PROSPECTS
Journal of Cellular Biochemistry 114:7–20 (2013)

Journal of Cellular Biochemistry

Recent Advances in Proteomic Technologies Applied to Cardiovascular Disease

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ABSTRACT

In recent years, the diagnosis of cardiovascular disease (CVD) has increased its potential, also thanks to mass spectrometry (MS) proteomics. Modern MS proteomics tools permit analyzing a variety of biological samples, ranging from single cells to tissues and body fluids, like plasma and urine. This approach enhances the search for informative biomarkers in biological samples from apparently healthy individuals or patients, thus allowing an earlier and more precise diagnosis and a deeper comprehension of pathogenesis, development and outcome of CVD to further reduce the enormous burden of this disease on public health. In fact, many differences in protein expression between CVD-affected and healthy subjects have been detected, but only a few of them have been useful to establish clinical biomarkers because they did not pass the verification and validation tests. For a concrete clinical support of MS proteomics to CVD, it is, therefore, necessary to: ameliorate the resolution, sensitivity, specificity, throughput, precision, and accuracy of MS platform components; standardize procedures for sample

Abbreviations: CVD, cardiovascular disease; MS, mass spectrometry; PTMs, post-translational modifications; 2D, two dimension; GE, gel electrophoresis; LC, liquid chromatography; CE, capillary electrophoresis; ESI, electrospray ionization; MALDI, matrix-assisted laser desorption/ionization; Q, quadrupoles; TOF, time-of-flight; FT-ICR, Fourier transform ion cyclotron resonance; QIT, quadrupole ion traps; LT, linear trap; MS/MS, tandem mass spectrometry; CID, collision-induced dissociation; ETD, electron transfer dissociation; QqQ, quadrupole–quadrupole; LTQ, linear trap-quadrupole; Q-TOF, quadrupole-time-of-flight; SCX, strong-cation-exchange; ITP, transient isotachophoresis; SPE, solid phase extraction; FASI, field amplified sample injection; CZE, capillary zone electrophoresis; iTRAQ, isobaric tag for relative and absolute quantitation; cICAT, cleavable isotope-coded affinity tags; DIGE, differential gel electrophoresis; COFRADIC, combined fractional diagonal chromatography; HPLC, high-performance liquid chromatography; IEF, isoelectrofocusing; SIM, single ion monitoring; MRM, multiple reaction monitoring; MIDAS, MRM-initiated detection and sequencing; 1D, one dimension; CPLL, combinatorial peptide ligand library; SILAC, stable isotope labeling by amino acids in cell culture; MudPIT; multidimensional protein identification technology; CRP, C-reactive protein; PAD, peripheral artery disease; β2M; β 2-microglobulin; ACS, acute coronary syndrome; CAD, coronary artery disease; AMI, acute myocardial infarction; MSIA, multiplexed MS immunoassay; HDL-C, high density lipoprotein-cholesterol; LDL, low-density lipoprotein; ApoJ, apolipoprotein J; 1STEMI, first episode of AMI; 2STEMI, second episode of AMI; cTnI, cardiac troponin I; CK, creatine kinase; CK-MB, creatine kinase MB; HSP, heat-shock protein; sTWEAK, soluble tumor necrosis factor-like weak inducer of apoptosis; AAA, abdominal aortic aneurysm; ILT, intraluminal thrombus; PRX-1, peroxiredoxin-1; PMN, polymorphonuclear neutrophils; LRG, leucine-rich α2-glycoprotein; CVE, cardiovascular events; SELDI, surface-enhanced laser desorption/ionization; SOD, superoxide dismutase; GST, glutathione-S-transferase; GDI, guanine nucleotide dissociation inhibitor; OGDH, 2-ketoglutarate dehydrogenase; LDH, lactate dehydrogenase; HCD, higher energy collisional dissociation; SNP, single nucleotide polymorphism.

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Manuscript Received: 30 April 2012; Manuscript Accepted: 26 July 2012

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 6 August 2012

DOI 10.1002/jcb.24307 •

collection, preparation, and analysis; lower the costs of the analyses; reduce the time of biomarker verification and validation. At the same time, it will be fundamental, for the future perspectives of proteomics in clinical trials, to define the normal protein maps and the global patterns of normal protein levels, as well as those specific for the different expressions of CVD. J. Cell. Biochem. 114: 7–20, 2013.

KEY WORDS: CARDIOVASCULAR DISEASE; MASS SPECTROMETRY PROTEOMICS; POST-TRANSLATIONAL MODIFICATIONS

In the post-genomic era, scientific attention is moving toward the ''omics'' studies, firstly to proteomics. This term signifies the identification, characterization, and quantification of the whole complement of proteins within a given biological sample, either a fluid, or a single cell, or a tissue [Tyers and Mann, 2003].

Proteomics has been used from basic scientific research, to applications in several fields, such as biomaterials research, ecotoxicology, plant biology, microbiology, livestock farming, food science, pharmacology, clinical diagnosis, therapeutics, and disease-associated biomarker discovery. Proteomics also analyzes protein post-translational modifications, turnover, and interaction with other proteins or ligands [Fields, 2001]. Proteomics is based on mass spectrometry (MS), which measures the molecular mass of charged molecules such as proteins or peptides. Proteins or protein fragments must be separated according to their physicochemical properties, charged, analyzed depending on their mass-to-charge ratios (m/z), and identified after producing spectra that are compared with databases through bioinformatic tools. Proteomics is a constantly and rapidly evolving science and utilizes different MS procedures. Particularly, clinical proteomics refers to the biomedical diagnostic applications of proteomics and aims at identifying novel disease biomarkers by correlating high-throughput analytical techniques, statistical analyses, epidemiology, and clinical chemistry [Ray et al., 2011]. A biomarker can be a molecule involved in the pathogenic mechanism, or a molecule that provides any other relevant information about the disease, like severity, therapy responsiveness, and prognosis. Proteomics is being increasingly applied to many areas of the medical science, from non-communicable degenerative diseases to infectious diseases.

Cardiovascular disease (CVD), the leading cause of death in the world [Murray and Lopez, 1997], has been successfully studied in the last 60 years with improvements in terms of understanding the major etiological co-factors, defining preventive and therapeutic treatments and reducing mortality [Lloyd-Jones et al., 2010]. Nevertheless, a significant fraction of cardiovascular events are not predictable on the basis of the established risk factors; furthermore the progression rate of the underlying pathological process, atherosclerosis, can present large and often indecipherable variations that result in early or late events during the lifetime. Therefore, there is still a compelling need to ameliorate the risk profile definition, to deepen the knowledge of molecular and cellular mechanisms involved in atherogenesis, to identify new therapeutic targets, and to achieve a personalized medical care based on a better definition of the individual gene–environment interaction.

Clinical proteomics holds promise for helping to achieve these goals [Kullo and Cooper, 2010; Van Eyk, 2011]. The present work gives a general overview of the most widely used proteomic technologies and an update of proteomic developments in the field of CVD.

PROTEOMICS TECHNOLOGIES

Proteomics is the study of the proteome, the full set of proteins present at a given time in a cell, a tissue, or a whole organism. Protein characterization comprises the processes of sequencing, identification of post-translational modifications (PTMs), and determination of conformational and structural features as well as cellular localization. Proteomic studies can be realized either by global proteomics approaches, involving the contemporary analysis of a multitude of proteins, or by the selection and analysis of specific types of proteins. Each approach involves the choice of the most appropriate experimental strategies and technologies.

MS, an analytical technique used for measuring the molecular mass of charged particles, is the core of proteomic studies. MS can be used for the characterization and sequencing of proteins and peptides. There are two main workflows in proteomic studies using MS: the top-down and the bottom-up. In the top-down procedure, intact proteins are pushed into MS-coupled systems and important data for protein characterization are provided, such as molecular mass, PTMs and isoforms. In the bottom-up procedure, proteins are purified, cut into fragments by chemicals or enzymes and analyzed with MS technologies (Fig. 1). The platforms for MS analysis consist of different units: the separation unit, which allows the separation of highly complex mixtures of analytes into single components; the ionization unit, which creates charged particles; the mass analyzer unit, which resolves ions formed in the ionization unit according to their m/z; the detector unit, which measures the electrical signal coming from the mass analyzer; the analysis unit, which records signals and converts them into MS spectra. The most representative instruments and methods in proteomic research are reported in Figure 2. Among the evolution of classical MS techniques, tandem mass spectrometry (MS/MS) supports the most powerful proteomic analyses. The simplest form of MS/MS consists of two analyzers, in general two quadrupoles, separated by a collision cell that fragments peptides, thus allowing to determine the mass of the intact individual samples and the derived fragments [Candiano et al., 2010]. Two frequently used methods for MS/MS molecule fragmentation are collision-induced dissociation (CID) and electron transfer dissociation (ETD). There are MS technologies that combine multiple on-line MS analyses such as matrix assisted laser desorption ionization-linear trap quadrupole (MALDI-LTQ) Orbitrap, resulting in a very high level of accuracy and resolution in mass determination. MS systems are extremely dynamic and modular; for this reason, they may be assembled by using different separation methods, ionization sources and mass analyzers.

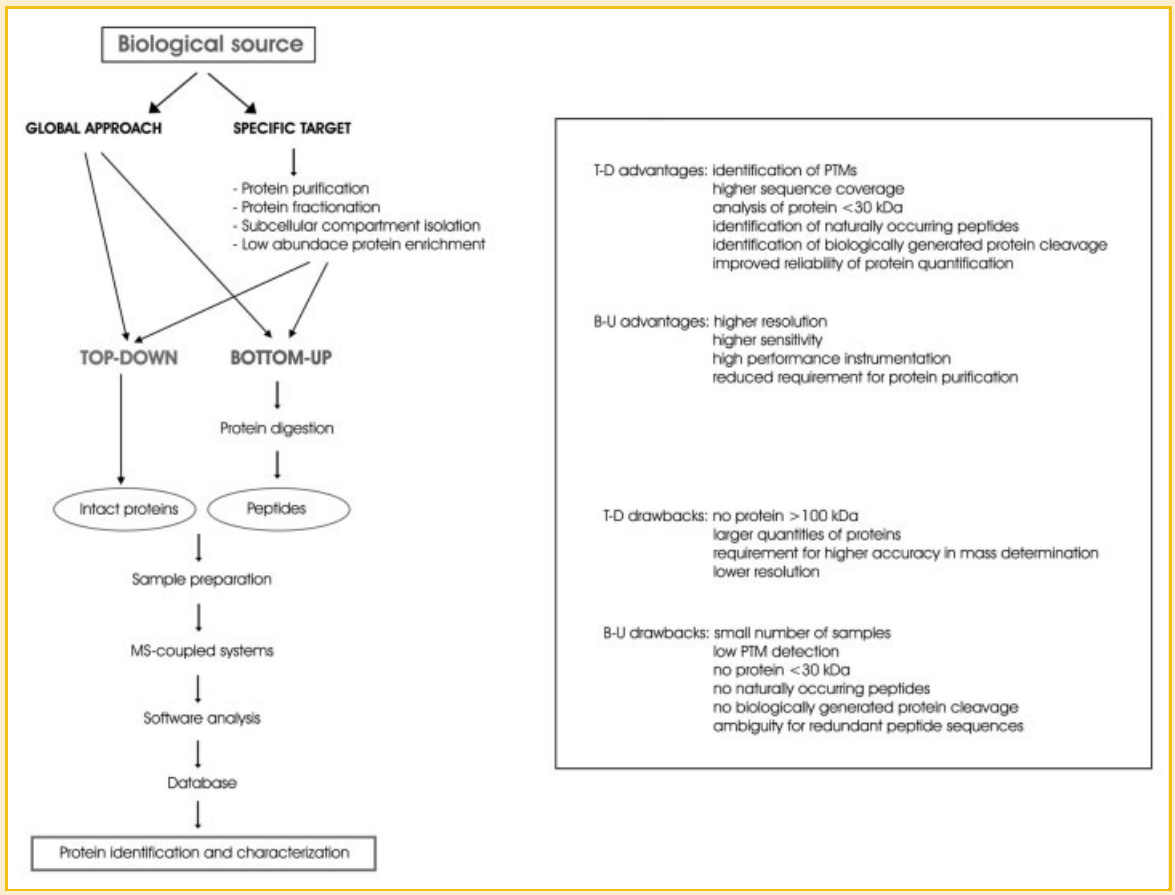

Fig. 1. Proteomic profiling workflow. Proteomic studies can be addressed either by a global or a targeted approach. In the global approach complex mixture of proteins are pushed into MS-systems, whereas in the targeted approach subsets of proteins are isolated from complex biological sources before MS analysis. Top-down (T-D) procedures allow analyzing intact proteins whereas bottom-up (B-U) procedures, protein fragments. The inset displays advantages and drawbacks of (T-D) and (B-U) proteomic approaches.

In spite of the current technological advancements, the vast amount of information deriving from proteomic studies can be difficult to analyze. Therefore, most proteomic studies are focused on subproteomic targets, such as cellular organelles, subsets of proteins (phosphorylated proteins, glycosylated proteins, etc.) or specific proteins (e.g., purified by affinity chromatography) [Dekkers et al., 2010].

Fractionation, pre-fractionation and pre-concentration methods allow to simplify the complexity of protein samples and to concentrate the analytes, making also the low-abundance proteins visible by MS or 2D-GE (deep proteome) [Fang et al., 2007]. This is the case of the cardiac muscle proteome, because of the high abundance of myofilaments and mitochondrial proteins that hinder the study of low-abundance proteins [Duan et al., 2009; Warren et al., 2010; Callipo et al., 2011]. In particular, immunodepletion and a bead-based library of combinatorial hexapeptide technology are two pre-fractionation methods, whereas strong-cation-exchange (SCX) chromatography is a peptide fractionation method.

Among the labeling techniques used for protein quantification, isobaric tag for relative and absolute quantitation (iTRAQ) seems to be more sensitive than the cleavable isotope-coded affinity tags (cICAT) and 2D-differential gel electrophoresis (DIGE) methods, but these post-isolation protein labeling techniques are biased towards large and abundant proteins [Vaughn et al., 2006; Wiese et al., 2007]. However, if iTRAQ labeling is coupled with highly efficient chromatographic techniques, a high-resolution separation of low-abundance peptides is obtainable, resulting in the identification of a greater number of proteins.

Combined Fractional Diagonal Chromatography (COFRADIC) is a protein/peptide chromatographic separation technique based on the principle of diagonal electrophoresis/chromatography. For peptide analysis and identification, it needs to be coupled to MS techniques, as LC–MS/MS. COFRADIC allows separating peptides according to the presence of specific amino acid residues/modifications. Therefore, it is particularly suited for the identification of PTMs. This technology has allowed the mapping of several PTMs and will be probably applied to the study of other PTMs.

Multidimensional protein identification technology (MudPIT) is a multidimensional high-performance liquid chromatography (HPLC)-MS protein identification technology, which combines two chromatographic separations, SCX column and reversed-phase column on-line. Because of its high-resolution power and loading capacity, this technique has been successfully applied to the study of complex peptide mixtures. Multiple cycles of MudPIT can be

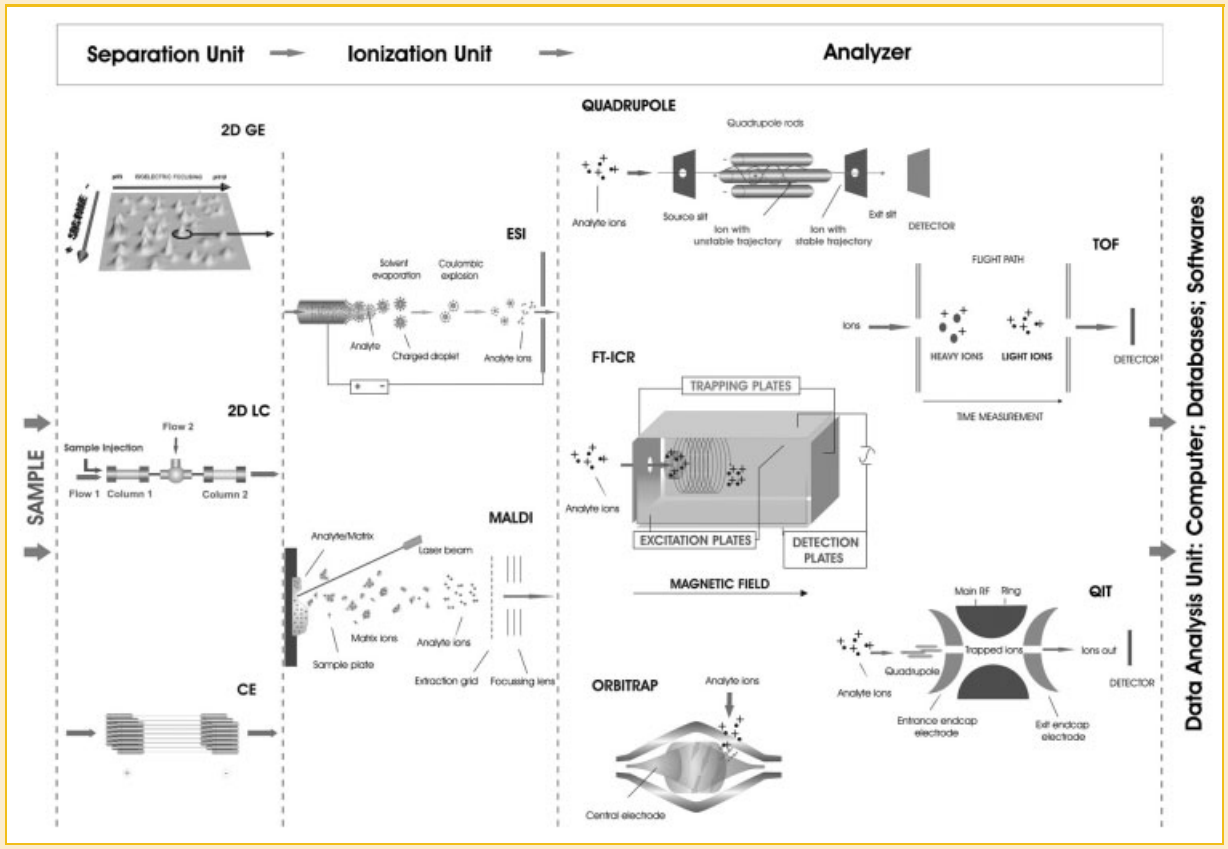

Fig. 2. MS platform components. The platform for MS analysis is composed of different units: the separation unit, to separate the mixtures of analytes into single components; the ionization unit, to charge the particles; the mass analyzer unit, to resolve the ions with respect to their mass-to-charge (m/z) ratios; the detector unit, to measures the signal from the mass analyzer; the analysis unit, to record and analyze the data.

performed to increase the number of identified proteins. It has been recently reported that this methodology can be used for quantitative measurement of peptides/proteins in complex protein samples by comparing spectral count numbers [Kislinger et al., 2005, 2006]. LC can be used for the separation of complex protein samples and combines different separation methods such as anion exchange, cation exchange, reversed-phase size exclusion and affinity chromatography. LC separation is based on specific protein characteristics including mass, isoelectric point, hydrophobicity, and biospecificity. Given these excellent properties, the LC–MS approach is replacing more and more 2D-GE in proteomics studies [Dekkers et al., 2010].

CE is a fast and highly efficient technique for the analysis of polar, charged, and chargeable molecules. The free zone mode, based on separation of the analytes according to the differences in charge-to-size ratio, has been a very useful CE technique for the analysis of proteins and peptides [Desiderio et al., 2010]. Due to the miniaturized size and material used for fabrication of conventional capillaries for CE, the amount of biological sample for each run are drastically reduced. Moreover, the complexity of samples makes it difficult to identify single components in heterogeneous samples. Therefore, CE has been coupled to MS, resulting in an extremely powerful system in clinical proteomics and biomarker discovering [Zimmerli et al., 2008; von Zur Muhlen et al., 2009]. The CE–MS analysis of proteins and peptides is compatible with both the top-down and bottom-up approaches. Until now, the most frequently used analyzers have been the TOF-MS because of their high speed of response and accuracy of the molecular mass determination, important requirement for the identification of biomarkers. The application of ionization sources, such as ESI and MALDI techniques, to CE–MS hyphenation widely enhances the range of applications of CE in proteomics. Capillary zone electrophoresis (CZE) and ESI and MALDI are the most frequently applied CE–MS hyphenated techniques. However, notwithstanding all the advantages deriving from the CE–MS approach, it is still not the preferred choice by clinical laboratories due to difficulties in interfacing CE to MS and the lack of routine methods in CE–MS [Desiderio et al., 2010].

FT-ICR MS is a type of mass analyzer that increases the sensitivity and the dynamic range of MS analysis due to the combination of electrophoretic mobility and molecular mass determination by MS/MS analysis. Although FTICR-MS is a very useful system for peptides characterization, it also lacks standardized protocols [Zurbig et al., 2006].

Although the analytical power of MS has been highlighted, there are pros and cons for each single MS procedure. Therefore, it is necessary to choose the appropriate proteomic analytical method for the specific purpose (Fig. 3). For example, 2D-GE technique is not

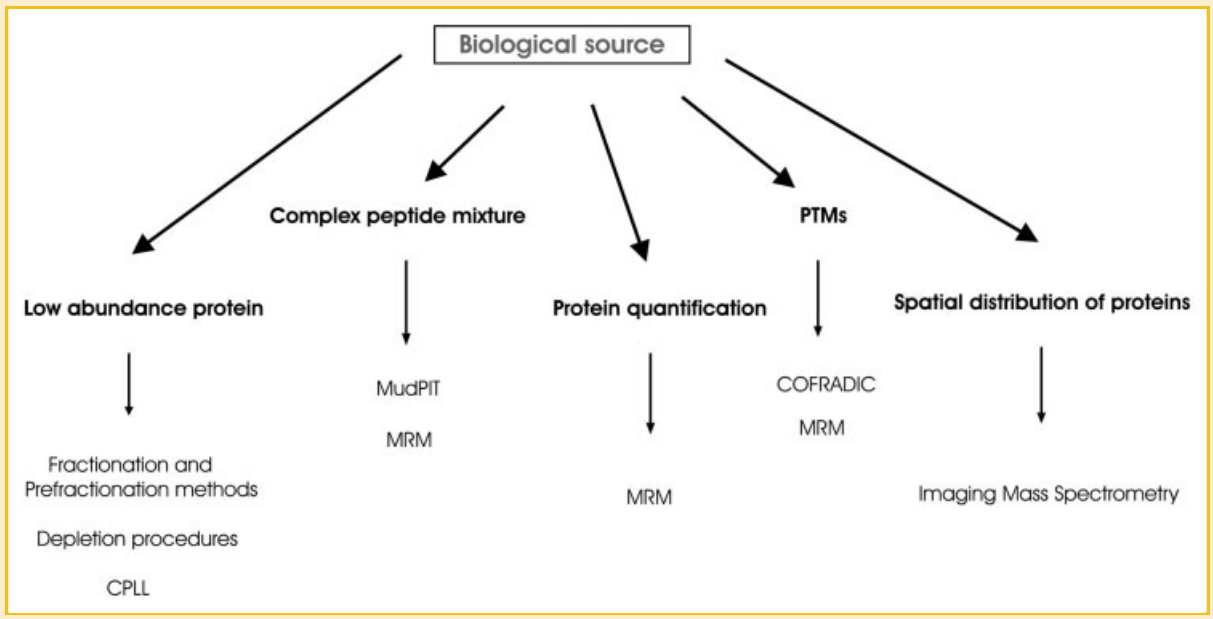

Fig. 3. MS procedure–aim association. Many MS approaches are available, and each of them is particularly suited for specific purposes.

very appropriate for studying membrane proteins, extremely acid and basic proteins, and low-abundance proteins. In order to overcome these limitations many other proteomics methods, such as 2 D-GE, isoelectrofocusing (IEF), LC, nano-LC and CE coupled on-line or off-line to MS can be used.

In order to overcome the side effects of sample homogenization before MS analysis, Imaging Mass Spectrometry has been developed. This technology enhances tissue and cell proteomics analysis, gives information about the spatial distribution of analytes inside the tissue slice or cell, and can be coupled with highly accurate and sensitive MS analyzers.

Multiple reaction monitoring (MRM) is a particular type of MS/MS where multiple fragments are simultaneously analyzed. It is an excellent system for quantification of protein isoforms, site specific PTMs and the analysis of limited-size samples [Van Eyk, 2011]. A new procedure has been recently developed to enhance MRM sensitivity by using antibodies against the targeted peptides [Dubois et al., 2011]. The use of antibodies in proteomic studies improved the detection and quantification of intact proteins; in fact, several companies are developing new generation of affinity reagents, as peptoids and aptamers [Uhlen and Hober, 2009; Kodadek, 2010].

MIDAS (MRM-initiated detection and sequencing) is a particularly sensitive approach that can be applied for the identification of PTMs and phosphorylation sites [Unwin et al., 2009].

All gel-based protein separation techniques, even if relatively simple to use, have several drawbacks as laboriousness and inability to be used in high-throughput experiments. On the contrary, gel-free protein separation methods, such as those based on HPLC, allow high-throughput proteomics and, therefore, might probably replace gel-based separation techniques.

The scientific community is now oriented toward the use of on-line and off-line multidimensional approaches for peptide identification. This strategy involves the combination of different technologies to further increase the sensitivity, the accuracy and the speed of analysis (CE, MALDI-TOF-MS, LC–MS/MS, nano-LCLTQ Orbitrap high resolution MS, quadrupole-TOF-MS etc.) [Candiano et al., 2010]. Each technology uses different separation criteria, ionization sources, mass analyzers and, therefore, they can complement each other resulting in a deeper characterization of proteins and identification of novel biomarkers. However, despite the described advantages of other techniques, 2D-GE-MS is still the most frequently adopted proteomic method in many laboratories.

PTMs

Part of the complexity of the proteome is due to PTMs, and their study has been encouraged by the improvements of proteomic technologies. Many proteins have multiple PTMs, which can significantly modify their structure, function and regulation; therefore, it is crucial to identify, quantify and map PTMs. It can be difficult to fully characterize the PTMs because they can be short-lived and often modify low-abundance proteins. Quantitative data on PTMs are also necessary for understanding proteome dynamic changes and their effects on cellular phenotypes. It has been reported that more than 50% of cellular proteins undergoes PTMs. Phosphorylation is the most frequently identified PTM, with more than 90% occurrence in proteins with a single PTM [Van Eyk, 2011]. The following methods are available for the analysis of phosphorylation and glycosylation: (1) 2D-GE, fluorescent Pro Q staining and MS analysis and (2) enrichments of phosphorylated or glycosylated peptides by means of metaloxide or lectin columns, respectively, 2D-GE, fluorescent Pro Q staining and MS analysis. However, 2D-GE and fluorescent Pro Q stain do not allow the identification of phosphorylated amino acid residues. Therefore, other techniques, such as immobilized metal affinity chromatography and TiO_2

affinity chromatography, can be used. Nowadays, ETD is the only way to obtain a direct mapping of phosphorylation and other labile PTMs. Indeed, ion fragmentation by ETD makes these PTMs more stable and results in higher quality of peptide sequences. Moreover, many different chemicals have been used to modify specific amino acid residues on the peptides allowing them to be differently labeled and specifically identified. For example, cysteine residues with different redox states can be selectively labeled with different alkylating tags by using specific chemical treatments [Wu et al., 2006].

SOFTWARE

Several softwares are available for protein data analysis, such as the most frequently used Sequest and Mascot (database search algorithms) and the less used algorithms Xhunter, X!Tandem, OSSMA and OLAV [Colinge et al., 2003; Van Eyk, 2011]. Data analysis is mainly based on the comparison between the MS spectra and the in silico spectra obtained by software digestion prediction [Van Eyk, 2011]. One of the most used analytical tools is MosaiquesVisu software which allows to convert the MS electropherograms in 3D plots (x, migration time; y, molecular mass; z, signal amplitude) and to identify peptides and/or proteins. 3D or 2D plots generated by these softwares give complex map of the sample proteome. Once standardized fingerprints are generated, it will be possible to use them for comparison and discrimination between healthy and pathological states [Desiderio et al., 2010].

CLINICAL PROTEOMICS AND CVD

The application of proteomic approaches to clinical practice, led to the development of a new field: clinical proteomics. This discipline is focused on the discovery of disease biomarkers for diagnosis or prognosis of pathologies and for monitoring medical treatments. Clinical proteomics exploits high-throughput platforms for detecting protein and peptide variations in body fluids, cells and tissues in different physiological and pathological conditions. However, it is important to distinguish the implications of MS proteomics applied to research studies or to routine clinical proteomics; in the latter case, the lack of standardization and validation of procedures and methods represents a strong limitation.

CE–MS showed very promising results in clinical proteomics, biomarker discovering, and identification of PTMs. Indeed, the use of the CE-ESI-TOF-MS approach led to the identification of protein and peptide disease biomarkers in urine [Coon et al., 2008; Metzger et al., 2009; Mischak et al., 2009; Zurbig et al., 2009]. However, also the lack of automation in CE–MS limits its use in clinical practice compared to more standardized liquid-based analytical methods such as LC or nano-LC-ESI-MS systems [Desiderio et al., 2010]. Together with CE–MS, LC–MS is the most powerful technique for protein detection and identification in human tissue samples. Although 2D-GE is still frequently adopted, the future of biomarker discovery by proteomic analysis will be probably based on multidimensional LC–MS/MS [Dubois et al., 2011]. Data of clinical proteomics studies will be also particularly useful for epidemiology, clinical chemistry, and many other medical disciplines. Clinical proteomics seems to be the future for biomarker discovering, but many factors, such as the sample pre-analytical items, have too many variables, and technological equipments are still so limited that its potential is reduced. To overcome these issues, analytical procedures should be standardized and the platform technologies ameliorated [Desiderio et al., 2010]. In CVD, great improvements have been introduced for the identification of protein markers in blood samples for the early detection of heart diseases, but the only accepted and available approach is the proteomic analysis of cardiac muscle specimens [Grant et al., 2009; Dekkers et al., 2010]. Moreover, in this field also the differential protein profiling approach by 2D-GE technique has been intensively used.

BIOMARKERS FOR CVD

CVD is the leading cause of mortality and morbidity in developed countries [Rosenson, 2010]. The diagnosis of CVD is mainly based on clinical, biochemical and instrumental parameters, and there are several useful techniques to establish or exclude the diagnosis of CVD hypothesized on the basis of a clinical suspicion.

In recent years, considerable progress in the ''omics'' tools have revolutionized the search for numerous putative biomarkers of CVD that could play a significant role in the development of new tools for diagnosis, prognosis, prediction of recurrences and monitoring of therapy in CVD [Balestrieri et al., 2008; Wilson, 2008]. Biomarkers can be measured in a biological sample (blood, urine, etc.) and are influenced by a variety of factors, including environmental factors, genetic predisposition, clinical or preclinical phase of disease. The clinical value of new biomarkers will depend on the accuracy and reproducibility of their identification procedures [Vivanco et al., 2008]. The discovery of new biomarkers is a very complex process, especially in CVD where several genes and different pathophysiological processes are involved [Zethelius et al., 2008]. The identification of individuals at risk of developing an acute ischemic event is still one of the great challenges of cardiovascular medicine. Indeed, the presence/absence of cardiovascular risk factors is not always able to accurately predict cardiovascular events [Gerszten and Wang, 2008]. The most common vascular diseases, such as atherosclerosis and aortic aneurysms, result from complex interactions between environmental risk factors and genetic predisposition. Changes in protein expression can reveal pathological progression. The function of many proteins is determined by PTMs, like oxidation, phosphorylation, and glycosylation. These modifications can alter protein activity, and therefore have an important pathological significance [Van Eyk, 2011]. Among the tissues that are most relevant to CVD, cardiac muscle is under intensive investigation relatively to its physiologic and pathologic proteome. Recently, the O-GlcNAcylation has emerged as an important PTM occurring in cardiac proteins and takes part to aging processes and disease development [Lima et al., 2009]. The proteome of cardiac muscle cells contains specific protein isoforms that are susceptible of tissue-specific PTMs and regulation. Even small changes in PTMs can profoundly alter protein activity; therefore, it is of primary importance to use, improve and develop

accurate techniques able to identify and quantify multiple PTMs. A major obstacle for cardiac muscle proteomic studies is that there are no dividing cell culture systems for ventricular cardiomyocytes. Approaches dependent on complete saturation metabolic labeling (such as stable isotope labeling by amino acids in cell culture also known as SILAC20) are, therefore, not possible. The proteome is a very dynamic system where proteins undergo continuous changes that need to be accurately identified and quantified in order to get the early markers for developing pathologies. For this purpose, it has been proposed to use strategies with unique metabolic labeling, adoption of new approaches and the use of multiple methods [Van Eyk, 2011].

In recent years, the proteomic approach for the study of CVD allowed the comparison of the expression of hundreds of proteins between two biological samples (healthy vs. affected subject) including cells, tissues or biological fluids. With the introduction of new proteomic techniques in protein separation (MudPIT), and their identification by MS, the evaluation of thousands of proteins in several samples at once is now possible. A list of recently identified biomarkers in CVD is reported in Table I.

PLASMA AS SAMPLE SOURCE FOR PROTEOMICS

At present, it is possible to apply the proteomic approach for the study of CVD to a variety of biological samples, including cells, biological tissues, atheroma, secretomes, or biological fluids such as serum or plasma and urine. These fluids represent the ideal samples for biomarker detection because of their easy accessibility [Ray et al., 2011].

Plasma biomarkers have been the main subject of numerous studies, and, despite a large number of molecules have been described as potential clinical biomarkers, the majority of them is still not used in clinical practice because they did not pass validation tests. Recently, a novel approach has been introduced to identify new and specific biomarkers in peripheral artery disease (PAD), by applying SELDI-TOF MS technique to plasma and comparing samples from affected subjects and controls [Cooke and Wilson, 2010]. A recent follow-up study of 540 high-risk individuals showed that the β 2-microglobulin (β2M) is a biomarker associated with PAD independently of other risk factors for CVD [Fung et al., 2008]. Indeed, the same study revealed that also cystatin C, hsCRP, and glucose were associated with PAD independently of traditional risk factors such as age, diabetes mellitus, hyperlipidemia, hypertension and tobacco use. More evidence about the correlation between plasma cystatin C and myocardial ischemia came from a study in which ESI–LC–MS/MS analysis detected cystatin C in the conditioned medium of cardiomyocytes isolated from hearts of rats and, subsequently, treated with H_2O_2 [Xie et al., 2010]. Moreover, in the same study cystatin C was detected in plasma of mice chronically treated with doxorubicin or in mice in which myocardial ischemia was generated by occlusion of the left anterior descending coronary artery. Furthermore, an increase of cystatin C was associated with the accumulation of fibronectin and collagen I/III in myocardial tissue from the ischemic area [Xie et al., 2010].

The identification of proteins directly involved in the pathophysiology of acute coronary syndrome (ACS) was performed using a proteomic approach with 2D-GE and DIGE on plasma of patients with ACS [Dardè et al., 2010]. Among 1,400 protein spots analyzed, 33 proteins were differentially expressed in patients with ACS compared with control subjects and patients with stable coronary artery disease (CAD). Some of the identified proteins had been previously associated with ACS, unlike α-1-B-glycoprotein, Hakata antigen, tetranectin, and tropomyosin 4 that were found altered in this disease for the first time [Dardè et al., 2010]. Recently, it has been shown that proteins derived from tissues can be detected in the plasma directly with MS or LC–MS/MS, providing a conceptual basis for plasma protein biomarker analysis. MALDI-TOF spectra of peptides from serum of patients with acute myocardial infarction (AMI) and healthy subjects produced models that could help in the diagnosis of AMI. The use of a multiplexed MS immunoassay (MSIA) for AMI diagnosis revealed the presence of two new markers such as serum amyloid A1α and S-sulfated transthyretin. Both proteins were subsequently validated in a cohort of patients [Kiernan et al., 2006].

A proteomic analysis by 2D-GE and MS of serum samples from patients with ischemic stroke showed four spots whose intensity was at least four times stronger in atherothrombotic than in cardioembolic patients. These spots have been identified as the haptoglobin related protein, serum amyloid A and haptoglobin α-chain. The subsequent use of ELISA techniques has validated the potential use of haptoglobin and serum amyloid A as markers for ischemic stroke. The levels of haptoglobin $> 1{,}040\ \mu g/ml$ identified atherothrombotic patients with 95% sensitivity and 88% specificity, while levels of serum amyloid A $> 160\ \mu g/ml$ identified atherothrombotic patients with 91% sensitivity and 83% specificity [Brea et al., 2009].

Plasma levels of high density lipoprotein-cholesterol (HDL-C) are inversely correlated to the incidence of CVD. Recently, the use of modern techniques of proteomics allowed the identification of about 50 different proteins associated with HDL particles. Gordon et al. used a size exclusion high-resolution chromatography, and identified 14 new phospholipid-associated proteins that co-migrate with HDL. Further characterization studies of proteins associated with HDL can result in the identification of particles with different degrees of cardioprotective activity [Gordon et al., 2010a]. In a significant proportion of patients with atherothrombotic ischemic syndrome, increased concentrations of inflammatory biomarkers such as CRP, serum amyloid A, myeloperoxidase, and interleukin-6 can be detected. Although biomarkers for the detection of ischemic myocardial lesions, such as cardiac troponin, are available, there is still the need for early marker detection in the first hours after the onset of AMI. In the group of new markers of atherosclerotic vascular diseases, biomarkers related to lipid metabolism, such as low-density lipoprotein- (LDL-), HDL-cholesterol, apolipoprotein AI and B, LDL-cholesterol oxidase, paraoxonase have achieved a significant relevance. A recent study using a proteomic approach reported that HDL carries protein families involved in complement activation, regulation of proteolysis, and acute-phase response processes. Therefore, the importance of HDL depends not only on cholesterol concentration but also on its composition, structure and

TABLE I. Biomarkers for Specific Forms of CVD Grouped According to Different Biological Sources and the Related Proteomic Technologies

Tissue	Protein identified	CVD	Proteomic technology	References
Plasma	β 2 microglobulin	PAD	SELDI-TOF MS	Fung et al. [2008]
	α-1-B-glycoprotein, Hakata antigen, tetranectin, tropomyosin	Acute coronary syndrome	2DE-DIGE	Dardè et al. [2010]
	Amyloid A1α, S-sulfate transthyretin	Myocardial infarction	MALDI-TOF	Kiernan et al. [2006]
	Haptoglobin α, serum amyloid A	Ischemic stroke	2D-DIGE-MS	Brea et al. [2009]
	Phospholipid-associated proteins	Atherosclerosis vascular disease	MS and density gradient ultracentrifugation	Gordon et al. [2010a], Gordon et al. [2010b], Davidsson et al. [2009], Vaisar et al. [2010], and Heinecke [2009]
	Apolipoprotein A1	Myocardial infarction	1D and 2D SDS–PAGE	Davidsson et al. [2009]
Serum	Apoliprotein J or clusterin	Myocardial infarction	2-DE-MALDI-TOF	Cubedo et al. [2011]
	cTnI, CK, CK-MB	Myocardial infarction	SELDI-TOF MS	Silbiger et al. [2011]
	HSP-27	Atherosclerosis vascular disease	2DE	Martin-Ventura et al. [2004]
	sTWEAK	Atherosclerotic plaques, PAD, Coronary artery disease, heart failure, myocardial infarction	2-DE-MALDI-TOF	Blanco-Colio et al. [2007], Blanco-Colio et al. [2011], and Urbonaviciene et al. [2011]
	CRP, C3a, C5a	Myocardial infarction, abdominal aortic aneurism	2D-DIGE	Distelmaier et al. [2009]
	Peroxiredoxin-1	Abdominal aortic aneurism	2D-DIGE	Martinez-Pinna et al. [2010]
	Leucine-rich α2-glycoprotein	Heart failure	MS	Watson et al. [2011]
Urine	Collagen α I and III and 17 urinary polypeptide	Coronary artery disease	CE–MS	von Zur Muhlen et al. [2009]
	α-1-antitrypsin, collagen types 1 and 3, granin-like neuroendocrine peptide precursor, membrane-associated progesterone receptor component 1, sodium/potassium-transporting ATPase γ and fibrinogen-α	Coronary artery disease	Capillary electrophoresis, micro time-of-flight spectrometry	Delles et al. [2011]
Atheroma	Annexin4, myosin regulatory light 2, smooth muscle isoform, ferritin light chain	Coronary artery disease	2D-DIGE	de la Cuesta et al. [2011]
	Osteopontin	Cardiovascular events	MS	de Kleijn et al. [2010]
	SOD3, GST, HSP20, and HSP27, annexin A10, Rho GDI	Atherosclerotic disease	2D-DIGE-MALDI-TOF, MS	Lepedda et al. [2008]
	SOD2, fibrinogen fragment D	Atherosclerotic disease	2D-DIGE-MALDI-TOF, MS	Lepedda et al. [2008]
	Hemorphin-7, cathepsin D	Abdominal aortic aneurism	SELDI-TOF/MS	Dejouvencel et al. [2010]
Secretome	14-3-3-protein	Abdominal aortic aneurism	2D-DIGE	Martinez-Pinna et al. [2010]
	Superoxide dismutase	Cardiovascular events	2D-DIGE	Liao et al. [2008]
	Gelsolin, vinculin, lamin A/C, and phosphoglucomutase 5	Atherosclerotic disease	LC–MS/MS	de la Cuesta et al. [2011]
Platelets	2-ketoglutarate dehydrogenase, LDH, γ-actin, 1B coronin, pleckstrin, β-subunit proteaosome 1, proteaosome subunit type 8	Coronary artery disease	2D-MS	Banfi et al. [2010]
Vascular smooth muscle cells	Prohibitin, vimentin	Abdominal aortic aneurism	2D-DIGE	Nordon et al. [2011]

function [Davidsson et al., 2009; Vaisar et al., 2010]. Moreover, HDL3 from patients with CAD is selectively enriched in apolipoprotein E, suggesting that the composition of HDL may be different in this disorder [Heinecke, 2009].

Apolipoprotein J (ApoJ) or clusterin is a disulfide-linked heterodimeric protein that is associated with ApoA-I in HDL. There are several isoforms of ApoJ that are differentially expressed in serum depending on the pathophysiological state. In a recent study by Cubedo et al. [2011], using a proteomics approach, significant changes in ApoJ were observed in patients with new-onset myocardial infarction. 2D-GE followed by MALDI-TOF showed a cluster of 13 spots with a different distribution among patients with AMI and healthy subjects. Moreover, 2D-GE shows a 25% decrease of glycosylated ApoJ in patients with AMI. The serum levels of ApoJ were lower in patients with AMI compared with controls in the early stage of stroke. These results showed that alteration of the proteomic profile of ApoJ in patients with AMI is due to a different glycosylation pattern within the first hours after the event. Studies by Silbiger et al. [2011] used this approach to discover new plasma biomarkers in the early phase of AMI. They analyzed two groups of patients with AMI, one group suffering the first episode of AMI (1STEMI) and the other group undergoing the second AMI (2STEMI), and a control group with similar risk factors for CVD and normal ergometric test. Unlike the controls, patients in the first group showed 510 peaks of proteins differently expressed in the first 48 h, while the second group presented 85% less protein differently expressed than in controls. Interestingly, among the 16 peaks present in both groups, 6 peaks are persistently down-regulated during the 48 h after AMI and are correlated with the serum protein markers (cTnI, CK, CK-MB) [Silbiger et al., 2011].

By using SELDI-TOF MS technology, soluble tumor necrosis factor-like weak inducer of apoptosis (sTWEAK) has been identified as an atherosclerotic marker released by plaques in culture [Blanco-Colio et al., 2007]. Patients with carotid stenosis have lower levels of sTWEAK compared to controls [Blanco-Colio et al., 2011]. In asymptomatic subjects, the plasma concentration of sTWEAK is negatively correlated with carotid intima-media thickness. Moreover, in patients with high risk for atherosclerotic disease because suffering from chronic kidney disease and/or type 2 diabetes, serum sTWEAK levels are low [Kralisch et al., 2008]. In a recent study, plasma levels of sTWEAK were a good predictive marker of mortality in hemodialysis patients [Carrero et al., 2009]. In patients with PAD, decreased concentration of sTWEAK and increased sCD163/sTWEAK ratio were significantly and independently associated with long term cardiovascular mortality [Urbonaviciene et al., 2011].

A decreased concentration of sTWEAK has been demonstrated in patients with carotid atherosclerosis, coronary artery disease, congestive heart failure, peripheral artery disease, or chronic kidney disease and in patients with abdominal aortic aneurysm (AAA) [Martín-Ventura et al., 2011]. Moreover, sTWEAK plasma levels predicted adverse outcomes in patients with heart failure, myocardial infarction and chronic renal failure [Blanco-Colio et al., 2011].

A recent proteomic study by Distelmaier et al. [2009] on plasma from patients with AMI showed a local activation of the complement system, with accumulation of CRP, C3a, and C5a as well as increased vascular occlusion in AMI due to neutrophil recruitment. In addition, studies by Pagano et al. [2009] have recently demonstrated the involvement of complement proteins in the development of AAA, using an elastase-induced mouse model of AAA. In the search for new biomarkers of AAA progression, proteins released from intraluminal thrombus (ILT) were analyzed using a differential proteomic approach with 2D-DIGE. Several differentially expressed proteins, involved in the pathological mechanisms leading to AAA (proteolysis, oxidative stress, and thrombosis), have been identified [Martinez-Pinna et al., 2010]. Among these, peroxiredoxin-1 (PRX-1) was released more by the luminal layer compared with the abluminal layer of the intravascular thrombus. In patients with AAA, serum levels of PRX-1 were found to be higher than those of healthy subjects. It was also found a direct correlation between AAA diameter and the circulating levels of PRX-1, plasmin-antiplasmin, and myeloperoxidase. Results suggest the potential use of PRX-1 as a biomarker for the development of AAA [Martinez-Pinna et al., 2011]. A proteomic approach with 2D-GE MS, LC–MS/MS has been applied to perform a proteomic characterization of the artery wall in patients with AAA and to relate it with the aneurism size and expansion rate. Six protein spots correlated with the aneurism size and the proteins contained in three of them were identified: vitronectin, calreticulin, albumin, collagen α-3 chain, and vitamin D binding protein. Based on the relationship between the expression of these proteins and the aneurism size, it was hypothesized that the proteins might be related to inflammatory or remodeling processes occurring within the affected tissue [Urbonavicius et al., 2010]. Another recent study by Májek et al. [2011] evaluated changes in the plasma proteome of patients with AMI, and unstable and stable angina pectoris. Particular attention was given to the plasma level and isoforms of apolipoprotein A1 that were estimated by using 1D- and 2D-GE, together with Western blotting. Interestingly, a high molecular weight fraction of apolipoprotein A1 was present only in the group of patients leading to hypothesize that these new high molecular weight isoforms of apolipoprotein A1 could be a potential new marker or risk factor for CVD. Using uniplex and multiplex tests, associations between the gender and/or ethnicity and 47 new circulating protein markers of CVD have been described. This study showed that the female sex is independently associated with higher levels of several inflammatory markers as well as lipoproteins, adipokines, natriuretic peptides, vasoconstrictor peptide, and markers of calcification and thrombosis.

The proteomic analysis with MS has been applied to discover differentially expressed proteins in patients with ventricular dysfunction and heart failure. The study showed that leucine-rich α2-glycoprotein (LRG) is a serum biomarker that accurately identifies patients with heart failure and is independent of age, sex, creatinine, ischemia, β-blockers, and BNP. In addition, LRG levels correlate with coronary sinus serum levels of tumor necrosis factor-α and interleukin-6 and with the expression of transforming growth factor-α and βR1-smooth muscle actin in myocardial tissue [Watson et al., 2011].

URINE AS SAMPLE SOURCE FOR PROTEOMICS

Urinary proteomics is emerging as a powerful non-invasive strategy for diagnosing and monitoring human diseases [Decramer et al.,

2008; Delles et al., 2011]. In clinical routine, urine of 67 patients with symptoms of CAD was analyzed by CE–MS. A group of 29 patients was studied to establish CAD and non-CAD-associated proteome patterns from plasma and urine. A significant discriminatory power was achieved in the urine, but not in plasma. By adding 38 additional patients to the study, a combination of 17 urinary polypeptides discriminated both groups in the test with 81% sensitivity, 92% specificity, and 84% accuracy. Sequencing of urinary marker peptides identified fragments of collagen α1 (I and III), which are expressed in human aortic atherosclerotic plaques. These results allowed associating specific CE–MS polypeptide patterns in urine with CAD, although the reliability of proteomic analysis of a urine sample as a screening method to improve diagnostic approaches for CAD should be further evaluated [von Zur Muhlen et al., 2009]. A previous study had shown that urinary proteomics can identify CAD patients with high confidence and might be useful in monitoring the effects of therapeutic treatments [Zimmerli et al., 2008]. Urine samples analysis of 623 individuals with and without CAD, by CE coupled online to micro TOF-MS, identified discriminatory polypeptides including fragments of α-1-antitrypsin, collagen I and III, granin-like neuroendocrine peptide precursor, membrane-associated progesterone receptor component 1, sodium/potassium-transporting ATPase γ chain and fibrinogen-α chain [Delles et al., 2010].

TISSUE SAMPLES

The proteomic analysis of the atheroma could provide valuable information for a better diagnostic and prognostic evaluation of CVD. However, it cannot be applied to the clinical routine. An alternative strategy to approach this study is to examine secreted proteins by atherosclerotic plaques that could be subsequently detected in blood [Alvarez-Llamas et al., 2008; Vivanco et al., 2008; Tuñón et al., 2010].

In one study, the intimal layer of human atherosclerotic coronary arteries was isolated by laser microdissection, thus reducing sample heterogeneity, and compared with non-atherosclerotic controls, using 2D-DIGE. The results showed 13 differently expressed proteins involved in vascular smooth muscle cell motility, extracellular matrix composition, coagulation, apoptosis, heat shock response and intraplaque hemorrhage deposition. Three proteins, annexin 4, myosin regulatory light 2 smooth muscle isoform and ferritin light chain, had not been previously identified in human atherosclerotic coronary intimal layer and, therefore, were validated by immunohistochemistry as novel markers of the atherosclerotic plaque. The study of tissue secretome of patients with atherosclerosis revealed key proteins involved in the arterial disease, such as gelsolin, vinculin, lamin A/C and phosphoglucomutase 5, which were not identified in the plasma [de la Cuesta et al., 2011ab]. The proteomic analysis of atheroma can help finding the molecular differences between those who develop adverse cardiovascular events and those that remain stable during follow-up. With this approach, and based on the concept that atherosclerosis is a systemic disease, a study by de Kleijn et al. [2010] showed that the analysis of osteopontin in a specific atherosclerotic lesion is a highly predictive biomarker of the occurrence of cardiovascular events in other vascular districts. MS imaging is another emerging technology for the study of whole tissues. MS is applied to thin tissue cryostat sections deposited on MALDI plates or protein chip surfaces (SELDI), thus highlighting the spatial distribution of proteins in tissue sections. With this technique, the presence of high amount of non-esterified fatty acids and vitamin E was demonstrated in human atheroma around areas with intimal accumulation of cholesterol [Mas et al., 2010].

Plaque stability versus instability has been studied with proteomic tools on extracts from human carotid atherosclerotic plaque [Lepedda et al., 2008]. In this study, authors have described several proteins, mainly involved in oxidative and inflammatory processes that were differentially represented in the stable or unstable plaques, suggesting that they may be involved in plaque stabilization. In particular, unstable plaque showed reduced abundance of: protective enzymes superoxide dismutase (SOD) 3 and glutathione-S-transferase (GST), small heat shock proteins HSP20 and hsp27, annexin A10, and Rho guanine nucleotide dissociation inhibitor (GDI). In unstable plaques, the more abundant proteins were: ferritin light subunit, SOD 2 and fibrinogen fragment D. Interestingly, while the previously described study used frozen samples from carotid endoarterectomies, proteomic analysis has been successfully applied also to paraformaldehyde-fixed, paraffin-embedded, arterial fragments, although approximately twice the number of proteins was identified from the frozen sections when compared with the paraformaldehyde-fixed sections [Bagnato et al., 2007].

Differential proteomic approach has been used to identify new biomarkers of atherothrombosis released from the diseased arterial wall into the plasma. In AAA, a principal feature is the presence of intraluminal thrombus (ILT) [Martinez-Pinna et al., 2010]. A recent study by Martinez-Pinna et al. [2010] analyzed peptides released from AAA specimens, characterized by an ILT by means of a SELDI-TOF/MS approach. A 1309-Da peptide, known as LVV-Hemorphin-7 generated from cathepsin D-digested hemoglobin was detected in large quantities in the newly formed luminal thrombus layer compared to the old layers. To identify the key proteins in the lesions of the aortic media, proteomic studies were done on segments of the ascending aorta obtained from patients with thoracic aortic dissection and normal subjects. The analysis of differentially expressed proteins by 2D-DIGE revealed 126 differentially expressed proteins, of which 26 were identified by mass spectrometry. Among the identified proteins, extracellular superoxide dismutase was more than 50% lower in patient samples compared with controls. All the cell types that make up the atheroma plaque are able to secrete proteins, thus contributing to the composition of the plaque secretome. For example, in the secretome of VSMCs in culture different proteins have been identified [Durán et al., 2007; Vivanco et al., 2007, 2008; Cecchettini et al., 2011]. In a recent study that evaluated changes in the vascular tissue proteome of patients with AAA showed decreased levels of prohibitin and an increase in a fragment of vimentin within the vascular smooth muscle cell layer [Nordon et al., 2011].

PLATELETS

Platelets play a critical role in hemostasis of the vessel wall. The proteome of platelets is particularly dynamic and activated platelets could be a source of plaque biomarkers. Indeed, in thrombin-activated platelets more than 300 proteins, not previously attributed to platelets, such as secretogranin have been identified [Senzel et al., 2009]. They were not present in normal arteries but were clearly identified in human atherosclerotic lesions. ACS is associated with platelet activation. A proteomic study on platelets from patients with ACS, compared with patients with stable CAD, showed that levels of proteins involved in cytoskeleton formation (F-actin capping, β-tubulin, α-tubulin isotypes 1 and 2, vinculin, vimentin, and two Ras-related protein Rab-7 isotypes), glycolyitic reactions (glyceraldehyde-3-phosphate dehydrogenase, lactate dehydrogenase and two pyruvate kinase isotypes), redox balance (manganese-SOD) were reduced in ACS. Moreover, GST expression and activity were decreased as well in ACS. Finally, also proteins associated with cell survival, such as the β subunit of the proteasome 1 are reduced in platelets from acute compared to stabilized patients [López-Farré et al., 2011]. Conversely, another study showed quite similar changes of platelet protein expression in stable CAD and ACS, thus suggesting a common mechanism that could be the underlying atherosclerosis affecting megakaryocytes proteomics [Porcelli et al., 2010]. By using proteomic techniques, six differentially expressed proteins have been identified: two involved in energy metabolism (2-ketoglutarate dehydrogenase [OGDH], and lactate dehydrogenase [LDH]), three associated with the cytoskeleton-based processes (γ-actin, 1B Coronin and Pleckstrin) and one involved in protein degradation (proteasome subunit type 8) [Banfi et al., 2010].

FUTURE PERSPECTIVES AND CONCLUSIONS

The modern medicine is evolving towards a type of ''niche personalized medicine'' [Dekkers et al., 2010]. It is foreseeable that proteomic analysis at the individual level, obtained by adding up the proteomics of the largest possible number of available tissues, will be instrumental to attain such type of tailored medicine. Also of paramount importance is early disease detection at the molecular level by identifying novel or previously unmeasurable disease biomarkers. Notably, proteomics approaches can virtually allow the identification of any specific biomarker. Especially in CVD, the ideal condition would be to have a routine, specific and sensitive proteomic approach to detect early biomarkers of the disease. For this purpose, further and continuous efforts are necessary to increase the power and efficacy of future proteomic methodologies that should allow a comprehensive picture of the individual physiological and pathological protein asset and its PTMs. In this respect, the use of a low-resolution SELDI-TOF proteomic method has been proposed [Dekkers et al., 2010]. In addition, COFRADIC is a very versatile and flexible technology, which is prone to further improvements for assessing the global protein profiling of PTMs [Witze et al., 2007]. All the improvements in proteomic analysis will allow further progress in drug discovery and therapeutic approaches in CVD. As in every technological system, each element of the proteomic methodology can be further ameliorated and combined with other systems, resulting in an increased detection power. For example, new peptide fragmentation technologies, such as ETD or higher energy collisional dissociation (HCD), can be coupled to FT-MS for the detection of phosphorylated and glycosylated residues [Syka et al., 2004; Balog et al., 2009; Hennrich et al., 2009; Lee et al., 2009; Takami et al., 2009]. In spite of continuous improvements, there are still obstacles to overcome in the field: MS provides incomplete detection (a result of inherent sampling bias, ionizability of individual peptides, and limited instrument sensitivity thus reducing the coverage of a protein) and the bioinformatic software and protein databases available still lack of resources. Therefore, it is needed to increase the MS system sensitivity, develop enhanced protein and peptide separation approaches, develop more powerful analysis software, extend databases, enhance quantitative methods, improve PTM mapping tools, create automated systems and make the instrumentations cost-effective. Another proteomic approach for the identification of disease risk is MRM to directly target specific disease-linked proteins, whose coding genes carry single nucleotide polymorphisms (SNPs) or mutations. In fact, the triple quadrupole MS instruments are routinely used in clinical laboratories for metabolite and drug quantification. In order to adopt MRM for clinical purposes it should be considered that each MRM peptide kit would be specific for a particular protein, isoform, mutant, SNP, or PTM, and would be therefore expensive. One step forward in the understanding of the proteome is the analysis of the complex network of protein–protein interactions by approaching the study of the interactome, which means the study of a protein and all its binding partners. The interactome adds a new dimension to the standard proteomics analysis, allowing a deeper comprehension of protein biochemical properties. Given the increasing complexity of information resulting from proteomics experiments, data evaluation and interpretation also require to be further enhanced.

Despite of the remarkable progress in understanding and curing CVD, yet more specific biomarkers are strongly needed to reduce the enormous burden of this disease on public health. The power of the evolving proteomic tools hold promise to identify such markers that, ideally, should be stable, easily detectable, specific, sensitive, and improve the current diagnostic and prognostic power. Present technological and bioinformatic achievements allow the analysis of single cells, tissues or body fluids, like plasma and urine, and boost the search for proteomic biomarkers of CVD. However, improvements are necessary to further increase the resolution, sensitivity and throughput of instruments and methods, and to reduce the complexity of samples, the time needed for the analyses and the costs of the runs. In parallel, it is critical to overcome also other limitations, such as the lack of standardized methods and the excessive time required for clinical biomarker verification and validation. Due to the large number of proteins analyzed within a single sample, the number of samples that can be analyzed is limited, thus highlighting the importance of carefully selecting the most informative ones. This means selecting the best available group of patients and controls and the most accurately collected specimens. To these aims it is crucial to set up a close synergy among the clinic and the clinical chemistry lab, the pathology lab, and the proteomic facility.

In the near future, the aims are: improvements and the integration of proteomics, genomics, metabolomics, and lipidomics to further reduce the incidence, morbidity, and mortality in CVD by developing personalized and targeted prevention and therapy.

In conclusion, proteomics seems to be very promising for tackling the study of different human diseases, in particular CVD. Moreover, it is noteworthy that this powerful approach can complement other robust analyses such as genomics. In fact, while the latter defines the individual predisposition to develop pathologies, proteomics provides information about the interaction between genetic and environmental factors the subject has been exposed to, and therefore about the real risk for the disease.

On the basis of the evidence so far collected, it is possible to hypothesize that proteomics will contribute to address some of the major still unsolved issues in CVD, such as prediction of severe CV events in low-risk patients and evolution of stable toward unstable atherosclerotic plaque.

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