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Contribution of proteomics to the management of vascular disorders

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ABSTRACT

Vascular disorders, and in particular atherothrombosis, are currently a leading cause of morbidity and mortality in Western societies. Proteomics research into these disorders has helped improving our knowledge of the underlying mechanisms involved in the development of atherothrombosis, as well as providing novel biomarkers to diagnose and for the prognosis of this disease. However, the application of these advances into clinical use has not followed this trend. In this review we explore the potential of Proteomics and Metabolomics for the management of vascular disorders, paying special attention to atherothrombosis and aiming to guide the reader from the experimental design of proteomic analysis through the initial discovery phase to the clinical implementation of biomarkers or therapeutic targets (Fig. 1), providing state-of-the-art proteomic studies to exemplify the concepts addressed.

1. Atherothrombosis: pathogenesis and clinical needs

Fig. 1 Clinical outcomes of atherothrombosis, including acute coronary syndromes (ACS), stroke and claudication from peripheral artery disease (PAD), represent the most important causes of mortality and morbidity in Western societies. Atherosclerosis originates through endothelial dysfunction, and sub-endothelial LDL (low density lipoprotein) deposition and oxidation, particularly at specific locations of the arterial tree (coronary, aorta, carotid, cerebral, renal and femoral arteries) [1]. Several risk factors predispose individuals to atherogenesis, including dyslipidemia, hypertension, tobacco use, diabetes and obesity. The high prevalence of these factors in the population, together with the increase in life expectancy, account for the overwhelming incidence of atherothrombosis in developed countries. For all these reasons, better clinical management of atherothrombosis will help decrease the death rate from cardiovascular disease (CVD) and improve the quality of life in the population. This goal can be achieved in part by increasing efforts in educational programs that make people aware of the benefits of a healthy lifestyle, namely a healthy diet, avoidance of smoking and regular physical activity [2]. On the other hand, a better understanding of the molecular mechanisms underlying atherothrombosis, and the incorporation of more efficient biomarkers of pathology, would benefit diagnosis, prognosis and may provide novel therapeutic targets, thereby improving clinical management of such patients.

1.1. Cardiovascular risk assessment

The biomarkers widely used to assess the risk of clinical outcomes derived from atherothrombosis are very often used in combination with the Framingham Risk Score (FRS). This score puts traditional cardiovascular risk factors together to calculate the 10-year risk of an adverse cardiovascular outcome, and constitutes the most internationally used predictor of CVD. First defined in 1998 [3], this score owes its name to the Framingham Heart Study, conducted on 2489 men and 2856 women aged 30–74 years old at baseline and over a 12-year follow-up. The FRS is calculated by adding or subtracting points in function of age, systolic blood pressure, LDL-cholesterol, HDL-cholesterol and smoking habit, evaluating the value according to gender. The higher the score, the greater the risk of CVD. The FRS allows populations to be stratified into three categories associated with the probability of developing cardiovascular events in the following 10 years: low (<10%), intermediate (10–20%) and high-risk (>20%). Nevertheless, and despite its great utility in the clinic, the imperfect discriminatory capability of FRS [4,5] requires further refinement of the algorithm in order to improve its value as a CV risk stratification tool.

In terms of molecular biomarkers for CVD, several soluble molecules are currently used to diagnose and predict future outcome, including C-reactive protein (CRP), an inflammatory marker used for CVD risk prediction [6], B-type natriuretic peptides, biomarkers of heart failure (HF) diagnosis [7], and

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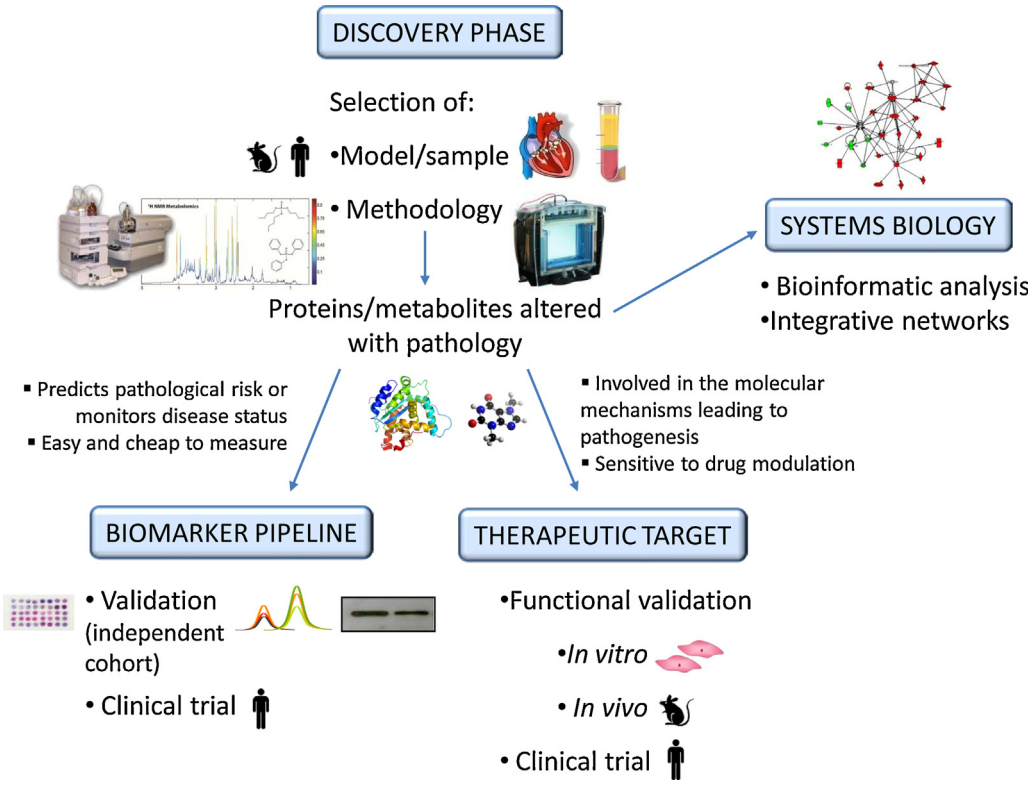

Fig. 1. A schematic view of the flowchart of a comprehensive proteomic/metabolomic analysis in the search for disease biomarkers and therapeutic targets.

cardiac troponins (cTnI, cTnT) to ensure the detection of acute myocardial infarction (AMI) [8] (Fig. 2). Although atherosclerosis is the underlying cause of the majority of cardiovascular events, none of the aforementioned biomarkers are specific biomarkers for the early diagnosis of atherothrombosis.

1.2. Biomarkers of plaque vulnerability

Markers of plaque vulnerability represent a useful tool for clinicians, since unstable plaques are more likely to rupture and provoke thrombosis. The development and vulnerability of an atherome plaque reflects multiple molecular processes associated with lipid accumulation, inflammation, proteolysis, angiogenesis, hypoxia, apoptosis and calcification. Among the biomarkers of plaque vulnerability, lipoprotein-associated phospholipase A2 (Lp-PLA_2) [9,10] and myeloperoxidase (MPO) [11–13] are probably those best demonstrated to be clinically useful. MPO is a heme protein involved in many secondary reactions that generate reactive species and in LDL oxidation, and it contributes to endothelial dysfunction and foam-cell formation [14]. This protein has been proven to predict risk and mortality in ACS patients [11,13], and it constitutes an early biomarker of atherosclerosis, as determined in a prospective study on healthy individuals [12]. Lp-PLA_2 is secreted by inflammatory cells and it binds to circulating LDL, and its deposition is greater in vulnerable plaques [15]. Thus, elevated levels of this protein in plasma have been associated with a higher risk of coronary heart disease [9,10]. Moreover, during atherogenesis, LDLs are deposited in the sub-endothelium and they are oxidized as a result of the pro-oxidative inflammatory milieu therein. Indeed, oxLDL levels are associated with advanced atherosclerosis and they constitute a well-established biomarker for outcome prediction [16].

Plaque rupture is frequently produced by the disruption of the fibrous cap from the atherome plaque, which is mediated by apoptosis of vascular smooth muscle cells (VSMCs) and proteolysis. Matrix metalloproteinases (MMPs) play a crucial role in these events as they degrade the extracellular matrix (ECM). Therefore, blood MMP-9 levels have been associated with cardiovascular risk in a variety of studies [14]. In addition, tissue factor (TF) is a pro-coagulant protein secreted by foam cells and VSMCs during plaque development and it initiates thrombosis after its release with plaque rupture. Elevated blood levels of TF have been associated with unstable angina [17] and increased blood thrombogenicity in type 2 diabetes mellitus [18]. One particularly interesting alternative is to evaluate TF-positive circulating extracellular microvesicles, which exert pro-coagulant ability and constitute a novel biomarker of thrombosis [19,20].

Recently, the fibrotic marker Gal-3 that has proved useful to predict heart failure [21], has been shown to modulate inflammation during the development of atherosclerosis [22], and its blood levels are associated with unstable angina [23] and increased cardiovascular mortality [24]. Moreover, several inflammatory biomarkers that are associated with plaque vulnerability have been shown to be useful to predict cardiovascular outcomes (sCD40L, IL-6, IL-18, MCP-1, etc.), although such results should be "handled with care", since underlying inflammatory pathologies may account for the observed changes in these biomarkers. Conversely, adhesion molecules that are over-expressed in endothelial dysfunction (VCAM-1, ICAM-1) are widely used as biomarkers of vascular function in the follow-up of high-risk cardiovascular patients [25].

Despite of the availability of these biomarkers, which are crucial for the diagnosis and risk assessment of CVD, more research focusing on the molecular mechanisms driving atherothrombosis, and on the identification of earlier, more discriminating and more specific biomarkers of the disease is still needed. In this sense, panels of biomarkers may be of great utility for diagnostic and prognostic purposes, providing better sensitivity and specificity. Indeed, the discrimination of particular patient sub-groups expressing specific panels of these biomarkers would increase

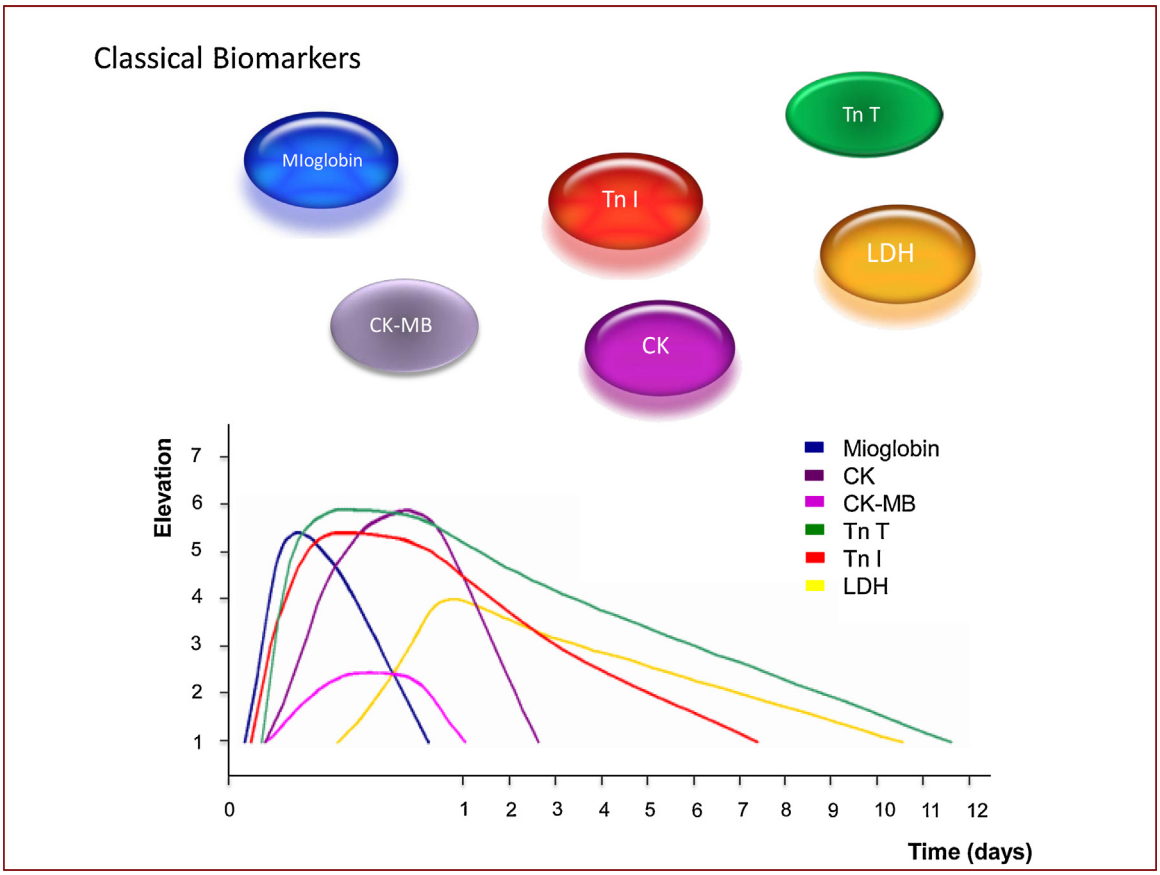

Fig. 2. From a clinical perspective, atherosclerosis has a silent and very progressive development, which constitutes the greatest issue for diagnosis. Unfortunately the symptoms only become evident when disease is in an advanced and irreversible state. Biomarkers such as CK-MB, troponins, and myoglobin provide the basis for the diagnosis of acute myocardial infarction. These biomarkers' levels rise twice within 3 6 h following symptom onset. Discovery of new biomarkers with real clinical value to predict the disease and stratifying individual cardiovascular risk are critical for early diagnosis of atherosclerosis.

their utility, as well as matching the outstanding desire of the clinical community to progress towards more personalized medicine.

2. "What to study?" Sample sources

With the rise of proteomics and other advances in molecular biology, biomarker studies have entered a whole new era and they hold particular promise for early diagnosis and effective treatment of many diseases. Different sample types can be used for this purpose, such as biological fluids (i.e. urine, blood) or tissue biopsies.

2.1. Biological fluids

Serum/plasma and urine and are the most commonly used biological matrices in cardiovascular research. A major goal in the field of clinical proteomics is to identify disease biomarkers in biological fluids that can be measured relatively inexpensively for early diagnosis of disease. Because urine can be obtained non-invasively in large quantities and it is more stable than other biofluids, it provides an attractive alternative to blood as a potential source of disease biomarkers. However, technical/methodological issues have hindered urinary proteomics from contributing significantly to our pathophysiological understanding of CVDs. Although proteinuria is an established risk factor for cardiovascular morbidity and mortality [26], the analysis of less-abundant and naturally existing urinary proteins and peptides in proteinuric patients still remains a challenge [27,28]. Serum and plasma have been the focus of extensive proteomics studies for decades [29]. Plasma/serum is one of the best clinical samples for diagnosis and prognosis, given the low cost and easy access to the sample. Furthermore, as a fluid of "communication" between cells and organs, most biological functions can be studied in this matrix. Indeed, the concentration of proteins involved in inflammatory processes and disease progression (interleukins, proteases, enzymes, etc.) often increases in plasma in the disease state, and this can be used to monitor the clinical status of patients. However, several limitations to study the plasma proteome exist, such as its complexity and the wide dynamic range of protein concentrations (more than ten orders of magnitude), a factor that makes proteomic analysis very challenging [29]. It is not uncommon that the identification of proteins truly secreted into biological fluids is not possible due to differences in the dynamics of release and clearance of proteins from circulation. In the context of stroke, cerebrospinal fluid (CSF) and brain extracellular fluid [30] are very good samples in which local biomarkers of disease can be studied, even though they involve invasive collection procedures.

2.2. Circulating cells and extracellular vesicles

An interesting alternative to plasma for vascular proteomic studies is to analyze circulating cells, which can be obtained from blood and therefore, in a similarly mildly invasive manner. Proteomic analyses of circulating cells like monocytes [31,32], platelets [33,34] or endothelial cells [35], have contributed to a better understanding of their role in atherothrombosis, besides

providing novel disease biomarkers. Moreover, circulating extracellular vesicles or microparticles have been widely shown to be relevant in thrombosis [36] and endothelial dysfunction [37], constituting a promising and easy-accessible source of potential biomarkers [38].

2.3. Tissues

Although most proteins are ubiquitously expressed, other proteins have a limited cellular or tissue distribution [39]. It is this latter group of proteins that is of potential interest for biomarker discovery, as they may reflect the physiological state of a specific cell population or tissue. Tissue proteomics in the context of atherothrombosis has allowed the atherosclerotic plaque [40–43], and the thrombus [44] to be characterized in greater depth. In comparison to blood, which may constitute an intermediary between organs and physiological activity, tissue proteome analysis is more directly linked to specific pathological states. Thus, this analysis has the potential to identify proteins that are deregulated in the disease state and which may potentially

Table 1
Compilation of studies reported so far in the field of proteomics in human atherosclerosis (Table 1 was modified from Cardiovascular proteomics by Vivanco et al. platelet proteomics: principles, analysis and applications. Garcia and Senis (2011), John Wiley & Sons. Inc.). New added references are indexed in the manuscript.

Sample source	Pathology	Methodology	Potential biomarkers
Serum/plasma	Ischemic stroke (atherothrombotic cardioembolic stroke)	2-DE	SAA haptoglobin
Serum/plasma	Peripheral arterial disease (PAD)	SELDI–TOF–MS	β-microgobulin
Carotid secretome	Atherosclerosis	SELDI–TOF–MS	TWEAK
Urine	Severe coronary artery disease (CAD)	Capillary electrophoresis + ESI-TOF/MS	Collagen α 1 (I), collagen α 1 (III)
Circulating cells (monocytes & T-cells)	AMI	Microarray	CD2, CD5, CD7, CD13, CD45, CD45RA, CD49e, CD52, CD64, CD66c
Circulating monocytes	ACS	2-DE	Protein profile
Circulating lipoproteins (LDL, HDL)	CAD	MS	Calgranulin A, lysozyme C, complement regulatory proteins, serine protease inhibitors
Platelets	Atherosclerosis	2-DE LC-MS/MS	14-3-3 ζ
Carotid	Atherosclerosis	2-DE	α_1-antitrypsin
Carotid	Atherosclerosis	2-DE	hsp27
Carotid	Atherosclerosis	2-DE	Fibrinogen fragment D, ferritin light subunit, SOD2, annexin A10, glutathione, transferase P1-1, hsp20, hsp27, Rho GDI, SOD3
Aorta	Atherosclerosis	2-DE	Annexin A5, decoy receptor 1, 14-3-3γ
Carotid	Atherosclerosis	Antibody arrays	TRAF4, Gads, GIT1, Caspase-9, c-src, TOPO-I I-α, JAM-1
Carotid	Atherosclerosis	Western arrays	TSP-2, MnSOD, apo B100, PTP1C, ALG-2, GSK-3β
Coronary	Atherosclerosis	LC-MS/MS LMD (layers) + LC-MS/MS	PEDF, periostin, MFG-E8, annexin I
Carotid	Atherosclerosis	Manual microdissection (intima) + proteoglycan extraction + LC-MS/MS	Lumican
Coronary	Atherosclerosis	LMD (intima) + 2-DE	Annexin 4, myosin regulatory light 2, ferritin light chain
Coronary	Atherosclerosis	LMD (media) + 2-DE	Filamin A, gelsolin, vinculin, vimentin
Coronary	Atherosclerosis	agLDL treated explants culture VSMCs + 2-DE	p-myosin RLC
Carotid secretome	Atherosclerosis	2-DE	HSP27, cathepsin D
Coronary secretome	Atherosclerosis	LC-MS/MS	Gelsolin, vinculin lamin A/C, phosphoglucomutase 5
Carotid and iliac secretome [52]	Atherosclerosis	Subtractive phage display + LTQ-ORBITRAP	Alpha 2 macroglobulin, annexin 5, caspase-14, junction plakoglobin, lipocalin-1, mucin 5A, serpin B3 etc
Plasma purified VLDL, LDL and HDL [53]	Atherosclerosis	2-DE	Apo J, ApoAIV, Apo D, Acute phase serum amyloid A protein, ApoCII, ApoCIII, Apo B100, Apo SAA etc
Coronary thrombus [44]	Myocardial infarction	2-DE MALDI MS/MS 1DE LC-MALDI MS/MS LC-ESI-MS/MS	DIDO 1
Circulating granulocytes [54]	Atherosclerosis	Two-dimensional LC-MS/MS	S100 proteins, myeloperoxidase, gelatinase, MMP9
Extracellular microvesicles [36]	Myocardial infarction	2D-DIGE-MS/MS	Alpha 2 macroglobulin, fibrinogen
Plasma [55]	Atherosclerosis	Quantitative proteomics	Vinculin
Plasma [56]	Aortic aneurysm	Protein array	IGFBP-1
Arterial thrombus [57]	Aortic aneurysm	LC-MS/MS	Thrombospondin, clusterin
Brain extracellular fluid [30]	Acute stroke	1D-MS/MS	Glutathione S-transferase, peroxiredoxin-1, protein S100-B
Carotid [58]	Atherosclerosis	Secretome LC-MS/MS	Thrombospondin-1, vitamin D binding protein, vinculin
Intraluminal thrombus [50]	Aortic aneurysm	LC-MS/MS	C3, C9
Atheroma derived SMC [59]	Atherosclerosis	2-DE-MS/MS	ATP synthase b, aldehyde dehydrogenase 2, annexin I
Brain tissue [60]	Hemorrhagic stroke	Bioinformatic-MS/MS	S100B, NSE, GFAP, α-Inx, MBP, NFM, β-Syn
Urine [61]	Atherosclerosis	CE–MS	Collagen type I, alpha(1)-antitrypsin, EGF

Table 2
Pros and cons of the different methodologies available for proteomic and metabolomic analyses.

Methodology		Pros	Cons
Proteomics			
Gel based			
Two-dimensional electrophoresis (2-DE)	Conventional	Inexpensive equipment, easy handling, isoforms information	Gel-to-gel variability (reproducibility), sample loading variability, time consuming, basic, hydrophobic, very high and very low molecular weight proteins under-represented
	Minimal labeling 2D-DIGE	(All conventional 2-DE pros) Internal standard for: 1 Normalization 2 Diminish gel-to-gel variability	Fluorochromes, scanner and image processing software price
	Saturation labeling 2D-DIGE	(All conventional 2-DE pros) Scarce samples analysis, redox analysis	Only 2 fluorochromes available: individual gels per sample
–			
Gel-free			
LC-MS/MS	MudPIT	Sensitivity, high-throughput	Specialized personnel required, high-cost equipment, resistance of certain peptides to ionization
	Label-free	Inexpensive, sample preparation ease	Quantification accuracy
	Isobaric labeling	Quantification accuracy, reproducibility (due to multiplexing)	Loss of individuality due to sample pooling, reactive costs
	SRM	High specificity, optimal for validation, multiple analytes in a single run	Targeted analysis, limited number of analytes
CE–MS		No interference of contaminants (i.e. urine), high separation efficiency	Moderate sensitivity
Arrays	Protein arrays	Characterization of specific pathways	Targeted analysis, limited number of analytes
	TMA	High sample throughput	One analyte per array, small tissue regions analyzed
MS-imaging		Spatial distribution of analytes, direct comparison with histology	Identification difficulties, incompatibility of staining protocols
–			
Metabolomics			
LC–MS		No derivatization, diversity of metabolites analyzed	Lack of spectral libraries
GC–MS		Reproducibility and robustness, spectral libraries available (NIST and Golm GC–MS)	Derivatization methods required, limited mass range
^{1}H NMR		Minimal sample preparation, sample preserved, reproducibility	Sensitivity, spectral resolution

serve as novel biomarkers if biopsy material is accessible or if the observed alteration is reflected in any biological fluid. Direct tissue proteomic studies may also enhance the understanding of the molecular mechanisms associated with the disease. However, obtaining sufficient human tissue for reliable proteomic analyses is often difficult, especially when considering control material required for comparative analyses.

2.4. Sub-proteomes: zooming in on specific proteomes

The complexity of samples often represents a challenge when searching a complete proteome for clinically useful molecules. In terms of tissue proteomics, the sample collected may be heterogeneous containing numerous cell types and stromal elements, or normal and abnormal cells), which may hamper the proteomic analysis. Tissue fractionation may be necessary when analyzing whole tissue in order to study the contribution of specific cell populations, or of cellular/extracellular components, to disease pathogenesis [42]. Moreover, immunohistochemistry can help to characterize the expression of proteins of interest, providing useful information for whole-tissue proteomic analyses. Nevertheless, proteomic analysis of cells isolated from tissues, without subsequent culture *in vitro*, can provide abundant information regarding an anatomic structure or cell type in the tissue, preserving the *in vivo* state. In this sense, non-contact laser microdissection (LMD) represents an ideal approach, since cells or regions in a tissue can be isolated by means of a laser beam that encompasses the delimited area of interest, preserving tissue integrity and avoiding sample overheating [45]. However, this approach has yet to be explored extensively in arterial proteomics. Nevertheless, in our experience coupling of LMD and proteomic analysis is now feasible due to the enhanced sensitivity of mass spectrometers and the appearance of fluorescent labeling dyes for two-dimensional electrophoresis (2-DE, e.g., saturation labeling DIGE [46,47]). Indeed, this has allowed us to perform differential layer-specific analysis of protein abundance in the atherosclerotic human coronary artery [41,42].

The aforementioned complexity is also applicable to the dynamic range in concentrations and in the number of proteins present in biological fluids, especially in blood serum and plasma. Thus, sub-fractionation provides access to less-abundant proteins, which might otherwise be masked in a complete proteome analyses. On the other hand, fractionation steps are detrimental to comparative analyses, potentially representing an additional

source of experimental error, which should be taken into account when evaluating the results obtained. Blood plasma constitutes the most complex proteomic sample, with more than 10,000 proteins identified to date and much more expected to be expressed within. With the goal of reducing the orders of magnitude of the dynamic range in protein concentrations within blood plasma, two different methods are widely used: (1) immunodepletion of highly abundant proteins; and (2) employing a combinatorial peptide ligand library (CPLL) to equalize the dynamic concentration range. The former consists in a pre-fractionation of the sample by means of affinity chromatography, in which the most abundant plasma proteins (1–20 proteins; depending on the manufacturer) are mainly eliminated through their binding to specific antibodies present on the affinity or spin column. The methodology associated to CPLLs is based on the use of a mixture of porous beads on which hexapeptides are covalently attached. When a plasma sample is exposed to the library, the beads with affinity for abundant proteins will become saturated, thereby leaving the rest of the molecules of this protein unbound. Conversely, less abundant proteins will not saturate their ligands and therefore all their molecules would be captured. As a result, plasma is equalized by reducing the number of copies of very abundant proteins while preserving those that are less abundant.

Another approximation to overcome the complexity of serum and plasma, as well as to focus on the site-of-injury, involves studying the proteins released by the damaged tissue in culture: the so-called secretome. Through this approach, proteins released into the blood from the vascular tissue can be discovered, constituting a potentially reliable source of disease biomarkers. In this sense, a characteristic secretome of atherosclerotic arteries [48], aortic valves with stenosis [49] and aortic intraluminal thrombi [50] has been reported, that is potentially relevant in the diagnosis of these pathologies and/or in the definition of novel therapeutic targets.

3. "How to perform such studies?" Methodologies

3.1. Sample preparation

Using an appropriate method to prepare the proteome/metabolome under study is critical for a successful molecular analysis, and the depth of such analysis may rely in this step. For this reason, several considerations must be taken into account when dealing with biomolecules, including degradation, solubility and sample storage. First of all, sample preparation should always be performed at low temperatures (4 °C) to avoid degradation and the inclusion of protease inhibitors is recommended. However, in metabolomics analyses elevated temperatures may be needed for GC separation methods, which may imply the need to incorporate a derivatization step when analyzing thermolabile metabolites. Depending on the sample origin, extraction may imply tissue dissociation and/or cell lysis, or direct solubilization of proteins/metabolites. Components of the lysis and extraction buffers should be carefully selected to ensure efficient tissue/cell disruption, protein/metabolite extraction, and compatibility with subsequent analytical techniques. In particular, the polarity of the metabolites under study will determine which are the most adequate extraction protocols. Storage of the extracts at −80 °C is mandatory to preserve the biomolecules present and repeated freeze/thawing should be avoided.

Obtaining optimal results requires selecting the appropriate experimental methodology, contemplating all aspects of the study, based on: (a) the characteristics of the analytes to be studied; (b) the selection of the technological platform, in terms of sensitivity, selectivity, specificity, linear dynamic range and throughput; and (c) the step in the biomarker research pipeline being addressed (discovery or validation).

3.2. Proteomics

A proteomic approach allows changes in protein expression between several conditions to be monitored in order to shed light on physiological or pathological processes, and it is a very useful tool in the search for biomarkers of CVDs [51]. With the appearance of the improved proteomic separation techniques, the evaluation of thousands of proteins at once is now possible. Such techniques include gel based methods like 2-DE, and non-gel based techniques like liquid chromatography tandem mass spectrometry (LC-MS/MS) and capillary electrophoresis-mass spectrometry (CE–MS). Table 1 identifies different studies in which such techniques were applied in recent years [30,36,44,50,52–61].

Gel based methods are techniques for the high-resolution separation of complex protein samples. As a genuine top-down analytical approach, 2-DE is an excellent tool but an improved method has appeared in the recent years, 2D-DIGE, which involves fluorescent labeling of protein mixtures and that allows two protein samples and an internal standard to be compared on a single gel. Although gel-free techniques have developed immensely, 2D-DIGE has been used for important studies focusing on the search for biomarkers involved in the development of atherosclerosis. In this sense, the study of the atherosclerotic plaque tissue has been carried out by means of 2D-DIGE by different groups focusing on either early lesion development [41,42] or plaque instability [40,43].

With the evolution of Proteomics, LC-MS/MS has become the method most commonly employed due to its sensitivity and high-throughput performance. Although several combinations of LC methods are available, a typical approach used when analyzing a complete proteome is 2D-LC-MS/MS. There are different alternatives to perform differential abundance analysis by LC-MS/MS, such as label-free LC–MS quantification and isobaric tags for quantification. The latter are gaining in popularity, and they include isobaric tags for relative and absolute quantification (iTRAQ, AB Sciex), and tandem mass tags (TMT, Protein Sciences). For example, increased protein levels were found in human brain extracellular fluids (ECFs) following acute stroke using TMT. These proteins could be of interest for the diagnosis and prognosis of stroke, indicating that ECF may be a useful source of blood biomarkers for this disease [30]. Recently, vinculin was identified as a novel candidate biomarker using iTRAQ, since elevated circulating levels of this protein were associated with atherosclerotic disease [55].

When interest focuses on analyzing a large number of heterogeneous samples that contain interfering compounds, such as lipids and precipitates, capillary electrophoresis coupled to mass spectrometry (CE–MS) is very useful [62]. In this sense, CE–MS constitutes an ideal technique for the proteomic analysis of urine, and it has enabled novel candidate biomarkers of atherosclerosis [61,63,64] and stroke [65] to be defined in the recent years in this non-invasive sample.

Many researchers have taken advantage of array-based techniques to search for biomarkers of atherosclerosis and a very recent study revealed that carotid plaque vulnerability is modulated by the up-regulation and down-regulation of pro-inflammatory and anti-inflammatory factors, respectively [66]. The levels of these proteins were measured on a multiplex bead array system and one of these proteins, pentraxin 3 (PTX3), may potentially be a predictive marker of plaque vulnerability. An advanced variant of protein arrays consists of paraffin blocks in which myriads of separate tissue cores are assembled in an array fashion to allow multiplex histological analysis, the so-called tissue

microarray (TMA) [67]. Using this approach in combination with transcriptomics, the proprotein convertase subtilisin/kexin type 6 (PCSK6) was associated with key processes in plaque rupture, such as inflammation and extracellular matrix remodeling [68].

An emerging platform to directly study the distribution of proteins and small molecules within tissues is imaging mass spectrometry (IMS). Only a few groups have utilized this method on cardiovascular tissues, although a few studies on atheroma plaques have been performed. By means of the imaging variant secondary ion mass spectrometry (SIMS), optimal for small molecule profiling, the distribution of lipids in human atherosclerotic plaque tissue has been correlated with the stability or vulnerability of a particular region of the plaque [69]. Moreover, IMS-based histopathological examination of atherosclerotic lesions from aortic roots of ApoE-deficient mice and of femoral arteries of humans with peripheral artery occlusive disease revealed characteristic peak profiles defining lipid localization, SMCs and calcification within the plaque [70]. Very recently, 5 sample preparation protocols for IMS analysis of human atherosclerotic and healthy arteries were evaluated, achieving remarkable spatial resolution (30 μm) and situating specific proteins to the intimal and medial layers [71].

Table 2 summarizes the pros' and cons' for a given proteomic technology.

3.3. Metabolomics

Although Metabolomics is a science *per se*, nowadays many groups include it in their proteomic projects since both disciplines together permit integrated and comprehensive analyses of metabolic routes involved in pathological processes. Metabolomics facilitates the unbiased analysis of many different molecules at a time, detecting and identifying the set of final products and by-products of metabolic pathways, thereby reflecting the metabolic state of the cell [72,73]. Different metabolomics approaches must be adopted to cover the broad range of metabolites that exists in terms of polarity, solubility and volatility. Fortunately, great advances are being made in high throughput technologies like mass spectrometry (MS) and nuclear magnetic resonance (NMR). On the one hand, this makes it easier for researchers to identify biomarkers and elucidate the mechanisms underlying disease. On the other hand, it allows clinicians to measure such molecules for diagnostic purposes [74]. NMR and MS, the latter coupled with a separation method such as liquid or gas chromatography (LC–MS or GC–MS), are the main platforms used in metabolomics analysis [75]. Metabolomics analyses can be divided into untargeted and targeted approaches, which primarily differ in the sensitivity and the number of metabolites detected. Untargeted or unbiased studies identify as many metabolites as possible, although their sensitivity is drastically affected. However, if there is any idea about the sub-group of compounds that are relevant to the pathology under study, these methods can be optimized to improve the limits of detection by restricting the number of molecules analyzed.

High resolution ^{1}H NMR spectroscopy, which detects the hydrogen atoms present in a molecule, is one of the preferred platforms to analyze urine and plasma [76]. NMR requires relatively little sample preparation, it is a non-destructive and very reproducible technique, and it provides detailed information on molecular structure. It also constitutes a valuable approach to identify unknown metabolites. However, NMR is limited in terms of sensitivity and spectral resolution, and thus, it is not a good technique to identify metabolites that are found in low concentration [77]. NMR has been widely used to study lipoprotein composition and its relationship with atherosclerosis [78–80]. Indeed, high-throughput metabolite quantification has allowed a risk prediction model for subclinical atherosclerosis to be defined based on a combination of lipoprotein lipids along with the novel biomarkers docosahexaenoic acid and tyrosine, in addition to non-laboratory risk factors [81]. NMR analysis of plasma has also been confirmed as a weak predictor of coronary artery disease [82].

In a typical metabolomics platform, MS is usually coupled to chromatographic methods that allow a wide number of metabolites to be analyzed with enhanced sensitivity. GC–MS is a first-rate choice to analyze volatile samples or when the expected compounds can be easily made volatile by derivatization. GC–MS analysis of human plasma has allowed a panel of biomarkers with utility in early diagnosis of acute coronary syndrome (ACS) to be identified [83]. Moreover, a characteristic metabolic fingerprint reflecting the oxidative and hypoxic stress that myocardial cells suffer in ACS was reported with a similar approach [84]. GC–MS and ^{1}H NMR have been used together to compare the plasma from patients with stable carotid atherosclerosis and healthy patients [85]. The association of both techniques provided complementary information regarding altered metabolic pathways and enabled a clearer picture of the metabolic state of patients with carotid atherosclerosis to be defined.

LC–MS has also been applied in studies to uncover metabolic pathways relevant to cardiovascular disease. Thus, three metabolites of the dietary lipid were identified as predictors of risk for cardiovascular disease in an untargeted LC–MS approach [86], and subsequent targeted studies validated this finding and the relationship between intestinal microbial metabolism and the development of atherosclerosis [87,88] (Table 3 shows different studies in which metabolomics has been applied in recent years [89,90]).

Table 2 summarizes the pros' and cons' for a given metabolomic technology.

4. Integrative bioinformatics analysis to build molecular networks: systems biology

The "omics" platforms offer a range of opportunities to study biological systems as a whole from different perspectives. An "omics" approach provides significant amounts of data at multiple biological levels from gene sequence and expression, to protein and metabolite patterns, all of which underpins the variability in cellular networks and activity in whole organ systems [91,92]. In this review, we focus on two such "omics" disciplines: Proteomics and Metabolomics. Proteins are the ultimate expression of genes and metabolites represent the end-products of the genome and proteome, providing an instantaneous snapshot of the physiology of a cell, tissue or organism. Given that atherothrombosis is a multifactorial disease, integrating "omics" data through a systems biology approach is a valuable means to identify protein and/or metabolite networks associated to atherothrombosis. Such a global approach, consider under the auspices of systems biology, has enabled protein networks or metabolites associated with CVD to be identified [93,94].

It is also important to consider the possibility of integrating our results with those of other groups, not only at the proteomic, transcriptomic or metabolomic level but also, taking into account that biological networks exist at higher levels such as organelles, cells and organs. In the case of atherothrombosis, interactions between multiple cell types (macrophages, endothelial cells, VSMC, lymphocytes, etc.) and organ systems (vascular, endocrine, adipose, renal . . .) have been described, with a myriad of interconnected molecules that are expressed by each of the different components. The existence of vast databases derived from high-throughput studies (e.g., GenBank sequence database [95], UniProt [96] or Golm Metabolome Database [97]) is particularly useful when comparing and integrating results from

Table 3
Compilation of studies reported so far in the field of animal and human metabolomics in atherothrombosis pathology. (Table 2 has been modified from Application of metabolomics to cardiovascular and renal disease biomarker discovery by Alvarez-Llamas et al. Applications of Advanced Omics Technologies: From Genes to Metabolites, 64, Elsevier B.V.; 2014 [chapter 11]). New added references are indexed in the manuscript.

Sample source	Pathology	Methodology	Potential biomarkers
Human plasma	NSTEACS	GC/MS	Citric acid, 4-hydroxyproline, aspartic acid, fructose, lactate, urea, glucose, valine
Human urine and plasma	Atherosclerosis	CE–MS	Collagen α1
Human aneurysm wall	Human intramural thrombosis	LC–QTOF–MS	Hippuric acid
Rat urine	Myocardial infarction	LC–QTOF–MS	Creatine, uridine, glutamate, pantothenic acid oxalosuccinic acid, nicotinamide mononucleotide, phenylacetylglycine, xanthosine, shexiang, baoxin, pill
Human blood samples	Myocardial infarction	Mass spectrometry-based metabolite profiling platform	Alanine, aminoisobutyric acid, hypoxanthine, isoleucine/leucine, malonic acid, threonine and trimethylamide N-oxide 1-methylhistamine, choline, inosine, serine, proline, xanthine taurine, ribose-5-phosphate DMPC, lactic acid, AMP, malic acid, succinic acid, glycertae-2-phosphate
Rat plasma and urine	Atherosclerosis	UFLC/MS-IT-TOF	Plasma: leucine, phenylalanine, tryptophan, acetylcarnitine butyrylcarnitine, propionylcarnitine, spermine. Ursodeoxycholic acid, chenodeocycholic acid, urine: 3-O-methyl-dopa, ethyl N_2-acetyl argininate, leucylproline, glucuronate, *N*(6)-(*N*-threnylcarbonyl)-adenosine methyl-hippuric acid, hippuric acid
Rabbit and rat myocardial cells and tissue	Atherosclerosis	ESI–MS	Plasmalogens
Human plasma	Cardiovascular status in healthy voluntaries	NMR	3-Hydroxybutyrate, A ketoglutarate, threonine dimethylglycine
	Atherosclerosis	NMR GC–MS	Glutamate, ketoglutarate, succinyl CoA, 4-OH-L-proline, creatine, pyruvate, malate, glycolate
Peripheral blood	Coronary artery disease	Quantitative mass spectrometry based metabolic profiling	Arginine, ornithine, alanine, proline, leucine/isoleucine, valine, glutamate/glutamine, phenylalanine glycine
Human plasma [89]	Coronary heart disease	NMR	Creatinine, serine, glucose, 1,5-anhydrosorbitol, trimethylamine N-oxide (TMAO), ornithine, citrate, glutamate, glycoproteins, an unsaturated lipid structure, valine
Human plasma [90]	Peripheral arterial disease	H NMR	Lipid molecules of lipoproteins, such as the eCH_3 group of triglycerides, cholesterols, phospholipids, and glycophospholipids

different studies. Moreover, a systems biology approach could be applied to biomarker discovery, situating putative biomarkers from previous experimental analysis in the context of a network of biological interactions, such as gene–gene, gene–protein or protein–protein interactions, subsequently performing different 'guilt-by-association' analyses [98]. Different bioinformatics tools are able to generate biological networks, most of them based on Cytoscape Web, a freely available network visualization tool that integrates biomolecular interaction networks with high-throughput expression data and other molecular states into a unified conceptual framework [99]. Moreover, the free web-accessible programs PANTHER [100] and DAVID [101], and the commercial software Ingenuity Pathways Analysis (IPA: Ingenuity® Systems, http://www.ingenuity.com) provide a comprehensive set of functional annotation tools (including gene function, ontology and pathways) to extract biological meaning from large lists of genes. These software tools allow enrichment analysis to be performed, which defines molecular/biological functions and pathways, and sub-cellular localizations significantly over-represented in a sub-set of proteins. Another useful software to build molecular networks is STRING, a database of reported and predicted protein interactions that includes direct (physical) and indirect (functional) associations [102].

Network analysis can also be used to design targeted experiments, which somehow constitutes an alternative "*in silico* discovery phase" to that of traditional proteomics analysis. Using this approach, the regulation of selected biomarkers belonging to pathways that are related to coronary artery disease (CAD) has been studied [103,104]. Specifically, network models based on regulatory transcription factors implicated in stress, inflammation, coagulation, oxidative stress, cell adhesion, obesity and renal function were developed (using STRING and Cytoscape), which were validated by transcriptomics alone [103], or in conjunction with proteomics [104], using plasma from CAD patients.

To summarize, with systems biology in mind, an integrative approach can provide a more holistic picture of the molecular mechanisms at play during the development of atherosclerosis.

5. The long and winding biomarker pipeline

Biomarkers are very important because they can be used in research studies to predict disease risk, monitor disease status and to provide information that might be useful for life-saving or health-promoting interventions. The clinical utility of molecular biomarkers relies on their specificity to predict pathological risk, although it must be considered that the biomarker must be accepted by the patient, it should be easy to interpret and able to explain a reasonable proportion of the outcome. Accuracy, reproducibility, availability, feasibility of implementation in a clinical setting and specificity are additional characteristics that must be fulfilled, and in this sense, panels of biomarkers are gaining acceptance as opposed to individual molecules [105].

In the discovery phase, proteomics gel-based platforms (2D-DIGE) and liquid chromatography (nLC-MS/MS) set-ups are most commonly used for protein analysis, although the combination of capillary electrophoresis with mass spectrometry (CE–MS) for peptidomes is gaining in popularity [106]. For those pathologies where the spatial distribution of proteins, peptides and metabolites is useful, mass spectrometry imaging (MSI) is the platform of choice. Differential metabolomics analysis is currently addressed through LC-MS/MS, gas chromatography on-line coupled to mass spectrometry (GC–MS) and nuclear magnetic resonance (NMR) [107].

Once a potential biomarker candidate has been discovered, its validation in a different cohort of samples must be undertaken. In this step, candidate biomarkers are analyzed in an independent and larger cohort of patients to that used in the discovery phase, preferably using an orthogonal technique, such as western blotting, ELISA or immunohistochemistry. Indeed, analysis by

selected reaction monitoring (SRM) is becoming more established in current proteomics platforms. This strategy is typically performed in a triple-quadrupole configuration of the MS apparatus to simultaneously monitor and quantify hundreds of molecules per sample by measuring specific fragments of the proteins/metabolites of interest [108]. A novel method, denoted "Stable Isotope Standards and Capture by Anti-Peptide Antibodies" (SISCAPA) was described in 2004 [109], which represented a step-forward in the fractionation of serum or plasma for the validation of biomarkers using targeted proteomics. In this approach, targeted peptide enrichment is achieved by nanoaffinity chromatography to improve quantification by SRM. Immuno-SRM provides an average 120-fold enrichment of peptide antigens and therefore, it constitutes a valuable method to perform the tedious task of quantifying proteins that are not very abundant in blood. Using this methodology, cTpn I and interleukin-33 have been efficiently quantified in human plasma with high precision, reproducibility and sensitivity [110].

Although limited, validation data can indicate whether the differences in protein levels observed might be associated with differences in the genetic background of the selected patients. Moreover, when validated in an adequate patient population, potential biomarkers may complete the final steps of the pipeline before being implemented in clinical practice.

The translation of a biomarker into routine clinical use is a clear step-forward, requiring the collaboration of the research laboratory, the diagnostics industry and the clinical laboratory [111]. Once the technological battle has been won and the sensitivity required for the detection of the specific candidates can be achieved, the final issue is to find a suitable, clinically relevant application and to gain the acceptance of industry. Achieving this will indicate that the benefit to patients, industry and society has reached its maximum expression. Careful design of the clinical trial (in terms of the number of patients, clinical characteristics of the cohort, measurement technique, etc.), as well as the collection of reliable results indicating an improvement in the discriminative capacity with respect to the available biomarkers, will determine whether a biomarker can be accepted by the scientific community.

6. Functional validation of therapeutic protein targets

Demonstrating the utility of a protein as a therapeutic target requires validating its actual implication in the disease. A range of strategies exists to modulate protein expression *in vitro* and *in vivo*. However, it is hoped that targeted discovery and validation will concurrently identify and validate therapeutic targets for the best intervention in human diseases.

6.1. In vitro

One of the abiding weaknesses of *in vitro* experiments is that they fail to replicate the precise cellular conditions in the organism. Such approaches can focus on the molecular mechanisms that regulate the process under study, and they are intended to investigate the mode of action and/or effects of a substance in relation to its desired therapeutic target. In the first level of the functional validation, potential protein targets can be validated using cell cultures of a known tissue affected by the disease. In our experience, *in vitro* studies allowed us to elucidate the role of the PDGF-BB protein in vascular tissue repair [112], which has been also implicated in vascular remodeling during atherosclerosis [113].

6.2. In vivo

Functional validation in animal models is the next step as it is closer to the reality of the human disease. Physiological processes, particularly those involving complex interactions of different cell types over time, can only be analyzed within the context of intact organisms. Animal models of atherosclerosis (apoE-mice, hypercholesterolemic rabbit) or ischemia/reperfusion (artery ligation models) allow the analysis *in vivo* of the effect of protein modulation. Furthermore, the generation of transgenic or knockout (KO) mice, allow the role of proteins to be studied in certain physiological/pathological conditions [114]. One attractive alternative to KO mice is the generation of transgenic mice expressing shRNAs that are subsequently processed to yield functional siRNAs inducing the silencing of specific mRNAs [115]. Since the expression of such shRNA constructs can be controlled by inducible and tissue specific inhibitors, it is conceivable that this transgenic RNAi system will become an interesting technology of choice to validate potential therapeutic targets *in vivo* [116].

To exemplify how proteomics can be used in the discovery and functional validation of a therapeutic target as discussed here, we provide the noteworthy example of the pharmacological enhancement of aldehyde dehydrogenase-2 by Alda-1 that was demonstrated to reduce ischemic damage in the heart [117].

7. The limitations of proteomics and metabolomics analyses

Although "omics" approaches are ideal to discover and characterize biomarkers of atherothrombosis, several limitations of the available proteomic and metabolomics platforms should be taken into account when evaluating the potential of our results. In the first place, mass spectrometry can identify thousands of proteins and metabolites per sample, yet it is not capable of defining the entire proteome or metabolome since particular peptides, proteins and metabolites may be difficult to detect given their resistance to extraction and ionization. The chemical complexity of metabolites constitutes a limitation in metabolomic analysis, which implies the need to employ diverse extraction methods for the extraction of the entire metabolome. Furthermore, enzymatic digestion of proteins prior to MS is not very reproducible, resulting in incomplete digestion and the appearance of missed-cleavages, which affects identification.

Concerning comparative studies, the limited reproducibility of the available quantification methods constitutes a major issue, which must be counteracted by employing normalization techniques, and which may indeed benefit from the use of internal standards.

Conflict of interest

There are no conflict of interest.

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