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REVIEW

Proteomic analysis of secretory proteins and vesicles in vascular research

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The release of proteins and membrane vesicles in the bloodstream regulates diverse vascular processes, both physiological, such as angiogenesis and haemostasis, and pathological, such as atherosclerosis and atherothrombosis. Proteomics, beside its canonical application for the expression profiling in cells and organs, can be applied to the study of secreted proteins and microvesicles, which play a significant role in the homeostasis of the vasculature, and the development of the atherosclerotic disease.

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1 Introduction

Atherosclerosis and its complications are a leading cause of morbidity and mortality worldwide. The formation of atherosclerotic lesions involves endothelial damage, smooth muscle cell proliferation and recruitment of lymphocytes, macrophages and platelets. In the process of plaque development, smooth muscle cells and macrophages accumulate lipids and undergo a phenotypical transformation promoted by the microenvironment of the atherosclerotic lesion [1]. The late stage of the pathological development consists of plaque erosion and rupture, which are followed by intraplaque thrombosis, vascular occlusion and atherothrombosis dissemination [2]. Proteins and microvesicles released by the cells in the atherosclerotic plaque play a pivotal role in the initiation and promotion of atherosclerosis. For example, growth and chemotactic factors are responsible for the recruitment and transformation of circulating and vascular cells [3]. The balance between extracellular matrix protein production and protease activity determines plaque stability [4].

Secreted or shed proteins coordinate many cellular processes in the vascular system, including growth, division, differentiation, apoptosis, migration and adhesion, thereby contributing to the homeostasis of the vasculature [5]. Thus, besides the identification of potential biomarkers in plasma, a new frontier of vascular proteomics is the analysis of the proteins released by vascular and blood cells. The release of proteins can occur *via* two main routes (Fig. 1) (i) direct secretion of proteins, and (ii) release of membrane vesicles. Extracellular secretory membrane bodies transport proteins either in their lumen or embedded in their membranes. They are increased in the atherosclerotic region and participate in the development of the pathology by regulating coagulation and immune response, facilitating cell-cell interactions, and transferring their content to the vascular lumen and/or to other cells in the atheroma.

The body of published work on the use of proteomics for the characterization of non-cellular fractions in vascular studies, in particular in the context of atherosclerosis, is the subject of this review. The first part underlines the current limitations of plasma proteomics and vascular biomarker research. The second part reviews the use of proteomics for the analysis of extracellular membrane vesicles shed *in vitro*

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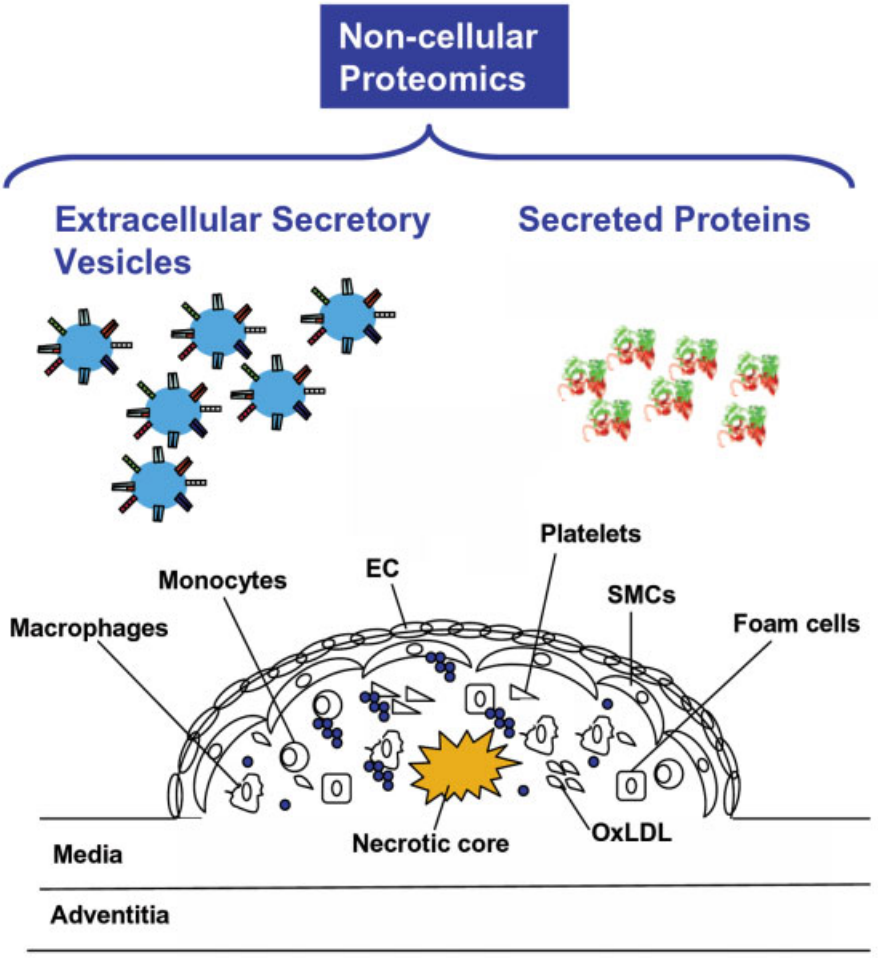

Figure 1. Schematic figure of the use of proteomics for the characterization of the non-cellular protein fractions relevant in atherosclerosis. The figure represents an atherosclerotic plaque and its cellular components. The cells involved in atheroma formation release soluble proteins and membrane bodies that modify the vascular microenvironment. Proteomics can be applied to the characterization of these non-cellular components of the atherosclerotic microenvironment.

and *in vivo* by different vascular cell types. The final part is a description of the vascular studies that utilised proteomics for the investigation of secretory proteins or peptides derived from *in vitro* cell and tissue cultures.

2 The limitations of plasma proteomics

The composition of plasma, the liquid component of blood, determines the biochemical environment of circulating and vascular cells, and ultimately regulates the functional responses of these cells. Therefore, plasma biomarkers offer the opportunity of assessing vascular risk before the manifestation of life-threatening pathologies. This would facilitate prevention and/or early intervention in vascular diseases. Hence, plasma and serum are routinely used for biomarker discovery in proteomics. However, attention needs to be drawn to the limitations and difficulties of plasma proteomics. Body fluids represent a formidable challenge for proteomic investigations owing to the high-abundance proteins, notably albumin and immunoglobulins, which together with haptoglobulin, antitrypsin and transferrin, typically constitute more than 90% of the total protein mass in human plasma [6]. On the other hand, proteins representing prospective biomarkers are found in the range of picogram to nanogram *per* millilitre, in sharp contrast to, for instance, albumin, which is present at approximately 35–50 milligrams *per* millilitre [7]. Unfortunately, current analytical techniques are still inadequate to resolve the dynamic range of protein concentration in human plasma.

Clearly, exploration of the plasma proteome is daunting with obstacles to overcome; nonetheless, the HUPO Plasma Proteome Project (HPPP) prepared and distributed reference specimens of human sera and plasma to multiple laboratories worldwide with the aim of creating a new human plasma proteome database. Through this collaboration, 3020 proteins were identified and subsequently classified in the context of what is known about human biology [8–10]. Despite the evident difficulties of plasma proteomics, several proteomic studies aimed at the discovery of predictive cardiovascular biomarkers have been published. Mateos-Caceres and co-workers [11] directly analysed crude plasma from patients with acute coronary disease and documented that

five major proteins were differentially present: α1 antitrypsin, apolipoprotein A-I, gamma chain of fibrinogen, heavy chain of immunoglobulin D and albumin. An industrial-scale proteomic discovery effort concentrated on coronary artery disease, where 53 male patients with the condition were compared against the same number of control subjects [12]. Overall, the differentially expressed proteins were linked with innate immunity and the natural defence system, growth, inflammation and coagulation. However, a significant limitation of this investigation was the pooling of plasma samples; although this increases the chance of detecting low abundant proteins, findings need to be validated on an individual-to-individual basis. Another research group has reported that at least 20 plasma proteins are differentially expressed between patients with atherosclerosis and healthy controls; but further validation and interpretation are required before the results of this investigation are communicated in full [13].

In general, the use of proteomics for the discovery of vascular biomarkers deserves careful consideration because of the limited ability of proteomics to detect low-abundance plasma proteins [14]. In addition, the reliability of biomarkers discovered by proteomics depends on the numbers of biological replicates, but given the technical challenges of plasma proteomics, the comprehensive analysis of large numbers of clinical samples remains a daunting task.

Apart from the more popular application of proteomics for biomarker discovery, the potential exists for this technology to be expanded to expose the pathways of disease development, and provide an in-depth knowledge of the metabolic and molecular modifications that occur in cardiovascular patients [15]. For example, to gain new insights in the role of lipoproteins in atherosclerosis, a proteomic approach was used to evaluate the composition of human low-density lipoprotein (LDL) and high-density lipoprotein (HDL). Beside previously known proteins three novel candidates, serum amyloid A-IV, calgranulin A and lysozyme C were identified in LDL particles [16, 17]. Interestingly, mice injected with endotoxin possessed apolipoprotein A-IV and apolipoprotein A-V in their HDL fraction, whilst control animals did not [18]. Such studies have highlighted the existence of new protein complexes that deserve further examination to establish their potential role in atherosclerosis. Alonso-Orgaz and co-workers focussed on hypercholesterolemia with the objective of mapping the changes in the proteome during statin administration [19]. Results obtained confirmed that statins modify the plasma expression of biomarkers associated with thrombosis, oxidative stress and vascular protection.

3 Proteomics of extracellular secretory vesicles

Cells are known to release secretory membrane bodies, such as apoptotic bodies, microparticles, exosomes and matrix vesicles (Fig. 2). Microparticles originate from the plasma membrane and have heterogeneous density and size (0.1–1.0 μm). Exosomes are smaller in size (diameter 30–90 nm) and are of endocytic origin. Matrix vesicles (diameter 30–300 nm) form at sites of interaction between different cell types and the extracellular matrix; they convey calcium, phosphate, lipids and annexins, and initiate biomineralisation of the matrix in a variety of tissues, including bone, cartilage, and dentin. Finally, apoptotic bodies, which are released during cell death, are the largest in terms of overall size (>1.5 μm) and characterised by nuclear content. In this review, the focus resides on microparticles and exosomes, as they are implicated in vascular pathophysiology and have already been the subject of proteomic studies.

3.1 Microparticles

Microparticles are secretory vesicles that originate from the plasma membrane and have attracted considerable attention in vascular research. They are shed from the surface of cells following apoptosis or activation by a variety of chemical stimuli, such as cytokines, thrombin and endotoxin, or physical stimuli, such as shear stress or hypoxia [20]. In the vascular context, microparticles are released by endothelial cells, smooth muscle cells, lymphocytes, monocytes, erythrocytes and platelets. Their importance is emphasized by the alteration of their plasma concentration in several vascular pathologies characterised by vascular dysfunction and inflammation. Plasma levels of microparticles are markedly elevated in patients with vein thrombosis [21], acute coronary disease [22, 23], ischemic stroke [24], diabetes [25], myocardial infarction [22], and hypertension [23, 26]. The understanding of microparticle activity has significantly improved, and several molecular mechanisms of action have been uncovered. Their pro-coagulant activity depends on the binding of coagulation factors on their surface and the accumulation of tissue factor [27–30]. Microparticles also show pro-inflammatory and pro-atherosclerotic activities. This patho-physiological role depends on their ability to (i) regulate vascular tone and reactivity by modulating the endothelial secretion of prostacyclin and nitric oxide [31, 32]; (ii) promote monocyte-endothelium interaction by direct transfer of arachidonic acid to the plasma membrane [31, 33]; and (iii) physically mediate leukocyte-leukocyte and leukocyte-endothelium interactions *via* direct binding of cell surface receptors [34].

Because of their critical patho-physiological role, the characterisation of which cellular proteins are represented in microparticles is pivotal to the understanding of their function. Proteomics is the instrument of choice for this kind of research, and some investigations into the microparticle proteome have appeared in the literature. The proteome of microparticles from human plasma has been mapped by 2-DE and subsequent MS [35]. Out of 169 high-abundance proteins identified in microparticle samples, actin cytoskeleton-associated proteins and integrins were the predominant classes. Also amongst the most represented proteins,

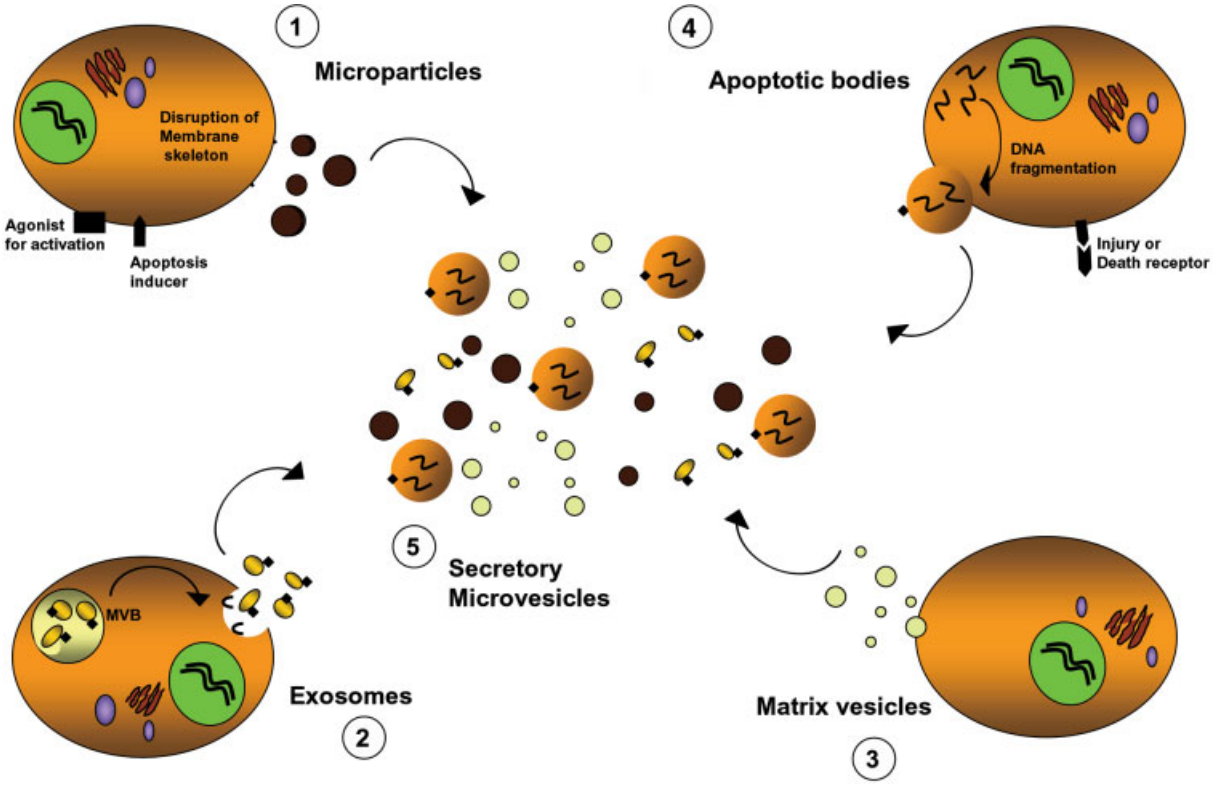

Figure 2. Schematic representation of extracellular secretory vesicles. (1) Microparticles are released from the plasma membrane of stimulated or apoptotic cells. Their protein composition may vary in response to different stimuli (high shear stress, apoptosis, *etc.*). (2) Exosomes are the smallest of the secretory membrane particles and are secreted as a consequence of the fusion of the plasma membrane with the multivesicular bodies (MVB). MVB are late components of the endocytic pathway. (3) Matrix vesicles are extracellular membrane particles observed in the initial stages of arterial calcification and contain high levels of calcium-binding acidic phospholipids. (4) Apoptotic bodies are large particles released from cells at the later stages of programmed cell death and characterized by large diameter, nuclear content, and surface ligands for phagocytic cell receptors. (5) Heterogeneous population or secretory microvesicles.

there were different subunits of IgM, possibly important for the clearance of microparticles by the reticuloendothelial system.

As for plasma proteomics, in the case of circulating microparticles, the main complication is the contamination by high-abundance plasma proteins. For example, when we compared the protein expression profile of isolated microparticles from peripheral blood and microparticle-depleted plasma using DIGE, plasma proteins still dominated the proteomic pattern of blood-derived microparticles (Fig. 3). Extensive washes, performed by successive cycles of centrifugation at high speed and resuspension in PBS of the microparticles, decreased the level of contamination by plasma proteins, but did not resolve the problem. All the proteins identified as selectively expressed in microparticles were high-abundance proteins, *e.g.* actin. On the other hand, haemoglobin, a ubiquitous high-abundance protein in blood samples, appeared to be predominantly present in the microparticle fraction, suggesting that abundant blood proteins can be genuine components also of the vesicular fraction. Thus, antibody-based depletion methods, as routinely employed for different applications of plasma proteomics, may result in the arbitrary alteration of the protein patterns, thereby impairing the validity of such methods.

Besides the investigation of the mixture of microparticles contained in human plasma, proteomics can be applied to the characterisation of microparticles released by a particular cell type *in vitro*. For example, platelet microparticles were analysed [36] using 1-D SDS-PAGE and LC coupled to a linear IT mass spectrometer. The authors of this study identified 578 proteins associated with the microparticles released by washed platelets activated *in vitro*. Amongst the hits, there were surface proteins typical of platelets, such as integrin αIIb, integrin β3 and P-selectin. The presence of several chemokines, such as CXCL4, CXCL7 and CCL5, suggested that platelet microparticles might play an important role in the regulation of leukocyte migration and differentiation. Moreover, the finding of 380 proteins not previously identified in platelets, suggested that the formation of microparticles is accompanied by selective enrichment of specific subsets of the proteome.

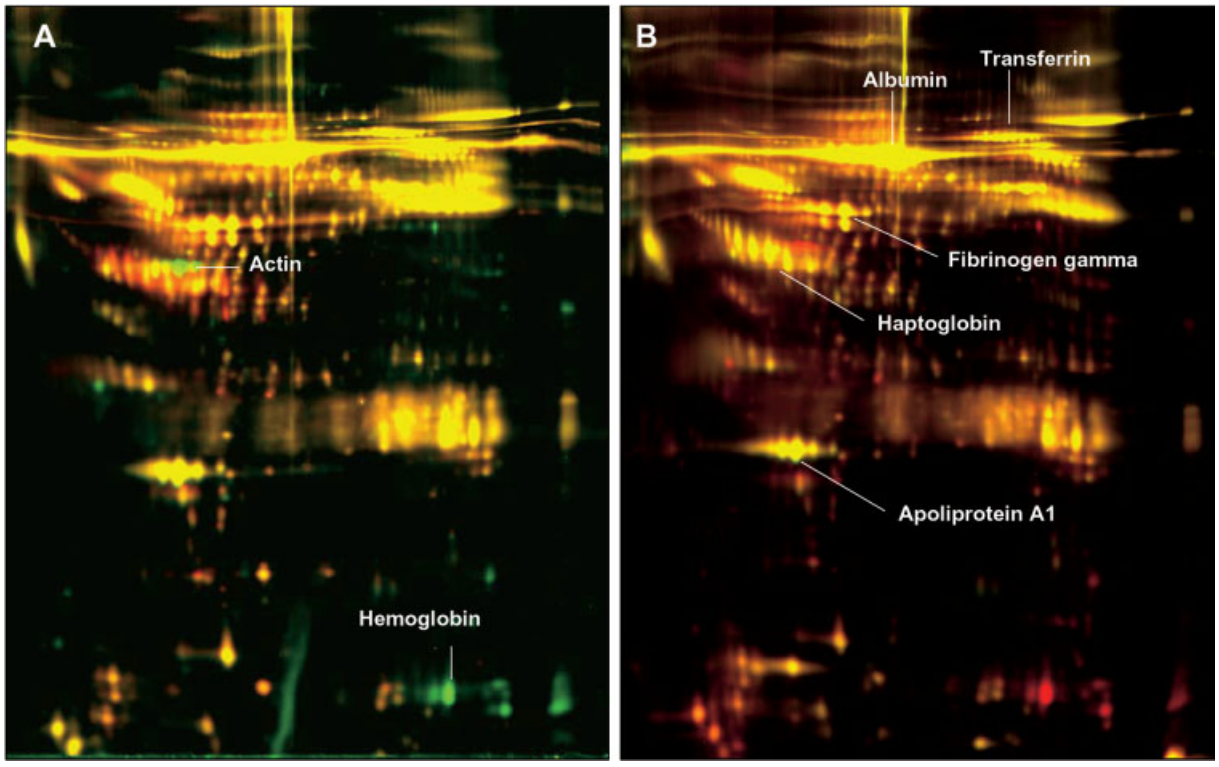

Figure 3. Proteomic analysis of protein expression in human plasma microparticles. Microparticles derived from the peripheral blood by centrifugation were lysed and labelled with Cydyes (green and red colour in A and B, respectively). Using DIGE, microparticle and microparticle-depleted plasma proteins were co-separated in large format 2-D gels. Images were acquired on a fluorescence scanner and proteins identified by LC-MS/MS. Actin and haemoglobin are enriched in microparticles, compared to microparticle-depleted plasma.

Endothelial cells are also known to release microparticles in response to stimulation with tumour necrosis factor α (TNFα). The composition of these microparticles has been investigated by protein fractionation using LC and MS/MS [37]. In this study, a conspicuous series of proteins was identified in the proteome of TNFα-induced endothelial microparticles, amongst which cytoskeleton and cytoskeleton-binding proteins (tubulin, actin, cofilin, vimentin, *etc.*), membrane-associated proteins that control transport and signalling (caveolin, annexins, dynein, *etc.*), and folding chaperones (calnexin, calreticulin, *etc.*) were predominant. Cytoskeleton- and plasma membrane-regulating proteins such as annexins, dynein and cofilin are likely to be responsible for the formation and secretion of microparticles. On the other hand, the abundance of adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1) and integrins β1, α5 and α2, suggests a role for endothelial microparticle in the mediation of cell-cell and cell-extracellular matrix interaction. This study is an example of how proteomics could function as a framework for understanding the biological activity of different microparticles, and perhaps, for discerning new therapeutic targets. So far, however, there is still a scarcity of published material on the proteome of microparticles from other cells relevant to vascular research.

3.2 Exosomes

Exosomes differ from microparticles by virtue of their size and their endocytic origin. Before secretion, these small vesicles are contained in large multivesicular endosomes and contain transferrin receptors as a relic of their endocytic origin. Proteins from the endocytic compartment and the cytoplasm, together with plasma membrane proteins, are represented in exosomes. About 80% of exosomal proteins are conserved among all species. Some protein classes typical of exosomes are tetraspanins, HSP and the MHC. Different blood cells produce exosomes, including erythrocytes, platelets, lymphocytes, mast cells and dendritic cells. The initial hypothesis regarding exosomes stated that their only function involves the clearance of redundant proteins (*e.g.* transferrin receptors); however, this view has recently been substituted by a more articulated interpretation of their relevance to vascular pathophysiology. It has become established that exosomes modulate immune response [38–40], and a possible role in the regulation of haemostatic balance has been proposed [41]. Exosomes prepared *in vitro* from mast cells support thrombin generation and induce expression and secretion of plasminogen activator inhibitor-1 by endothelial cells, thereby attenuating fibrinolysis and promoting pro-thrombotic conditions [41].

Besides their known functions, as in the case of microparticles, exosomes might be of relevance in vascular research, due to their ability to be absorbed to the cell surface and mediate cell-cell interactions in the cardiovascular system. Unfortunately, there is little uniformity in the way to prepare exosomes. For example, the preparation of the exosomal fraction not always contains a centrifugation step aimed at the elimination of microparticles. Therefore, the samples analysed often contain different types of microvesicles and their proteomic analysis leads to results that deserve careful evaluation. Nonetheless, some valuable efforts to characterize the proteome of exosomes have already been made. For example, the characterization of the proteome of dendritic cell-derived exosomes has been reported [42]. As expected, endocytic proteins were abundant components of the proteome of exosomes. In addition, 21 new exosomal proteins were identified, including cytoskeleton-related proteins, such as cofilin, profilin I or elongation factor 1α, and intracellular membrane transport proteins, such as annexins, rab7 and 11, rap 1B, and syntenin. In addition, a series of apoptosis-related proteins was detected, including thioredoxin peroxidase II, Alix, 14-3-3, and galectin-3. As anticipated, mast-cell derived exosomes regulate the secretion of plasminogen activator inhibitor-1 by endothelial cells [41]. To establish which exosomal components are responsible for this phenomenon, the authors of this study used a proteomic approach. 2-DE combined with ESI-MS/MS allowed the identification of three candidates for the regulation of endothelial cell secretion of plasminogen activator inhibitor-1, namely the prothrombinase complex, TNF-α and angiotensinogen precursors. This discovery might shed new light on the atherogenetic role of mast cells, which increase in numbers in atherosclerotic lesions, where they might contribute to the pathological process by inhibiting fibrinolysis.

4 Proteomics of the secretome

The complex collection of proteins secreted by a particular type of cell - often maintained *in vitro* - is referred to as the "secretome". In the vascular field in particular, the analysis of the secretome of different blood and vascular cell types could be of critical importance in the clarification of heterogeneous cell-cell interactions and their regulation by autocrine and paracrine factors. A method for studying the secretome of isolated atherosclerotic lesions was also described [43] and has been recently reviewed. The limited complexity of the secretome makes it suitable for the application of a proteomic approach [44–48]. Nevertheless, it also presents formidable challenges. First, although the conditioned medium is usually sampled after extensive washing [49] and during incubation in serum-free medium, it is difficult to completely avoid cross-contamination with proteins of the serum supplement commonly used in cell cultures. Even a minor serum contamination of the sample can constitute a severe hindrance for the mass spectrometric identification of low-abundance secreted proteins, as the concentration of peptides from secreted proteins can be below the detection capabilities of the equipment in the presence of high abundant ions. Secondly, any variation in the carry-over of serum proteins has a profound impact on quantitative comparisons, as it is not uncommon in shotgun proteomic analysis that abundant serum proteins constitute >10% of the total spectra in conditioned medium [49]. Thirdly, cell death and cytoplasmic protein release in the culture medium is a source of false positives, which further impairs the reliability of proteomic analysis of the secretome. Consequently, a series of technical innovations have been proposed to improve the capability of secretome analysis, which include pulse-chase metabolic-labelling [50], protein-enrichment by precipitation (*e.g.* carrier-assisted TCA precipitation [51]), high-abundance serum protein depletion (*e.g.* sodium chloride/ethanol precipitation [52]), LC fraction (*e.g.* RP tC2 Sorbent [47]) and dialysis/ultrafiltration methods [53].

4.1 Secretome of vascular cells

Amongst the first vascular cells, for which a proteomic analysis of the secretome was reported, were rat aortic smooth muscle cells [54]. The authors of this study identified proteins secreted by vascular smooth muscle cells upon stimulation by ROS, which are implicated in the pathogenesis of atherosclerosis and hypertension. Proteins identified by capillary chromatography and ESI-MS/MS were HSP90-α and cyclophilin B. Interestingly, immunodepletion of HSP90-α inhibited the ability of the conditioned medium from ROS-stimulated vascular smooth muscle cells to activate MAPK kinase signalling, namely ERK1/2.

In a more recent study [55], the authors described a method for the separation by 2-DE and identification by MS of proteins secreted by human arterial smooth muscle cell primary cultures. In the collection of secreted proteins identified in this study, some are of special interest, *i.e.* matrix metalloproteinase-1 (MMP-1) or interstitial collagenase. This protease, previously shown to be secreted by vascular smooth muscle cells [56], has the ability to degrade collagen, which is responsible for maintaining the mechanical integrity of the fibrous cap of atherosclerotic lesions. Another exciting candidate from the authors' list of proteins is plasminogen activator inhibitor-1 (PAI-1) [57]. This protein is an important regulator of fibrinolysis and in general, proteolysis of the extracellular matrix. Elevated levels of PAI-1 have been detected in diseased aortas [58], where accumulation of extracellular matrix components may contribute to aortic occlusion and aneurysmal disease.

A lot is known about the secretory activity of endothelial cells, which consists of the release of relevant regulatory factors, such as platelet-derived growth factor (PDGF), von Willebrand factor, prostacyclin, nitric oxide, endothelin-1 and chemokines. Such secretory functions regulate fundamental physiological and pathological phenomena, such as hae-

mostasis, angiogenesis, thrombosis and inflammation. A method for the proteomic profiling of the secretome of primary cultures of rat arterial endothelial cells has recently been published [49]. It has also been reported that cell senescence leads to changes in the secretory profile of endothelial cells [59]. Nonetheless, an exhaustive proteomic study of the endothelial secretome and its modification in senescent/unhealthy conditions has not yet been described.

4.2 Secretome of macrophages

The secretory activity of macrophages plays a central role in the physiological function of these circulating cells; their better-characterised secreted components are proteases involved in the remodelling of the extracellular matrix. An attempt to describe the secretome of macrophages from *in vitro* cultures of monocytes has been reported [60]. In this study, the authors used a method based on 2-DE and MS to identify 38 different secreted proteins. Recently, a procedure based on carrier-assisted TCA protein precipitation has been utilized to identify proteins secreted by the J774 macrophage cell line into the culture medium [51]. This technique improved the sensitivity of the proteomic analysis and allowed the identification of proteins secreted at low concentrations, such as growth and chemotactic factors. A recent publication described a further advance for the characterisation of the secretome of macrophages using LC-MS/MS-based approach [61] to compare the secretion profile of macrophages with or without infection by HIV-1.

4.3 Secretome of platelets

Platelets are critical in the initiation and progression of the atherosclerotic process [62]. Proteomics has been successfully applied to platelet investigations due to the wide availability and limited protein repertoire of this cell type. Several good reviews based on proteomic investigations of platelets are available [63–65]. Upon activation, platelets release several classes of factors known to regulate not only haemostasis, but also inflammation and atherosclerosis. Amongst the components of the platelet releasate, CXC and CC chemokines are particularly important. The CXC chemokine platelet factor 4 (PF4) and β-thromboglobulin are produced by activated platelets and promote leukocyte migration and differentiation, thereby regulating inflammation, angiogenesis and atherosclerotic plaque formation [66]. Beside classical approaches, proteomics has also been applied to the analysis of platelet secretome. A particularly successful study on the supernatants from low-dose thrombin-activated platelets using multidimensional chromatography to separate tryptic digests prior MS, led to the identification of 82 platelet-secreted proteins [67]. Sixty percent of the identified proteins were not previously known to be secreted by platelets. Three of the proteins, namely secretogranin III, cyclophilin A and calumenin, are potential therapeutic targets for atherosclerosis, being expressed in atherosclerotic plaques but not in normal blood vessels [67]. Interestingly, MS has also been applied to study how aspirin, a widely used drug for cardiovascular diseases, influences the secretory activity of platelets [68].

4.4 Secretome of vascular progenitors

Atherosclerosis development and progression depend on a dynamic imbalance between endothelial injury and repair. Until the discovery of progenitor cells it was thought that, following endothelial injury, repair was mediated by undamaged endothelial cells from adjacent areas of the vessel. It was postulated that these mature cells would proliferate and migrate in order to restore endothelial integrity and function in areas of injury. However, the discovery of vascular progenitors has led to a major paradigm shift regarding vascular repair. By now, numerous studies have demonstrated that vascular progenitor cells are present in circulating blood and have the capacity to differentiate into endothelial and smooth muscle cells [69, 70]. Circulating progenitors may contribute to vascular repair, remodelling, and atherosclerotic lesion formation by physical incorporation into the vessel wall. Nonetheless, only limited numbers of progenitor-derived cells were found in human vessels [71–73], thus it has been suggested that progenitor cells may mediate their effect in a paracrine manner [74]. Moreover, precise phenotypic definition of endothelial progenitor cells (EPC) remains controversial as they lie in a spectrum of development between stem cells and mature endothelial cells. The use of a combination of antigenic markers has been the principal method of identification, but it is acknowledged that even this approach is not specific for identifying EPC [75, 76]. Given the recent evidence that some of the methods used to harvest EPC might isolate angiogenic macrophages rather than cells that can *de novo* form vessels *in vivo*, it seems imperative to use the potential of proteomics to better characterize the isolated stem cell population, i.e. by sampling their secretome rather than just measuring the expression of a handful of marker proteins. A secretome analysis would not only provide essential information of the cell phenotype, but also of the possible presence of other cell types, which might have unexpectedly contaminated the cell preparation. Besides, secreted factors by stem cells may correlate to their activation state and might help to assess their repair capacity in patients. We therefore envisage that the characterisation of stem cells by proteomics will become an emerging topic in vascular research [77].

5 Conclusions

Discovering biomarkers of disease and understanding the targets of therapeutic intervention are highly desirable goals for modern medicine. Rapid progress towards a comprehensive mapping of the plasma proteome will certainly help

towards the achievement of these goals, nonetheless, plasma proteomics is still hindered by technical limitations, and the task ahead remains challenging. Besides its more common application for biomarker identification, we prefer to use proteomics for gaining mechanistic insights into the pathophysiology of cardiovascular disease [15, 78–82]. Considering the importance of protein secretion in vascular homeostasis, we believe that secretory proteomics is an exciting area, that will contribute to a better understanding of vascular diseases.

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