by a multiple lineal regression model (stepwise selection of variables) including those variables statistically significant in the bivariate analysis to assess the most parsimonious model. Minimal required sample size was calculated and validated using the JavaScript-based method for simple power/sample size calculation when two independent groups are compared, provided in http://www.stat.ubc.ca/~rollin/stats/ssize/n2.html.

Receiver-operating characteristic (ROC) analysis was used to obtain the sensitivity and specificity of the assays. Statistical analysis was performed with the Stat View 5.0.1 and IBM SPSS Statistics v 19.0 software. N indicates the number of subjects tested. A p-value ≤ 0.05 rsionwas considered significant.

IV. RESULTS

1. Paper 1

“Changes in thrombus composition and profilin-1 release in acute myocardial infarction”.

Ilaria Ramaiola, Teresa Padro´, Esther Peña, Oriol Juan-Babot, Judit Cubedo, Victoria Martin-Yuste, Manel Sabate, Lina Badimon

Eur Heart J. 2014 Sep 12. pii: ehu356. [Epub ahead of print]

The *objective* of this study was to investigate the evolving composition of human coronary thrombus in ST-segment elevation myocardial infarction (STEMI) in function of elapsed time of ischaemia.

In *conclusion*, our results show a dynamic evolution of coronary thrombi both in structure and cell composition as a function of ischaemia time. Aged ischaemic thrombi were more likely to have reduced Pfn-1 content releasing Pfn-1 to the circulation. Onset-of-pain-to-PCI elapsed time in STEMI patients and hence age of occlusive thrombus can be profiled by Pfn-1 levels found in the peripheral circulation.

Eur Heart J. 2014 Sep 12. pii: ehu356. [Epub ahead of print]

Changes in thrombus composition and profilin-1 release in acute myocardial infarction.

Ilaria Ramaiola, Teresa Padró, Esther Peña, Oriol Juan-Babot, Judit Cubedo, Victoria Martin-Yuste, Manel Sabate, Lina Badimon

Ramaiola I, Padró T, Peña E, Juan-Babot O, Cubedo J, Martin-Yuste V, Sabate M, Badimon L. Changes in thrombus composition and profilin-1 release in acute myocardial infarction. Eur Heart J. 2015 Apr 21;36(16):965-75. doi: 10.1093/eurheartj/ehu356

2. Paper 2

"High-molecular-weight kininogen and the intrinsic coagulation pathway in patients with de novo acute myocardial infarction".

Judit Cubedo, Ilaria Ramaiola, Teresa Padró, Victoria Martin-Yuste, Manel Sabate-Tenas, Lina Badimon

Thromb Haemost. 2013 Dec;110(6):1121-34

The *objective* of this study was to investigate the coordinated changes in coagulation-related proteins in the evolution after an acute myocardial infarction (AMI).

In conclusion, our results demonstrate an active exchange between HMWK forms and a decrease in FXI indicative of intrinsic pathway activation, together with an increase in fibrinogen gamma turnover and D-dimer formation in the early phase post-AMI. Moreover, coronary thrombi showed a dynamic evolution in fibrinogen composition depending on the duration of ischemia influencing serum fibrinogen-related products content.

Thromb Haemost. 2013 Dec;110(6):1121-34. doi: 10.1160/TH13-05-0381. Epub 2013 Aug 29.

High-molecular-weight kininogen and the intrinsic coagulation pathway in patients with de novo acute myocardial infarction.

Judit Cubedo, Ilaria Ramaiola, Teresa Padró, Victoria Martin-Yuste, Manel Sabate-Tenas, Lina Badimon

Cubedo J, Ramaiola I, Padró T, Martin-Yuste V, Sabate-Tenas M, Badimon L. High-molecular-weight kininogen and the intrinsic coagulation pathway in patients with de novo acute myocardial infarction. Thromb Haemost. 2013 Dec;110(6):1121-34. doi: 10.1160/TH13-05-0381

3. Paper 3

"Imbalance in stress-homeostasis related proteins in the proteomic profile of in-stent thrombus"

Ilaria Ramaiola , Judit Cubedo, Teresa Padró, Javier Goicolea, Ana Blasco , Lina Badimon

Manuscript in preparation

The *objective* of this study was to to characterize in-stent thrombus (IST) in comparison to thrombus formed on native coronary arteries, identifying differencial proteins with potential relevance of clinical-outcome.

In c*onclusion*, our results reveal important structural differences in IST versus thrombi in native vessels with a highly cellular profile. Moreover the detected imbalance in redox-related proteins could induce an accumulation of H_2O_2 contributing to an increased oxidative damage in in-stent thrombus.

Imbalance in stress-homeostasis related proteins in the proteomic profile of in- stent thrombus

Running title: Thrombus Proteome in Stent-Thrombosis

By

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ABSTRACT

Background The incidence of in-stent thrombosis has been reported in 0.5-2%, but despite being a quantitatively minor problem, in-stent thrombosis has a major clinical impact owing to a high risk of myocardial infarction and death. Thus, mortality due to in-stent thrombosis has been reported to be as high as 40%. Moreover, 20% of patients with a first in-stent thrombosis experience a recurrent in-stent thrombosis episode within 2 years. The objective of the present study was to characterize intra-stent thrombus in comparison to thrombus formed on native coronary arteries.

Methods Nineteen patients clinically treated as per guidelines and thromboaspiration (within the first 6 hours after the onset of the event) were included in the study. Nine patients were intervened by PCI because of in-stent thrombus (IST) and ten because of thrombus formation on native coronary arteries (CT). The retrieved thrombi were frozen and the thrombus proteomic profile was characterized by 2D-electrophoresis and mass-spectrometry.

Results Identified proteins were mainly related to the vessel and coagulation system (52%), followed by structural proteins (23%), and proteins with enzymatic activity (7%) binding and transport

related proteins (6%). IST showed a higher content of structural-related proteins such as gelsolin, actin cytoplasmic 1, tropomyosin, and myosin, without changes in fibrin(ogen) related products. IST thrombus showed an imbalance in redox-homeostasis related proteins with an increase in superoxide dismutase and a decrease in peroxoredoxin-2. Moreover, superoxide dismutase thrombus content was positively correlated with platelets.

Conclusions Our results reveal important structural differences in in-stent thrombus versus spontaneous thrombi in native vessels with a highly cellular profile. Moreover the detected imbalance in redox-related proteins could induce an accumulation of H_2O_2 contributing to an increased oxidative damage in in-stent thrombus.

INTRODUCTION

Management of acute ST-elevation myocardial infarction (STEMI) has undergone dramatic improvements during the past three decades and in-hospital and 30-day mortality rates have tremendously decreased from 15–20% in the pre-thrombolytic area to 4.5% by the use of primary percutaneous coronary intervention (PPCI) in controlled trials by experienced centres under optimal trial conditions.[1] PPCI following STENT implantation has improved survival as compared to thrombolysis,[2-4] even though concerns still remain regarding the risk of in-stent thrombosis in the setting of STEMI.[5 6] Several studies have reported an incidence of in-stent thrombosis (ST) of 0.5-2%, but despite being a quantitatively minor problem, ST has a major clinical impact owing to a high risk of myocardial infarction and death. Thus, mortality due to ST has been reported to be as high as 40 % in subacute ST and 9% in very late thrombosis.[5, 7-9] Moreover diabetes, post-procedural TIMI 0–2 flow and an ischemia time of more than 6 hours have been shown to be independent predictors of ST.[5]

Mechanical procedures may explain the high risk of ST in STEMI. Stent strut penetration of an underlying necrotic core may augment inflammation and fibrin deposition and inhibit neointimal growth,

resulting in uncovered stent struts. Furthermore, reduced epicardial flow and vasoconstriction may lead to stent undersizing, with incomplete stent apposition and underexpansion during the index procedure.[5] Several studies have demonstrated associations of coronary thrombus characteristics with myocardial perfusion,[10-14] restenosis,[15] left ventricular function/remodeling,[12, 13] ischemia time,[16] and long term mortality.[17, 18] Moreover the cellular thrombus composition has been associated with redox imbalance in diseased arterial tissues[19] and oxidative stress has been linked to an impaired myocardial reperfusion in STEMI patients.[13] However, until now, there are no studies comparing the proteomic composition of in-stent thrombus with those formed in native coronary arteries, making unclear the potential molecular pathways linking the high rate of mortality in ST and thrombus composition.

Herein we have performed a differential proteomic analysis of in-stent thrombus in comparison with those formed on native coronary arteries to underscore the potential pathways behind thrombus formation and the high risk in ST patients.

MATERIALS AND METHODS

Study population

The study population comprised a group of 19 patients admitted with ST-elevation myocardial infarction who had undergone percutaneous coronary intervention (PCI) associated with thromboaspiration followed by stent implantation.

Ten patients were intervened by PCI because of in-stent thrombus (IST) and ten because of thrombus formation on native coronary arteries (CT). To investigate the effect of stent in the thrombus composition, thrombi retrieved from patients with in-stent thrombosis (IST; n = 9) and those formed on native coronary arteries (CT; n = 10) were comparatively investigated.

Loading dose of clopidogrel and aspirin was 600 and 300 mg respectively. From 2011 to the patients was administrated prasugrel: loading dose of 60 mg and maintenance of 10 mg/day. All the patients received heparin before PCI (5000 UI) and 1000 UI every 30 minutes during procedure. Dosages of GPIIb/IIIa were bolus of 180 µg/kg i.v. and 2 µg/kg /min infusion for 12 h.

Demographic and clinical characteristics of both groups of patients are provided in **Table 1.**

The studies were conducted according to the principles of Helsinki's Declaration. All patients gave written informed consent before enrolment.

A schematic view of the workflow of the study is shown in **Supplemental Figure 1.**

Thrombus collection and sample preparation

Thrombi aspired from the coronary artery through the thrombectomy device were immediately frozen. Previously to protein extraction thrombi were washed with PBS solution to eliminate un-attached superficial blood cells. Extraction was performed using urea/detergent buffer (7 mol/L urea; 2mol/L thiourea; 2% CHAPS) as previously described.[20] Protein concentration was measured with 2D-Quant Kit (GE Healthcare, Uppsala, Sweden). All processed samples were stored at -80ºC until used.

Proteomic analysis

Analysis of differential protein expression patterns and protein identification was performed by a proteomic approach using two-

dimensional electrophoresis and mass-spectrometry identification, as previously described.[16, 20]

Two-dimensional gel electrophoresis (2-DE): For analytical and preparative gels, 100 μg and 300 μg of protein of the urea/chaps thrombi extracts were respectively loaded in 17-cm dry strips (pH 3-10 linear range, GE Healthcare, Uppsala, Sweden) and the proteins were separated by electrofocussing according to their isoelectric point (pI). Second dimension was resolved in 12% SDS-PAGE gels. Electrophoresis was performed using an Ettan Daltssix System (GE HealthCare, Uppsala, Sweden). Gels were developed by fluorescent Flamingo staining using a Typhoon 9400 (GE-HealthCare, Uppsala, Sweden).

Analysis for differences in protein patterns was performed with the PD-Quest 8.0 (Bio-Rad Laboratories, Hercules, CA), using a single master that included all gels. Each spot was assigned a relative value that corresponded to the single spot volume compared to the volume of all spots in the gel, following background subtraction and normalization between gels.

Mass spectrometry analysis: For mass spectrometry analysis, proteins were identified, by matrix-assisted laser

desorption/ionization time-of-flight (MALDI-TOF) using an AutoFlex III Smartbeam MALDI-TOF/TOF (Bruker Daltonics, Bremen, Germany). Samples were applied to Prespotted AnchorChip plates (Bruker Daltonics, Bremen, Germany) surrounding the calibrants provided on the plates. Spectra were acquired with flexControl on reflector mode, (mass range 850-4000 m/z, reflector 1: 21.06 kV; reflector 2: 9.77kV; ion source 1 voltage: 19 kV; ion source 2: 16.5kV; detection gain 2.37x) with an average of 3500 added shots at a frequency of 200 Hz. Each sample was processed with flexAnalysis (version 3.0, Bruker Daltonics, Bremen, Germany) considering a signal-to-noise ratio over 3, applying statistical calibration and eliminating background peaks.

For identification, peaks between 850 and 1000 (m/z) were excluded, since they mainly refer to matrix peaks. Spectra were analyzed by the BioTools software (version 3.2, Bruker Daltonics, Bremen, Germany) and proteins were identified using the MASCOT search on Swiss-Prot 57.15 database [Taxonomy: Homo Sapiens, Mass Tolerance 50 to 100, up to 2 trypsin miss cleavage, Global Modification: Carbamidomethyl (C), Variable Modification: Oxidation (M)]. Identification was accepted with a score higher than 56.

Western Blot validation

Thrombus protein extracts (25 μg of total protein) were resolved in 12% SDS-PAGE under reducing conditions and electrotransferred to nitrocellulose membranes in semidry conditions (TransBlot Turbo transfer system, BioRad). Detection was performed with a polyclonal antibody against superoxide dismutase (1:1000 dilution, Calbiochem), and monoclonal antibodies against peroxiredoxin-2 (1:2000 dilution, Abcam), and CD61 (1:1000 dilution, Millipore). After the addition of the secondary anti-mouse antibody (1:10000 dilution, Dako) chemiluminescence detection was performed with ChemiDoc XRS (BioRad). Band intensity quantification was performed using ImageLab v4.0 software (BioRad). Protein load was normalized with total protein signal.

In-silico bioinformatic analysis

The statistically significant neural network and canonical pathway in which the identified proteins were involved were generated through the use of IPA (Ingenuity System, www.ingenuity.com).

Functional analysis of a network: The functional analysis of a network identified the biological functions and/or diseases that were

most significant to the molecules in the network. The network molecules associated with biological functions and/or diseases in the Ingenuity Knowledge Base were considered for the analysis. Right-tailed Fisher's exact test was used to calculate a p-value determining the probability that each biological function and/or disease assigned to that network is due to chance alone.

Canonical pathway analysis: Canonical pathway analysis identified the pathways from the IPA library that were most significant to the data set. The significance of the association between the data set and the canonical pathway was measured in 2 ways: 1) A ratio of the number of molecules from the data set that maps to the pathway divided by the total number of molecules that maps to the canonical pathway is displayed. 2) Fisher's exact test was used to calculate a p-value determining the probability that the association between the genes in the data set and the canonical pathway is explained by chance alone.

Statistical analysis

Data are expressed as median and interquartile range [IQR] unless stated. N indicates the number of subjects tested. Normality was assessed by using Shapiro-Wilks test. Categorical variables were

compared using $\chi 2$ test. Statistical analysis was performed with Stat View5.0.1 software. Mann–Whitney testing was used for comparison between stent (IST) and no-stent thrombosis (CT) groups. A p-value ≤ 0.05 was considered significant.

RESULTS

Clinical characteristics of the study population

There was no significant difference in demographic characteristics and cardiovascular risk factors between IST and CT-patients (**Table 1**). IST-patients showed significant higher levels of CRP, CPK, and Troponin I. More than 80% of PCI procedures were successful.

Proteomic profile of thrombus samples

As depicted in **Figure 1A** most of the identified proteins in thrombus samples were secreted (54%) or had cytoplasmic localization (34%). Proteins involved in 8 different functional categories were identified in the thrombus proteome (**Figure 1B).** Vascular and coagulation-related proteins were the most abundant ones (52%), followed by structural proteins (23%), proteins with enzymatic activity (7%), and binding/transport related proteins (6%). Minor functional groups were proteins involved in signal transduction, chaperone activity, mitochondrial signalling and DNA interaction. Several proteins related to the 4 main functional categories showed a differential proteomic profile in IST thrombi when compared to CT (**Figure 2; Table 2 and Supplemental Table 1**).

Moreover, the IPA analysis revealed that those differential proteins were related to the functional networks of cardiovascular disease, haematological system, oxidative stress and cell death and survival (**Figure 3**).

Stent influences the proteomic profile of coronary thrombi

Up-regulation of structural proteins in IST

The main structural related protein identified in thrombus samples was actin cytoplasmic 1 that was detected as a single spot with apparent molecular mass of 42 kDa and pI of 5.2 and as a cluster of 4 spots with a molecular mass range of 25.5-23.2 kDa and pI range of 5.2-5.5. Total actin intensity depicted a significant increase in the IST group compared with CT-patients ($p<0.05$; **Figure 4A**).

Another differential structural protein was myosin regulatory light chain (MLC) that was identified as two spots with a molecular mass of 18.7 and 16.9 kDa and a pI of 4.7 and 5.0.

The total intensity of MLC was also significantly increased in IST-patients ($p<0.05$; **Figure 4B**).

The strongest change in structural proteins was detected in tropomyosin. This protein was identified as a cluster of 5 spots with an apparent molecular mass between the range of 28.2 and 27.1 kDa

and a pI range between 4.7 and 4.9. Total tropomyosin intensity evidenced a 3.2-fold increase in the IST group ($p<0.05$; **Figure 4C**). Gelsolin, that was identified as a single spot with an apparent molecular mass of 54.1 kDa and a pI of 5.1, showed an increasing trend in IST-patients when compared to the CT group although not reaching significance (**Figure 4D).**

Bioinformatic analysis using data mining and the IPA software of the structural identified proteins revealed significant changes in the cell motility canonical pathway between the studied groups ($p<0.0001$; **Supplemental Figure 2**).

Changes in proteins with enzymatic activity and transport/binding properties

Within the differential proteins with enzymatic activity, flavin reductase that was identified as a single spot of 22 kDa and pI of 7.13 and carbonic anhydrase-1 that was identified as a single spot of 28.8 kDa and pI of 6.59 showed the most important change with a 3.3-fold decrease in IST-patient when compared to the CT group ($p<0.05$ for both; **Figure 5A and 5B** respectively). Alpha enolase that was identified as a single spot of 47 kDa and a pI of 7 showed a decreasing trend in the IST group although not reaching significance (10-fold mean decrease). The transport protein

hemoglobin beta chain was identified as a single spot with an apparent molecular mass of 15.4 and pI of 7 that depicted an increasing trend in IST-patients (3-fold mean increase) when compared to CT-patients.

Redox-homeostasis imbalance in IST

The differential proteins involved in redox homeostasis showed an opposite behaviour in IST-patients. Superoxide dismutase (SOD) that was identified as a single spot of 21.6 kDa and pI of 7.3 showed a significant increase in IST-patients when compared to the CT-group (p<0.05; **Figure 6A**). On the contrary peroxiredoxin-2 (PRX-2) that was identified as a single spot of 22 kD and pI of 5.66 showed a significant decrease in IST when compared to CT thrombus samples (p<0.05; **Figure 6B**). This imbalance between SOD and PRX-2 levels in IST could lead to the accumulation of hydrogen peroxide and to an increased oxidative stress in these thrombi (**Figure 6C**).

Western blot validation confirmed an increasing trend in SOD (**Figure 7A**) and the significant decrease in PRX-2 in IST-patients (p=0.038; **Figure 7B**). In order to analyze the impact of the platelet content in the redox-imbalance in IST samples the amount of CD61 intensity was analyzed by WB analysis. Although the increasing

trend in CD61 intensity in IST samples did not achieve significance (**Figure 7C**), there was a direct and significant correlation between SOD and CD61 intensities (**Figure 7D**) suggesting the potential contribution of platelets to the redox-imbalance in IST.

Fibrinogen mapping in IST and CT thrombus

As previously described,[20] beta fibrinogen (FBB) was identified as 5 clusters of different molecular weight and pI that represented the 73% of total fibrinogen (**Supplemental Figure 3A**). FBB3 cluster corresponded to the whole fibrinogen beta chain, whereas FBB4, FBB5, FBB6, and FBB7 were identified as different forms of the fragment-D of fibrinogen **(Supplemental Figure 3A and Supplemental Table 1)**. IST-patients did not show significant differences in the FBB distribution profile when compared to CT group **(Supplemental Figure 3B and Table 2)**. Two gamma fibrinogen clusters were detected in coronary thrombus corresponding to entire fibrinogen gamma form and fibrinogen gamma fragments **(Supplemental Figure 4A and Supplemental Table 1)**. There were no differences in FBG distribution between IST and CT-patients (**Supplemental Figure 4B, C and D, and Table 2)**.

DISCUSSION

In the last years, mechanical reperfusion has proven to be a superior treatment strategy in STEMI patients, leading to a better myocardial reperfusion and lower risk of distal embolization improving late clinical outcome.[2, 3, 21] Nevertheless, concerns still remain regarding the risk of in-stent thrombosis (ST) in the setting of STEMI, and the high risk of mortality that reaches the 40% in the subacute in-stent thrombosis, ten times higher than in native coronary arteries in STEMI patients.[5] Until now the high risk of ST in STEMI has been mainly attributed to the mechanical procedure of stent placement and the biological response of the artery to this procedure such as changes in the dimension of the vascular wall.[22] But whether thrombi grown in stent have a differential protein composition that may explain their higher aggressiveness remains to be elucidated.

Herein we have evaluated the possible molecular mechanisms that could explain the high risk of adverse events in stent thrombosis by applying differential profiling proteomic and system biology approaches to analyze the composition of in-stent thrombus (IST). Our results have demonstrated that in-stent thrombus have a different protein composition compared with thrombi formed in native coronary artery. Specifically, IST show a coordinated

increase of cell-related structural proteins such as actin, tropomyosin, myosin, and gelsolin. Moreover, the specific marker for erythrocytes, hemoglobin, was also increased in IST. These changes underscore an increase in the cellularity of thrombi developed in the stent compared to those formed in native coronary arteries. Indeed, endothelial injury and foreign body stent placement have shown to induce platelet activation and deposition at the site of the lesion, with the recruitment of circulating leukocytes. Leukocyte-platelet interactions are critical in the initiation and progression of neointimal formation which is followed by the activation of chemokines and cytokines leading to the proliferation and migration of smooth muscle cells (SMCs) within the media and the intima.[22] Previous studies have pointed out to platelets, leukocytes and erythrocytes as sources of oxidative stress in vascular disease.[19] In fact, platelet-leukocyte interaction is accompanied by ROS production causing oxidative damage. Moreover, Yunoki and colleagues correlated erythrocyte-rich thrombus with impaired myocardial reperfusion in association to oxidative stress.[13] In line with these results the detected increase in hemoglobin in IST could induce oxidative stress by the deregulation of the heme/iron metabolism. Indeed it has been reported that

oxidation of hemoglobin leads to non-functional methaemoglobin provoking an ischemia/hipoxia condition in the tissues [23] [24] Interestingly the enzymatic reduction of methaemoglobin has been attributed to flavin reductase[25] [26, 27] which is an anti-oxidant enzyme that is decreased in IST. This decrease could contribute to the accumulation of methaemoglobin increasing the oxidative stress in IST. Additionally we have detected a coordinated decrease of another anti-oxidant enzyme, peroxiredoxin-2 which has also shown to be decreased in IST. This enzyme is mainly involved in peroxide degradation .[28] [29] Moreover, IST have also shown an increase of the redox-homeostasis enzyme superoxide dismutase. This enzyme is responsible for the transformation of O_2^- to produce H_2O_2,[30] therefore the increase in SOD could induce an increase in H_2O_2 in IST thrombi probably due to an increase in plts content as suggested by the positive correlation between CD61 and SOD band intensities in WB validation analysis. In normal condition the excess of H_2O_2 is eliminated by the action of peroxiredoxin-2. Thus, in IST, the imbalance between superoxide dismutase and peroxiredoxin-2 activities could lead to the accumulation of H_2O_2 contribution oxidative damage.[19] The decrease of carbonic anhydrase, protein that protects cells from hydrogen peroxide-induced apoptosis could

contribute to the oxidative damage induced by hydrogen peroxide accumulation.[31]

Previous studies have reported the involvement of H_2O_2 in collagen induced platelet activation[32] and the propagation of platelet aggregation.[33] Therefore, the imbalance in redox-homeostasis could contribute to a higher aggressiveness of IST thrombi by an increased H_2O_2-induced platelet response.

Moreover, alpha enolase that has been related to hypoxia tolerance in endothelial cells[34] showed a 10-fold decrease in IST. The hypoxia state generated by metahemoglobin favours reactive oxygen species formation and increases the oxidative damage. Therefore, the detected decrease in alpha enolase could induce an increased susceptibility to oxidative stress in the milieu of the thrombi developed in the stent contributing to the higher aggressiveness of this type of thrombus.

On the other hand, IST thrombi did not show changes in fibrinogen proteomic profile, which has been previously shown to be affected by other parameters such as ischemia time.[20] Nevertheless, but the increase of the oxidant environment present in stented arteries could lead to oxidative post-translational modification of fibrinogen

influencing both its structure and function, phenomenon that has been associated with vascular disease pathogenesis.[35 36]

CONCLUSION

Overall our results reveal important differences in the protein composition of in-stent thrombus versus spontaneous thrombi in native vessels with an increase in thrombus cellularity that represents a source of oxidative stress. This change in thrombus composition together with the weakening of the antioxidant machinery favours the formation of a highly oxidative stress environment probably contributing to the increased aggressiveness of in-stent thrombus finally leading to worse prognosis of IST-STEMI-patients.

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Table 1. Background description of STEMI-patients

	CT (n = 10)	IST (n = 9)	*p*-value
Age	66±15	61±10	ns
Male (%)	80	78	ns
Risk factors (%)			
Active smoker	30	33	ns
Diabetes	10	11	ns
Hypertension	50	56	ns
Dyslipemia	60	56	ns
Ischemia time			
Symptom onset to PCI (min)	255±137	334±180	ns
Biomarkers on admission			
Troponin I (µg/L)	72±45	218±204	ns
CPK (U/L)	1798±1117	3716±45	ns
CRP (mg/L)	12±83	78±109	p=0.05
Previous treatment (%)			
Statin	50	100	p=0.01
Anti-HT	70	100	ns
GPIIb/IIIa antagonist (%)	90	89	ns
Reperfusion assessment (%)			
TIMI flow grade 3 before PCI	0	0	ns
TIMI flow grade 3 after PCI	80	89	ns

Data are expressed as mean ± SD unless stated. Anti-HT=antihypertensive; CPK = creatine phosphokinase; CRP= C-reactive protein; PCI = percutaneous coronary intervention; TIMI = thrombolysis in Myocardial Infarction (0= no perfusion, 1= penetration without perfusion, 2= partial reperfusion, 3= complete reperfusion).

Table 2. Changes in the identified proteins in the 2-DE analysis of thrombus samples of IST and CT-patients.

Spot/ cluster No.	Protname	Biological function	Subcellular location	Ratio IST/CT	*p*-value
1	Fibrinogen gamma chain	coagulation and haemostasis	secreted	0.9	ns
2	Fibrinogen gamma chain	coagulation and haemostasis	secreted	1.1	ns
3	Fibrinogen beta chain	coagulation and haemostasis	secreted	1.6	ns
4	Chain B, Crystal structure of Fibrinogen fragment D	coagulation and haemostasis	secreted	1.2	ns
5	Chain B, Crystal structure of Fibrinogen fragment D	coagulation and haemostasis	secreted	1.0	ns
6	Chain B, Crystal structure of Fibrinogen fragment D	coagulation and haemostasis	secreted	0.5 ↓	ns
7	Chain B, Crystal structure of Fibrinogen fragment D	coagulation and haemostasis	secreted	1.0	ns
8	Gelsolin	structural	cytoplasm	2.2 ↑	ns
9	Tubulin	structural	cytoplasm	1.0	ns
10	Actin cytoplasmic 1	structural	cytoplasm	2.3 ↑	≤0.05
11	Tropomyosin alpha chain	structural	cytoplasm	3.2 ↑	≤0.05
12	Myosin light chain	structural	cytoplasm	2.2 ↑	≤0.05
13	Alpha enolase	enzymatic activity	cytoplasm	0.1 ↓	ns
14	Carbonic anhydrase 1	enzymatic activity	cytoplasm	0.3 ↓	≤0.05
15	Flavin reductase	enzymatic activity	cytoplasm	0.3 ↓	≤0.05
16	Protein MICAL 3	enzymatic activity	cytoplasm	1.2	ns
17	Cytochrome P450	enzymatic activity	membrane	1.1	ns
18	Peroxiredoxin-2	Redox homeostasis	cytoplasm	0.5 ↓	≤0.05
19	Superoxide dismutase [Mn]	Redox homeostasis	mitochondrion	2.9 ↑	≤0.05
20	Beta Hemoglobin	Transport/Binding	red blood cells	3.2 ↑	ns

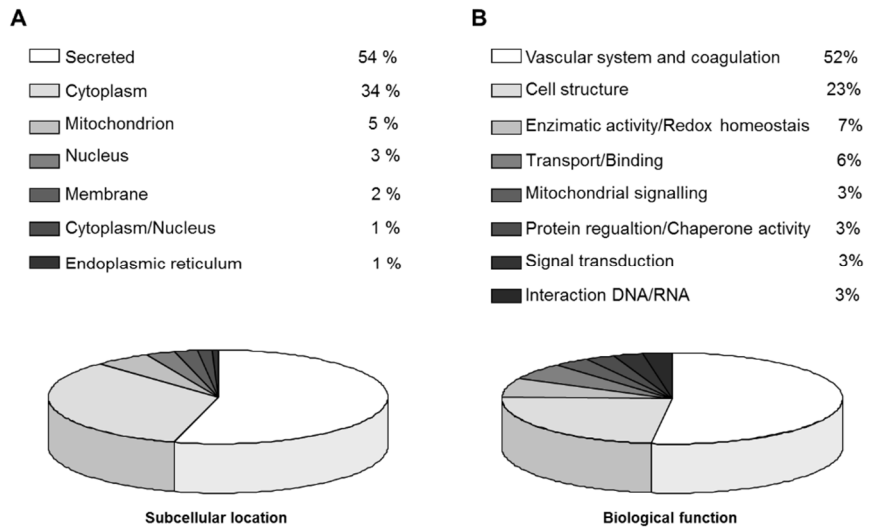
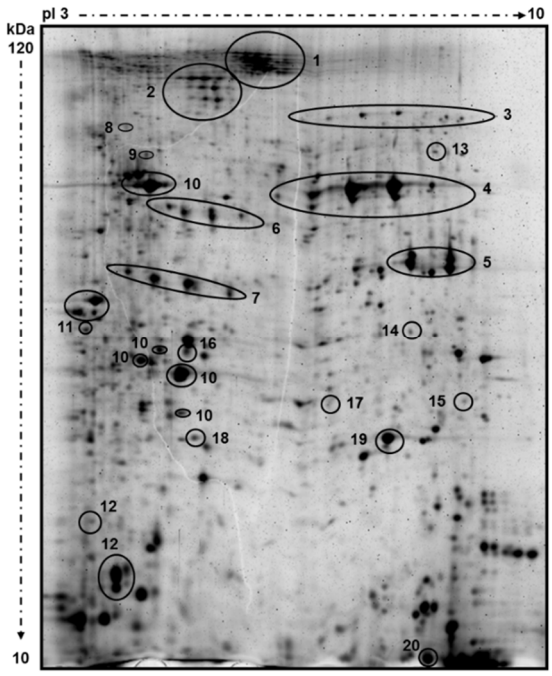


Figure 1. Pie charts representing the (A) biological function and (B) the subcellular location distribution of the proteins identified in thrombus samples.



Spot No.	Protname
1	Fibrinogen gamma chain
2	Fibrinogen gamma chain
3	Fibrinogen beta chain
4	Chain B, Crystal structure of Fibrinogen fragment D
5	Chain B, Crystal structure of Fibrinogen fragment D
6	Chain B, Crystal structure of Fibrinogen fragment D
7	Chain B, Crystal structure of Fibrinogen fragment D
8	Gelsolin
9	Tubulin
10	Actin cytoplasmic 1
11	Tropomyosin alpha chain
12	Myosin light chain
13	Alpha enolase
14	Carbonic anhydrase 1
15	Flavin reductase
16	Protein MICAL 3
17	Cytochrome P450
18	Peroxiredoxin-2
19	Superoxide Dismutase [Mn]
20	Haemoglobin beta

Figure 2. Thrombus proteomic profile. Representative image of the 2-DE profile of thrombus samples in a pI range between 3 and 10 showing the mainly identified proteins.

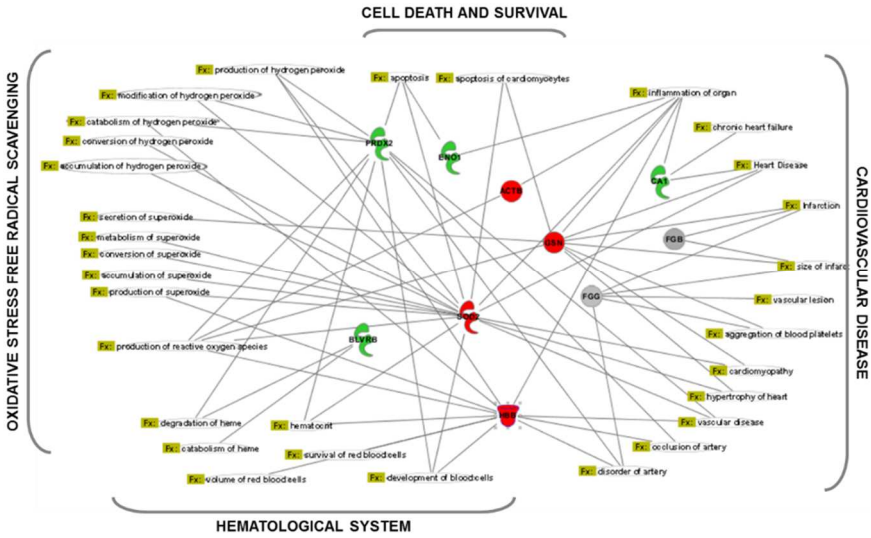


Figure 3. Ingenuity Pathway Analysis Network. The identified proteins were mainly related to the functional networks of: cardiovascular disease, haematological system, oxidative stress, and cell death and survival. Proteins in red are up-regulated and proteins in green are down-regulated in IST. Proteins in grey are not modified between groups.

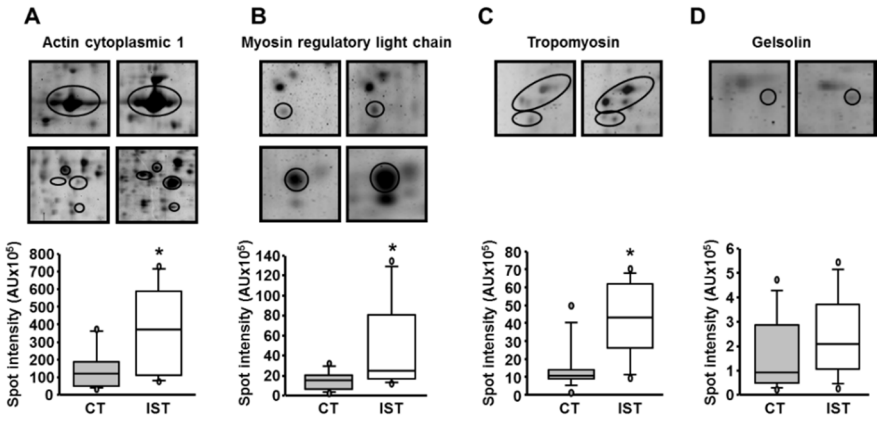


Figure 4. Changes in structural proteins in IST. (A) Representative 2-DE image and box plot showing the significant increase in the intensity of actin cytoplasmic 1 in IST thrombus samples compared to CT. **(B)** Representative 2-DE image and box plot showing the significant increase in the intensity of myosin regulatory light chain in thrombus samples of IST-patients when compared to CT group. **(C)** Representative 2-DE image and box plot showing the significant increase in tropomyosin intensity in IST thrombus samples compared to CT. **(D)** Representative 2-DE image and box plot showing a 2.2-fold increase in gelsolin intensity in IST thrombus samples compared to CT. *$p < 0.05$; Mann–Whitney test.

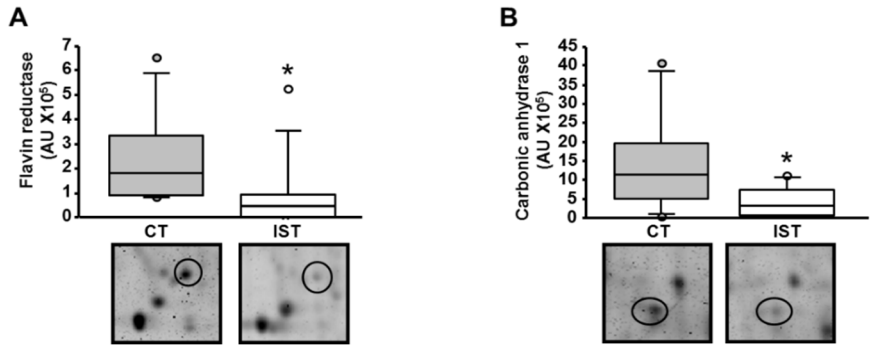


Figure 5. Differential proteomic profile of enzymatic proteins in IST. Box plots and representative 2-DE images showing the significant decrease in the intensity of flavin reductase **(A)** and carbonic anhydrase **(B)** in IST thrombus samples compared to CT. *p < 0.05; Mann–Whitney test.

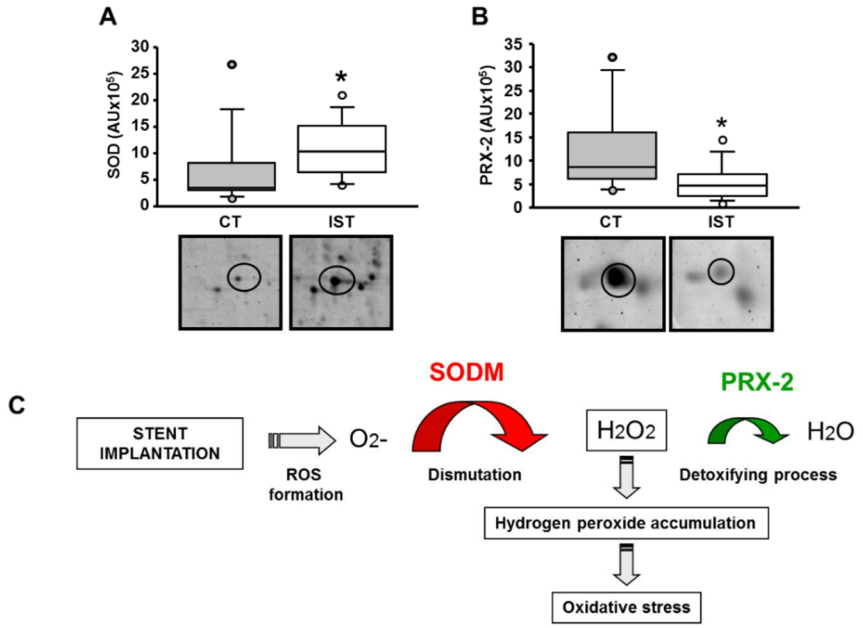


Figure 6. Imbalance in proteins involved in redox-homeostasis in IST. Box plots and representative 2-DE images showing **(A)** the significant increase in the intensity of superoxide dismutase (SOD); and **(B)** the significant decrease in the intensity of peroxiredoxin-2 (Prx-2) in IST thrombus samples compared to the CT group. **(C)** Schematic diagram of the imbalance between SOD and Prx-2 that could favour the accumulation of hydrogen peroxide in IST thrombus.

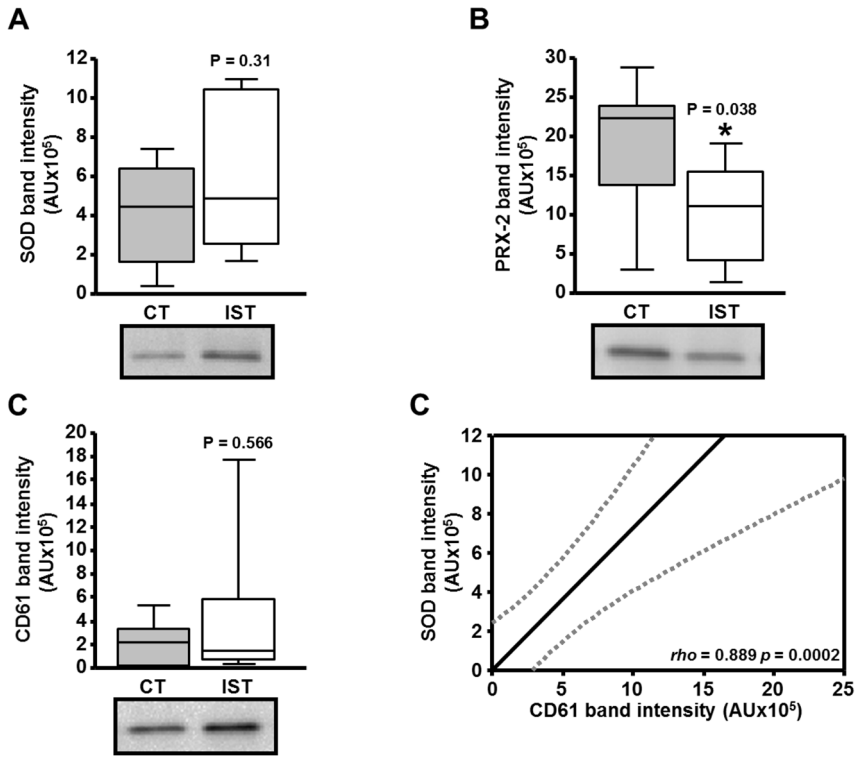


Figure 7. Western blot validation of redox-homeostasis-related proteins. Box plots and representative WB images showing **(A)** the increasing trend in superoxide dismutase (SOD); **(B)** the significant decrease in the intensity of peroxiredoxin-2 (Prx-2); and **(C)** the increasing trend in CD61 in IST thrombus samples compared to the CT group. **(D)** Regression plot showing the significant and positive correlation between SOD and CD61 in thrombus samples.

SUPPLEMENTAL MATERIAL

Imbalance in stress-homeostasis related proteins in the proteomic profile of in- stent thrombus

Running title: Thrombus Proteome in Stent-Thrombosis

By

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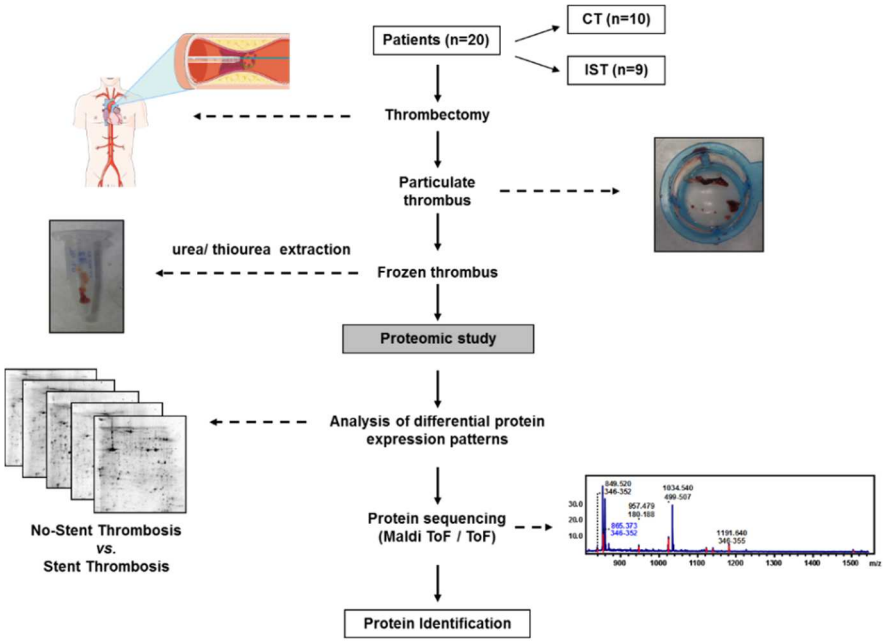
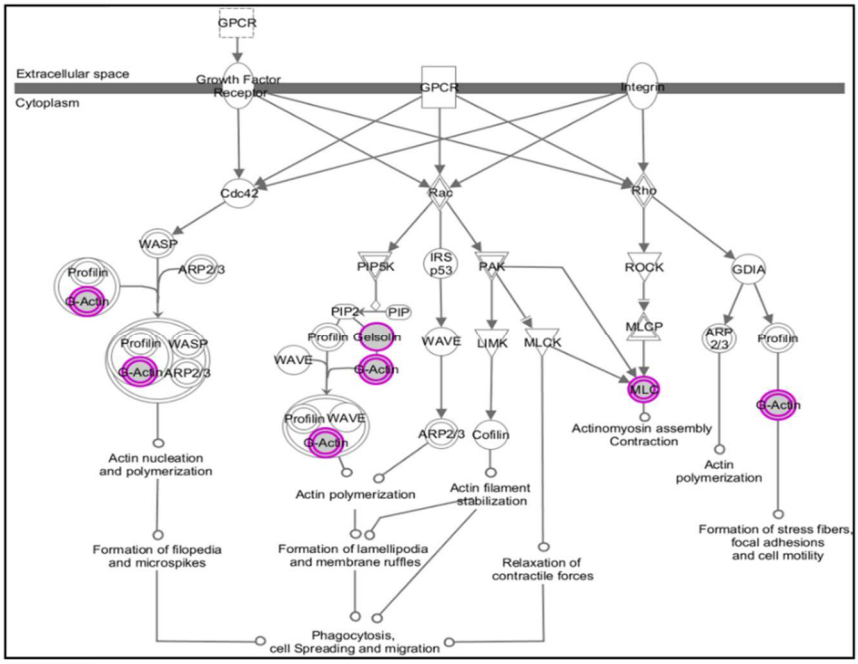
Supplemental Table 1. Proteins identified by MALDI-TOF/TOF in thrombus samples after 2-DE analysis.

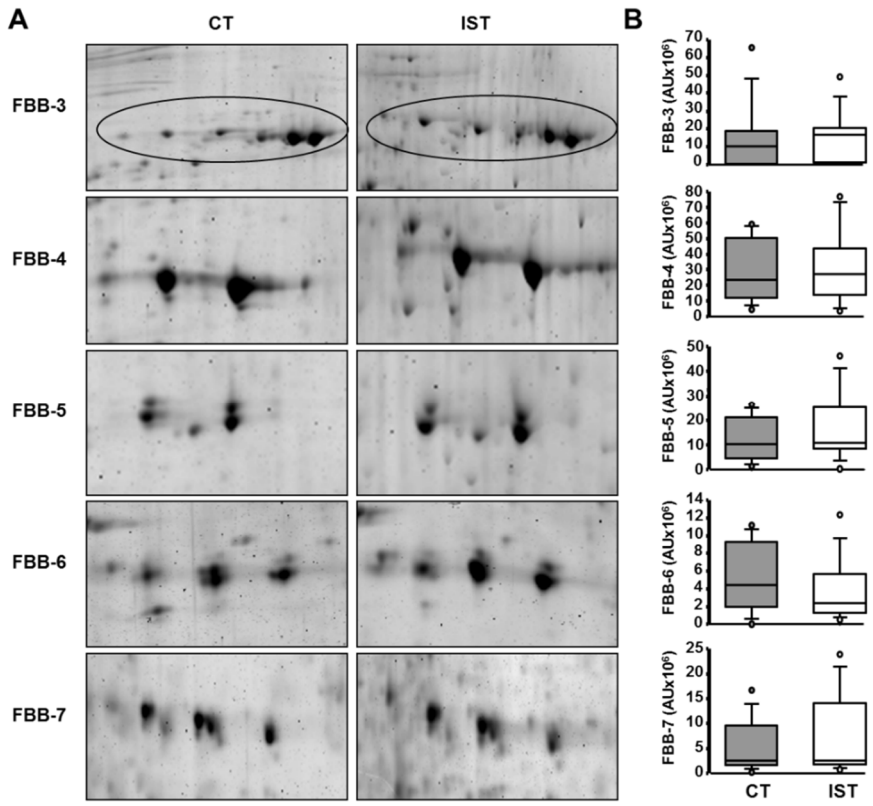
Spot/ cluster No.	Protname	Swiss-Prot No.	MW range (kDa)	pI range	Mascot score **	Sequence coverage (%) **	MS/MS Pico p
1	Fibrinogen gamma chain	P02679	67-82	5.7-6.6	91	28	
2	Fibrinogen gamma chain	P02679	57-64	5.4-5.8	93	17	
3	Fibrinogen beta chain	P02675	53-54	6.3-8.6	138	33	
4	Chain B, Crystal structure of Fibrinogen fragment D	GI:2781208*	41	6.4-7.4	97	25	
5	Chain B, Crystal structure of Fibrinogen fragment D	GI:2781208*	30.31	7.1-7.8	72	16	
6	Chain B, Crystal structure of Fibrinogen fragment D	GI:2781208*	36-37	5.3-6.1	80	19	
7	Chain B, Crystal structure of Fibrinogen fragment D	GI:2781208*	29-30	5.1-5.8	94	23	
8	Gelsolin	P06396	54.1	5.1	41		1349.6
9	Tubulin	Q9H4B7	50.4	5.2	85		1334.7
10	Actin cytoplasmic 1	P60709	42	5.2	93	25	
			25.5-23.2	5.2-5.5	89	23	
11	Tropomyosin alpha chain	P67936	28.2-27.1	4.7-4.9	60	17	
12	Myosin light chain	P19105	18.7	4.7	87	50	
			16.9	5.0	47		1382.79
13	Alpha enolase	P06733	46.9	7.4	67	21	
14	Carbonic anhydrase 1	P00915	27.2	7.2	62		985.5
					68		1034.55
15	Flavin reductase	P30043	22.5	7.9	63		1161.6
16	Protein MICAL 3	Q7RTP6	25.5	5.5	60	19	
17	Cytochrome P450	Q6UW02	22.8	6.7	60	13	
18	Peroxiredoxin-2	P32119	21.7	5.4	68	24	
19	Superoxide dismutase [Mn]	P04179			68	25	
20	Beta Hemoglobin	P68871	15.3	7	60	58	



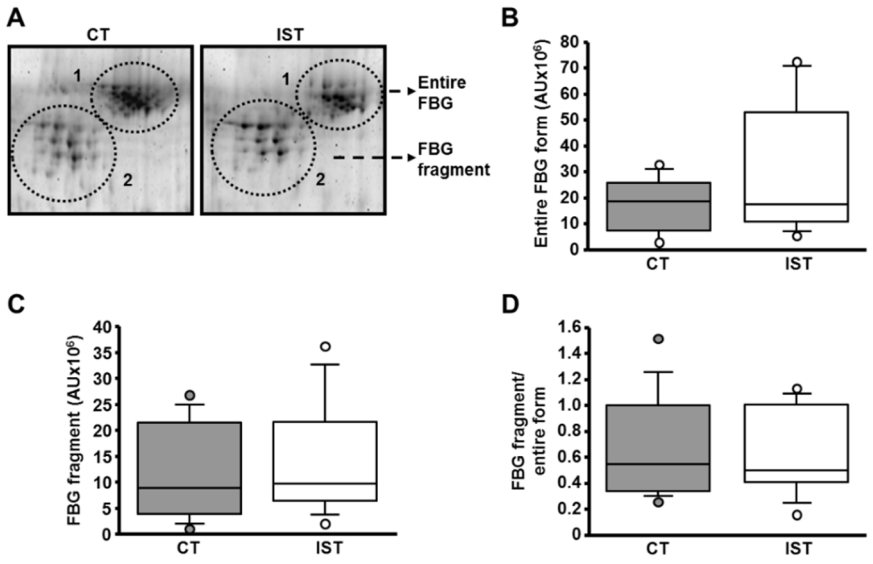
Supplemental Figure 1. Schematic diagram representing the overall approaches of this study.



Supplemental Figure 2. Cell motility canonical pathway. Bioinformatic analysis using the Ingenuity System Pathway Analysis showing significant changes in the cell motility canonical pathway between IST and CT thrombus samples ($p<0.0001$).



Supplemetal Figure 3. Beta fibrinogen distribution profile in PCI thrombus proteome. (A) Representative image of the 2-DE profile of beta fibrinogen (FBB) clusters in IST and CT samples. **(B)** Box plots showing the lack of differences in FBB clusters between groups.



Supplemetal Figure 4. Gamma fibrinogen distribution profile in PCI thrombus proteome. (A) Representative image of the 2-DE profile of gamma fibrinogen (FBG) clusters in IST and CT samples. Box plots showing the lack of differences between groups in the intensity of: **(B)** the entire form of FBG, **(C)** FBG fragments, and **(D)** the ratio between FBG fragments and the entire form.

V. DISCUSSION

Atherothrombosis is a major cause of clinical cardiovascular events such as acute coronary syndromes. Among these, and despite currently available therapies, ST-segment elevated myocardial infarction (STEMI) is a leading cause of mortality in developed countries. In the recent years the time of myocardial ischaemia defined as the time from the symptom onset to successful reperfusion, has been considered the most important factor to improve clinical outcomes. For this reason there is an increasing need for the identification of new biomarkers for an early detection of atherothrombotic events such as acute myocardial infarction. Thrombi obtained by PCI from coronary artery have facilitated the investigation of real time *in vivo* human coronary thrombi.

In this thesis the changes in thrombus structure in relation to onset-of-pain-to-PCI elapsed time were analyzed comparing thrombi of 3 h to thrombi of 6 h of evolution. These two groups of thrombi were retrieved from patients presenting similar characteristics in demographics, risk factors and in antithrombotic treatment.

This thesis demonstrates that the onset-of-pain-to-PCI elapsed time significantly influences the cellular and protein composition of the coronary thrombi. Although we cannot conclude from our work how plaque type has affected thrombus characteristics because we do not have plaque characterization, these studies have shown that platelet deposition and fibrin mass change rapidly within the first hours of evolution.

The coronary thrombus is rich in soluble fibrinogen and fibrinogen-derived fragments. By investigating the fibrinogen distribution profile of thrombus from PCI patients time-dependent fibrinogen distribution changes and a differential evolution profile of both, beta and gamma fibrinogen have been detected. In order to validate the influence of the elapsed time of ischaemia on the fibrinogen turnover found in the thrombus, a serum proteomic profiling approach was performed in an independent patient population. In line with the detected changes in the

thrombus proteomic profile, increased fibrinogen gamma levels in serum in the early phase of the event have been found. Moreover, AMI patients within the first 6 h after the onset of pain showed a high fibrinogen gamma turnover rate. Previous studies have reported increased levels of fibrinogen gamma in AMI patients within the first 24 h after the event (Mateos-Caceres et al., 2004). The increase in fibrinogen levels in the early phase post-AMI is clinically relevant as it has been demonstrated that fibrin clots formed in the presence of fibrinogen gamma chain variant are resistant to fibrinolysis (Collet et al., 2004). Interestingly, while 3 days after the event fibrinogen gamma levels were still higher than those found in control subjects, fibrinogen gamma fragments were nearly undetectable, highlighting a decrease in fibrinogen turnover.

Thrombus formation is a dynamic process, and the intravascular fibrinolytic system continually tries to lysate the developing thrombus, leading to the release of fibrin degradation products (van der Putten et al., 2006). To this respect D-dimer is the primary degradation product of cross-linked fibrin and, therefore, serves as a direct marker of ongoing coagulation with fibrinolysis (Hunt et al., 1985). Interestingly, the most evident change detected in the serum proteomic profile of AMI patients is the very early presence of D-dimer within the first 6 h after the event. The relationship between serum D-dimer levels and ACS is unclear, and published data are contradictory (Bayes-Genis et al., 2000; Gurfinkel et al., 1995; Lee et al., 1997). The assay technique utilized for D-dimer measurement is a well-recognized source of variability as traditional assays detect different fibrin compounds including D-dimer, HMW fibrin degradation products and fibrin X-oligomers (Lippi et al., 2008). Importantly, the proteomic approach together with the mass spectrometry identification allows the specific analysis of D-dimer changes in the thrombus without the interference of other fibrin-related degradation products. Previous studies have reported the presence of D-dimer in

plasma early after an AMI (Ieko et al., 2009; Orak et al., 2010). The results of present work have demonstrated that D-dimer is already detected within the first 6 h after the onset of the event before the raising of the conventional markers of myocardial necrosis, the troponins. Moreover, at this time point, D-dimer levels correlate with the extent of myocardial necrosis.

Besides being a useful marker for early diagnosis, D-dimer is also a risk factor for the development of MI complications. It has been demonstrated a relation between increased D-dimer levels and thrombotic complications in AMI patients (Lee et al., 1997). In the proteomic analysis, AMI patients showed nearly undetectable D-dimer traces 3 days after the event highlighting, once more, a decrease in fibrinogen turnover at this time point. Indeed, it has been described that D-dimer levels reflect the extent of fibrin turnover and active coagulation (Saigo et al., 2004). Therefore, serum profiling allowed to demonstrate that the increase in fibrinogen-derived soluble forms clearly reflect the thrombus fibrinogen composition changes and the ongoing fibrinolytic process.

Furthermore, in coronary thrombus there is a dynamic change with time in the infiltration of leucocytes subtypes. While neutrophils and monocytes were already present at 3 h of thrombus evolution, T-lymphocytes and B-lymphocytes appeared in thrombus of longer evolution. Similarly, these aged occlusive thrombi (T6) showed infiltration of CD105+ progenitors and CD34+cells that were not present in 3 h occlusive thrombi.

Together with the detected changes in cellularity, by applying proteomic approaches, we have discovered one of the major findings of this thesis. We have reported for the first time that a significant presence of Pfn-1 is found in the coronary thrombi. Interestingly, the Pfn-1 content in thrombi is inversely related to ischaemic thrombus age. Thus a significantly lower content of Pfn-1 is found in T6 thrombi (6 h pain-to-PCI). Profilin-1 is an actin-binding protein that has been associated with atherosclerosis and

smooth muscle cell proliferation (Caglayan et al., 2010; Cheng et al., 2011; Elnakish et al., 2012; Romeo et al., 2007), regulation of the microfilament system and signaling pathways in mesenchymal cells (Bae et al., 2010; Yun et al., 2011). Formerly known as an inhibitor of actin polymerization, it has been shown that it also promotes actin depolymerization (Yarmola and Bubb, 2009). In nucleated cells, Pfn-1 interacts with a multitude of ligands including various phosphoinositides and proteins containing proline-rich motifs that are involved in actin cytoskeletal regulation, endocytosis, and gene transcription, so playing an important role in processes such as cell motility, development, signaling, and membrane trafficking(Yarmola and Bubb, 2006). In platelets, Pfn-1 had been associated with shape change cytoskeleton proteins changes (Goldschmidt-Clermont et al., 1991; Hartwig et al., 1989; Kasina et al., 2006; Volpi et al., 2012). Data obtained by confocal microscopy suggested to us that Pfn-1 is released by fully activated and aggregated platelets that are recruited in the initial stages of thrombosis on the culprit plaque because intrathrombus Pfn-1 levels significantly decrease with thrombus ageing.

Previous studies had shown that vascular smooth muscle cells express Pfn-1. In order to discriminate the source of the released Pfn-1 we performed experiments that showed how Pfn-1 was released by platelets. We evidenced that, Pfn-1 is found both in the supernatant and in the PRP clots (devoid of erythrocytes and leucocytes) and it is not found neither in the supernatant nor in clots prepared with PFP, suggesting that Pfn-1 is secreted by fully activated platelets. Indeed we observed that Pfn-1 is released from platelets when they are activated with thrombin but not with collagen, having ADP a minor effect. Interestingly, a proteomic study of the secretoma of thrombin-activated platelets identified Pfn-1 as one of the components of the platelet releseates (Piersma et al., 2009).

Because of the changes seen between T3 and T6 coronary thrombi, the release of Pfn-1 into coronary blood was investigated. Indeed Pfn-1was found in blood aspirated from the culprit coronary lesion, suggesting secretion of Pfn-1 upon platelet aggregation in the coronary thrombus mass. We observed that Pfn-1 levels in plasma inversely correlate with Pfn-1 content in thrombi and that the longer the thrombus is occluding the coronary artery (aged thrombi) the higher Pfn-1 levels are found in the systemic circulation because platelets become depleted of Pfn-1. No differences were detected in Pfn-1 plasma levels between T3 patients and controls supporting that Pfn-1 release is strongly dependent on the elapsed time of ischaemia. Receiver-operating characteristic analysis predicts a cut-off level of Pfn-1(320 pg/mL) in the peripheral circulation that indicated longer than 6 h of onset-of-pain-to-PCI time. Interestingly, 85% of the patients that had major adverse cardiac events (MACE) within 2 weeks after PCI had plasma Pfn-1 levels above the median value of the entire STEMI-population. Moreover, the few cases of patients with *exitus* within the first 24 h after PCI had extremely high Pfn-1 plasma levels in their peripheral blood at 3 h (1400 pg/mL vs. 200 pg/mL average for the group).

All together these data show that Pfn-1 is released from fully activated platelets (as seen with thrombin stimulation) in coronary thrombus where the main trigger for thrombus formation is tissue factor (TF) exposed by atherosclerotic plaques that leads to *in situ* thrombin formation (Badimon et al., 1999; Toschi et al., 1997).

Indeed the implication of TF-dependent extrinsic coagulation pathway in atherosclerosis has been widely studied, and it is known that TF plays an essential role contributing to thrombosis. Therefore, several coagulation-related markers have been investigated as to their use in early diagnosis (Shand et al., 2010). However, available data on the relevance of the intrinsic coagulation pathway along the progression of AMI are limited as,

traditionally, major attention has been focused on the extrinsic coagulation pathway. In this thesis, by applying proteomic technologies and systems biology analysis, a time-dependent differential expression profile of the intrinsic coagulation pathway after an AMI with an increase in serum levels of coagulation proteins during the early phase, followed by an attenuation of those changes 3 days after the onset of the event has been found. Prior studies have demonstrated an increase in plasma of specific coagulation proteins in the setting of ACS (Bayes-Genis et al., 2000; Danesh et al., 2001), yet, this is the first study showing a coordinated change in several coagulation-related molecules by proteomic approaches in relation to the time after the onset of AMI. Two different molecular mass forms of HMWK have been identified. These refer to the upstream effector of the intrinsic coagulation pathway, which is cleaved into several LMW forms in the initiation of the coagulation cascade (Mauron et al., 2000). Specifically, kallikrein excises HMWK, releasing two bioactive molecules: bradykinin and cleaved HMWK (Lalmanach et al., 2010). Besides being a non-enzymatic cofactor in the initiation of the contact phase, kininogen has also been associated with vascular injury, inflammation, and complement activation. This thesis has shown a decrease in the HMW form of HMWK, validated by ELISA, and a coordinated increase in the LMW form suggesting an active cleavage of HMWK in the early phase post-AMI and the activation of the intrinsic coagulation pathway. Previous studies have reported changes in HMWK describing decreased levels in relation to AMI and unstable angina (Tanaka and Suzuki, 1994; Vaziri et al., 1992). In order to validate the intrinsic pathway activation, FXI serum levels were further analized. Specifically, an assay that measures the homodimer FXI zymogen that circulates in blood previous to its activation through the contact pathway when is cleaved to form FXIa has been used. A significant decrease in FXI serum levels in the early phase post-AMI has been found suggesting

its active cleavage and pointing out once more to the activation of the intrinsic coagulation pathway. Moreover, both effectors, HMWK and FXI returned to control levels 3 days after the event suggesting an attenuation of the intrinsic pathway and highlighting a time dependent effect in its activation. The detected differences in HMWK changes in AMI patients 3 days after the event between proteomic and ELISA analysis were due to the sample size used in each analysis. Indeed, when only the patients used in proteomic studies were analyzed by ELISA no significant changes were detected between the moment of admission and 3 days after. Importantly, in this study, besides the detected changes in the HMW form, a correlation between the LMW kininogen form 3 days after admission and the necrosis of the myocardium has been seen, highlighting a possible implication of this form in the degree of myocardial injury.

Within the identified coagulation-related proteins two anti-coagulant factors were found, alpha-2-macroglobulin (A2MG) and antithrombin III. A2MG is an acute phase protein with an endogenous inhibitor activity of serine proteases, such as thrombin (Guzzetta et al., 2006) and protein C (Esmon, 1992; Hoogendoorn et al., 1991). Specifically two A2MG forms with different molecular mass have been identified in the present work, with the LMW form decreased in AMI patients 3 days after admission. Until now different results have been reported for A2MG levels in the setting of myocardial infarction, with studies showing no changes within the first 8 h after the onset (Vaziri et al., 1992)and increased A2MG levels 2 days after the event (Crook et al., 1994). Moreover, increased A2MG levels have been suggested as an indicator of the progression of coronary artery disease (Mori et al., 1995). On the other hand, no significant changes were detected in the present study in relation to antithrombin III levels as previously reported (Vaziri et al., 1992).

In addition, this study has demonstrated for the first time a progressive decrease in histidine-rich glycoprotein (HRG) levels after a new onset

AMI. Like kininogen, HRG is a type 3 cystatin (Lee et al., 2009), which is able to interact with several coagulation-related molecules such as plasminogen/plasmin (Poon et al., 2009) and fibrinogen (Vu et al., 2011). Specifically, by its interaction with fibrinogen HRG can be incorporated in fibrin clots retarding the rate of conversion of fibrinogen to fibrin (Leung, 1986). Moreover, HRG can also neutralize anticoagulant activity of heparin by preventing the formation of heparin-antithrombin III complexes that inhibit activated coagulation factors such as thrombin (Lijnen et al., 1983; Lijnen et al., 1984). Therefore, the observed changes in relation to AMI might have a direct impact in haemostasis as HRG has a dual role being able to interact with both the coagulation and the fibrinolytic system. Thus, the coordinated changes in serum coagulation-related proteins in the early phase post-AMI could reflect the ongoing processes in the thrombus site and as a result may have a direct impact on the clinical evolution of AMI patients.

Among patients with AMI, those who present a recurrent event with in-stent thrombosis show a worst clinical outcome, being the risk of mortality ten times higher than thrombosis in native coronary arteries (De Luca et al., 2013). Until now, the high risk of in-stent thrombosis (IST) in STEMI has been mainly attributed to the mechanical procedure of stent placement and the biological response of the artery to this procedure including changes in the dimension of the vascular wall (Chaabane et al., 2013). However, up to now, it is not known whether the thrombi grown in stent have a differential protein composition that may explain their higher aggressiveness.

As part of this thesis the possible molecular mechanisms that could explain the high risk of adverse events in stent thrombosis have been evaluated by applying differential profiling proteomic and system biology approaches to analyze the composition of in-stent thrombus. The obtained results have demonstrated that in-stent thrombus have a different protein

composition compared with thrombi formed in native coronary arteries. Specifically, thrombi generated after stenting show a coordinated increase of cell-related structural proteins such as actin, tropomyosin, myosin, and gelsolin. Moreover, the specific marker for erythrocytes, hemoglobin, was also increased in IST. These changes underscore an increase in the cellularity of thrombi developed in the stent compared to those formed in native coronary arteries. Indeed, endothelial injury and foreign body stent placement have shown to induce platelet activation and deposition at the site of the lesion, with the recruitment of circulating leukocytes. Leukocyte-platelet interactions are critical in the initiation and progression of neointimal formation which is followed by the activation of chemokines and cytokines leading to the proliferation and migration of smooth muscle cells (SMCs) within the media and the intima (Chaabane et al., 2013). Previous studies have pointed out to platelets, leukocytes and erythrocytes as sources of oxidative stress in vascular disease (Martin-Ventura et al., 2012) .In fact, platelet-leukocyte interaction is accompanied by ROS production causing oxidative damage. Moreover, Yunoki and colleagues correlated erythrocyte-rich thrombus with impaired myocardial reperfusion in association to oxidative stress (Yunoki et al., 2012). In line with these results, the detected increase in hemoglobin in IST could induce oxidative stress by the deregulation of the heme/iron metabolism. Indeed, it has been reported that oxidation of hemoglobin leads to non-functional methaemoglobin provoking an ischemia/hipoxia condition in the tissues (Hare et al., 2012; Quach et al., 2012). Interestingly the enzymatic reduction of methaemoglobin has been attributed to flavin reductase (Juni et al., 2013; Nagai and Yoneyama, 1983; Yubisui et al., 1980) which is an anti-oxidant enzyme that is decreased in IST. This decrease could contribute to the accumulation of methaemoglobin increasing the oxidative stress in IST. Additionally, a coordinated decrease of another anti-oxidant enzyme, peroxiredoxin-2,

has been detected in IST. This enzyme is mainly involved in peroxide degradation (Mohanty et al., 2014; Rhee et al., 2012). Moreover, IST have also shown an increase of the redox-homeostasis enzyme superoxide dismutase. This enzyme is responsible for the transformation of $O2^{-}$ to produce H_2O_2, (Fukai and Ushio-Fukai, 2011). Therefore the increase in SOD could induce an increase in H_2O_2 in IST thrombi probably due to an increase in platelets content as suggested by the positive correlation between CD61 and SOD band intensities in the western blot validation analysis. In normal condition the excess of H_2O_2 is eliminated by the action of peroxiredoxin-2. Thus, in IST, the imbalance between SOD and peroxiredoxin-2 activities could lead to the accumulation of H_2O_2 and its contribution to oxidative damage (Martin-Ventura et al., 2012). The decrease of carbonic anhydrase, protein that protects cells from hydrogen peroxide-induced apoptosis could contribute to the oxidative damage induced by hydrogen peroxide accumulation (Raisanen et al., 1999).

Previous studies have reported the involvement of H_2O_2 in collagen induced platelet activation (Pignatelli et al., 1998) and the propagation of platelet aggregation (Violi and Pignatelli, 2012). Therefore, the imbalance in redox-homeostasis could contribute to a higher aggressiveness of IST thrombi by an increased H_2O_2-induced platelet response.

Moreover, alpha enolase that has been related to hypoxia tolerance in endothelial cells showed a 10-fold decrease in thrombi after stenting when compared to native thrombi. The hypoxia state generated by metahemoglobin might potentiate the formation of reactive oxygen species and the increase of oxidative damage. Therefore, the detected decrease in alpha enolase could induce an increased susceptibility to oxidative stress in the milieu of the thrombi developed in the stent contributing to the higher aggressiveness of this type of thrombus.

On the other hand, IST thrombi did not show changes in fibrinogen proteomic profile. Nevertheless, the increase of the oxidant environment

present in stented arteries could lead to oxidative post-translational modification of fibrinogen influencing both its structure and function, phenomenon that has been associated with vascular disease pathogenesis (Martinez et al., 2012; Martinez et al., 2013).

These results reveal important differences in the protein composition of in-stent thrombus compared to spontaneous thrombi in native vessels with an increase in thrombus cellularity and an impaired oxidative environment.

In summary, the results obtained from the different sub-studies included in this thesis provide novel information about the evolving composition of coronary thrombus both in cellular and protein content, in relation to the time of ischemia. Moreover, changes in circulating protein levels reflect the process ongoing in the coronary arteries, which may have a potential value in the assessment of the evolution of patients after an event. In addition, this work brings important knowledge about the structural differences in in-stent thrombus compared to thrombi in native vessels, highlighting the imbalance in redox-related proteins as a potential mechanism behind the worst prognosis of patients with IST

VI. CONCLUSIONS

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

1. **The elapsed pain-to-PCI time has impact in the composition of STEMI thrombus**

- Coronary thrombi show dynamic changes in cellular composition as a function of elapsed pain-to-PCI time:
  - Older intracoronary thrombi are able to recruit circulating inflammatory innate and adaptive immunity cells
  - In aged coronary thrombi, but not in the early phase of ischemia, progenitor cells are found within the thrombus.
  - The recruitment of platelets is an early event. Thrombi of more than 6 h of evolution show a reduction in the number of recruited platelets
- The structure of fibrin changes with pain-to-PCI time. Aged occlusive thrombi present an increase in fibrin network complexity
- Pfn-1 in thrombi is inversely related to ischemic thrombus age and inversely correlates with the platelet content
- Pfn-1 is secreted from fully activated platelets. Pfn-1 levels are increased in coronary and peripheral circulation in patients with more than 6h of ischemia independently of the antiplatelet treatment.

2. **There are time-dependent changes in the serum profile of coagulation related proteins in the early phase post-AMI.**

- Within the first 6 hours after the onset of pain there is a dynamic exchange between HMWK and its cleaved form, and a decrease in FXI levels indicative of intrinsic coagulation pathway activation,

together with an increase in fibrinogen gamma turnover and D-dimer formation.

- The elapsed time of ischemia affects the fibrinogen distribution profile in the evolving coronary thrombus with decreased fibrinogen beta fragment formation and increased fibrinogen gamma turnover in the early phase post-AMI directly influencing serum fibrinogen content.
- Fibrin degradation products are found in coronary thrombi but not in *in vitro* generated clots

3. In-stent thrombi show differences in the protein composition when compared to thrombi in native coronary arteries:

- In-stent thrombi have a highly cellular profile.
- IST showed coordinated change in enzymatic proteins related to oxidative stress.

These findings may have clinical implications on:

– Defining the age of the occlusive thrombus and its prognostic implications

– The discovery of new biomarkers of the early phase of ischaemic process

– **Indicating that the interaction between the changing thrombi and the vessel wall may affect the progression of the underlying vascular disease**

VII. BIBLIOGRAPHY

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