Platelet proteomics in cardiovascular diseases

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ARTICLE INFO

Article history:
Received 7 July 2014
Received in revised form 16 September 2014
Accepted 20 September 2014
Available online 7 October 2014

Keywords:
Platelet clinical proteomics
Cardiovascular disease

ABSTRACT

In recent years, platelet proteomics has been applied successfully to the study of cardiovascular diseases (CVDs). It is very well known that platelets play a pivotal role in the pathophysiological mechanisms underlying many CVDs, especially acute coronary syndromes (ACSs), since they are implicated in thrombus formation after atheroma plaque rupture. This is the reason why molecules involved in platelet activation and aggregation are primary targets for treatment of ACSs. Many efforts are aimed at finding drugs that inhibit platelet activation; however it is difficult to separate the therapeutic benefits from harmful effects because pathophysiological and physiological functions of platelets are due to the same mechanisms. Given that platelets lack a nucleus, proteomics is regarded as an ideal method to approach their biochemistry. Current platelet proteomic studies are focusing on the identification of proteome events and functional changes in normal and pathological states, enriching the comprehension of platelet biological function, and screening for new biomarkers and antiplatelet agents. In the present article, we introduce the reader to platelet biology and function, and revise recent advances in platelet proteomics applied to the study of CVDs, including a special emphasis on sample preparation requirements for proteome analysis of platelet clinical samples.

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Abbreviations: ACS, acute coronary syndrome; ADP, adenosine diphosphate; ANG, angiotensin; ARB, angiotensin II receptor blocker; ASA, Acetyl Salicylic Acid; ATP, adenosine triphosphate; CAD, coronary artery disease; CEP, collagen-related peptide; CVD, cardiovascular disease; GPS, gray platelet syndrome; GPV, glycoprotein VI; IPA, ingenuity pathway analysis; LDL, low-density lipoprotein; MK, megalakoyote; MP, microparticle; MS, mass spectrometry; MV, microvesicle; NO, Nitric oxide; NP-40, Nodinet P-40; NSTEMI, Non ST-Segment Myocardial Infarction; PCI, percutaneous coronary intervention; PFP, platelet-free plasma; PGJ2, prostaglandin I2; PRP, platelet-rich plasma; PS, phosphatidyl serine; SA, stable angina; SCAD, stable coronary artery disease; STEMI, ST-Segment Myocardial Infarction; TCA, trichloroacetic acid; TRAP, thrombin receptor activating peptide; TXA2, thromboxane A2; UA, unstable angina.

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http://dx.doi.org/10.1016/j.trprot.2014.09.002
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1. Introduction

1.1. Platelet definition, biogenesis and subpopulations

Platelets are specialized anucleate mammalian blood cells averaging 2–3 μm in diameter and 0.5 μm in thickness. They have discoid shape under normal physiological conditions, and their half-life is 10 days in peripheral blood. Platelets derive from megakaryocytes (MKs), which are unique polyploid hematopoietic cells that are found only in mammals and are specialized to produce and release platelets into the blood circulation. Although MKs arise in the bone marrow, they can migrate and nest at non-marrow sites, reason why platelet biogenesis (thrombopoiesis) has been proposed to take place not only in the bone marrow but also in lungs and blood. Different stages of platelet development have been observed in all these three locations.

For a long time it has been questioned whether the MKs present in the bone marrow, in the lungs or in the blood, are responsible for the greater production of platelets. For example, the lungs are reservoirs for megakaryocytes and platelets, releasing them in response to certain stimuli. Platelets continuously circulate through pulmonary vessels, contributing to lung defense, disease, and remodeling, being the effectors of injury in a variety of pulmonary disorders and syndromes [1]. The estimated contribution of pulmonary MKs to total platelet production remains unclear, but in theory platelets and MKs may reach the pulmonary circulation completing their development into platelets in lung capillaries.

Among the two critical functions of platelets, which are adhesion at site of vascular injury and promotion of thrombin generation, it is assumed that all platelets are equally able to satisfy the former one. Nevertheless, in the last decade some studies suggest that some platelets have a greater ability to promote thrombin generation. Within a thrombus, there are clearly distinguishable platelets with different surface properties. Different platelet populations and their functions were recently reviewed by Heemskerk et al. [2], and are briefly described below.

Close to collagen fibers, there is a population of highly activated platelets arranged as patches around the thrombus. These platelets, known as procoagulant platelets, have a rounded structure, expose PS in their surface, have a sustained calcium-induced morphology, the ability to bind coagulation factors, and produce procoagulant microvesicles (MVs).

Coated-platelets are another subpopulation formed after platelet stimulation with collagen plus thrombin, although some studies demonstrated that an indistinguishable subpopulation of cells can be produced by stimulation with other agonists pairs or even with single agonists [3–6]. Coated-platelets show high levels of several α-granule procoagulant-proteins on their surface (i.e., FV, fibrinogen, thrombospondin, vWF), expose high levels of surface PS, are functional in their ability to bind FXa and generate an active prothrombinase complex, bind serotonin-conjugated proteins, and also shed MVs. The physiological significance of coated-platelets is still unknown, although it is speculated that they could be significant contributors to thrombotic process. The potential clinical relevance of coated-platelets in hemostasis may be the contribution to a hemorrhagic or thrombotic phenotype. Thus, a low level of coated-platelets is correlated with the appearance of spontaneous intracerebral hemorrhages [7] and with bleeding diathesis [8], while a high amount of them seems to correlate with transient ischemic attack and ischemic stroke [9,10], and with patients with a history of arterial or venous thrombosis [11]. Coated-platelets also appear to have an impact on the bleeding phenotype in severe hemophilia [12] among other diseases.

In a thrombus, there are also aggregate-forming platelets. These platelets are characterized by the presence of active αIIbβ3 in their surfaces and fibrinogen binding to this receptor. Under coagulant conditions, procoagulant platelets produce FXa and thrombin outside the platelet plug, whereas aggregating and clot-retracting platelets are responsible for plug consolidation and clot retraction.

A final category of platelets are apoptotic and necrotic platelets, characterized by an apoptotic PS exposure, which appears to rely on caspase activation. Although several in vitro studies have reported that these platelets have a coagulant potential and support thrombin generation, their role in physiological conditions is questionable, as they are rapidly taken up by scavenging cells present in the blood circulation. Moreover, it has been suggested that apoptotic and necrotic platelets are activated by a necrotic cell death pathway [13].

1.2. Platelet function

Platelets main function in hemostasis was recognized more than a century ago [14]; indeed, low platelet count is responsible for hemorrhage and prolonged bleeding time, even despite normal coagulation [15]. Nowadays some other non-hemostatic crucial biological functions are known in relation to platelets, such as inflammation [16], immunity [17,18], malignancy [19] and maintenance of vascular integrity [20].

1.2.1. Role of platelets in hemostasis, thrombosis and cardiovascular disease

Under normal conditions, endothelial cells of the vessel walls synthesize prosstaglandin I2 (PGI2) and nitric oxide (NO) as inhibitors of platelet function, acting synergistically [21–23]. They have also ecto-ADPase (CD39) activity, which metabolizes ADP (platelet activator) to adenosine (platelet inhibitor) [24,25]. These three mechanisms working coordinately inhibit platelet activation. When the endothelium is damaged, these mechanisms are altered and subendothelial matrix is exposed undergoing potent platelet-activation and shape change, leading to platelet plug formation in order to avoid excessive bleeding. Thrombus generation starts with a platelet plug formation followed by stabilization of this plug through fibrin deposition (coagulation). Primary platelet adhesion
to the damaged vessel wall is initiated by subendothelial matrix-proteins exposition, which engages specific receptors on the platelet surface. Firm adhesion of platelets is associated with granule secretion and extensive cytoskeletal remodeling, resulting in the exposure of a highly reactive surface promoting the recruitment of additional platelets. This process is principally influenced by the mobilization of intracellular calcium, considered the principal initiator of fibrinogen-receptor activation (integrin αIIbβ3) and granule secretion. The content of these granules, along with thrombin generated on the platelet surface, act as a positive feedback signals to attract more platelets to the site of injury and to activate them. Factors secreted from platelets include high concentrations of secondary agonists such as ADP, adrenaline, serotonin and thromboxane A2 (TXA2), each of which binds to specific receptors of circulating platelets contributing to this positive-feedback.

Under pathological conditions exaggerated platelet activation can occur, leading to a pathological thrombosis, which can develop in coronary artery disease (CAD), stroke and peripheral vascular occlusions [26]. The development of a platelet-rich thrombus at the site of atherosclerotic plaque can lead to occlusion of blood vessels, underlying cause of acute cardiovascular events. Cardiovascular diseases (CVDs) are the leading cause of death all over the world [27] and this is the reason why cardiovascular research has huge relevance. In the last decades many basic and clinical studies have been done due to the urgent need to shed light into the complex mechanisms that underlay these diseases and their progress. CVDs include a number of conditions which affect structure or function of the cardiovascular system. They comprise CADs, vascular diseases, arrhythmias, heart failure, heart valve disease, congenital heart disease, cardiomyopathy, pericardial disease and aorta disease. In the last few years much progress has been made in the study of CVDs, but there is still a great need to understand deeply the physiopathology of this set of diseases.

Coronary artery disease is the most common type of heart disease and cause of heart attacks. The primary cause of CAD is the disruption of the atherosclerotic plaque in a coronary artery followed by the formation of a thrombus in the site of injury blocking totally or partially the blood flow to the heart. Patients suffering from CAD can be divided into two groups; those suffering from stable angina (SA) which is a predictable pattern of chest pain due to narrow of a coronary artery, and acute coronary syndromes (ACS) which refers to a set of clinical symptoms related to acute myocardial ischemia.

Acute coronary syndromes are subdivided into ST-elevated myocardial infarction (STEMI), non-ST elevated myocardial infarction (NSTEMI) and unstable angina (UA). There are differences in the composition of the coronary artery thrombi on each condition. NSTEMI initiates with severe coronary artery narrowing, due to development of platelet-rich white thrombi which is normally not completely occlusive [28]; it is defined by absence of ST-segment elevation in the electrocardiogram and elevation of cardiac biomarkers. STEMI starts with complete and prolonged coronary artery narrowing involving formation of a fibrin rich red clot superimposed to the subjacent white thrombi [28]. It is probably due to a higher plaque rupture [29] and characterized by ST-segment elevation in the electrocardiogram and elevation of myocardial necrosis biomarkers. UA characterizes by non-occlusive thrombi [30] and absence of detectable release of the enzymes and biomarkers of myocardial necrosis. A good diagnosis and stratification of patients is crucial for the management of patients with ACS. In each case, the clinician is challenged to identify the most effective and safest option of therapeutic treatments available.

There are many risk factors associated with CVDs such as family history, ethnicity and age, which cannot be changed. Other risk factors that can be modified include smoke, hypertension, high cholesterol, obesity, physical inactivity, diabetes, unhealthy diets, and harmful use of alcohol.

As mentioned above, platelets play a pivotal role in the pathophysiological mechanisms underlying many cardiovascular diseases, especially ACSs, since they are implied in thrombus formation after atheroma plaque rupture [31]. This is the reason why molecules involved in platelet activation and aggregation are the primary targets for treatment of ACSs [32]. Many efforts are aimed at finding drugs that inhibit platelet activation; however quite often it is difficult to separate the therapeutic benefits from harmful effects because both pathological and physiological functions of platelets are due to the same mechanisms. Here lies the importance of investigation progress on antiplatelet drugs to treat CVDs [33].

There exist some antiplatelet agents routinely used in the treatment of ACSs. Cyclooxygenase-1 inhibitors (such as aspirin) drastically reduce the mortality due to myocardial infarction, the risk of subsequent cardiovascular events and ischemic complications due to percutaneous coronary interventions [34,35]. Other drugs include thienopyridines (such as clopidogrel) which are antagonists of ADP receptor P2Y12, and glycoprotein IIb/IIIa inhibitors (such as abciximab or eptifibatide). Combination of dual antiplatelet drugs (Cyclooxygenase-1 inhibitors and thienopyridines) is routinely used in the prevention and treatment of ACSs [36]. Current guidelines on percutaneous coronary intervention recommend the administration of dual antiplatelet therapy [37]. However, prolonged treatment with dual or triple antiplatelet drugs can develop either poor platelet responses, which lead to antiplatelet resistance [38,39], or hyper platelet responses resulting in an increasing risk of bleeding [40]. For evident reasons the study and development of novel antiplatelet agents, such as more potent P2Y12, GPVI, and glycoprotein IIb/IIIa antagonists, are now booming in order to reduce recurrent atherothrombotic events without increasing bleeding risk.

1.2.2. Role of platelets in immunity

In the same way that platelets sense danger by recognizing exposed subendothelial molecules after endothelial damage, they can also sense danger by noticing the presence of pathogens or pathogens-derived structures during infection and inflammatory situations. It is known that there is a close relationship between platelets and immunity, due to the sophisticated interplay of platelets and bacteria. Thrombocytopenia (relative decrease of platelets in blood) is a frequent occurrence in sepsis [41] and sepsis is a significant risk factor for thrombocytopenia in critically ill patients [42]. During bacterial infection, direct interactions between platelets and bacteria, leading to enhanced macrophage, may contribute to thrombocytopenia.

In this sense, platelets can be categorized as innate immune cells specialized in pro-inflammatory activities. Platelets, by inheritance from its predecessors (MKs), express ligands that bind foreign structures, express receptors that ease the ingestion of pathogens to destroy or inhibit them, secrete a large range of factors involved in inter-cellular communication to mediate local actions such as inflammation, and also are able to detect and respond to different signaling molecules adapting their response depending on the danger they face [18]. The ability of distinguishing between different pathogens is due to the presence of pathogen sensors and ligands on and in platelets. Functional pathogen recognition receptors allow platelets to bind foreign microbial invaders and combat them specifically. It has been recently demonstrated that human platelets can discriminate between various bacterial lipopolysaccharides isomers, leading to the differential secretion of soluble signaling/effective molecules from platelets [43].
1.2.3. Role of platelets in inflammation

In addition to their main role in hemostasis and thrombosis, there are increasing evidences that platelets actively promote the inflammatory process. Inflammation causes endothelial activation which triggers leukocyte extravasation and adhesion to endothelial cells, in a receptor-dependent process. After this, platelets are rapidly recruited to sites of injury and infection. Inflammation leads to an imbalance between procoagulant and anticoagulant properties of the endothelium. The procoagulant effect is mediated by TNF-α, the first pro-inflammatory cytokine released at the site of infection, while the anticoagulant effect is mediated by NO a strong inhibitor of platelet activation.

The first suggestion that platelets have a role in inflammation came from observations related to atherosclerosis. The accumulation of subendothelial LDL initiates the activation of endothelial cells, characterized by loss of vascular integrity, cytokine production, and the expression and shedding of leukocyte adhesive molecules [44], thus triggering the subsequent process of inflammation. The role of platelets in the early stages of atherosclerosis was first recognized by Steinhibl and Moliterno [45] and reinforced by the characterization of the platelet releasate comprising many proteins with significant pro-inflammatory properties [46]. Moreover, several studies have been focused on the adhesion of quiescent platelets to activated endothelial cells endorsing the role of platelets in atherosclerosis [47–49].

The contribution of platelets to inflammation associated with other disorders has been less studied. However, platelets have been related to many other inflammatory conditions such as rheumatoid arthritis, inflammatory bowel disease, systemic lupus erythematosus, migraine, inflammatory pulmonary disease and psoriasis. This topic was extensively reviewed a few years ago by McNicol and Israels [50].

1.2.4. Role of platelets in malignancy

The association between malignancy and platelets was first described in 1865 when Armand Trousseau observed that migratory thrombophlebitis could be an indicator of malignancy [51]. Since then, numerous work has focused on the study of the role of platelets in malignancy. Nowadays, there is a wide knowledge of the bidirectional interaction of platelets and tumor cells. Platelets promote tumor survival and progression, by forming platelet–tumor aggregates which confers a survival advantage to malignant cells [52,53], protecting them from immune system surveillance [54,55] and enhancing metastatic potential [56]. Moreover platelets facilitate vessel wall adhesion [57] and tumor cell invasion by penetration of the vessel wall [58]. Further than this, platelets release growth factors that may enhance tumor cell growth [59–61] and angiogenesis growth factors, which are required for tumor angiogenesis, growth and metastasis [57,62,63]. On the other hand, tumor cell-expressed proteins are able to activate platelets, and the potential to activate them varies among tumor cell lines [50].

1.2.5. Role of platelets in maintenance of vascular integrity

Platelets are known to support the semi-permeable function of the endothelium being considered as critical guardians of vascular integrity. First evidence of this platelet function was described by Danielli in 1940 when he noted that platelets help to preserve the barrier function of endothelium during organ perfusion [64]. Many subsequent studies demonstrated that platelets continuously support the barrier function of the resting endothelium [65,66] and, in inflammation, they prevent or heal vascular injuries caused by the infiltrating leukocytes. The platelet mechanisms of maintaining vascular integrity not only involve the process of platelet plug formation, but also the secretion of the various platelet granules. Further information on this and other aspects of the role of platelets in vascular integrity can be found in a recent review by Ho-Tin-Noé [67].

1.3. Platelet proteomics

Since platelets do not have nucleus, proteomics is an ideal tool to approach their biochemistry. During the last decade proteomics allowed the discovery of many platelet receptors and signaling proteins, some of which are being studied as antithrombotic drug targets. Initial studies were with platelets from healthy individuals but in the last few years studies started focusing on the identification of platelet functional changes in normal and pathological states, enriching the comprehension of platelet biological function, searching for platelet biomarkers and new antiplatelet agents [68]. Platelet proteomics has been recently applied to understand the development of serious diseases, such as hematological diseases and coronary heart disease. Fractionation of samples in subproteomes (i.e. secretomes, exosomes and microvesicles) is a good strategy to simplify the complexity of the samples and to increase the success when searching for new biomarkers [68]. Moreover, new technologies and strategies of analysis (i.e. high sensitivity quantitative proteomics or depletion of high abundant proteins) are being developed to access the low abundance proteins. All this together encourages us to think that a new era of biomarker and drug target discovery is starting to take off, and it moves us one step closer to the practical application of proteomics to the diagnosis and prognosis of CVDs as well as its medical treatment [69]. Indeed, Burkart et al. performed a recent study revealing that >85% of the platelet proteome shows no variation between healthy donors using an elaborate quantitative analytical strategy [68], which is of great importance. This strategy may be useful in the search for novel biomarkers, drug targets or key mediators of platelet function. This reinforces the idea that platelet proteomic studies can address issues of higher clinical relevance such as differences in the response to antiplatelet treatments or in the pathological potential of platelets in the genesis of CVDs [70]. The following section will provide a guide for sample preparation in platelet clinical proteomic studies.

2. Sample preparation for platelet clinical proteomic studies

2.1. Pre-analytical step

In platelet clinical proteomic studies it is essential to have a very well established method to prepare samples. There are many preparative steps from patient selection and blood collection to platelet isolation and protein extraction, separation and analysis (Fig. 1). All preparative steps have to be well defined and comply with strict quality control standards, in order to minimize the technical variation among the specimens included in a study.

In patients or donors selection, it is important to establish very well defined and stratified groups with the least variability. Attention should be paid to the drugs taken by the patients/donors because they may have important effects in platelet function [71], particularly antiplatelet drugs, and others such as anti-inflammatory and antidepressive drugs, which may alter platelet activation and aggregation [72]. Physical state and stress may also influence platelet reactivity so – if possible – it is advisable to take blood at the same time each day because circadian rhythms alter the aggregability and adhesiveness of platelets [73]. Patients included in a study must be defined according to strict clinical or laboratory criteria to avoid mixed populations which may cause confusing or misinterpreted results. It is also important to consider the limited amount of blood normally available from patients. Moreover, all the necessary approvals by clinical ethics committees must be in place before starting the study.
In the case of platelets, it is important to bear in mind that they have a highly sensitive nature and that minimal changes in the moment of blood collection and preparation can activate or deteriorate them. It is therefore of high importance to take all necessary precautions during sample collection for platelet proteomic studies, especially in the case of clinical biomarkers.
research. There are several studies focusing on the step of platelet isolation avoiding any platelet preactivation; some of them reviewed by Everts et al. [74]. Blood should be taken from the same source each time (i.e. arterial or venous blood) and when the individual is at rest. Zellner and Oehler [75] recently reported the recommended parameters for blood extraction prior to a platelet proteomics study. They advise to withdraw blood from the median cubital vein, discarding the first 2 mL, using a 21-gauge needle and a light tourniquet. Collection tubes must not only prevent coagulation but also preserve from spontaneous platelet activation. Tubes may influence platelet functions and proteomic studies subsequently, so they must comply with several criteria. They should be plastic-made and ideally contain sodium citrate (3.8%) as anticoagulant. Tubes should be gently but slowly mixed immediately after blood extraction. Sample processing should be done as soon as possible, ideally within the first 1–2 h after blood collection. Also environmental conditions must be adequate because platelets are very sensitive to temperature and pH changes, so we must be careful with these parameters during transport and manipulation. Temperature must be between 20–25°C and pH must be controlled around 6.6 with acid-citrate dextrose (ACD) in order to avoid aggregation. Another parameter to consider is platelet concentration after isolation; it should never exceed 10^9 platelets/mL, otherwise spontaneous activation may occur.

All measures must be taken to minimize contamination with proteins from other sources, since cellular contamination can introduce artifacts on the proteome analysis. For this reason, platelet isolation avoiding contamination with other cells or plasma proteins is crucial for a successful proteome analysis. A key point in platelet purification is the separation of the platelet-rich plasma (PRP) from white and red blood cells. This step can be performed by blood centrifugation and careful separation of the superior phase of PRP, or using leukocyte removal filters [76–78]. There is no standardized method for this, and one must be aware of the drawbacks of both techniques. In the former case mentioned, there is a risk of leukocyte contamination, while in the later a loss of up to 25% of platelets is non-acceptable when it comes to clinical samples. In our hands, mild centrifugation at 200 × g during 20 min results in a clear layer of leukocytes easily avoided, just pipetting the upper third of the PRP [79]. A new development in platelet isolation recently reported is cell sorting using magnetic microbeads against the integrin β3 subunit [80].

The following step consists in platelet separation from plasma and two strategies can be followed, one based on gel-filtration and other based on successive centrifugations. The first method is softer but it has other disadvantages as it does not allow concentration of platelets. The second method is based on platelet pelleting by centrifugation and resuspension in a suitable buffer. In this method any centrifugation must be made in presence of inhibitors such as protacyclin (final concentration 1 μM) to avoid platelet activation. Different times and centrifugal forces are used, but our and other groups have a great experience applying 1000 × g for 10 min [79]. Finally, it is advisable to resuspend platelet pellets in a physiological buffer; we use modified Tyrode’s buffer (134 mM NaCl, 0.34 mM Na₂HPO₄, 2.9 mM KCl, 12 mM NaHCO₃, 20 mM HEPES, 5 mM glucose, 1 mM MgCl₂, pH 7.3). A final centrifugation step at the same speed is advisable to reduce plasma proteins to a minimum level and obtain washed platelets. All centrifugation steps must be performed at room temperature because cooling may result in platelet activation [81]. Platelet counting can be done ideally using an electronic particle analyzer such as a Coulter particle counter. Once quantified, platelets are resuspended in an appropriate volume of an adequate buffer (suitably modified Tyrode’s buffer) to leave them at the desired concentration.

2.2. Whole proteome and subproteome analysis

2.2.1. Whole proteome

Once platelets are isolated, the next step is to snap them in order to prepare protein samples. The method selected determines the results of proteomic analysis and depends on the downstream process of the assay.

When it comes to whole platelet-proteome studies, disruption of the platelet membranes is a mandatory step. For this, two options are available, using hypotonic buffers containing Nodinet P-40 (NP-40) [78], Triton X-100 [82] or SDS [83] that cause cells swell and burst; or lysing cells in liquid nitrogen. In each case it is necessary the utilization of protease and phosphatase inhibitors in order to avoid protein degradation by intracellular proteases and phosphatases which remain active [84]. Platelet-membrane residues are removed at high speed centrifugal forces (>10,000 × g, 5 min) and the supernatant is used for proteome analysis. Following lysis, platelet proteins can be precipitated to purify and concentrate them. In our hands, protein precipitation with 20% trichloroacetic acid (TCA) in acetone (final concentration) is a good option [79], but it can be also made with an ethanol/dialysis method or commercial precipitation kits. Zellner et al. reported that proteomic findings vary depending on the precipitation method used [85], showing that the proper choice on the method is of great importance. Beyond precipitation, proteins may be solubilized in the appropriate buffer for further studies. A representative 2D-map of the total platelet proteome is shown in Fig. 2A.

2.2.2. Subproteome analysis

In some cases, and in order to increase the coverage of the proteome analysis and the reduction of sample complexity, pre-fractionation of the samples prior to electrophoresis must be done. The study of subproteomes (i.e. exosomes, microvesicles, releasate, organelles and membrane proteome) is of high interest and may provide novel information on low-abundant proteins masked in the whole proteome, thereby increasing the number of proteins identified [86]. Some recommendations for subproteome analysis are indicated below.

In the first platelet proteome studies, based on 2-DE, membrane proteins were several underrepresented due to their low abundance and low solubility in the 2-DE sample buffers. The platelet membrane proteome encompasses all proteins embedded in or associated with the various platelet lipid bilayers. Its study is of high interest, since it includes receptors (which mediate cellular responses on binding of a ligand), transporters (which move substances across the membranes) and enzymes [87]. Identifying the complete repertoire of membrane-proteins on resting and activated platelets has relevant implications for understanding the physiological and pathophysiological role of platelets. Interestingly, it is estimated that more than 70% of all drug targets are localized in plasma membranes [88]. Several techniques are used to enrich integral membrane proteins, including chemical precipitation and/or density gradient centrifugation, affinity chromatography and membrane shaving. The utilization of those techniques followed by 1D SDS–PAGE and LC–MS/MS, MudPIT or COFRADIC, greatly increased the power of membrane proteins identification. A study by Senis et al. took advantage of three different membrane enrichment techniques, in combination with LC–MS/MS analysis, to identify 136 transmembrane proteins expressed in human platelets [89]. A more recent study by Lewandrowski et al. [90] reported the highest number of platelet membrane proteins never found (626).

The total platelet releasate consists of all the proteins and other biomolecules released by activated platelets, including microvesicles and exosomes contents. Platelet–granules contents contribute to thrombosis, coagulation, atherosclerosis and
inflammation. The relevance of the study of the platelet releasate is explained by the evidence that proteins released by platelets were found in human atherosclerotic plaques, which indicates that they could be contributing to the pathogenesis of atherosclerosis [46,91]. Moreover, a recent report has revealed that platelet secretion is kinetically heterogeneous in an agonist-responsive manner [92], which is in line with our own data when comparing the releasate from collagen – vs thrombin – stimulated platelets [106].

Some studies address the investigation of the whole platelet releasate, while others focus on a specific part. In the case of whole releasate studies (including MVs, and exosomes), firstly, platelets are stimulated with agonists to induce protein secretion, avoiding lysis. The releasate is then obtained by centrifuging platelet samples after stimulation, in order to pellet and fully eliminate them. Before centrifugation it is important to add prostacyclin to prevent spontaneous aggregation during centrifugation (we recommend 1 µM), and also protease and phosphatase inhibitor cocktails to avoid sample degradation. We recommend performing two centrifugation steps, the first one at 1000 × g for 10 min, followed by a second centrifugation at 10,000 × g for 2 min, both at 4°C. Supernatants containing the releasate can be concentrated in Amicons to reduce sample volume (we use a 3 kDa Amicon, centrifuging at 4000 × g for 1 h and 15 min at 4°C, and concentrating the sample to a final volume of about 100 µL). Samples should be finally precipitated to purify and concentrate proteins and solubilize them in the desired buffer. In our hands, precipitation with 20% TCA in acetone (final concentration) is a good option, but other precipitation methods can also be used. Depending on the interest of the study, MVs can be removed prior to analysis. To remove MVs, an extra ultracentrifuge step must be done before protein precipitation; 1 h at 4°C and 50,000 × g is a good option [46].

Proteome analysis of the platelet releasate can be carried out by different strategies. Coppinger et al. in 2004 [46] published a MudPIT-based study of the platelet releasate in response to thrombin. The same group published another study using a standard GeLC approach followed by MS/MS leading to the conclusion that aspirin has a general moderating effect on the amount of protein released regardless of the agonist [72]. Della Corte et al. in 2008 published a quantitative analysis of platelet releasate using 2D-DIGE [93]. A more recent study of the TRAP-induced platelet releasate employed GeLC followed by LTQ-FT resulting in a measurement of peptide fragments at a higher mass accuracy than in any previous comparable study [94]. A representative 2D-map of the platelet releasate, including microvesicles, is shown in Fig. 2B.

As it was mentioned above, platelet-derived microvesicles are part of the platelet releasate, but they can also be studied independently by proteomics. Among 90% of the plasma microvesicles are platelet-derived [95] and released into the circulation, along with smaller vesicles of endosomal origin called exosomes. MVs represent an important mode of intercellular communication by serving as vehicles for transfer between cells of membrane and cytosolic proteins [96], but are also involved in many other functions such as coagulation, inflammation, intercellular communication and tumor progression. MVs are known to have important roles in the pathogenesis of various inflammatory diseases such as atherosclerosis [97], and their number is increased in the blood of patients with acute coronary syndromes [98,99]. Since microvesicles are released into plasma, they are typically prepared from it before or after platelet stimulation.

Plasma MVs are normally isolated for proteomic studies from platelet-poor plasma (PPP), whereas platelet MVs are isolated after activation of purified platelets. In any case, elimination of residual
cells is fundamental, and for this purpose we recommend to perform two consecutive centrifugations, the first one for 10 min at 1500 × g and the second one for 2 min at 15,000 × g, both at 4 °C. Finally, the cell-free sample is subjected to ultracentrifugation to pellet microvesicles. Ultracentrifugation forces and times vary widely among groups, because there is no consensus protocol; some groups apply 150,000 × g for 90 min at 4 °C [100,101], 100,000 × g for 1 h at 4 °C [102], 250,000 × g for 1 h and 4 °C [103], for 200,000 × g 2 h at 4 °C twice [104]. We made a comparative study to isolate plasma MVs including several of those protocols and obtained the best result making centrifugations as follows: PPP has to be processed until obtain platelet-free plasma (PPP), we recommend two centrifuge steps for that as indicated above (10 min at 1500 × g and 2 min at 15,000 × g at 4 °C). PPP is then diluted in HEPES buffer (10 mM HEPES, 5 mM KCl, 1 mM MgCl2/136 mM NaCl2 (pH 7.4)) in a ratio of 1/2.5 and centrifuged for 90 min at 200,000 × g and 4 °C. After supernatant removal, the MVs pellet is resuspended in 0.25 M KBr in order to eliminate soluble serum proteins, and incubated on ice for 20 min. Samples are then spun down for 90 min at 200,000 × g and 4 °C. Supernatant is removed again and MVs pellet resuspended in PBS 1X and spun down at the same conditions. The supernatant is finally removed and the MVs pellet air dried before resuspension in the desired buffer. It is important to notice that all the ultracentrifugation protocols mentioned in this section not only sediment MVs, but also exosomes. A representative 2D-map of the plasma MVs proteome is shown in Fig. 2C.

The first study of platelet-derived microvesicle proteome was carried out by García et al., after platelet activation with ADP. They performed a conventional 1-D SDS–PAGE followed by nano-LC–MS/MS, identifying a total of 578 distinct proteins in these MVs [100]; the complete list is available from the American Chemical Society (http://pubs.acs.org). Two years later, Smalley et al. published a proteomic comparative study of platelet-derived and plasma-derived MVs using spectral count analysis and isotope-coded affinity tag (ICAT) labeling of proteins. This analysis revealed 21 proteins expressed in human plasma-derived but not platelet-derived MVs [101]. In a very recent study by Capriotti et al., the platelet-derived microvesicle proteome was analyzed again after platelet activation with ADP. This study, based in a shotgun proteomics approach, led to the identification of 603 proteins, 243 of which were not previously identified [105]. Besides this basic proteomic works, some clinical proteomic studies related to platelet-derived or plasma-derived MVs have been done in the last few years [104,106] because of the relevance of MVs in the pathogenesis of several diseases.

As it happens with in the case of microvesicles, platelet-released granules can be isolated and studied independently. The release of platelet granules into blood has large consequences for thrombosis, atherosclerosis and other pathological processes. Both types of granules, α- and dense granules, are derived from MK-multivesicular bodies [107]. When platelets are stimulated with strong agonists, both types of granules are secreted following calcium mobilization from intracellular stores, releasing their contents into the plasma and contributing to the increase in platelet membrane surface due to granule membrane fusion. Regarding their function, platelet granules contribute to propagation of platelet activation, and moreover, to processes such as coagulation, host defense, angiogenesis, wound healing, and inflammation [108]. Storage pool deficiencies are rare genetic disorders which may affect predominantly α-granules, dense granules or both, and their symptoms illustrate perfectly the importance of platelet secretory granules in hemostasis.

In a very recent study by Zufferey et al., platelets underwent subcellular fractionation for granule enrichment, using sucrose gradients, and centrifugal forces of 96,800 × g for 135 min. They identified 827 proteins, constituting the largest platelet granule proteome qualitative dataset to date [109].

Alpha-granules contain a wide range of proteins and some studies reported that there are distinct subsets of α-granules with differential sorting of selected proteins into each [110]. Despite being aware of the importance of α-granules, only one group has specifically studied their proteome in detail. It was Maynard’s group, in 2007, who addressed this challenge. They isolated α-granules using a linear sucrose gradient method that allowed them to identify 284 proteins [111]. Three years later the same authors applied the same method to analyze the platelet proteome of one patient suffering from gray platelet syndrome (GPS), a rare inherited disorder characterized by mild to moderate thrombocytopenia with bleeding tendency and a marked decrease or absence of platelet α-granules, and compared this patient to healthy α-granules [112]. They found that soluble, biosynthetic cargo proteins were severely reduced or undetected in GPS platelets, whereas the packaging of soluble, endocytic cargo proteins was only moderately affected.

Dense granules contain predominantly small molecules, such as ADP, ATP, serotonin, calcium and polyphosphate [113]. A specific study of the platelet dense granule content was done by Hernández-Ruiz et al. [91]. Dense granules were isolated by centrifugation using density columns. The low number of proteins identified (40) is not surprising since dense-granules cargo are mostly composed by small molecules.

Regarding quality controls, contamination with other blood cells must be analyzed in the case of whole platelet proteome studies. This can be done by immunoblotting using antibodies against specific red or white cell proteins. The electronic particle analyzer mentioned above can also give an indication of the presence of other blood cells, based on counting cells above platelet size. In the studies of subproteomes, quality controls must be made in order to ensure that there are no proteins from other subproteomes in the isolated subproteome. Moreover, in order to characterize specific subpopulations such as exosomes or MVs, there are several useful tools to check their purity, such as immunoblotting, FACS, immunogold labeling or electron microscopy.

3. Platelet proteomics in cardiovascular disease-related studies

Over the last decade, the potential of proteomics has become extensively recognized and its use for the study of complex diseases has increased exponentially. There are hundreds of platelet-proteomic studies, but it was only in the last few years when several groups focused on platelet proteomics applied to cardiovascular and arterial thrombosis diseases. In this section we revise the most relevant proteomic studies on platelet-related cardiovascular diseases and some other related conditions (Table 1 and Fig. 3).

3.1. CAD platelet proteome: searching for biomarkers and drug targets

To the best of our knowledge, there are four proteomic studies searching for platelet biomarkers in CAD, two of them from our group.

In the last few years our group started to study the functional change experienced by platelets in ACSs applying 2-DE-based proteomics combined with MS. Our aim was to expand the knowledge about these functional changes and also search for novel platelet biomarkers and potential drug targets for these diseases. We performed two studies; the first one focused on NSTEMI patients [114] and the second on STEMI ones [115].

The first study included 18 NSTEMI patients and 10 stable coronary artery disease (SCAD) controls, matched by sex, age and treatments. 2-DE analysis (24 cm, pH 4–7 and 10% SDS–PAGE)
Table 1
Platelet proteomic studies in CVD.

<table>
<thead>
<tr>
<th>Authors</th>
<th>Source sample</th>
<th>Proteomic approach</th>
<th>Main findings</th>
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<tr>
<td>Corrao et al. 2010</td>
<td>NSTEMI patients vs SCAD controls and healthy donors.</td>
<td>2DE MALDI-TOF/TOF</td>
<td>Variation in signaling, cytoskeletal and secreted proteins. Involvement of signaling pathways (GPVI and αIIbβ3). Sustain the idea of pre-activated platelets in acute patients.</td>
</tr>
<tr>
<td>Parguiña et al. 2010</td>
<td>STEMI patients vs SCAD controls and healthy donors.</td>
<td>2DE MALDI-TOF/TOF</td>
<td>Variation in signaling, cytoskeletal and secreted proteins. Involvement of signaling pathways (GPVI, αIIbβ3 and αIIbβ1). Higher activation of GPVI in STEMI patients. Uplift the idea of pre-activated platelets in acute patients. Changes in cytoskeleton, energy metabolism and protein degradation. Alterations might be produced at MK level.</td>
</tr>
<tr>
<td>Banfi et al. 2011</td>
<td>NSTEMI and SA patients vs SCAD controls and healthy donors.</td>
<td>2DE LCQ-IT</td>
<td>Down-regulation of cytoskeleton, antioxidant system and energy metabolism. Support the idea of decrease on platelet predisposition to activation and limited platelet activity. Alterations might be produced at MK level.</td>
</tr>
<tr>
<td>López-Farré et al. 2011</td>
<td>ACS patients vs CAD controls.</td>
<td>2DE MALDI-TOF/TOF</td>
<td>Changes of proteins related to energetic metabolism, cytoskeleton, oxidative stress and cell survival. Platelets from ASA-resistant patients may have a higher apoptotic state.</td>
</tr>
<tr>
<td>Mateos-Cáceres et al. 2010</td>
<td>SCAD ASA-resistant vs ASA-sensitive patients.</td>
<td>2DE MALDI-TOF/TOF</td>
<td>Cytoskeletal, oxidative stress and energy metabolism changes. Clopidogrel interrupt exocytosis and control enhanced platelet activation.</td>
</tr>
<tr>
<td>Volpi et al. 2012</td>
<td>SCAD patients (SA) before coronary intervention, after clopidogrel administration and after PCI.</td>
<td>2DE MALDI-TOF/TOF</td>
<td>Dual antiplatelet therapy did not enhance the effect of aspirin. Patients have lower platelet reactivity after dual antiplatelet therapy.</td>
</tr>
<tr>
<td>Azcona et al. 2012</td>
<td>SCAD patients suffering from type II diabetes mellitus and treated with ASA or ASA + clopidogrel.</td>
<td>2DE MALDI-TOF/TOF</td>
<td>Changes in platelet activation, after clopidogrel administration and after PCI.</td>
</tr>
<tr>
<td>Hypertension</td>
<td></td>
<td></td>
<td>Hypertensive patients taking or not Olmesartan medoxomil.</td>
</tr>
<tr>
<td>Sacristán et al. 2008</td>
<td></td>
<td>2DE MALDI-TOF/TOF</td>
<td>Olmesartan medoxomil alters the expression of some platelet proteins. Up-regulation of a gelsolin precursor suggests reduction in platelet activation. Decrease of several other proteins suggests reduced antioxidative requirement of platelets in treated patients. Alteration may be produced at MK level.</td>
</tr>
<tr>
<td>Gebhard et al. 2011</td>
<td>Rats induced angiotensin II-dependent hypertension vs normotensive rats.</td>
<td>2D-DIGE MALDI- TOF/TOF FT-ICR</td>
<td>Changes in calpain substrates and several other proteins not previously related to calpain. Conclude that calpain inhibition may be effective to decelerate atherothrombosis development preserving platelet function.</td>
</tr>
<tr>
<td>Diabetes</td>
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<tr>
<td>Randriamboavonjy et al. 2012</td>
<td>Type 2 diabetic mielitits patients before and after treatment with pioglitazone</td>
<td>2D-DIGE MALDI- TOF/TOF</td>
<td>Increasing of 14-3-3Σ forming patches in the luminal side of atherosclerotic plaques in abdominal aortic aneurism patients. This protein plays a relevant role in the progression of atherosclerosis.</td>
</tr>
<tr>
<td>Subproteomes</td>
<td>Platelet-derived dense granules from healthy donors. Includes abdominal aortic aneurism patients.</td>
<td>2DE MALDI-TOF/ TOF LC-MS/MS</td>
<td>Changes in proteins related to inflammation, thrombosis and cardiovascular disease.</td>
</tr>
<tr>
<td>Hernández-Ruiz et al. 2007</td>
<td>Plasma-derived MVs from STEMI patients vs SCAD controls and healthy donors.</td>
<td>2D-DIGE MALDI- TOF/TOF LC-MS/MS</td>
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</table>

revealed 40 protein features differentially regulated. All these features were successfully identified by MS (MALDI-TOF/TOF), and corresponded to 22 ORFs. A systems biology approach using ingenuity pathway analysis (IPA) revealed that 16 of these proteins are interconnected in a network related to cell assembly and organization and cell morphology. Fourteen of those proteins are either signaling or cytoskeletal and nine are known to play a major role in platelet activation by αIIbβ3 and/or GPVI receptors, suggesting that these pathways may play a relevant role in platelet activation associated with NSTEMI. We validated two signaling proteins by 2-DE western blotting whose differences were due to PTMs. We also validated the secreted protein SPARC and found it down-regulated in NSTEMI patients; which is consistent with platelet secretion associated to platelet activation in NSTEMI patients. Moreover this study includes patient monitoring during a 6-month follow-up revealing that those proteins returned to normal levels after that time. Our data suggest higher platelet activation in NSTEMI patients.

In the second study, the platelet proteome of STEMI patients (N = 11) and SCAD controls (N = 15) was compared. The analytical approach was the same than in the previous study: 2-DE (24 cm, pH 4–7 and 10% SDS–PAGE) followed by MS (MALDI-TOF/TOF). In this case, we found 56 differences between groups, corresponding to 42 ORFs. IPA analysis revealed that 35 of the 42 differentially regulated proteins identified are interconnected in a network related to tissue and cell morphology and the top molecular and cellular functions are cell-to-cell signaling and interaction, cellular assembly and organization and cell morphology (as in the NSTEMI study). We validated p-Src and Crkl. The up-regulation of the active form of Src (Src-PThr418) in STEMI suggests a primary role of pathways related to GPVI, αIIbβ1 and αIIbβ3. Moreover, IPA revealed 3 platelet signaling pathways related to platelet activation in ACSs: acting cytoskeleton signaling, integrin-linked kinase signaling, and GPVI signaling. Furthermore, this study included an independent experiment of activation with the GPVI-specific agonist collagen-related peptide (CRP) in healthy, SCAD and STEMI groups (6 patients per group) revealing a higher activation of GPVI signaling in platelets from STEMI patients. Additionally we performed a 6-month follow-up analysis, as in the NSTEMI study, which revealed a decrease in the number of differences with time.

Besides the above studies, in 2011 Banfi et al. [116] published a study comparing the platelet proteome of patients with NSTEMI (N = 14) and SA (N = 12) with healthy donors (N = 10). The analysis was based on 2-DE (17 cm, pH 3–10 and 7–17% SDS–PAGE) followed by MS (LCQ-IT). They identified 6 differentially expressed proteins: two involved in energy metabolism (OGDH and LDH), three related to cytoskeleton-based processes (γ-actin, coronin 1B...
and pleckstrin) and one associated to protein degradation (PSB8). OGDH and γ-actin levels were significantly increased in platelets from both patient groups compared to the healthy control group, whereas LDH was higher only in patients with NSTEMI. Coronin 1B and PSB8 were less expressed in platelets from both patient groups, and basic forms of pleckstrin exhibited the same behavior. In agreement with our results [114], the differences they found were related to cytoskeletal remodeling, platelet activation and proteolytic activity. They propose that platelets from NSTEMI patients suffer from an increase in the energy metabolism and attribute the differences to the atherosclerotic process. Authors suggest that alterations might be produced at MK level.

Finally, and also in 2011, López-Farré et al. [117] compared the platelet proteome of a group of patients with ACS (N = 16) and CAD controls (N = 26), combining 2-DE (18 cm, pH 3–10 and 10% SDS–PAGE) with MS (MALDI-TOF or MALDI-TOF/TOF). They focalized their study on proteins related to glycolysis, because platelet energetic production is mainly dependent on glycolysis, on proteins involved in cytoskeleton (that regulates most of platelet activities), and on the platelet antioxidat system (protects platelets from oxidant agents, which are important platelet stimulators). 2-DE analysis revealed that proteins involved in activities aforementioned were downregulated in platelets from ACS patients compared to CAD platelets. From a biological point of view, a decrease of the antioxidat system in ACS platelets might predispose those patients to easier platelet activation by prooxidant agents produced during acute coronary event, turning platelets unable to prevent their activation by oxygen free radicals [118]. Moreover, reduction on the expression of both cytoskeleton and energetic-metabolism-related proteins, may suggest a decrease on platelets predisposition to be activated. Broadly, they conclude that differentially expressed proteins on platelets from ACS patients seem to reflect easier platelet activation (diminished antioxidat system) and a limited platelet activity (reduction in the expression of cytoskeleton and glycolytic-related proteins). Therefore, the authors hypothesize that some of the inflammatory compounds released from the inflamed plaque may play as signals to produce these “bewildered” platelets from megakaryocytes, days before the clinical onset of the acute event.

3.2. CAD platelet proteome: influence of treatment

Recent platelet proteomic studies in cardiovascular diseases were also made to assess how the platelet proteome varies depending on the treatment used. There are several evidences that some ACS patients do not respond well to the treatment with acetyl salicylic acid (ASA). The reason why these patients show inadequate platelet inhibition by ASA is still unclear and this is why Mateos-Cáceres et al. performed a platelet proteome analysis including 51 stable coronary ischemic patients [119]. They compared ASA-resistant (N = 25) and ASA-sensitive (N = 26) patients by 2-DE (18 cm, pH 3–10 or pH 4–7 and 10% SDS–PAGE) followed by MS (MALDI-TOF/TOF). They found that platelets from those patients differ in terms of the expression of proteins related to energetic metabolism, cytoskeleton, oxidative stress and cell survival. ASA-resistant showed a downregulation on three proteins related to cytoskeleton (gelsolin precursor types 2 and 3, F-actin capping) which control the assembly and disassembly of actin filaments essential in platelet shape change. In addition proteins involved in energetic metabolism (disulphide isomerase isotype 1, glutathione S-transferase, 1,6-bisphosphate aldolase) were downregulated in ASA-resistant patients, unless glyceraldehyde 3-phosphate dehydrogenase, which suggests that platelets from these patients have a reduced protection against oxygen free radicals which makes them more prone to activation. Regarding stress and cell survival, they found that disulphide isomerase isotope 1 was increased in ASA-resistant patients whereas some heat shock proteins were decreased. No changes were observed in inflammatory proteins. Taken all these results together, authors conclude that platelets from ASA-resistant patients may have a higher apoptotic state than ASA-sensitive ones.

Clopixogrel, which is a thienopyridine-class antiplatelet agent used to inhibit blood clots in CVDs, is widely used as platelet inhibitor for coronary angioplasty procedures [120], such as
percutaneous coronary intervention (PCI). Volpi et al. [121] focused their attention on the fact that ACS patients undergoing clopidogrel treatment and elective PCI may have altered platelet reactivity, and decided to study the platelet proteome in order to shed light over the molecular mechanisms that regulate platelet reactivity in this context. The proteome study was based on 2-DE (18 cm, pH 3–10 and 12.5% SDS–PAGE) followed by MS analysis (MALDI-TOF/TOF), and included 20 SCAD patients suffering from stable angina. This analysis was based on the comparison of platelets taken at three time points: before coronary angiography (T0), 12 h after 600 mg of clopidogrel administration (T1) and 24 h after PCI (T2). Moreover, they included a study of clopidogrel response and platelet reactivity (using flow cytometry and aggregation tests) which showed that a clopidogrel-loading dose produced a significant inhibition in all markers of platelet activation. Among the 24 proteins found differentially expressed, 18 were identified by MS analysis. These included oxidative stress-related proteins (heat shock 70 kDa protein 5, snit-stress induced phosphoprotein 1), energetic metabolism-related proteins (nucleoside diphosphate kinase B, protein-isoaspartate(α-aspartate)-O-methyltransferase, ubiquitin-like modifier-activating enzyme 1) and cytoskeleton rearrangement proteins (profilin-1, calphin, thrombospondin, α-soluble NSF attachment protein). These proteins seem to be announcing changes associated with platelet activation and clopidogrel response, like controlling enhanced platelet activation in SCAD patients or interrupting platelet exocytosis processes.

In the same year, Azeona et al. [122] compared the effect of dual antiplatelet therapy (clopidogrel + aspirin) with respect to aspirin treatment, on the platelet proteome of type-2 diabetic patients with stable coronary artery disease. This study included 57 patients randomized to receive dual (N = 29) or single (N = 28) aspirin platelet therapy. A 2-DE (18 cm, pH 3–10 and 10% SDS–PAGE) was performed for each patient. Identifications were by MS (MALDI-TOF or MALDI-TOF/TOF) focusing on the study of platelet proteins associated with cytoskeleton, energetic metabolism, oxidative stress and inflammation. Between the two different antiplatelet strategies, differences were found in actin-binding protein isotypes 2 and 5 (cytoskeleton and contractile system-related proteins) which were found upregulated in single antiplatelet therapy. Also lactate dehydrogenase (protein associated with energetic metabolism and oxidative stress) was found upregulated in platelets from patients with single antiplatelet therapy. Similarly, FBC isotope 5, immunoglobulin heavy chain and RAB7B isoforms 1 and 6 (all of them related to inflammation) were found upregulated in platelets from patients treated with aspirin. They also tested plasma levels of platelet factor 4 (PF4), a marker of platelet activity, without finding any differences between both groups. On the basis of PF4 circulating levels, it seems that dual antiplatelet therapy did not enhance the effect of aspirin. Taken in mind all before mentioned, authors suggest that there exist lower platelet reactivity after dual antiplatelet therapy but further studies are needed to assess the implications of these changes in platelet functionality.

3.3. Hypertension: variations in the platelet proteome and influence of treatment

Hypertension is the major risk factor of cardiovascular problems. Platelets from patients suffering from hypertension show a basal state of pre-activation and a tendency to aggregate [123], and it is known that some angiotensin II receptor blockers (ARBs) inhibit human platelet activation in vitro [124]. This is the reason why several researchers conducted their efforts to the study of platelet-proteomics in hypertension.

Sacristán et al. [71] focused on the study of the platelet proteome of moderate hypertensive patients before and after treatment with Olmesartan medoxomil, which is a new highly potent and selective ARB, which exhibits pleiotropic effects that are not fully understood. This study included 13 patients with moderate hypertension who were randomized to have or not a 6 month treatment with Olmesartan medoxomil. They found that patients treated with this drug showed significantly reduced systolic blood pressure, proteinuria, total cholesterol and LDL-cholesterol in plasma. Moreover this treatment did not modify the circulating plasma levels of different pro-inflammatory proteins, but changed the expression of some platelet proteins. Using 2-DE (pH 3–10 or pH 4–7 and 10% SDS–PAGE) combined with MS (MALDI-TOF or MALDI-TOF/TOF) they demonstrated that the drug altered the expression of some platelet proteins. They found an increase in the expression of gelsolin precursor isotype 4, suggesting a reduction in platelet activation. Also it was found a decrease of tropomyosin-β chain isoforms 1–5, leukocyte elastase inhibitor and chloride intracellular channel-protein isotype 1, which suggests a reduced antioxidative requirement of platelets in treated patients. They conclude that this differential expression may be related to some action of the drug predictably at megakaryocyte level.

Another platelet-proteome study related to hypertension, but in rats, was done by Gebhard et al. [125]. They induced angiotensin II-dependent hypertension in rats following two strategies: feeding rats with indole-3-carbinol (IC3, N = 10) or subcutaneously infusing angiotensin II (ANG II, N = 7). Fourteen days later, authors compared the platelet proteome of these rats with a control group of normotensive rats after placebo treatment (N = 7 and N = 10, respectively). Proteomic analysis was based on 2D-DIGE (pH 4–7 and 12.5% SDS–PAGE) and MS (MALDI-TOF/TOF or FT-ICR). Sixty-six altered proteins were found in feeding IC3 rats and 157 in ANG II infused rats, 45 of which were common to both groups. Proteins differentially regulated were mostly cytoskeletal protein fragments, which might result from ongoing signaling in the platelet. This is in agreement with the observation that platelet function is increased in hypertensive patients [126]. This protein degradation could also produce acceleration in the aging of platelets. These changes on the platelet-proteome of rats were consistent and reversible.

3.4. Diabetes: variations in the platelet proteome and influence of treatment

Although diabetes is not a CVD itself, their relationship is very well known, since platelets from diabetic patients are hyper reactive and have increased adhesiveness, aggregation, degranulation and thrombus formation. All of these characteristics make diabetes vulnerable to vascular disease development. In this context Randriamboavonjy et al. [127] developed a platelet proteome study of type 2 diabetic miellitus patients. Among other experiments they performed a 2D-DIGE analysis (18 cm, pH 3–10 and 12% SDS–PAGE) of platelet proteins from the same diabetic patient before and after treatment with Pioglitazone, which increases insulin sensitivity and has deep effects on platelet proteome. Protein identification was by MALDI-TOF/TOF. Among the proteins identified in the analysis, there were calpain substrates (i.e. talin-1 and filamin A) and some other proteins not previously linked with calpain (i.e. septin-5, ILK and glycerol-3-phosphate dehydrogenase 2). With these results, those obtained in mice, and the studies that show a beneficial effect of calpain inhibition in vascular disease, the authors conclude that since diabetes-induced platelet dysfunction is mediated largely by calpain activation, calpain inhibition may be an effective way to decelerate atherothrombosis development and to preserve platelet function.
3.5. Platelet subproteome analysis in CVD

As it was mentioned earlier in the sample preparation section, the study of subproteomes is of great interest in order to obtain novel information masked in whole proteome analyses by high abundant proteins. In this regard, some proteomic studies are targeting platelet subproteomes. This is the case of the study published in 2007 by Hernández-Ruiz et al. [91] who performed a proteomic study of platelet-derived dense granules. This study was made in healthy donors by 2-DE (11 cm, pH 3–10 or 5–8) followed by MS (MALDI-TOF or LC-ESI MS/MS). They excised 20 protein spots from the gel which correspond to 40 proteins, most of them previously described as platelet proteins, but without knowledge of their place of origin. Among those proteins they focused their attention on the study of 14–3–3ζ which is a protein released by activated platelets [46]. This protein is also associated with intercellular signaling and regulation in platelets in conjunction with vWF receptor, GPIb–V–IX [128]. Moreover a recent study has identified 14–3–3ζ as a platelet signaling protein that is tyrosine phosphorylated following GPVI activation [129]. Hernández-Ruiz et al. confirmed the presence of high levels of this protein in platelet dense granules by western blot, immunofluorescence and functional analysis of platelet secretion. In addition, as it had been shown that some proteins secreted by platelets contribute to atherosclerosis progression, they evaluated the localization of 14–3–3ζ in sections of the aorta wall from 14 patients with abdominal aortic aneurism, which present atherosclerotic plaques. Results show that those patients have 14–3–3ζ forming patches on the extreme luminal side of atherosclerotic plaques, which suggests that this protein may come from activated platelets in the cavity of the artery. Overall, authors suggest that 14–3–3ζ might play a relevant role in the progression of atherosclerosis.

Microvesicles are known to be increased in the blood of patients with acute coronary syndromes, and STEmi in particular [130], among many other pathological conditions, moreover inflammatory disease. Plasma microvesicles are constituted primarily by platelet derived MVs. With that in mind, our group decided to study the proteome of plasma-derived MVs from patients with STEmi compared to stable coronary artery disease (SCAD) controls [106]. Ten STEmi patients and 10 matched SCAD controls were used for proteomic analysis, which was based on high-resolution 2D-DIGE (24 cm, pH 4–7 and 11% SDS–PAGE) and MS (LC–MS/MS). We found 117 protein features differentially regulated, and from those we successfully identified 102 corresponding to 25 ORFs. Most proteins identified were related to inflammatory response, thrombosis and cardiovascular disease. Indeed 11 proteins could be located in a network related to infarction and 10 in a network related to atherosclerosis, as it revealed the systems biology approach (IPA). Among the proteins identified, we report that α2-macroglobulin isoforms, fibrinogen (both related to thrombogenesis), and viperin (related to atherosclerosis) are up-regulated in plasma-derived MVs from STEmi patients; whereas ITIH4, an anti-inflammatory protein, is down-regulated. Validations were by 1D- and 2D-western blotting using an independent cohort of patients (15 STEmi, 13 SCAD and 10 healthy donors). The up-regulation of α2-macroglobulin isoforms and fibrinogen indicates that MVs might be playing a relevant role at local level in the atherothrombotic events that lead to a myocardial infarction. Meanwhile, the detection of viperin in atherosclerotic lesion may be linked to an active immune status in the lesion.

3.6. Key conclusions from platelet proteomic studies related to CVD

The studies mentioned above reflect the hyperreactive state of platelets in CVD, especially in CAD. The studies carried out on platelets from ACS patients reflect an up-regulation of signaling and cytoskeletal proteins, as well as a down-regulation of antioxidant proteins. These data are in agreement with increased platelet activation levels in ACS, which might be responsible for the atherothrombotic events related to the acute event. Regarding CAD studies related to the effect of drug treatments, proteome studies show that dual antiplatelet treatment seems to diminish platelet reactivity, as expected. On the other hand, results indicate that platelets from ASA-resistant patients may have a higher apoptotic state than ASA-sensitive ones.

Regarding other CVD-related pathologies, such as hypertension and diabetes, results are also in line with the hyper-reactive state of platelets in those pathologies. In the case of diabetes, results seem to indicate that platelet dysfunction is mediated by calpain activation so calpain inhibition is proposed as a way to decelerate atherothrombosis development and preserve platelet function.

Studies related to subproteomes, primarily focused on MVs, are also in line with the platelet studies. Thus, the proteome analysis of MVs from myocardial infarction patients shows the up-regulation of proteins involved in atherothrombosis on those patients. This suggests that activated platelets are releasing MVs at local level that contribute to the atherothrombosis responsible for an ACS.

Overall, the studies carried out to date are pretty coherent and give clues on novel potential platelet drug targets to prevent/treat atherothrombosis-related CVD. Current data pave the way for future studies that should focus on validating the results obtained so far in larger cohorts of patients and pathological conditions.

4. Concluding remarks

With the beginning of the 21st century and the sequencing of the human genome a new era began for biomedical research. Rapid advances in –omics technologies and systems biology allowed a thorough analysis of biological samples both from patients and healthy individuals. The enormous expectations and optimism present in the early days were followed by caution and skepticism because a lot of money was invested in –omics research and yet results were not being obtained so rapidly. Analytical problems appeared that had, and still have, to be tackled; a good example of this is the proteomic analysis of plasma and serum. As a consequence, the initial expectations led to some sort of frustration as the number of biomarkers and drug targets identified by these –omic technologies, including of course proteomics, was not as high as initially expected. Time is needed to address all the various challenges that appeared on the way, and this is true especially for proteomics where recent advances in mass spectrometry-based technologies for protein analysis and quantitation are bringing new prospects to the field and allowing solving many of the analytical problems mentioned above.

Platelet proteomics also faced the same challenges than proteomics in general had to face. The fact platelets lack of a nucleus makes them easier to prepare for proteome analysis. Besides whole proteome analysis, subcellular fractionation and the analysis of signaling pathways, microvesicles and the releasate have provided additional relevant information so we can today say that an important proportion of the platelet proteome has been uncovered. This definitely paves the way for future quantitative studies can be applied to biomarker discovery. Indeed, it has been only in the last 7 years that platelet proteomics has been applied to clinical studies, and more precisely to cardiovascular research. This review tried to show not only recent advances present in the literature but also some of the analytical challenges that the study of the platelet proteome has to face when dealing with clinical samples. The task is not easy but with appropriate methodological standardization procedures for platelet preparation, subcellular fractionation and releasate/microvesicles isolation, there is no doubt it will be easier to deal with these delicate samples for downstream
proteomic analyses. This will also allow better inter-lab reproducibility for a given assay. Recent advances in proteomics will also allow obtaining promising results for biomarker and drug target discovery related to platelets, as already shown by our group and by other recent studies mentioned in this review. Obviously, inter-lab reproducibility regarding proteomic results is more difficult given the high variety of proteomic approaches that can be used depending on the proteomic platform present in a particular laboratory; nevertheless, the aim should be that laboratories with the same platform yield similar results. We are still in the early days of platelet proteome applied to clinical studies, and more precisely to the study of cardiovascular disease; however, recent advances in the field allow us to be moderately optimistic in the hope that this time expectations will not be frustrated.

Acknowledgements

The authors would like to acknowledge the support given by the Spanish Ministry of Industry and Competitiveness (MINECO) (grant no. SAF2013-45014-R, co-funded by the European Regional Development Fund (ERDF)) and the Sociedad Española de Trombosis y Hemostasia (SETH).

References


According to recent studies, the development of personalized therapeutic strategies in treating cardiovascular diseases has become an area of intense research. Among these strategies, the use of proteomics and metabolomics has emerged as powerful tools for understanding the complex biological processes underlying cardiovascular diseases. These technologies enable the comprehensive analysis of biological samples, allowing for the identification of novel biomarkers and therapeutic targets.

Proteomics, as a subfield of proteomics, involves the systematic identification and characterization of proteins and their post-translational modifications in biological systems. This approach has been instrumental in the discovery of proteins that are dysregulated in various cardiovascular diseases, such as atherosclerosis. For instance, Pfluger and colleagues (2015) demonstrated that a specific set of proteins, including extracellular matrix proteins, were upregulated in a mouse model of atherosclerosis, providing insights into the pathophysiology of the disease.

Metabolomics, on the other hand, focuses on the comprehensive analysis of low-molecular weight metabolites in biological samples. This field has been particularly fruitful in understanding the metabolic perturbations associated with cardiovascular diseases. For example, a study by Li et al. (2013) revealed that certain metabolic pathways were dysregulated in patients with chronic kidney disease, highlighting the potential of metabolomics in identifying novel therapeutic targets.

In summary, the integration of proteomics and metabolomics approaches offers a promising avenue for the development of novel therapeutic strategies in cardiovascular diseases. Continued advancements in these technologies will likely lead to the identification of new biomarkers and therapeutic targets, ultimately improving patient outcomes.
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1538-7836.2009.03387.x.
10.1021/pr070380o.
S1040-8428(98)00044-4.
Blair,
Italiano
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Drwal,
characterization
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J
in
peripheral
(2013)
Reed,
23420871.
Porto,
a
of
19450911.
Wortelkamp,
stroke
membrane
microparticles,
release
22664782.
Nolli,
Vito,
García,
38,
2013
doi:http://dx.doi.org/10.1161/
Grigorian-Shamagian,
Rosa,
and
megakaryocytes
regulated
dx.doi.org/10.1074/jbc.M406261200.
17200773.
Docampo,
Horne,
blood
spectrometry,
proteins
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microparticles,
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significance
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colloidal
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