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Overview of Current Proteomic Approaches for Discovery of Vascular Biomarkers of Atherosclerosis

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1. Introduction

Cardiovascular diseases are the leading cause of mortality and morbidity in developed countries being atherosclerosis the major contributor. Atherosclerosis is a form of chronic inflammation characterized by the accumulation of lipids and fibrous elements in medium and large arteries (Libby, 2002). The retention of apoB-100 containing lipoproteins (mainly LDL and Lp(a)) in the subendothelial space and their subsequent oxidation is thought to be the leading event in the development of atherosclerotic lesions (Williams & Tabas, 1995). The degree of inflammation, proteolysis, calcification and neovascularization affects the stability of advanced lesions. Plaque rupture and thrombosis are the most important clinical complications in the pathogenesis of stroke, coronary arteries and peripheral vascular diseases (Lutgens et al., 2003). So, the identification of early biomarkers of plaque presence and susceptibility to ulceration could be of primary importance in preventing such a life-threatening event. Disease aetiology is very complex and includes several important environmental and genetic risk factors such as hyperlipidemia, diabetes, and hypertension. In this regard elevated plasma levels of LDL cholesterol and low levels of HDL cholesterol have been long associated with the onset and development of atherosclerotic lesions. Although enormous efforts have been done to elucidate the molecular mechanisms underlying plaque formation and progression, they are not yet completely understood. In the last years, proteomic studies have been undertaken to both elucidate pathways of atherosclerotic degeneration and individuate new circulating markers to be utilized either as early diagnostic traits or as targets for new drug therapies.
This chapter will provide an overview of latest advances in proteomic studies on atherosclerosis and some related diseases, with particular emphasis on vascular tissue proteomics and lipoproteomics.

2. Application of proteomic technologies to the study of atherosclerosis

Atherosclerosis is a very complex pathology in terms of cell types involved, inflammatory mechanisms and multifactorial aetiology. Many efforts have been done to shed light on the mechanisms underlying atherogenesis and to identify new circulating biomarkers which, along with traditional risk factors, will help in early diagnosis and prevention as well as in

monitoring the effects of pharmacological agents. To address these issues, proteomic studies have been focused on different matrices such as vascular cell/tissues, looking at both proteomes and secretomes, plasma/serum, urine, and purified plasma lipoprotein fractions (fig. 1).

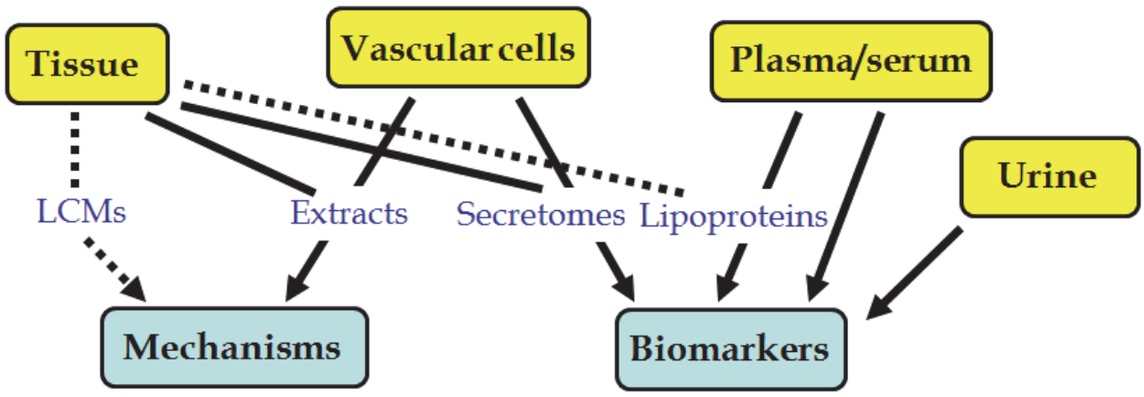

Fig. 1. Overview of the main targets of proteomic studies searching for both mechanisms of atherogenesis and biomarkers of atherosclerotic lesion presence and progression. Dotted lines represent almost unexplored paths. LCMs, laser-captured microdissections

To date, several proteomic approaches, such as 1D-2D electrophoresis (1DE-2DE) followed by mass spectrometry (MS) analyses, western arrays, protein arrays, and gel-free MS based proteomics, have been applied in the search of vascular biomarkers of atherosclerosis. Often, classical biochemical methods, mainly western blotting (WB), ELISA, and immunohistochemistry (IH) have been used to validate the proteomic results.

2.1 Vascular tissue proteomics

Even though tissue analyses frequently provide useful data, there are major drawbacks in analysing human atherosclerotic specimens. Atherosclerotic plaques are quite complex in terms of vascular cells and extracellular components. In this respect, besides vascular smooth muscle cells (VSMCs) and endothelial cells (ECs), they are composed of inflammatory cells, filtered plasma proteins, new-formed extracellular matrix, cellular debris and end-products of lipid and protein oxidation. Another critical point in the *in situ* analysis of protein expression within atherosclerotic plaques is the choice of the appropriate control. It would be desirable to utilize control specimens from the same vascular district of the same patient, in order to minimize intrinsic tissue differences, and from surgical endarterectomy rather than from post-mortem material, to avoid the occurrence of proteolytic modifications prior to analysis. Also the availability of a significant number of human specimens could be limiting. Because of the complexity of advanced lesions (Stary, 2000; Virmani et al., 2000) in terms of necrotic core dimension, fibrous cap thickness, inflammatory and proteolytic components, careful histochemical classification is needed. Moreover, results from different advanced lesion typologies are difficult to interpret because they could be either associated to the lesion development or merely a consequence of the advanced condition. In the latest years proteomic technologies have been applied to human diseased tissues to both characterize mechanisms of advanced atherosclerotic plaque development, mainly those responsible for its instability, and to identify markers useful in

diagnosis and patients treatment. Compared to tissue specimens of human origin, animal models, mainly rodents, have been utilized to study the mechanisms underlying the early stages of lesion formation.

2.1.1 Studies on animal models

Apolipoprotein E-deficient mouse is the most popular murine model in cardiovascular research and has revealed important insights into mechanisms affecting atherogenesis. Mayr et al. analysed aortic lesions from apolipoprotein $E^{-/-}$ and wild type mice classified as light, medium, and severe according to lesion-covered areas on the aortic surface (Mayr et al., 2005). As expected, authors found an increase of inflammatory cells, a decrease of VSMCs, and an accumulation of serum proteins associated to an impaired endothelial barrier function with lesion progression. Interestingly, immunoglobulins, that were barely detectable in apolipoprotein $E^{+/+}$ mice, accumulated even in aortas of young apolipoprotein $E^{-/-}$ mice. The authors identified 79 differentially expressed spots. Moreover, they suggested an increase in oxidative stress with lesion progression evaluating the ratio between the oxidized and the reduced forms of peroxiredoxin, the former resulting in a charge shift toward a more acidic isoelectric point. Overall, they found a linear relationship between the degree of peroxiredoxin-Cys oxidation and the extent of lesion formation in aortas of apolipoprotein E-deficient mice. Almofti et al. applied 2DE coupled to matrix-assisted laser desorption/ionization time of flight (MALDI TOF) MS analysis to a rat model of atherosclerosis. They induced atherosclerosis by a single dose of vitamin D3 associated with a high fat diet and identified 46 proteins differently expressed in diseased tissues. Among them, 18 proteins, including a group of oxidization-related enzymes, were found to be up-regulated, while 28 proteins were found down-regulated (Almofti et al., 2006). Vascular endothelium plays important physiological roles in vascular homeostasis, coagulation, inflammation, as well as tissue growth and repair. Impairment of the endothelial function is an early event in atherosclerotic lesion formation leading to overexpression of adhesion molecules as well as secretion of pro-inflammatory and chemotactic cytokines. An affinity-based proteomic approach was used by Wu et al. (Wu et al., 2007) to identify vascular endothelial surface proteins differentially expressed in aortic tissues of apolipoprotein E deficient mice. After *in situ* perfusion of vascular bed with a solution containing a biotin-derivative, biotinylated endothelial proteins were extracted, purified by affinity enrichment with streptavidin-agarose beads, and resolved by SDS-PAGE. The whole gel lanes were cut into slices that were subjected to tryptic digestion for nano liquid chromatography (LC) MS/MS analysis. In this way, 454 proteins, mainly extracellular or associated to cell membrane, were identified. Among them, there were cell adhesion molecules, accounting for the largest category, followed by proteins involved in signal transduction and transport. Interestingly, proteins associated with immune and inflammatory responses were more than doubled in atherosclerotic aorta (13%) in comparison to normal aorta (6%). On the other hand, proteins involved in lipid metabolism were decreased by 34% in atherosclerotic aorta. A rat model has been recently used for a proteomic study on the effects of blood shear stress on atherogenesis (Qi et al., 2008). It is well known that blood shear stress affects endothelial cell shape and orientation, as well as vascular wall permeability. Indeed, regions of arterial branching or curvature, where blood flow is not uniform, are preferential sites for lesion formation. By comparing homogenates of aortas kept under two levels of shear stress in a perfusion culture system for 24 hours, Qi et al. detected a reduced expression of protein Rho-GDP dissociation inhibitor alpha (Rho-GDIα) in low shear stress conditions and

demonstrated, by siRNA technology, that this reduction enhances VSMC migration and apoptosis.

2.1.2 Studies on human tissues

As from 2003, 14 researches on human atherosclerotic plaque proteomics have been published; the diseased tissues used as matrices were coronary arteries (2/14), carotid arteries (11/14), and aortas (1/14).

Most of them were conducted by using two-dimensional electrophoresis coupled to mass spectrometry as analytical method (10/14). The sample source, the methodology applied, and the most relevant findings of these studies are summarized in table 1.

In 2003, You et al., by analysing 10 diseased (coronary artery disease, CAD) and 7 normal autoptic coronary arteries, reported about 2 fold increase of the ferritin light chain in the pathological specimens (You et al., 2003). Quantitative analysis by real-time PCR showed a decrease in ferritin light chain mRNA expression in diseased tissues suggesting that the increased expression of ferritin light chain in CAD coronary arteries may be related to increased protein stability. This result highlights the importance of protein expression analysis in studying disease-associated gene expression. Donners et al. analysed 5 stable plaques and 6 lesions with a thrombus from patients undergoing carotid endarterectomy, classified according to Virmani et al. (Donners et al., 2005). By 2DE analysis, they identified vinexin-β and α1-antitrypsin as differentially expressed. However, neither immunohistochemistry nor western blotting confirmed vinexin-β differential expression underlining the importance of validating proteomic results by other biochemical methods. Conversely, western blotting of 2D gels revealed, in lesions with a thrombus, the expression of six isoforms of the acute phase protein α1-antitrypsin, one of which was uniquely expressed in thrombus-containing plaques. Sung et al. analysed non-diseased and atherosclerotic specimens from 7 patients undergoing aorta bypass surgery. They identified a panel of 27 proteins differentially expressed in the atherosclerotic aorta involved in a number of biological processes, including calcium-mediated processes, migration of VSMCs, matrix metalloproteinase activation and regulation of pro-inflammatory cytokines (Sung et al., 2006). A different approach was adopted by Martin-Ventura et al. who analysed the protein secretion profiles obtained from 35 cultured atherosclerotic plaques (10 femoral, 25 carotids) and 36 control arteries (24 mammary, 12 radial) in the search of new biological markers potentially released by the arterial wall into the plasma (Duran et al., 2003; Martin-Ventura et al., 2004). In particular, they isolated and analysed the secretomes from non-complicated and ruptured/thrombosed areas of the same cultured carotid plaque so avoiding the variability of the control specimens. They showed that, compared to control arteries, heat shock protein 27 (HSP27) secretion into the culture medium was significantly lower in atherosclerotic plaques and barely detectable in complicated plaque supernatants, as confirmed by WB analysis. They also evidenced a 20-fold reduction in HSP27 levels in the plasma of patients with carotid stenosis respect to healthy controls so identifying HSP27 as a possible marker of atherosclerosis. The same research group evaluated the effects of incubation with atorvastatin, a 3-hydroxy-3-methylglutaryl CoenzymeA reductase inhibitor, on the secretomes of cultured atherosclerotic plaques (Durán et al., 2007). They identified 24 proteins that were increased and 20 proteins that were decreased in atherosclerotic plaque supernatants compared to controls. Interestingly, the presence of atorvastatin in culture medium reverted secretion of 66% proteins to control values. In this report, authors identified cathepsin D as a potential target for therapeutical treatment of atherosclerosis.

Human tissues (Methods)	Results	Known functions	Ref.
10 coronary arteries from CAD patients vs 7 normal autoptic coronary arteries (2DE of homogenates, LC-MS/MS, WB, rt-PCR)	↑ ferritin light chain ↓ ferritin light chain mRNA	modulation of oxidation	You et al., 2003
6 carotid plaques containing a thrombus vs 5 advanced stable lesions (2DE of homogenates, MALDI-TOF/TOF MS, LC-MS/MS, WB, IH)	↑ α1-antitrypsin	acute-phase protein	Donners et al., 2005
7 atherosclerotic aortic specimens vs biopsies of the normal tissue from the same patients (2DE of homogenates, MALDI-TOF MS, WB)	↑ 39 proteins (27 identified)	signal transduction angiogenesis MMP activation regulation of pro-inflammatory cytokines	Sung et al., 2006
35 atherosclerotic endarterectomies (10 femoral, 25 carotids) vs 36 control endarteries (24 mammary, 12 radial) (2DE of secretomes, MALDI-TOF MS, LC-MS/MS and IMAC combined with MALDI Q-TOF MS/MS, WB, ELISA, IH)	↓↓ HSP27 secretion ↓↓ HSP27 plasma levels	anti-inflammatory down-regulation of the apoptotic signaling pathway	Duran et al., 2003; Martin-Ventura et al., 2004
21 stenosing complicated carotid regions with/without atorvastatin treatement vs fibrous regions (ex vivo) (2DE of secretomes, MALDI-TOF MS, LC MS/MS, WB)	↑ 24 proteins ↓ 20 proteins Treatment reverts the differential protein secretion	modulation of oxidation and inflammation structural signaling pathway cholesterol metabolism	Durán et al., 2007
29 unstable carotid plaques vs 19 stable carotid plaques (2DE of extracts, MALDI-TOF MS, WB)	↑ ferritin light subunit ↑ superoxide dismutase 2 ↑ fibrinogen fragment D ↓ superoxide dismutase 3 ↓ glutathione S-transferase ↓ Rho GDP-dissociation inhibitor 1 ↓ annexin A10 ↓ HSP 20 ↓ HSP 27	modulation of inflammation and oxidative stress	Lepedda et al., 2009
10 complicated segments in the internal carotid artery (ICA) vs 10 stable segments in the common carotid artery (CCA) (2-D DIGE of homogenates, LC-MS/MS, IH)	↑ 6 proteins ↓ 11 proteins 2 proteins with isoform dependent distributions	signal transduction transport cell growth metabolism	Olson et al., 2010

Human tissues (Methods)	Results	Known functions	Ref.
10 carotid plaques vs reference synthetic gel (2DE of homogenates, LC-MS/MS)	Identification of proteins exclusive to plaque		Terzuoli et al., 2007; Porcelli et al., 2010)

IMAC, immobilized metal affinity chromatography. ↑, increase. ↓, decrease.

Table 1. 2DE coupled to MS studies on the human atherosclerotic plaque.

Since carotid plaque rupture and thrombosis are the most important clinical complications in the pathogenesis of cerebro-vascular diseases, many efforts have been done to elucidate mechanisms underlying plaque vulnerability and to identify reliable specific markers of plaques prone to rupture. In a previous study we provided evidence for a wide fragmentation of some apolipoproteins and arterial proteoglycans and for a pro-inflammatory microenvironment in unstable and much less in stable endarterectomy carotid plaques (Formato et al., 2004). Recently, we evaluated differential protein expression in a considerable number (n=48) of plaques obtained from carotid endarterectomy classified by immunohistochemistry in stable and unstable (Lepedda et al., 2009). Our study was carried out on the premise that plaque stability/instability is associated with distinct patterns of protein expression. We analysed extracts from finely minced tissues in order to allow enrichment in both topically expressed and filtered/retained proteins. A total of 57 distinct spots corresponding to 33 different proteins were identified in both stable and unstable plaques by peptide mass fingerprinting (PMF) analysis, most of which were of plasma origin (about 70%). This suggested the existence of an impaired endothelial barrier function independent from plaque typology. Compared to stable plaques, unstable ones showed reduced abundance of protective enzymes superoxide dismutase 3 and glutathione S-transferase, small HSP 27 and 20, annexin A10, and Rho GDP-dissociation inhibitor and a higher abundance of ferritin light subunit, superoxide dismutase 2 and fibrinogen fragment D. These proteins are described to play a role in either oxidative or inflammatory processes and in the formation and progression of the atherosclerotic plaque. Our proteomic approach, trying to differentiate unstable from stable human carotid plaques, identified, in the former, a panel of proteins with pro-oxidant and pro-inflammatory potentials according to our current understanding of the molecular basis of the atherosclerotic process.

To overcome inter-individual variations in protein expression, Olson et al. applied 2-D differential in gel electrophoresis (2D DIGE) in combination with MS/MS to compare protein distribution in 10 complicated segments located in the internal carotid artery (ICA) with that in 10 more stable segments in the common carotid artery (CCA) from the same patient (Olson et al., 2010). In this way, they identified 19 proteins with differential distribution between ICA and CCA segments. To overcome the problem of plaque heterogeneity, Terzuoli et al. proposed a method for selecting proteins exclusive to plaque by constructing a reference synthetic gel (Terzuoli et al., 2007; Porcelli et al., 2010). This gel, obtained by averaging the positions, shapes and optical densities of spots in 2DE maps from 10 carotid plaque samples was compared with an equivalent synthetic gel constructed using 10 plasma samples from the same carotid surgery patients. The comparison allowed discriminating between plasma and plaque proteins, the latter being potential markers of plaque vulnerability.

Some alternative proteomic approaches have been applied to date in the search of new biomarkers of the atherosclerotic process (table 2).

Human tissues (Methods)	Results	Known functions	Ref.
12 carotid endarterectomy specimens vs 7 non-atherosclerotic mammary arteries (Western array (823 Abs), WB, rt-PCR, IH)	↓↓ apoptosis-linked gene 2 ↑↑ Thrombospondin-2, Mn superoxide dismutase, apolipoprotein B-100, protein-tyrosine phosphatase 1C, apolipoprotein E ↓↓ glycogen synthase kinase-3β	mediator of apoptosis	Martinet et al., 2003
4 pooled unstable carotid plaques vs 4 pooled stable carotid plaques (protein microarray analysis of the expression of 512 proteins)	↑ 21 proteins ↓ 3 proteins	modulation of inflammatory, angiogenic, proliferative, and apoptotic pathways	Slevin et al., 2006
Histological sections from 35 coronary vessels in paraffin or frozen blocks (direct tissue proteomics AQUA methodology)	806 unique proteins identified with high confidence		Bagnato et al., 2007
Carotid plaques from 80 patients that had a secondary cardiovascular event vs 80 sex and age matched event-free patients (during a 3-year follow-up) (LC MS/MS)	Strong positive association between osteopontin and the occurrence of new vascular complications		de Kleijn et al., 2010

↑, increase. ↓, decrease.

Table 2. Alternative approaches in proteomics of the atherosclerotic plaque.

High-throughput western blot analysis, also called western array, was used to screen cell lysates from 12 carotid endarterectomy specimens and 7 non-atherosclerotic mammary arteries, obtained during bypass surgery, with 823 monoclonal antibodies mainly directed against signal-transducing proteins (Martinet et al., 2003). Western arrays showed a highly reproducible pattern of protein expression but also a high rate of false-positive signals (differential protein expression of only 7 of the 15 proteins detected by using this method was confirmed by standard immunoblot assay). A strong down regulation of apoptosis-linked gene 2 (ALG-2) was found, suggesting a novel mechanism inhibiting cell death in human advanced atherosclerotic plaques.

By using microarray technology, Slevin et al. compared the expression of 512 proteins associated with inflammatory, pro/anti-apoptotic, and angiogenesis pathways between 4 pooled fibrous stable carotid plaques and 4 pooled ulcerated, hemorrhagic unstable plaques (Slevin et al., 2006). In spite of the high sensitivity of protein microarrays, allowing detecting nanogram quantities, errors can occur because of weakly reacting or nonspecific antibodies, degraded proteins, and/or the efficiency of sample fluorescent labelling. However, western blotting analyses confirmed differential expression between stable and unstable plaque pools for all 11 proteins selected suggesting a high level of specificity of the array antibodies and the usefulness of this proteomic approach in the study of plaque pathogenesis.
Atherosclerotic tissue could also be laser-microdissected, which would allow one to compare different areas of the plaque such as necrotic core and shoulders/fibrous cap, providing valuable spatial information. Bagnato et al. applied the direct tissue proteomic (DTP) approach to paraffin or frozen blocks from 35 coronary atherosclerotic lesions classified by histopathological examination in early, intermediate, and advanced (Bagnato et al., 2007). In particular, different plaque regions were laser-microdissected (LCMs) from both paraffin and frozen sections and subjected to tryptic digestion followed by LC MS/MS to obtain area-specific proteomic information. Frozen sections were also homogenized and proteins resolved by SDS PAGE and analysed by LC MS/MS. Moreover, they used AQUA (absolute quantitation) methodology to quantify Stromal Cell-derived Factor 1 α (SDF1-α) and growth factors not detected by the above mentioned methods. These multiple approaches allowed them to identify 806 unique proteins with high confidence so obtaining a large scale protein profile of human atherosclerotic coronary arteries.
Recently, de Kleijn et al. analysed carotid endarterectomy specimens from 80 patients that had a secondary cardiovascular event in the 3-year follow-up and 80 sex and age matched event-free patients, by two HPLC fractionations coupled to MS (de Kleijn et al., 2010). They identified osteopontin as potential biomarker and validated data by assaying its level in a group of 574 patients that underwent carotid endarterectomy and a group of 151 patients that underwent femoral endarterectomy included in the follow-up. Osteopontin resulted highly predictive for secondary manifestations of cardiovascular events in other vascular territories.
Atherosclerosis is characterized by high oxidative and proteolytic activities. This process may lead to a pathological remodelling of aorta characterized by dilatation that can evolve toward vessel wall rupture (aortic aneurysm). Recently, Dejouvencel et al. focused their attention on intraluminal mural thrombus that develops in human abdominal aortic aneurysm (Dejouvencel et al., 2010). In particular, they analysed the protein released from three different aortic layers of the intramural thrombus (luminal, intermediate and abluminal), after 24 hours incubation on RPMI medium, by surface-enhanced laser desorption/ionization (SELDI) TOF MS profiling. They identified a peptide that was largely abundant in newly formed luminal layer respect to the other areas as hemorphin 7, a proteolytic fragment of the hemoglobin. The levels of this peptide were confirmed (by ELISA) to be higher in sera of abdominal aortic aneurysm patients respect to controls, and positively correlated with the volume of the thrombus. This peptide has been suggested as a potential marker of pathological vascular remodelling.

2.2 Vascular cell proteomics

As mentioned above, the study of atherosclerotic specimens are complicated by both the heterogeneous cellular composition and the inflammatory/proteolytic environment. In this respect, cell culture systems could represent a useful tool to partially overcome drawbacks

of tissue analyses, allowing researchers to study single aspects of the atherosclerotic process in very controlled conditions. In the last years, studies on proteome (the intracellular proteins) and secretome (the proteins released into the cell culture medium) by 2DE and MS of ECs (Bruneel et al., 2003; Chen et al., 2007; Tunica et al., 2009), VSMCs (McGregor et al., 2001; Dupont et al., 2005; Lee et al., 2006), and monocytes/macrophages (Dupont et al., 2004; Fach et al., 2004; Slomianny et al., 2006; Zhang et al., 2007; Zhao et al., 2009) have been performed. Moreover, in the attempt to help in elucidating the mechanisms of atherogenesis, several proteomic studies have been carried out on vascular cells cultured in different experimental conditions (table 3). The most applied proteomic methodologies were 1DE-2DE coupled to MS analyses. Very few studies applied gel free proteomic approaches such as LC MS/MS (Fach et al., 2004; X.L. Wang et al., 2007; Zhao et al., 2009; Tunica et al., 2009; Zimman et al., 2010) and microarrays (Sukhanov et al., 2005).

2.3 Plasma/serum proteomics

Plasma is one of the most useful matrices to investigate for identifying new biomarkers. It carries resident proteins that represent the majority of plasma proteins, together with proteins released from vascular cells and other tissues. In this respect, variations in plasma proteome could reflect directly or indirectly a cardiovascular disease or other pathological conditions. Moreover, monitoring plasma proteome could be successful in patients follow up and in relation to drug therapies. Besides plasma, also serum is widely investigated, although its proteome is known to be drastically affected by blood coagulation. Plasma proteomic studies are challenging due to both the high dynamic range of protein concentrations and the number of low expressed proteins. Plasma is composed for up to 99% by 21 most abundant proteins and for the remaining 1% by about 50,000 low expressed protein variants, representing the so called "deep proteome" (Righetti et al., 2005). Plasma protein levels range from 40-50 mg/ml for albumin to less than 10 ng/ml for interleukins, chatepsins and peptide hormones. Therefore, differential proteomics of unfractionated plasma provides only limited information. In this regard, several pre-analytical depletion systems that imply affinity/immunoaffinity steps for simultaneous removal of the most represented plasma proteins have been set up. However, due to non-specific binding, many other proteins could be depleted. Besides depletion systems, also protein enrichment technology has been developed. This relies on solid-phase combinatorial ligand libraries, made of hexapeptides, to reduce the high dynamic range of protein concentration preventing the co-depletion of low-abundance proteins (Boschetti et al., 2007). Since variations in plasma protein expression can provide useful information on both physiological and pathological conditions of the different tissues in the body, many efforts have been made in characterizing the entire human plasma proteome. In this regard, during 2003-2005, 55 laboratories worldwide participating to the Human Plasma Proteome Project (HPPP) analysed reference specimens by using emerging technologies in the field of proteomics, and generated integrated databases for proteins detectable and identifiable in human serum and plasma (http://www.hupo.org/research/hppp/). They confidently identified 3020 proteins with a minimum of two high-scoring MS/MS spectra that have been searched for relevance to cardiovascular function and disease using PubMed search engine and specific keywords. On the basis of the current knowledge, the study individuated a subset of 345 proteins showing cardiovascular-related functions (markers of inflammation and/or cardiovascular disease, proteins implicated in coagulation, signalling, growth, differentiation, and vascular remodelling) (Berhane et al., 2005).

Experimental conditions	Endothelial cells	Smooth muscle cells	Monocytes/macrophages
Senescence	Kamino et al., 2003		
Shear stress	X.L. Wang et al., 2007; Huang et al., 2009	McGregor et al., 2004	
Pro-inflammatory conditions	Lomnytska et al., 2004; Pawlowska et al., 2005; González-Cabrero et al., 2007	Boccardi et al., 2007	
Oxidized/aggregated LDL	Chen et al., 2007	Sukhanov & Delafontaine, 2005; Padró et al., 2008	Fach et al., 2004; Conway & Kinter, 2005; Dupont et al., 2004; Kang et al., 2009; Burillo et al., 2009; Y.L. Yu et al., 2003a; Y.L.Yu et al., 2003b
Antioxidant/oxidant	Ha et al., 2005; Zimman et al., 2010	Jang et al., 2004; Lee et al., 2006	
Cholesterol loading	T. Wang et al., 2006		
HSP27 over-expression	Trott et al., 2009		
Drug treatment	M. Yu et al., 2004; Bieler et al., 2009; Millioni et al., 2010	Won et al., 2011	Barderas et al., 2009
PKCδ-/-		Mayr et al., 2004	
Hyperinsulinemia		Y. Wang et al., 2010	
Lipopolysaccharide or phorbol myristate			Gadgil et al., 2003; Sintiprungrat et al., 2010
ACS vs CAD			Barderas et al., 2007

ACS, acute coronary syndrome; CAD, coronary artery disease

Table 3. Literature overview of experimental conditions adopted in proteomic studies on ECs, VSMCs and monocytes/macrophages.

In the latest years, proteomics has been applied to plasma/serum to identify early diagnostic markers in relation to several cerebro-cardiovascular pathologies such as stroke (Allard et al., 2004; Kiga et al., 2008; Brea et al., 2009; Prentice et al., 2010), acute coronary syndrome (Mateos-Cáceres et al., 2004; Dardé et al., 2010), angiographic coronary disease (Donahue et al., 2006), acute myocardial infarction (AMI) (Distelmaier et al., 2009), coronary heart disease (CHD) (Prentice et al., 2010), coronary artery bypass grafting (CABG) (Banfi et al., 2010), aortic atherosclerotic plaque (Tabibiazar et al., 2006), and peripheral arterial disease (Wilson et al., 2007). Moreover, differential proteomic analysis has been addressed to

elucidate effects on plasma proteome of different pharmacological treatments (Alonso-Orgaz et al., 2006; López-Farré et al., 2007). In this regard, the most applied proteomic methodologies were gel electrophoresis coupled to mass spectrometry analyses followed by gel free proteomic approaches such as LC MS/MS (Donahue et al., 2006; Wilson et al., 2007; Prentice et al., 2010) and microarrays (Tabibiazar et al., 2006). Almost all of these analytical approaches were preceded by one or more fractionation steps, mainly immunoaffinity depletion of the most abundant plasma protein species and ion exchange chromatography, to reduce the high complexity of plasma samples in terms of both number and dynamic range of protein species. Since many genetic and environmental factors affect atherosclerosis aetiology, one of the main drawbacks in differential analysis is the choice of a proper control group. In particular, results could be affected by coexisting pathological conditions such as dyslipidemia, hypertension and diabetes.

2.4 Urine proteomics

Urine is an easily accessible body fluid, stable against proteolytic degradation even after long storage times, and it represents a rich source of information. Urine protein and peptide composition results from glomerular filtration and proximal tubular absorption of circulating proteins (30%) and from the kidney and the urinary tract (70%) (Decramer et al., 2008). Urine proteomics is emerging as a powerful tool for identifying new biomarkers useful in diagnosis and monitoring of several human diseases. However, the urinary proteome analysis is not a simple task because the urine shows low protein concentration and high levels of salts or other interfering compounds. Moreover, urinary proteome is highly influenced by both inter-individual and intra-individual variability, the latter due to physical training, diet, drugs, caffeine consumption, etc. One of the priorities in this field during the coming years is to optimize sample preparation methods for urine proteomics (Thongboonkerd V., 2007). 2DE coupled with MS has represented for years the technique of choice for the analysis of urine proteins. Recently, Candiano et al. resolved 1118 spots in normal urine samples, 275 of which were characterized as isoforms of 82 proteins, 30 (108 spots) corresponding to typical plasma components (Candiano et al., 2010). However, the identity of most of the proteins found in normal urine by 2DE remains to be determined, the majority being low-molecular weight proteins (<30 kDa). By means of 1DE and HPLC as fractionation methods, and nano LC MS/MS and MS^3 as analytical methods, Adachi et al. identified 8041 peptides corresponding to 1543 proteins, probably representing the most advanced proteomic approach to urine characterization (Adachi et al., 2006).

The study of urinary proteome in relation to atherosclerosis is in its infancy but, in the last years, many efforts have been done. In particular, multiple urinary biomarkers of CAD have been described (Zimmerli et al., 2008; von Zur Muhlen et al., 2009; Delles et al., 2010) by means of capillary electrophoresis coupled online to micro time-of-flight mass spectrometry. A pattern of 238 CAD-specific polypeptides (49 of which have been sequenced) that identifies patients with high sensitivity and specificity has been defined.

2.5 Lipoproteomics

In 1995 Williams and Tabas published the "response-to-retention" hypothesis. According to this theory, early events in atherogenesis are related to a selective retention of LDL in the sub-endothelial space by means of specific interactions with some extracellular matrix components (Williams & Tabas, 1995). The molecular mechanisms underlying these processes are not completely understood, but knowledge of lipoprotein structure,

apolipoprotein composition and their post-translational modifications could help in this respect. There are several types of lipoproteins differing for chemical compositions, physical properties and metabolic functions. They may be classified according to their densities in chylomicrons (d< 0.95 g/ml), very low density lipoproteins (VLDL, d< 1.006 g/ml), low density lipoproteins (LDL, 1.019<d<1.063 g/ml), high density lipoproteins (HDL, 1.063<d<1.21 g/ml) (Vance & Vance, 2008). A further class of lipoprotein particles is known as lipoprotein(a) (Lp(a)). Lp(a) is a LDL-like particle that carries, linked to apoB100 by a single disulfide bond, an heavily glycosylated multi-kringle protein named apolipoprotein(a). The physiological role of Lp(a) is unknown, although it is considered a risk factor for cardiovascular disease. Lipoproteins have attracted a great deal of interest because of their implication in the development of cardiovascular diseases, such as atherosclerosis. Although it is well known that high LDL-cholesterol and low HDL-cholesterol are positively correlated with the risk for the development of cardiovascular disease, clinical studies suggest that levels of apo B-100 and apo A-I may be better predictors (Walldius et al., 2001). Since the protein component of these particles is largely responsible for carrying out their various functions, detailed information about the apolipoprotein composition and structure may contribute to reveal their role in atherogenesis and to develop new therapeutic strategies for the treatment of lipoprotein-associated disorders. Applying proteomics to the study of lipoproteins seems to contribute significantly to the achievement of this goal. Indeed, recent proteomic studies have revealed that lipoproteins carry an array of proteins previously unsuspected. Among proteomic approaches, 2DE was applied to the study of lipoprotein particles for the first time in the 1970s (Emes et al., 1976) and allowed to reveal several protein isoforms (Zannis, 1986). With the improvement of 2DE technologies, due to the advent of immobilized pH gradient strips, in the last ten years several studies have been done in the attempt to elucidate the apolipoprotein cargo of the different lipoprotein species. In this respect, besides 2DE, several gel-free mass spectrometry based proteomics have been applied. So far, 31 proteomic studies on VLDL, LDL and HDL have been published, while no proteomic studies on chylomicrons or Lp(a) are present in literature. Among them, only 9 focused on lipoproteomics in relation to atherosclerosis in humans (table 4).

Before overviewing lipoproteomic studies, it is worth mentioning that the method used to isolate lipoproteins significantly affects the protein content of the resulting particles. Traditional methods, established in the 1950s (Havel et al., 1955), imply ultracentrifugation in high-salt media containing KBr or NaBr. Several lipoproteomic studies have been published using these procedures of lipoprotein isolation (Banfi et al., 2009; Davidson et al., 2009; Green et al., 2008; Heller et al., 2005, 2007; Hortin et al., 2006; Karlsson et al., 2005a, 2005b; Khovidhunkit et al., 2004; Mancone et al., 2007; Mazur et al., 2010; Rezaee et al., 2006; Vaisar et al., 2007, 2010; Alwaili et al, 2011). However, the high ionic strength and the high centrifugal field forces might cause either the dissociation of proteins or their exchange between different lipoprotein classes, altering the pattern of associated exchangeable apolipoproteins. Indeed, some of these studies reported a loss of proteins after a second step of ultracentrifugation (Banfi et al., 2009; Davidson et al., 2009; Mancone et al., 2007). Some others employed two ultracentrifuge procedures, using both salts and other compounds, such as sucrose and iodixanol (Bondarenko et al., 1999; Sun et al., 2010), reporting comparable results. By the way, Stahlman et al. reported that deuterium oxide (D_2O) is to be preferred over salts at least for LDL and HDL, since for VLDL isolation, the ionic strength of the solution is not so relevant (Ståhlman et al., 2008). Alternatively, lipoprotein can be isolated by means of

Subjects analysed	Purification methods Proteomic methods	Results	Ref.
VLDL			
1 hyperlipidemic subject vs 3 healthy subjects	ultracentrifugation in sucrose or in NaBr density gradient MALDI-TOF and ESI-TOF MS	↑ apo C-III	Bondarenko et al., 1999
LDL			
10 subjects with metabolic syndrome and subclinical carotid atherosclerosis vs 10 healthy controls 21 patients with type 2 diabetes and atherosclerosis vs 23 healthy controls.	ultracentrifugation in D_2O density gradient (small dense LDL) SELDI-TOF MS 1DE MALDI-TOF/TOF WB	Proteins differentially expressed in small dense LDL: ↑ apo C-III (3 isoforms), ↓ apo C-I (2isoforms), ↓ apo A-I, ↓ apo E	Davidsson et al., 2005
HDL			
20 control subjects for total HDL analysis 7 CAD subjects vs 6 control subjects for HDL_3 analysis	ultracentrifugation or affinity chromatography LC-ESI MS/MS	48 proteins identified in total HDL HDL_3 analysis: ↑apo C-IV, ↑PON1, ↑complement C3, ↑apo A-IV, ↑apo E	Vaisar et al., 2007
6 CAD subjects treated with niacin and atorvastatin for 12 months vs 6 non treated CAD subjects	sequential salt ultracentrifugation (HDL_3) LC–Fourier transform Ion Cyclotron Resonance –MS	↑PLTP, ↑apo F, ↑apo J, ↓apo E	Green et al., 2008
18 men with established CAD vs 20 apparently healthy men	sequential salt ultracentrifugation (HDL_2) MALDI-TOF MS and pattern recognition analysis LC-MALDI-TOF/TOF	↑apo C-III, ↓apo C-I ↑apo A-I peptides containing oxidized methionine	Vaisar et al., 2010
7 hypercholesterolemic subjects vs 9 normolipidemic subjects	ultracentrifugation in salt density gradient Shotgun LC-ESI MS/MS	↓apo A-I, ↑apo C-I, ↑apo C-III, ↑apo E	Heller et al., 2007
3 subjects having low HDL-cholesterol vs 3 subjects having high HDL-cholesterol	ultracentrifugation in salt density gradient (HDL_3) Top-down Differential Mass Spectrometry	380 peaks ↑ two forms of apo C-III	Mazur et al., 2010

Subjects analysed	Purification methods Proteomic methods	Results	Ref.
10 subjects having low HDL-cholesterol vs 10 subjects having high HDL cholesterol challenged with lipopolysaccharide (24 hours follow up)	apo A-I immunocapturing SELDI-TOF MS	profound changes in 21 markers in both groups	Levels et al., 2011
10 ACS subjects vs 10 stable CAD vs 10 healthy control subjects	KBr sequential ultracentrifugation 1D LC-MS/MS WB ELISA	67 proteins identified ↓ apo A-IV ↑SAA ↑complement C3	Alwaili et al., 2011

↑, increase. ↓, decrease.

Table 4. Overview of the lipoproteomic studies related to atherosclerosis in humans reviewed in the chapter.

immunopurification methods that rely on antibodies specific for the dominant protein of each class (Levels et al., 2007, 2011; Ogorzalek Loo et al., 2004; Rashid et al., 2002; Rezaee et al., 2006). Although this procedure does not lead to loss of weakly associated protein, it tends to nonspecifically co-purify associated proteins as serum contaminants and other lipoprotein fractions having the same antibody target (e.g. apolipoprotein A-I is the main HDL apolipoprotein but it is also present in both VLDL and LDL fractions). Other lipoprotein isolation methods that have been applied in lipoproteomic studies, involve electrophoretic techniques, specifically free solution isotachophoresis (Böttcher et al., 2000), and chromatographic techniques, such as fast protein liquid chromatography (Collins & Olivier, 2010; Richardson et al., 2009) and size exclusion/affinity chromatographies (Gordon et al., 2010).

2.5.1 VLDL

Both 2DE coupled to MS and gel-free MS approaches have been applied to the study of VLDL protein composition. Mancone et al., by using 2DE coupled to MALDI TOF/TOF MS analysis, provided a detailed map of VLDL, isolated by the classical ultracentrifugation method, from a plasma pool of 3 healthy volunteers. They identified two newly VLDL-associated proteins, namely apo L-I and prenylcysteine lyase that were known to be associated with HDL, and some post-translational modifications of Apo E (Thr^{212}glycosylations) and apo L-I (Ser^{296}phosphorylation) (Mancone et al., 2007). Sun et al. used two different analytical approaches to compare the protein content of VLDL and LDL isolated from pooled samples of healthy subjects by either NaBr or iodixanol gradient ultracentrifugation. By using a gel-free approach based on LC coupled to MS/MS analysis of tryptic digests labeled with iTRAQ (isobaric tag for relative and absolute quantitation) tags, they revealed 15 proteins differentially expressed in the two classes of lipoproteins. By using 2DE coupled with LC MS/MS, they further revealed 6 proteins differentially expressed as well. Moreover, the 5 apo A-I isoforms were found to be phosphorylated. This study, besides describing the VLDL and LDL lipoproteomes, provided insights into the

metabolic changes, in terms of protein composition, during physiological VLDL to LDL transition (Sun et al., 2010). While the studies described above focused on the human lipoproteome of mature VLDL particles, other researches tried to shed light on VLDL assembly and maturation in animal models. For example, Rashid et al. immunopurified apo B from rat liver microsomes treated with chemical crosslinkers. Then, using LC MS/MS technology, they identified 99 unique proteins that co-immunoprecipitated with apo B, many of which were ribosomal proteins (Rashid et al., 2002).
Bondarenko et al. applied MALDI TOF and ESI TOF MS techniques to identify low molecular weight proteins constituting VLDL from 3 healthy subjects and 1 hyperlipidemic subject without previous tryptic digestion. By this approach they identified 15 apo C isoforms and 1 apo E isoform and observed higher level of apo C-III in the hyperlipidemic subject (Bondarenko et al., 1999).

2.5.2 LDL

LDL particles have been studied using different proteomic approaches. Karlsson et al. provided a 2DE map of LDL-associated proteins from a pooled plasma of 4 healthy subjects purified by KBr density gradient ultracentrifugation. Their results confirmed the presence of proteins known to be associated with LDL particles, showing that many of these were present in different isoforms. In particular, they detected three proteins not previously identified in LDL: serum amyloid A-IV, calgranulin A, and lysozyme C. To confirm that the proteins identified were truly associated with LDL rather than adsorbed during the isolation procedure, LDL was also purified by size-exclusion chromatography (Karlsson et al., 2005a). Moreover, they described three isoforms of apo M that were characterized for PTMs in a following work (Karlsson et al., 2006). Stahlman et al. applied 2DE coupled to MALDI TOF/TOF and SELDI TOF MS as well, to compare VLDL, LDL and HDL isolated from pooled plasma of 5 healthy donors by ultracentrifugation using either KBr or D_2O/sucrose to generate the gradient. VLDL profiles obtained with the two procedure of isolation were almost identical. Conversely, 2DE maps and SELDI TOF profiles of LDL and HDL were qualitatively similar, but differed in relative abundance of some protein species. Moreover, a reduced protein-lipids ratio was detected in LDL and HDL fractions purified by using KBr indicating that in the D_2O buffer the lipoproteins retained a higher content of exchangeable apoproteins (Ståhlman et al., 2008). LDL-associated proteins have also been studied using other proteomic approaches. Banfi et al. applied liquid-phase IEF and 1DE coupled with LC MS/MS to characterize the proteome of LDL isolated by density gradient ultracentrifugation from healthy subjects. They identified LDL-associated proteins not previously described, including prenylcysteine lyase (PCL1), orosomucoid, retinol-binding protein, and paraoxonase-1. The authors analysed PCL1 distribution in all the lipoprotein classes isolated by ultracentrifugation from 6 healthy subjects showing a decline from VLDL to LDL to HDL and its absence in lipoprotein-depleted plasma. Due to the oxidizing role of PCL1, they hypothesized that lipoproteins can themselves generate pro-oxidant species, thus suggesting a new role for lipoprotein in the development of atherosclerosis (Banfi et al., 2009). Bancells et al. analysed the proteome of LDL subfractions isolated by anion exchange chromatography after sequential ultracentrifugation of pooled healthy subjects plasma. Proteomic analysis, performed by LC MS/MS method, revealed the presence of 28 proteins most of which were involved in inflammation, coagulation and innate immunity, besides apolipoproteins involved in lipid metabolism. They observed that electronegative LDL, a minor subfraction of LDL fraction, has a higher content of minor proteins, especially apo F

and apo J, compared to electropositive LDL (Bancells et al, 2010). Collins et al. performed a proteomic analysis, applying LC MS/MS, to compare the lipoprotein-associated proteins derived from plasma and serum samples. They isolated both HDL and LDL from healthy subjects by means of fast protein liquid chromatography-size exclusion chromatography (FPLC-SEC). 16 proteins, several of which were complement subcomponents, were found only in the LDL fraction. 65 proteins were identified to be unique to HDL, while another list of proteins was found to overlap between the two lipoprotein fractions. Regarding the differences between plasma- and serum-derived LDL and HDL particles, the authors reported that the most relevant differences regarded fibrinogen proteins which were depleted in serum. Therefore, they stated that, apart from significantly higher levels of apo B-100 in LDL purified from serum samples, comparative proteomic analysis of plasma and serum gives similar results (Collins & Olivier, 2010).

Up to date, only few studies on LDL proteomics and atherosclerosis have been reported. 2DE coupled with LC MS/MS and label-free quantitative MS (LFQMS) was applied by Richardson et al. to the analysis of LDL in the early stages of atherosclerosis in an animal model. LDL was isolated by fast protein LC (FPLC) from non-diabetic hyperlipidemic, diabetic dyslipidemic, diabetic dyslipidemic under exercise training, and healthy Yucatan pigs (Richardson et al., 2009). They identified 28 unique proteins and detected several differential expression patterns for apo E, A-I, C-III, fibrinogen, apo B, adiponectin, alpha-2-macroblobulin, complement C1q, ficolin, and apo J. Since LDL was isolated from pigs in the early stages of atherosclerosis, the alterations observed might be involved in the initiating stages of the disease. LDL-associated proteins have also been studied using other proteomic approaches. For example Davidsson et al. applied SELDI TOF technologies to compare LDL associated proteins from atherosclerotic patients (having either metabolic syndrome or diabetes) to that from healthy subjects. They focused on small dense LDL isolated by means of gradient ultracentrifugation using D_2O. The results showed that LDL from patients had lower content of apo A-I, apo C-I and apo E and higher content of apo C-III, the latter responsible for higher affinity for arterial proteoglycans that could facilitate LDL in situ oxidative modifications (Davidsson et al., 2005).

2.5.3 HDL

HDL is the most studied among lipoprotein particles, probably because of its anti-atherogenic functions. Proteomic studies in humans succeeded in identifying, besides the known apolipoproteins involved in the lipoprotein metabolism, other associated proteins such as acute-phase response proteins, proteinase inhibitors, and members of the complement activation. Therefore, characterizing the HDL proteome should help in the identification of novel anti-inflammatory and cardioprotective actions of HDL and could provide insights into lipid therapy. The most used among the several proteomic approaches that have been applied to characterize the HDL-associated proteins is 2DE coupled with MS. Böttcher et al. applied two-dimensional non-denaturing gradient gel electrophoresis (2D-GGE) and immunoblotting to analyse HDL subfractions isolated from healthy subjects. By means of free solution isotachophoresis (FS-ITP), they separated 3 HDL subfractions, namely fast (fHDL), intermediate (iHDL) and slow-migrating (sHDL). Proteomic analysis showed compositional differences in HDL subfractions. In particular, they observed that fHDL and iHDL contained the bulk of HDL and of apo A-I. Apolipoproteins other than apo A-I and apo A-II were not detectable in fHDL, while sHDL contained several minor apolipoproteins such as apo A-IV, apo D, apo E, apo J, and factor H. Apo C-III was found

mainly in iHDL and sHDL with only little apo C-III in fHDL (Böttcher et al., 2000). A study performed by Ogorzalek Loo et al. suggests that a synergy between classical 2D gels and virtual 2D gels can be useful for studying HDL protein composition. Virtual 2DE is based on combining a first-dimensional isoelectric focusing (IEF) separation on polyacrylamide gels with MALDI MS surface scanning of the dried gel. In such a way, a virtual 2D gel can be created, generating an image in which mass spectrometry substitutes the second-dimension SDS-PAGE separation. By this approach the authors examined HDL isolated from human sera by selected-affinity immunosorption of apo A-I and revealed 42 unique masses for protein species with isoelectric points between pH 5.47–5.04 (Ogorzalek Loo et al., 2004). Heller et al. by using multiple proteomic approaches such as native or denaturing PAGE coupled with LC MS/MS, shotgun LC MS/MS and MALDI TOF MS profiling, analysed the protein complement of HDL_3, HDL_2, HDL_2/LDL and LDL/VLDL enriched fractions, isolated from a plasma pool of 10,000 healthy donors by density gradient ultracentrifugation. Therefore, they were able to characterize comprehensively the protein composition of the purified lipoprotein fractions (Heller et al., 2005). Karlsson et al. provided a detailed 2DE map of HDL_2 and HDL_3 isolated by salt gradient ultracentrifugation from pooled plasma of 4 healthy volunteers. Besides several isoforms of apolipoproteins already described to associate with HDL, they identified new proteins such as α-1-antitrypsin, two isoforms of salivary-α-amylase in HDL_2 and a glycosylated apoAII in HDL_3 (Karlsson et al., 2005b). By using 1DE and 2DE MALDI TOF MS and isotope-coded affinity tag (ICAT), Rezaee et al. detected many more proteins than Karlsson et al. in ultracentrifugally isolated HDL. This was the first study employing an ICAT method to identify lower abundance proteins. The overall identified proteins are known to be involved in different functions, such as lipid transport and metabolism, inflammation, immune system, hemostasis and thrombosis (Rezaee et al., 2006). The higher number of identified proteins could be ascribed to the use of ICAT method, that improve the sensitivity of the detection, as well as to the use of a single step of ultracentrifugation to isolate HDL. Khovidhunkit et al. investigated changes in proteins associated to HDL during inflammation by means of 2DE and LC MS/MS in an animal model. For this purpose, they analysed HDL isolated by salt gradient ultracentrifugation from sera of mice injected with normal saline or with endotoxin so detecting increased levels of SAA, apo E, apo A-IV and apo A-V and decreased levels of apo A-I and apo A-II in acute-phase HDL (Khovidhunkit et al., 2004).

Besides 2DE analyses, several groups have applied different gel-free proteomic approaches to characterize HDL proteome in healthy subjects. One of the first attempts was performed by Bondarenko et al. that used MALDI TOF MS and ESI TOF MS techniques to the analysis of intact protein of HDL isolated by density gradient ultracentrifugation in sucrose solution. They observed forty-nine peaks in the MALDI spectrum and 11 species in the ESI MS spectrum corresponding to the most abundant apolipoproteins, such as apo A-I, apo A-II, apo C-I, apo C-II, and apo C-III showing different isoforms due to post-translational modifications (Bondarenko et al., 2002). Applying immobilized pH gradient isoelectric focusing coupled with MALDI TOF MS, Farwig et al. were able to detect also SAA-IV in HDL isolated by ultracentrifugation in CsBiEDTA. They developed a successful method for recovering the apolipoproteins from immobilized pH gradient gels prior to MALDI analysis, demonstrating the analytical power of linking the IPG pI profile with MALDI TOF MS analysis (Farwig et al., 2003). Hortin et al. focused on HDL-associated low molecular weight peptides. By using HPLC and MALDI TOF MS or HPLC-ion trap mass spectrometry,

68 peptides in the 1-5 kDa size range were identified in ultracentrifugally isolated HDL. Among these, 19 were fragments derived from well-known HDL-associated protein while others were derived from non-lipoprotein plasma proteins as fibrinogen, α1-proteinase inhibitor, and transthyretin, suggesting that HDL particles may represent significant reservoirs of small peptides in the circulation (Hortin et al., 2006). Levels et al. applied SELDI TOF MS technologies to HDL isolated from normolipidemic individuals by means of immunocapturing directly on a SELDI protein chip covalently bound with anti-apo AI or anti apo-AII antibodies. In this way, 95 peaks in the 3–50 kDa molecular mass range and 27 more peaks between 50 and 160 kDa were detected (Levels et al., 2007). Gordon et al. applied MS-based proteomic approaches to the analysis of HDL purified from healthy subjects by means of gel filtration chromatography. To overcome problems related to nonspecific co-purification, they isolated only phospholipid-containing particles using calcium silicate hydrate (CSH), that were subjected to trypsin digestion while still bound to the CSH for identification by means of LC MS/MS. By this approach 47 proteins were identified. Among these, 14 were described as newly discovered HDL-associated proteins that support roles for HDL in complement regulation and protease inhibition (Gordon et al., 2010). To investigate the role of specific subspecies in the anti-atherogenic effects of HDL, Davidson et al. applied LC MS/MS to investigate the distribution of associated proteins across 5 subpopulations of HDL from healthy human volunteers. Subjecting one set of samples to sequential ultracentrifugation followed by salt gradient ultracentrifugation, and the other one to a single step of salt gradient ultracentrifugation they identified 22 and 28 proteins, respectively. Among them, the majority were apolipoproteins already known to be associated with ultracentrifugally-isolated HDL, while several complement factors and protease inhibitors already documented in other proteomic studies were not detected. By using peptide counts determined by MS, they monitored the relative abundance of a given protein across the HDL subfractions. Some proteins were found to associate preferentially to a specific subclass, while others were uniformly distributed across the subpopulations. This finding supports the proposal that HDL is composed of distinct subpopulations of particles that have discreet biological properties (Davidson et al., 2009).

A limited number of studies have focused on HDL proteomes in relation to atherosclerosis. Vaisar et al. used a shotgun LC MS/MS approach to identify proteins associated to total plasma HDL isolated from 20 healthy individuals. In this way, they described 48 proteins, 13 of which not yet known to associate to HDL. Moreover, they compared plasma HDL_3 fraction isolated from 6 healthy donors and 7 CAD patients. By means of Gene Ontology (GO) Consortium analysis, they were able to associate the array of HDL proteins to biological processes. Members of the complement pathway and endopeptidase inhibitors were found, suggesting that HDL plays also roles in regulating the complement system and protecting tissue from proteolysis. Thereafter, they found that some proteins associated to HDL_3 were upregulated in CAD patients, in particular apo C-IV, PON1, complement C3, apo A-IV, and apo E. Interestingly, they found three of these proteins also in HDL isolated from human carotid atherosclerotic tissues, being apo E the most abundant (Vaisar et al., 2007). In another study, they investigated whether combined statin and niacin therapy, which increase HDL cholesterol levels and reduce CAD risk, could reverse the changes in the protein composition observed in HDL_3. For this purpose HDL_3, isolated from 6 CAD patients before and 1 year after combined therapy, were subjected to LC–Fourier Transform Ion Cyclotron Resonance MS. By means of spectral counting and extracted ion chromatograms they found that treatment decreased apo E levels and increased apo J, apo F,

and phospholipid transfer protein levels (Green et al., 2008). In a successive study, they investigated if protein composition was altered in HDL_2 isolated from CAD patients. Ultracentrifugally isolated HDL_2 was digested with trypsin and analysed by MALDI TOF MS and pattern recognition analysis. The most significant informative features were then subjected to LC MALDI MS/MS for identification. This analysis revealed that HDL_2 of CAD subjects carried a distinct protein cargo with increased levels of apo C-III and decreased levels of apo C-I, two apolipoproteins involved in the metabolism of HDL particles. Moreover, they found increased levels of apo A-I peptides containing oxidized methionine indicating the occurence of oxidative processes in CAD patients (Vaisar et al., 2010). Heller et al. used a shotgun LC MS/MS approach to characterize HDL protein composition of 7 hypercholesterolemic subjects and 9 normolipidemic ones. They used the peptide match score summation index, based on probabilistic peptide scores for absolute protein quantitation. By this approach, they found that in hypercholesterolemic subjects apo A-I levels were reduced while apo C-I, apo C-III, and apo E levels were increased, suggesting that HDL protein composition could be altered in lipemic disease (Heller et al., 2007). Mazur et al. applied differential top-down mass spectrometry to compare HDL_3 protein profiles between 3 subjects having low HDL cholesterol and 3 subjects having high HDL cholesterol. Differently from the so called "bottom up" proteomic methods that are based on the digestion of proteins into short peptides, "top-down" proteomic techniques characterize intact proteins. In this study HDL_3 samples were analysed by a reverse-phase nano-HPLC coupled to a linear trap quadrupole Fourier transform (LTQ-FT) hybrid mass spectrometer. The authors found 380 peaks that changed significantly in protein abundance between high HDL-c and low HDL-c subject groups demonstrating that this approach is suitable for the detection of quantitative differences in proteins and protein isoforms in human HDL samples (Mazur et al., 2010).

Very recently, Levels et al., applied SELDI TOF MS to HDL isolated, by apo A-I immunocapturing, from healthy subjects having low HDL-c and high HDL-c challenged with an endotoxin for 24 hours. Overall they observed profound changes in 21 markers in both study groups proteome irrespective of HDL cholesterol levels (Levels et al., 2011). Alwaili et al. applied 1D followed by LC-MS/MS to HDL isolated by sequential ultracentrifugation from male control, stable CAD, and ACS subjects (n=10/group). They identified 67 HDL-associated proteins involved in lipid binding, acute-phase response, immune response, and endopeptidase/protease inhibition. By means of spectral counting they found that nine proteins were differently abundant. Among them, apo A-IV was significantly reduced, whereas serum amyloid A and complement C3 were significantly increased in ACS patients compared to either controls or CAD subjects, as confirmed by western blotting and ELISA (Alwaili et al, 2011).

Recently, our research group started to study lipoproteomic profiles in relation to atherosclerosis (Formato et al., 2011). For this purpose we purified plasma VLDL, LDL, and HDL from patients undergoing carotid endarterectomy and from healthy normolipidemic donors by single isopycnic salt density gradient ultracentrifugation, followed by a second step of ultracentrifugation. Samples were subjected to 2DE followed by PMF analysis as reported in figure 2. In this way, we identified 21 spots corresponding to about 96% of 52 protein spots detected in VLDL, 22 spots corresponding to about 92% of 43 spots in LDL, and 20 spots corresponding to about 96% of 60 spots in HDL. The relative abundance of several identified lipoprotein-associated proteins differed between patients and healthy subjects (paper in preparation).

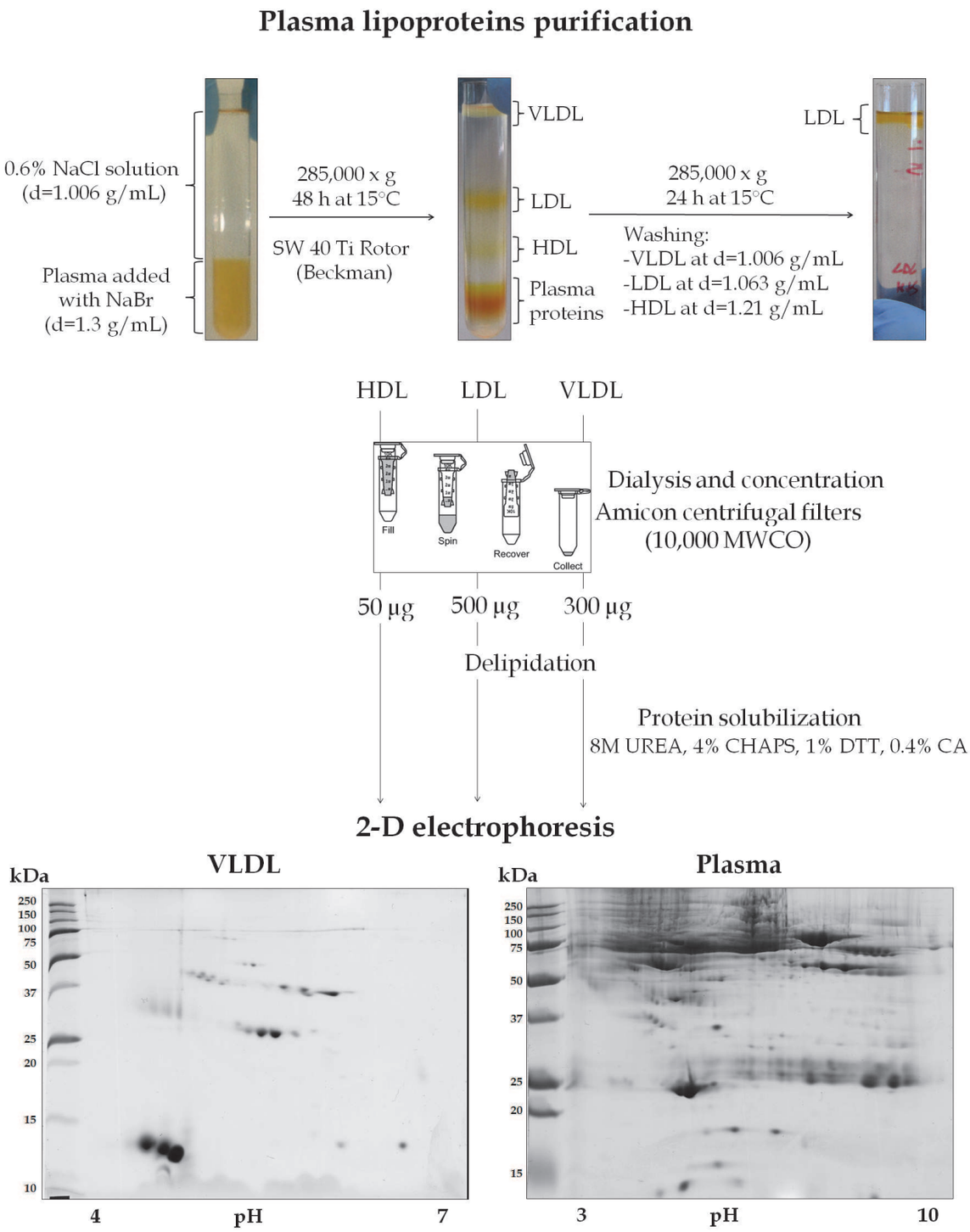

Fig. 2. Schematic workflow of the proteomic analysis of plasma lipoproteins adopted in our laboratory. Representative 2D maps of isolated VLDL and whole plasma are reported.

3. Conclusions

Plaque rupture and thrombosis are the most important clinical complications in the pathogenesis of vascular diseases. To date, many efforts have been done to elucidate mechanisms underlying plaque vulnerability and to identify reliable specific markers of plaques prone to rupture. In the last years, with the improvement of proteomic tools, large-scale technologies have been proved valuable in attempting to unravel pathways of complex diseases and biomarkers for early diagnosis and patients follow up. Collecting multiple biomarkers would be preferable over single markers in terms of higher sensitivity and specificity for the diagnosis of cardiovascular diseases. In this chapter, we have reviewed a great deal of information obtained by applying proteomics to the study of proteome/secretome from atherosclerotic tissues and plasma lipoproteins. In tissue proteomics, major drawbacks such as the plaque complexity, tissue sampling and availability, and the choice of the proper controls could affect the analysis. Even though results reported above seem to be quite promising, large-scale clinical studies are required to validate the usefulness of newly identified biomarkers. Moreover, there are several aspects of the atherosclerotic process that deserve further investigation. The analysis of laser captured microdissections by proteomics is still in its infancy but it could reveal valuable topological differences between specific areas of such a heterogeneous environment. Atherosclerotic plaques are characterized by the presence of an imbalance between oxidant and antioxidant species toward the former, leading to deep protein modifications. In this respect, recent advances in protein post-translational modifications analysis by mass spectrometry could be helpful. To date, many studies have been performed on proteome of purified plasma lipoproteins focusing mainly on HDL and LDL due to their association with atherosclerosis. As far as we know, no proteomic analyses have been performed on Lp(a). Since it is well known that elevated Lp(a) plasma levels are an important risk factor in atherogenesis, it would be of great interest to elucidate its apolipoprotein composition in relation to cardiovascular diseases. Another promising topic for future investigations is the characterization of proteomes of lipoproteins retained in atherosclerotic plaque. Finally, besides the great deal of work to be done in the future in both improving proteomic technologies and providing clues for the many aspects not yet investigated, it will also be necessary to put efforts on a comprehensive analysis of the huge quantity of data provided by the several proteomic studies.

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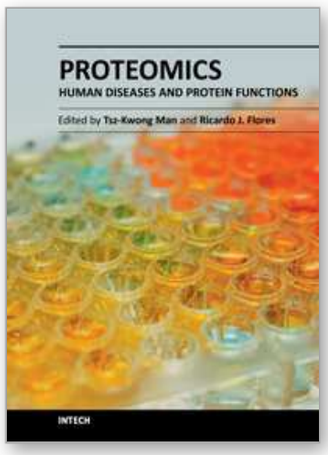

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Biomedical research has entered a new era of characterizing a disease or a protein on a global scale. In the post-genomic era, Proteomics now plays an increasingly important role in dissecting molecular functions of proteins and discovering biomarkers in human diseases. Mass spectrometry, two-dimensional gel electrophoresis, and high-density antibody and protein arrays are some of the most commonly used methods in the Proteomics field. This book covers four important and diverse areas of current proteomic research: Proteomic Discovery of Disease Biomarkers, Proteomic Analysis of Protein Functions, Proteomic Approaches to Dissecting Disease Processes, and Organelles and Secretome Proteomics. We believe that clinicians, students and laboratory researchers who are interested in Proteomics and its applications in the biomedical field will find this book useful and enlightening. The use of proteomic methods in studying proteins in various human diseases has become an essential part of biomedical research.

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