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Proteomic analysis of plasma apolipoproteins in relation to atherosclerosis

Direttore
Prof. Bruno Masala

Tutor
Prof.ssa Marilena Formato

Dottoranda
Dott.ssa Elisabetta Zinellu

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1 INTRODUCTION

Cardiovascular diseases (CVD) are the leading cause of death and illness in developed countries, being atherosclerosis the most important contributor. Atherosclerosis is a chronic inflammatory condition that can be converted into an acute clinical event by plaque rupture and thrombosis. Indeed, vascular inflammation not only plays a major role in the development of atherosclerosis, but also contributes to the acute onset of thrombotic complications (Libby, 2002).

The early lesions of atherosclerosis are called “fatty streak” and consist of accumulations of macrophages stuffed with lipids. These lesions, not clinically significant, are the precursor of more advanced lesions of lipid-rich necrotic debris and smooth muscle cells (SMCs). Such lesions typically have a “fibrous cap” consisting of SMCs and extracellular matrix that encloses a lipid-rich necrotic core and eventually lead to clinical manifestations. Common risk factors for atherosclerosis include hypercholesterolemia, familial predisposition and high blood pressure. In addition, smoking, obesity, diabetes and sedentary lifestyles all contribute to the increasing prevalence of atherosclerosis. These risk factors may contribute to the initiation of plaque formation, but the biological processes underlying atherogenesis is very complex (Lusis, 2000).

Anyway, it is well known that high levels of low density lipoprotein (LDL) cholesterol and low levels of high density lipoprotein (HDL) cholesterol strongly associate with an increased risk of CVD. Clinical studies suggest that apolipoprotein B100 and apolipoprotein AI levels, the major protein structural component of very low density lipoprotein (VLDL)/LDL and HDL respectively, could be better predictors of CVD risk (Walldius et al., 2001). Other protein components of LDL and HDL play important roles in lipid metabolism, energy homeostasis, and inflammation. Therefore, obtaining detailed information about apolipoprotein composition and structure may contribute to reveal their role in atherogenesis and eventually to develop new therapeutic strategies for the treatment of lipoprotein-associated disorders. The knowledge of lipoprotein protein composition has been improved in the last years especially with the improvement of proteomics technologies. However, the majority of the lipoproteomic

studies have been devoted so far to the characterization of the lipoprotein protein composition in healthy subjects, while few studies exist in relation to atherosclerosis.

1.1 ATHEROSCLEROSIS

Atherosclerosis is a progressive disease characterized by the accumulation of lipids and fibrous elements in the large arteries. The etiology of atherosclerosis is very complex and, as revealed by several epidemiological studies, numerous risk factors are involved either with a genetic component or environmental. Factors with a strong genetic component are: elevated levels of LDL, VLDL and lipoprotein (a) (Lp(a)), reduced levels of HDL, elevated levels of homocysteine, hypertension, diabetes and obesity, family history, systemic inflammation, and gender. Environmental factors involved are: high-fat diet; smoking; lack of exercise, and infectious agents (Lusis, 2000). There is also a strong body of evidence indicating that elevated triglycerides (TG) are an independent risk factor for atherosclerosis (Boullart et al., 2011; Sarwar et al., 2007).

The earliest and presumably initiating event in atherogenesis is the selective retention of circulating apo B100 containing lipoproteins, particularly LDL and Lp(a), in the subendothelial space, by means of specific interactions with artery wall proteoglycans (Williams & Tabas, 1995; Skalen et al., 2002). The retained lipoproteins induce a series of biological responses that lead to atherosclerotic lesion formation (figure 1). These responses are: (a) endothelial alterations; (b) recruitment of macrophages and lymphocytes to the intima which in turn induces an inflammatory state in the arterial wall; (c) accumulation of cholesteryl fatty acyl esters in macrophages and, to a lesser extent, smooth muscle cells (so-called 'foam cell' formation); and (d) medial-to-intimal migration of smooth muscle cells that produce a fibrous cap of extracellular matrix over the lesion (Lusis, 2000).

Lipoproteins diffuse passively in the subendothelium space through endothelial cells junctions; therefore the accumulation of LDL in the subendothelium depends on the circulating level of LDL. Regions of arterial branching or curvature, where flow is disturbed and cells have polygonal shapes and no particular orientation, show increased permeability to macromolecules such as LDL and are preferential sites for

lesion formation (Gimbrone et al., 1999). Once retained, lipoproteins are more susceptible to oxidative and enzymatic modifications. One of the modifications most significant for early lesion formation is lipid oxidation that gives rise to 'minimally oxidized' LDL species that have pro-inflammatory activity (Lusis, 2000). Mice lacking lipoxygenase have considerably diminished atherosclerosis, suggesting that this enzyme may be an important source of reactive oxygen species (ROS) in LDL oxidation (Cyrus et al., 1999). Under the stimulus of minimally oxidized LDL, the endothelial cells (ECs) produce a number of pro-inflammatory molecules, including adhesion molecules, chemotactic proteins such as monocyte chemotactic protein-1 (MCP-1), and growth factors such as macrophage colony-stimulating factor (M-CSF), resulting in the recruitment of monocytes to the vessel wall (Libby, 2002; Dzau et al., 2002). Oxidized LDL can also inhibit the production of nitric oxide (NO), a chemical mediator with multiple anti-atherogenic properties, including vasorelaxation. Among endothelial cell adhesion molecules likely to be important in the recruitment of leukocytes are ICAM-1, P-selectin, E-selectin, and VCAM-1 which bind to carbohydrate ligands on leukocytes (Cybulsky et al., 2001; Dong et al., 1998; Collins et al., 2000). As a result of the action of ROS produced by ECs and macrophages and of various enzymes such as sphingomyelinase (SMase), secretory phospholipase 2 (sPLA2), and myeloperoxidase (MPO), highly oxidized aggregated LDL is formed in the vessel. Such modified LDL is recognized by macrophage scavenger receptors such as SR-A, CD36 and CD68 whose expression is mediated by cytokines such as tumor necrosis factor-α (TNF-α) and interferon-ϒ (IFN-ϒ). The highly modified LDL is rapidly taken up by macrophages to form foam cells that finally undergo apoptosis leaving behind a growing mass of extracellular lipids and other cell debris (Libby, 2002). The uptake by macrophages is mediated in particular by 'scavenger' receptors, such as SR-A and CD36 that appear to be of primary importance. It has been shown that mice lacking these receptors show a modest reduction in atherosclerotic lesions (Suzuki et al., 1997; Febbraio et al., 2000). Macrophages and T cells secrete cytokines and growth factors that stimulate SMC migration/proliferation and extracellular matrix production. The intimal SMCs secrete extracellular matrix and give rise to a fibrous cap whose maintenance reflects matrix

production and degradation. The products of inflammatory cells are likely to influence these processes. For example IFN-ϒ produced by T cells inhibits the production of matrix by SMCs and macrophages produce various proteases that degrade extracellular matrix (Libby et al., 1996). The composition and vulnerability of a plaque is likely to be more important than the severity of stenosis in the development of thrombus mediated acute events. The most prominent features of vulnerable plaques include: thinning of the fibrous cap, a 'necrotic' or 'lipid' core consisting of dead macrophages, macrophage debris, and extracellular lipid and a state of inflammation, consisting of activated leukocytes and inflammatory cytokines (Aikawa & Libby, 2004; Libby, 2002). It is likely that thinning of the fibrous cap directly promotes plaque breakdown leading to dreadful acute clinical events.

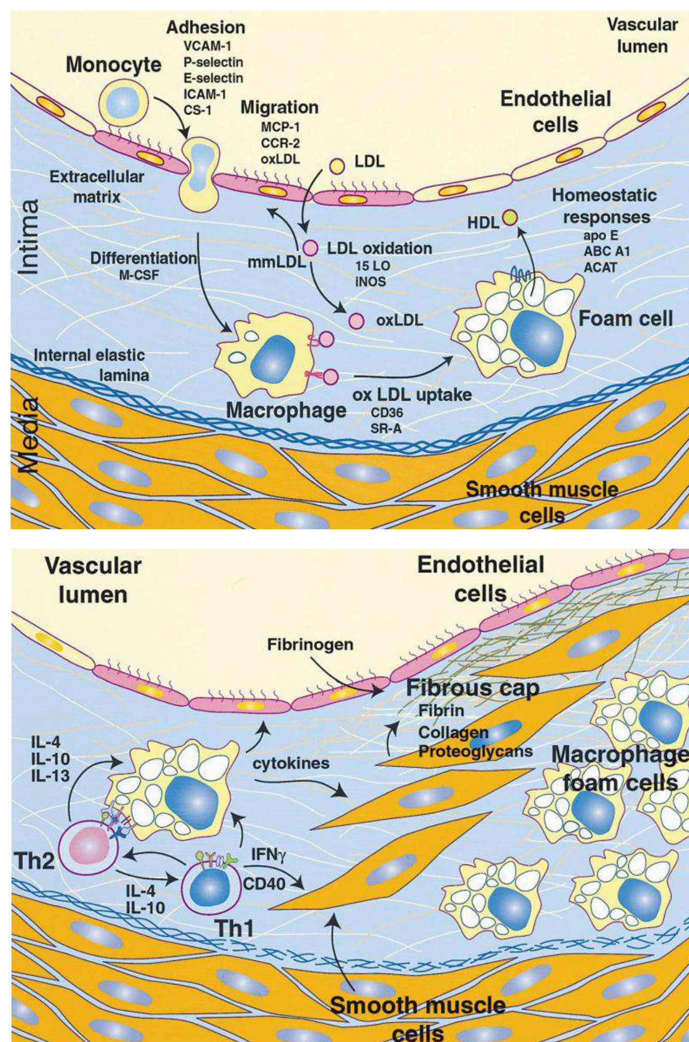


Figure 1. Atherosclerotic lesion development (Glass and Witztum, 2001)

1.2 LIPOPROTEINS

Lipoproteins are supramolecular complexes that deliver insoluble lipids from the tissues where they are synthesized to those that utilize, or store them. They consist of hydrophobic molecules, particularly triacylglycerol and cholesteryl esters, stabilized with a coat of amphipathic compounds: phospholipids, unesterified cholesterol and proteins, referred to as apolipoproteins (apo) (Vance & Vance, 2008).

The apolipoproteins have more than a stabilizing role, allowing lipoproteins to be recognized by specific receptors on cell surfaces, and acting as regulator of the activity of enzymes involved in lipoprotein metabolism (Bolanos-Garcia & Miguel, 2003).

There are several types of lipoproteins differing for chemical compositions, physical properties and metabolic functions. They may be classified according to their densities that depend on the lipid/protein ratio within the particle: in general the higher this ratio, the lower the density and the bigger the diameter. From lowest to highest density, lipoproteins are: chylomicrons (CM), very low density lipoproteins, low density lipoproteins and high density lipoproteins. An additional class of intermediate density lipoproteins (IDL) is sometimes included between VLDL and LDL. Chylomicrons and VLDL particles are often grouped as the triacylglycerol-rich lipoproteins, whereas LDL and HDL are more important as carriers of cholesterol. It is important to emphasize that lipoproteins, within each class, are heterogeneous in terms of their density, size, lipid and apolipoprotein compositions, as well as in their functional properties. Moreover, the lipoprotein particles are extensively remodeled in the plasma compartment (Gurr et al., 2002; Vance & Vance, 2008).

A further class of lipoprotein particles is known as lipoprotein(a) that displays a higher density than LDL. Its structure is that of an LDL particle, to the apo B-100 of which is covalently attached, by a single disulfide bond, a glycosylated multi-kringle protein, apolipoprotein(a) or apo(a) (Scanu, 2005). The physiological role of Lp (a) is unknown, although it is considered a risk factor for cardiovascular diseases (Scanu, 2001).

1.2.1 LIPOPROTEIN METABOLISM

Chylomicrons (diameter >100 nm; d< 0.95 g/ml) are the largest and least dense among lipoproteins and their function is to transport lipids of exogenous origin, principally triacylglycerols. The TG core is stabilized with a surface monolayer of amphipathic molecules: phospholipids, unesterified cholesterol and proteins. The core lipid also contains some cholesteryl esters and minor fat-soluble substances like vitamins and carotenoids. Their major apolipoprotein is apo B-48. Chylomicrons are secreted by the intestine and are abundant in plasma only after a meal. They are metabolized in several districts, such as the liver, the adipose tissue and the skeletal muscle (Gurr et al., 2002). As the chylomicron particles, secreted from enterocytes, enter the plasma, they interact with other particles and acquire other apolipoproteins, especially apo CII, apo CIII and apo E. In the capillaries of a number of extra-hepatic tissues, chylomicrons come into contact with the enzyme lipoprotein lipase (LPL), anchored to the luminal side of the endothelial cells (Redgrave, 2004). LPL, of which apo CII is an essential cofactor, hydrolyses the triacylglycerol core in the particle (Mead et al., 2002). The non-esterified fatty acids liberated during hydrolysis are used as energy source by various cells or are taken up by adipocytes and stored as triglycerides, while surface components dissociate and are acquired by other lipoproteins. The resulting particle is known as a chylomicron remnant that is removed from plasma and taken up by the liver through interaction with cell-surface receptors, probably lipoprotein receptor-related protein (LRP), a process that requires apo E (Redgrave, 2004; Cooper, 1997).

Very low density lipoproteins, (30 nm <diameter<90 nm; d< 1.006 g/ml), are the main vehicle for neosynthesized lipids, especially TGs, that they transport from the liver to various tissues, mainly muscles and adipose tissues. Together with TGs, VLDL particles contain also some free cholesterol and cholesterol esters (CE). Each VLDL particle is assembled around one molecule of apo B100 and contains other apolipoproteins especially apo B, E, and C (Cushley & Okon, 2002). VLDL particles are assembled in the liver using free fatty acid derived from chylomicrons or from de novo production. Within the plasma compartment, as described for chylomicrons, VLDL particles interact with LPL that hydrolyses their triacylglycerol core. Through successive interactions

with lipoprotein lipase they lose their triacylglycerols and subsequently their surface components (Gurr et al., 2002). A proportion of the resulting particles, namely the IDL (d=1.006-1.019 g/ml), are removed from the circulation by the hepatocytes through the interaction with LDL receptor (LDLR), while the remaining part is converted to LDL following a further reduction of the TG component by hepatic lipase (Perret et al, 2002).

Low density lipoproteins, (diameter ≈20 nm; d=1.019-1.063 g/ml), represent the end product of VLDL catabolism and are the major cholesterol-transporting lipoproteins in the plasma. The main structural apolipoprotein component is apo B100 that has an important role in the recognition of LDL by cells. Circulating LDL particles are removed by LDL-receptors on cell surfaces in the sub-endothelial space. The major site for removal of LDL particles is normally the liver although the LDL-receptor is expressed by most cells being a way by which cells can acquire cholesterol (Vance & Vance, 2008). The LDL-receptor is also known as the B/E receptor because it recognizes homologous regions on apo E and on apo B 100. The expression of LDL-receptors is finely regulated to maintain cellular cholesterol homeostasis and mutations in this receptor cause loss of function resulting in elevation in the plasma LDL-cholesterol concentration (familial hypercholesterolaemia) (Soutar & Naoumova, 2007). Once synthesized, the receptor is exported to specialized regions of the cell membrane where the cytoplasmic leaflet is coated with the protein clathrin. After binding of an LDL particle through interaction between its apolipoprotein B 100 molecule and the ligand-binding domain of the LDL-receptor, the receptor-LDL complex is internalized by endocytosis of the coated pit. Inside the acidic environment of the endosome, the LDL particle and receptor dissociate and the receptor is recycled back to the cell surface. The remainder of the particle is transferred to lysosomes, where the cholesteryl esters are hydrolysed by lysosomal acid hydrolases (Gurr et al, 2002).

High density lipoproteins (diameter 10 nm; d=1.063-1.21 g/ml) are responsible for the reverse transport of cholesterol in excess from different tissues to the liver and steroidogenic tissues for metabolism and excretion. Its main apolipoprotein component is apo AI. HDL maturation is a complex process involving the secretion of lipid-poor

particles and extracellular lipid acquisition, mainly cholesterol and phospholipids (Rader, 2006)

Immature lipid-poor HDL particles acquire unesterified cholesterol interacting with cell membranes. The protein involved in this case is a member of a family of cell-membrane-associated transporter proteins that bind ATP, (this particular protein is now known as ABCA1) (Oram, 2002; Oram & Heinecke, 2005). The unesterified cholesterol acquired by the particles is esterified with a long-chain fatty acid by the HDL associated enzyme lecithin-cholesterol acyltransferase (LCAT), activated principally by apo AI (Yokoyama et al., 1980), and also by others apolipoproteins such as AIV, CI and E (Yokoyama, 1998). This enzyme catalyses the transfer of a fatty acid from phosphatidylcholine, present in the HDL particle, to cholesterol to form a cholesteryl ester. The hydrophobic cholesteryl ester moves to the core of the particle, which becomes spherical as more cholesterol is acquired and esterified. This is probably the origin of the bulk of HDL-cholesterol in plasma, although some is also acquired from the surface components of chylomicrons and VLDL particles when their triacylglycerol is hydrolyzed by lipoprotein lipase (Gurr et al., 2002). When HDL-cholesterol reach the liver, offloads the cholesteryl ester content interacting with the scavenger receptor SR-BI (Krieger, 1999). Another mechanism for delivering HDL-cholesterol to the liver involves a plasma protein known as cholesteryl ester transfer protein (CETP), which is secreted from the liver and from adipose tissue. CETP, of which physiological inhibitor are known to be apo F (Wang et al., 1999) and apo C-I (Dumont et al., 2005), mediates the exchange of triacylglycerol and cholesteryl esters between particles, according to concentration gradients. When HDL particles become enriched in cholesteryl esters, they may exchange these esters for triacylglycerol carried in the triacylglycerol-rich lipoproteins, i.e. chylomicrons and VLDL.

A schematic overview of lipoprotein metabolism is reported in figure 2.

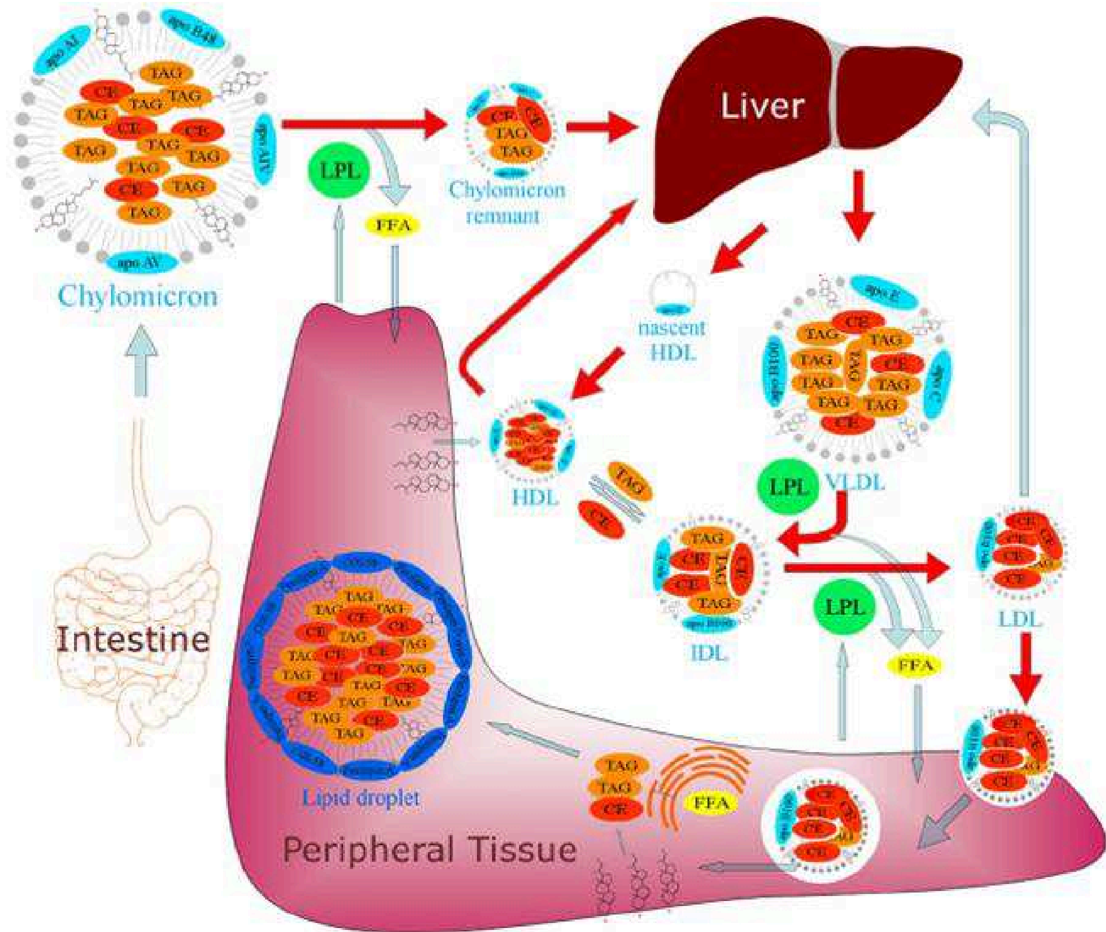


Figure 2. Schematic overview of lipid uptake and transport (Schittmayer & Birner-Gruenberger, 2009).

1.3 THE APOLIPOPROTEINS

The apolipoproteins found in plasma are classified into two broad types: the non-exchangeable and the exchangeable apolipoproteins. Some apolipoproteins are known as exchangeable because of their ability to move and exchange between lipoprotein particles. Apo B100, the principal protein components of LDL, VLDL, Lp (a), and apo B-48, the principal protein component of CM, are non-exchangeable apolipoproteins. They are very large and water-insoluble proteins that are assembled with lipids at their site of synthesis in the endoplasmic reticulum of liver or intestinal cells. These apolipoproteins circulate bound to the same lipoprotein particle through various metabolic transformations in plasma, until they are cleared via specific lipoprotein receptors. In contrast, the exchangeable apolipoproteins have much smaller molecular

masses than apo B100 or apo B48, have limited solubility in water in their delipidated states, can transfer among lipoprotein particles, and can acquire lipids while in circulation (Mahley et al., 1984).

The common function of all apolipoproteins is to help solubilize neutral lipids in the circulation but they have also roles in receptor recognition and hence in targeting of lipoproteins to specific destinations. Moreover several apolipoproteins are known to participate in lipoprotein metabolism modulating enzymatic activity (Bolanos-Garcia & Miguel, 2003).

1.3.1 EXCHANGEABLE APOLIPOPROTEINS

- Apolipoprotein AI

Apolipoprotein AI is a single polypeptide of about 28 kDa composed of 243 amino acids of known sequence (Brouillette et al., 2001). There are two major sites of synthesis: the intestine and the liver. It is the major protein component of HDL and promotes efflux of phospholipid and cholesterol from cell membranes, playing a central role in reverse cholesterol transport (RCT) (Frank & Marcel, 2000).

Apo AI is involved in RCT taking part to different steps: transfer of free cholesterol to acceptors mediated by ATP-binding cassette transporter A1 (ABCA1); esterification of free cholesterol to form cholesteryl esters by the HDL-associated enzyme LCAT; delivery of cholesterol esters to the liver mediated by scavenger receptor class B type I (SR-B1). Apo AI interacts with ABCA1 and accepts free cholesterol in the first step (Oram, 2002), acts as a cofactor for LCAT in the second one (Jonas et al., 2000) and mediates interaction of HDL with SR-B1 in the third one (Krieger, 1999). Moreover HDL and apo AI play a significant role in both modulation of endothelial function and inhibition of low-density lipoprotein induced monocyte chemotaxis (Barter et al., 2004).

- Apolipoprotein AII

Apolipoprotein AII is the second most abundant protein component of HDL and its major site of synthesis is the liver. In humans, apo AII is composed of two identical polypeptide chains, each containing 77 amino acids, connected by a single disulfide bridge at position 6 in the sequence (Brewer et al., 1972; Brewer et al., 1986). It has been

shown that apo AII is involved in HDL particles remodeling displacing apo AI from the surface (Lagocki & Scanu, 1980). This could result in the impairment of reverse cholesterol transport mediated by apo AI. Apo AII also plays a crucial role in triglyceride catabolism by regulating LPL activity (Julve et al., 2010).

- Apolipoprotein AIV

Apolipoprotein AIV is a glycoprotein containing 376 amino acid residues with a molecular weight of 46 kDa (Weinberg & Scanu, 1983). It is synthesized primarily in the enterocytes of the small intestine and it is secreted into the circulation through the lymphatic system. Apo AIV may facilitate and/or mediate lipid absorption, transport, and utilization. Its synthesis and secretion rates increase after consuming a fat-rich meal especially in triglyceride rich lipoproteins concomitant with a decrease in HDL (Kalogeris et al., 1994; Dallongeville et al., 1997). Apo AIV may participate in reverse cholesterol transport by activating lecithin-cholesterol acyltransferase (Steinmetz & Utermann, 1985) and cholesterol ester transfer protein (Main et al.1996), and facilitating cholesterol efflux out of cells from different tissues (Steinmetz et al., 1990).

- Apolipoprotein CI

Apo CI is a 57 amino acid polypeptide with a molecular weight of 6.6 kDa (Jong et al., 1999). It is synthesized principally in the liver and has several roles in lipoprotein metabolism as the other members of the apolipoprotein-C family. Apo CI inhibits lipoprotein lipase (Berbee et al., 2005) as well as the apo E-mediated hepatic remnant clearance via the low-density lipoprotein receptor (LDLr) (Sehayek & Eisenberg, 1991) and the LDL receptor-related protein (LRP) (Weisgraber et al., 1990). These roles have been associated with hypertriglyceridemia. Moreover apo CI is involved in the modulation of the inflammatory response improving the presentation of lipopolysaccharide to macrophages, as revealed in vitro and in mice (Berbee et al., 2006).

- Apolipoprotein CII

Apolipoprotein CII is synthesized in liver and intestine as a 79 amino acid polypeptide with a molecular weight of 8.8 kDa (Jong et al., 1999). Apo CII, the first apolipoprotein for which a specific function was found, activates LPL as a cofactor (LaRosa et al., 1970).

It has been shown that the helical structure close to the C-terminal end of apo CII is important for the activation of LPL (Shen et al, 2010). Lack of functional apo CII leads to hypertriglyceridemia (Breckenridge et al., 1978).

- Apolipoprotein CIII

Apolipoprotein CIII is synthesized in liver and intestine as a 79 amino acid protein with a molecular weight of 8.8 kDa (Jong et al., 1999). Apo CIII inhibits lipoprotein lipase activity (Wang et al., 1985) and impairs clearance of apo B lipoproteins from the circulation by interfering with their binding to hepatic lipoprotein receptors (Clavey et al., 1995), so impairing catabolism of triglyceride-rich lipoproteins. A number of studies demonstrates that increased plasma apo CIII concentration predicts risk for coronary heart disease (CHD) (Sacks et al., 2000; Scheffer et al., 2008) and that this increase is associated with increased levels of small, dense LDL (Shin & Krauss, 2010). Moreover, it has been shown that apo CIII content of apo B-containing lipoproteins enhances their ability to bind to the vascular proteoglycan biglycan (Olin-Lewis et al, 2002). Apart from its role in lipoprotein metabolism, there are evidences that apo CIII may directly contribute to the development of atherosclerosis by being involved in diverse inflammatory responses such as enhancement of human monocytic cells adhesion to endothelial cells (Kawakami et al., 2006).

- Apolipoprotein E

Apolipoprotein E is synthesized in many tissues, primarily liver, brain, skin and tissue macrophages. It is a glycoprotein of 299 amino acids with a molecular mass of 34 kDa (Mahley & Rall, 2000). Apo E is involved in normal lipoprotein metabolism, especially in targeting remnant lipoproteins for removal from circulation being a ligand of LDL receptor and LDL receptor-related protein. Moreover it has been described a role in reverse cholesterol transport for apo E being an activator of LCAT (Yokoyama, 1998). It is a polymorphic protein, and this matter influences its functional and structural properties. The three common allelic isoforms, apo E2, apo E3 and apo E4, differ at positions 112 and 158. Apo E3, the most common isoform, contains cysteine and arginine, respectively, whereas apo E2 has two cysteines and apo E4 has two arginines at these positions (Weisgraber et al., 1981). Although these isoforms differ from one

another by only a single amino acid substitution, the changes have profound effects at the structural and functional level. For example, the binding of the different isoforms to LDL receptors is different: apo E3 and apo E4 bind LDL receptors with similarly high affinity, but the binding of apo E2 is weaker (Weisgraber et al., 1982). A number of studies have investigated the impact of apo E polymorphisms on cardiovascular disease, often yielding conflicting results. Several studies link the apo E4 with greater risk for cardiovascular disease, while the possible relation between the apo E2 and CVD risk is uncertain (Kolovou et al, 2003; Eichner et al, 2002). In addition to cardiovascular disease, apo E polymorphisms have been investigated as a risk factor for other pathophysiological conditions, including Alzheimer's disease. In this regard it has been shown that, of the three common allelic isoforms, apo E4 confers the greatest risk of developing Alzheimer's disease (Farrer et al., 1997).

- Apolipoprotein J

Apolipoprotein J (clusterin) is a ubiquitous disulfide linked heterodimeric glycoprotein composed of two 40 kDa subunits (de Silva et al., 1990a). It circulates associated to high density lipoprotein bound to apo AI (de Silva et al., 1990b) and, in complex with apo A-I, has cholesterol ester transfer activity (de Silva et al., 1990 a,b). Apo J is believed to protect cell membranes from damage and to participate in wound-repair processes (Rosenberg & Silkensen, 1995). It has been shown that Apo J accumulates in the artery wall during the development of atherosclerosis together with paraoxonase (PON) and Apo AI (Mackness et al., 1997). These proteins are the components of a specific HDL subspecies that has been implicated in the prevention of peroxidative damage. Moreover, it has been shown that apo J has a protective role against neointimal hyperplasia inhibiting VSMCs migration and restores endothelial nitric oxide synthase expression suppressed by TNFα (Kim et al, 2009).

- Apolipoprotein D

Apolipoprotein D is a protein component of human plasma lipoprotein particles, with maximum concentration in the high-density lipoprotein fraction. It belongs to the lipocalin superfamily that comprises a heterogeneous family of lipid-binding proteins, including fatty acid binding proteins (FABP), plasma retinol-binding proteins (RBP),

and apo M. In humans, apo D is a glycoprotein of 169 amino acids with an apparent molecular weight that varies from 19 to 32 kDa (Rassart et al., 2000). The heterogeneity in the apparent molecular weight has been attributed to differential glycosylation of apo D (Weech et al., 1986). As regard to lipoprotein metabolism, apo D contributes to improve triglyceride metabolism enhancing its hydrolysis and clearance (Perdomo et al., 2010). Moreover, it has been observed an increase in the cholesteryl esterification activity of LCAT in presence of apo D. This suggests a possible role for apo D in reverse cholesterol transport (Steyrer & Kostner, 1988).

➢ Apolipoprotein M

Apolipoprotein M (apo M) is a novel apolipoprotein belonging to the lipocalin protein superfamily whose members exhibit diverse properties such as lipid binding, transport, and immunological functions. Human apo M contains 188 amino acid residues, has a predicted molecular mass of 21 kDa and it is highly expressed in the liver and kidney (Hu et al, 2010). Human apo M contains a glycosylation site and both non-glycosylated and glycosylated forms of apo M are present in human plasma (Duan et al, 2001). It has been shown that apo M is required for HDL maturation and for cholesterol efflux to HDL and thereby it is implicated in the inhibition of atherosclerotic lesions formation (Wolfrum et al, 2005). Moreover, in healthy individuals, the plasma concentration of apo M was found to correlate positively with total cholesterol suggesting that apo M is linked to plasma cholesterol metabolism (Hu et al, 2010).

➢ Serum amyloid A protein (SAA)

SAA is a family of acute-phase proteins synthesized primarily in the liver in response to stimulation by cytokines. It is transported in plasma mainly associated to HDL, in particular to the denser subpopulation HDL3 (Coetzee et al, 1986), even though different studies describe an association with VLDL and LDL (King et al., 2011). During an acute-phase response, SAA becomes the major apolipoprotein on HDL, displacing apo A-I (Coetzee et al, 1986). Moreover, a number of different cell types known to have a role in atherogenesis have been reported to express SAA, but whether locally synthesized SAA may have different biological effects than systemic SAA is unclear (King et al, 2011). Different studies have reported that lipid-free SAA, but not SAA

complexed with HDL, exerts biological activity (Furlaneto & Campa, 2000; Wilson et al., 2008).

SAA has been shown to have proatherogenic effects, such as promotion of monocytes and neutrophils chemotaxis and stimulation of proinflammatory cytokines (Furlaneto & Campa, 2000; Badolato et al., 1994). Moreover it has been shown that overexpression of SAA could stimulate the synthesis of vascular proteoglycan with increased LDL-binding affinity (Wilson et al., 2008).

1.4 LIPOPROTEOMICS: STATE OF THE ART

The knowledge of lipoprotein protein composition has been improved in the last years especially with the improvement of mass spectrometry (MS) technologies. Indeed, recent mass spectrometric studies have revealed that lipoproteins carry an array of proteins previously unsuspected. MS is easily linkable to gel electrophoresis and liquid chromatography (LC). Moreover, quantification can be realized by MS with appropriate labeling techniques, e.g. isotope coded affinity tags (ICAT) and isobaric tag for relative and absolute quantitation (iTRAQ).

Two general approaches have evolved for protein analysis and have been applied to the study of apolipoproteins in VLDL, LDL and HDL: gel-based proteomics and gel-free proteomics (Vaisar, 2009).

The first methodology is widely used and involves electrophoretic gel-based separations, primarily one-dimensional (e.g., SDS-PAGE) or two-dimensional electrophoresis (2-DE). 2-DE allows resolving proteins based on their isoelectric point (pI) in the first dimension, and on their molecular size in the second one. The resulting protein spots can be proteolytically or chemically cleaved into peptides that, in turn, can be extracted from the gel and analyzed by MS or MS/MS. 2-DE can be useful for quantification of relatively abundant proteins and is also a powerful tool for identifying different protein isoforms or studying posttranslational modifications (Görg et al, 2004); the second approach consists of the separation of a mixture of either proteins ("top-down" approach) or peptides ("bottom-up" approach) by liquid-phase separation techniques, primarily liquid chromatography (LC), coupled with electrospray

ionization and tandem MS analysis (ESI-MS/MS). These techniques are powerful and suitable for the rapid identification of the components of complex sample mixtures and for the comparative quantitative analysis of the proteins contained in different samples (Vaisar et al, 2007).

Before overviewing lipoproteomic studies, it is important to note 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 ultracentrifugation 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 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 proteins, it tends to nonspecifically co-purify associated proteins as serum contaminants and other lipoprotein fractions having the same antibody target (e.g. apolipoprotein AI 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).

Several proteomic studies have been published so far on VLDL, LDL and HDL. The major part of these studies has been performed in healthy subjects, in the attempt to elucidate the apolipoprotein cargo of the lipoprotein species using different proteomic approaches. Conversely, only a few focused on lipoproteomics in relation to atherosclerosis.

1.4.1 LIPOPROTEOMICS IN HEALTH

Both 2-DE coupled to MS and gel-free MS approaches have been applied to the study of VLDL, LDL, and HDL protein composition in healthy subjects. These studies are summarized in table 1 and discussed below.

- VLDL

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 LI and prenylcysteine lyase that were known to be associated with HDL, and some post-translational modifications of Apo E (Thr^{212} glycosylations) and apo LI (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 2-DE coupled with LC MS/MS, they further revealed 6 proteins differentially expressed as well. Moreover, the 5 apo AI 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).

- LDL

Karlsson et al. provided a 2-DE map of LDL-associated proteins from 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, 2-DE 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 1-DE 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 B100 in LDL purified from serum samples, comparative proteomic analysis of plasma and serum gives similar results (Collins & Olivier, 2010).

- HDL

HDL is the most studied among lipoprotein particles, probably because of its anti-atherogenic functions. Proteomic studies in humans succeed 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 2-DE 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 AI. Apolipoproteins other than apo AI and apo AII were not detectable in fHDL, while sHDL contained several minor apolipoproteins such as apo AIV, apo D, apo E, apo J, and factor H. Apo CIII was found mainly in iHDL and sHDL with only little apo CIII in fHDL (Böttcher et al., 2000). A study performed by Ogorzalek Loo et al. suggests that a synergy between classical 2-D gels and virtual 2-D gels can be useful for studying HDL protein composition. Virtual 2-DE 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 AI 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 HDL3, HDL2, HDL2/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 2-DE map of HDL2 and HDL3 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 HDL2 and a glycosylated apo AII in HDL3 (Karlsson et al., 2005b). By using 1-DE and 2-DE 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.

Besides 2-DE 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 AI, apo AII, apo CI, apo CII, and apo CIII showing different isoforms due to post-translational modifications (Bondarenko et al., 2002). Applying immobilized pH gradient isoelectric focusing (IPG IEF) 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 either HPLC-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).

Table 1. Summary of lipoproteomic studies performed in healthy subjects. Studies on animal models are indicated in italics.

Subject analyzed	Lipoprotein purification methods Proteomic method	Results [n° of protein isoforms]	Ref.
VLDL			
a pool of 3 healthy volunteers	two step NaBr density gradient ultracentrifugation 2DE MALDI-TOF/TOF	Proteins identified: apo E [5], apo LI [7], apo CIV [3], apo AIV, apo AI, apo M, apo CIII and prenylcysteine lyase [2]	Mancone et al., 2007
2 pooled samples from normolipidemic healthy volunteers	Two step density gradient ultracentrifugation with either iodixanol or NaBr to isolate VLDL and LDL iTRAQ labeling and LC-nESI MS/MS; WB 2DE and LC-nESI-MS/MS; WB	Proteins differentially expressed: (VLDL vs LDL): ↑apo CI, ↑apo CII, ↑apo CIII, ↑apo CIV, ↑SAA IV, ↑apo E , ↑apo (a), ↓apo M, ↓apo F, ↓paraoxonase 1, ↓albumin, apo B100, apo AII, apo AI, apo L1 Proteins identified: apo CIII [3], apo CII [3], SAA [2], SAA IV [5], apo M [4], apo AI [5], apo D [4], apo E [6], apo J [3], apo LI [7], apo AIV [3], paraoxonase 1 [2], α-1-antitrypsin [2], prenylcysteine oxidase 1 [5], and albumin [2]	Sun et al., 2007
microsomes prepared from rat liver	*solubilized proteins were coimmunoprecipitated with antibody against apoB* *LC-ESI-MS/MS*	*99 unique proteins many of which were ribosomal proteins. Moreover many hepatic enzymes, some known chaperone proteins and ferritin heavy and light chains.*	*Rashid et al., 2002*
LDL			
plasma pool from 4 healthy volunteers	two-step KBr density-gradient ultracentrifugation 2DEMALDI-TOF-MS	Proteins identified: apo B100, apo CII, apo CIII [3], apo E [4], apo AI [2], apo AIV, apo J and apo M [3], SAA IV [2], calgranulin A, lysozyme C	Karlsson et al., 2005a

plasma pool from 5 healthy donors	D2O/sucrose procedure; KBr-based fractionation 2DE MALDI TOF/TOF and SELDI-TOF-MS WB	Proteins identified: α1 anti-trypsin, apo J, apo E, apo AI, apo CII, apo CIII, SAA IV	Ståhlman et al., 2008
6 healthy volunteers	two step density gradient ultracentrifugation liquid phase IEF, PAGE and LC-ESI-MS/MS. WB	Proteins identified: apo M, apo D, paraoxonase 1, apo AI, apo B100, apo AIV, apo E, apo J, apo H, SAA, prenylcysteine lyase	Banfi et al., 2009
pooled plasma from healthy normolipidemic subjects. LDL (-) vs LDL (+)	sequential ultracentrifugation. Anion exchange chromatography to fractionate LDL into LDL(+) and LDL (-) LC-ESI MS/MS analysis Immunoturbidimetry ELISA WB	28 proteins identified in both fractions: proteins belonging to the apolipoprotein families and proteins involved in innate immunity, inflammation and coagulation. Proteins differentially expressed: ↑apo E, ↑apo AI, ↑ apo CIII, ↑apo D, ↑apo AII, ↑apo F, ↑apo J	Bancells et al., 2010
plasma and serum of healthy volunteers	FPLC-SEC nano-HPLC ESI-MS	16 newly identified proteins in LDL fraction	Collins & Olivier, 2010
HDL			
healthy normolipidemic volunteers	FS-ITP 2D-GGE with subsequent immunoblotting ELISA WB	Apo AI ,apo AII, apo AIV, apo E, apo D, apo CIII, apo J, and factor H	Böttcher et al., 2000
healthy, young volunteers serum	density gradient ultracentrifugation in sucrose solution MALDI-TOF-MS ESI-TOF-MS	49 peaks in the MALDI spectrum 11 species in the ESI spectrum corresponding to different isoform of apo AI, apo AII, apo CI, apo CII, and apo CIII	Bondarenko et al., 2002.

Serum of a normolipidemic subject	ultracentrifugation in CsBiEDTA IPG-IEF MALDI TOF MS	apo AI, apo AII, apo CI, apo CII, apo CIII and SAA IV	Farwig et al., 2003
human sera	immunoaffinity chromatography Virtual 2-DE- MALDI-MS	42 unique masses for protein species with isoelectric points between pH 5.47-5.04	Ogorzalek Loo et al., 2004
human plasma pool of more than 10 000 donors	salt gradient ultracentrifugation pooling of lipoprotein in fractions: HDL3, HDL2, HDL2/LDL, LDL/VLDL Native/SDS, 2D PAGE and LC-MS/MS; native PAGE and LC-MS/MS; shotgun LC-MS/MS; MALDI-TOF MS	Proteins identified: α-2-macroglobulin, serotransferrin, albumin, α-1 antitrypsin, apo A-I, isocitrate dehydrogenase, α-2-HS glycoprotein, vitamin D-binding protein, α-1 acid glycoprotein, paraoxonase, apo AIV, apo L, apo J, haptoglobin related protein, apo D, galectin 7, apo E, apo F, serum amyloid A 4, apo AII, apo CI, apo CIII, aortic smooth muscle actin, apo CII, apo B, apo(a)	Heller et al., 2005
Blood samples obtained from a pool of four healthy volunteers	HDL2 and HDL3, were isolated by two-step KBr density-gradient ultracentrifugation 2DE-MALDI-TOF MS	apo AI [6], apo AII, apo AIV, apo CI, apo CII, apo CIII [2], apo E [5], apo M [2], SAA [2], and SAA-IV [6], α-1-antitrypsin, salivary α-amylase, apo L and a glycosylated apo A-II in HDL3	Karlsson et al., 2005b
400 mL of blood freshly collected from a healthy donor	KBr density gradient ultracentrifugation HPLC and MALDI-TOF MS, HPLC-ion trap MS	more than 100 peptide components were found in the size range from 1000 to 5000 Da 19 peptides derived from HDL-associated proteins; 49 peptides of proteins not considered to be components of HDL (fibrinogen, transthyretin, α1-proteinase inhibitor, α1B-glycoprotein, and inter-α-trypsin inhibitor).	Hortin et al., 2006

normal healthy volunteers	one-step salt gradient ultracentrifugation 1-DE and 2-DE MALDI-TOF, ICAT and LC-MS/MSMS. WB ELISA	Proteins identified: 56 HDL-associated proteins including all known apolipoproteins, proteins involved in hemostasis and thrombosis and proteins of the immune and complement system. Furthermore, growth factors, receptors and hormone-associated proteins were found.	Rezaee et al., 2006
normolipemic individuals	apo AI or apo AII immunocapturing SELDI-TOF MS	95 peaks were detected in the 3–50 kDa molecular mass range. Between 50 and 160 kDa, 27 more peaks were detected.	Levels et al., 2007
plasma pool from five healthy donors	D2O/sucrose procedure; KBr-based fractionation 2-DE and SELDI-TOF-MS WB	Proteins identified: α1 anti-trypsin, apo E, apo -I, SAA [2], apo CII, apo CIII, apo AII	Ståhlman et al., 2008
pool from 20 healthy normolipidemic donors 9 healthy normolipidemic donors	sequential ultracentrifugation and isopycnic density gradient ultracentrifugation isopycnic density gradient ultracentrifugation LC-ESI/MS	22 proteins from pooled samples were identified: apo AI, apo B, apo E, apo AII, apo M, SAA IV, apo CI, apo D, apo CIII, apo L1, apo CII, SAA (1 and 2), apo F, PON1, apo C-IV, PLTP, PAF AH IB, apo(a), apo J, PON 3, α1 anti-trypsin, apo A-IV. 6 more proteins from individual donors were identified: albumin, fibrinogen α chain, haptoglobin related protein, plated related protein, prothrombin, transthyretin	Davidson et al., 2009

3 healthy normolipidemic male donors	Gel filtration chromatography and purification of phospholipid-containing particles using CSH LC-ESI-MS/MS	Proteins identified: 47. 14 of them were described for the first time as HDL associated protein: complement factor B, heparin cofactor 2, Ig lambda chain C regions, complement C5 precursor, pigment epithelium-derived factor, α-1- antichymotrypsin, insulin like growth factor binding protein acid labile subunit, antithrombin III, kallistatin, lumican precursor, plasma protease CI inhibitor, tetranectin, complement C1s subcomponent, complement C2.	Gordon et al., 2010
plasma and serum of healthy volunteers	FPLC-SEC nano-HPLC ESI-MS	Proteins identified: 65 unique proteins.	Collins & Olivier, 2010

↑, increase. ↓, decrease.

1.4.2 LIPOPROTEOMICS IN RELATION TO ATHEROSCLEROSIS

Only few studies have been published on the study of VLDL, LDL, and HDL protein composition in relation to atherosclerosis. These studies are discussed in detail below and summarized in table 2.

- VLDL

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 CIII in the hyperlipidemic subject (Bondarenko et al., 1999).

- LDL

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, AI, CIII, fibrinogen, apo B, adiponectin, alpha-2-macroglobulin, 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 AI, apo CI and apo E and higher content of apo CIII, the latter responsible for higher affinity for arterial proteoglycans that could facilitate LDL in situ oxidative modifications (Davidsson et al., 2005).

- HDL

A limited number of studies have focused on HDL proteomes in relation to atherosclerosis.

Khovidhunkit et al. investigated changes in proteins associated to HDL during inflammation by means of 2-DE 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 AIV and apo AV and decreased levels of apo AI and apo AII in acute-phase HDL (Khovidhunkit et al., 2004). 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 HDL3 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 HDL3 were upregulated in CAD patients, in particular apo CIV, PON1, complement C3, apo AIV, 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 HDL3. For this purpose HDL3, 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 following study, they investigated if protein composition was altered in HDL2 isolated from CAD patients. Ultracentrifugally isolated HDL2 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 HDL2 of CAD subjects carried a distinct protein cargo with increased levels of apo CIII and decreased levels of apo CI, two apolipoproteins involved in the metabolism of HDL particles. Moreover, they found increased levels of apo AI 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 AI levels were reduced while apo CI, apo CIII, 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 HDL3 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 HDL3 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 AI 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 independently from HDL cholesterol levels (Levels et al., 2011). Alwaili et al. applied 1-D 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, three of which were confirmed by western blotting and ELISA: levels of apo AIV were found to be significantly reduced, whereas levels of serum amyloid A and complement C3 were significantly increased in ACS patients compared to controls and CAD subjects (Alwaili et al, 2011).

Table 2. Summary of lipoproteomic studies performed in relation to atherosclerosis. Studies on animal models are indicated in italics.

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 CIII	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 CIII (3 isoforms), ↓ apo CI (2isoforms), ↓ apo AI, ↓ apo E	Davidsson et al., 2005
non-diabetic hyperlipidemic, diabetic dyslipidemic, diabetic dyslipidemic under exercise training vs healthy Yucatan pigs	*fast protein LC (FPLC)* *2DE LC MS/MS label-free quantitative MS (LFQMS)*	*28 unique proteins*	*Richardson et al., 2009*
HDL			
sera of mice injected with endotoxin vs sera of mice injected with normal saline	*salt gradient ultracentrifugation* *2-DE and LC MS/MS*	*↑SAA,* *↑apo E,* *↑apo AIV* *↑apo AV* *↓apo AI* *↓apo AII*	*Khovidhunkit et al., 2004*
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	Proteins identified in total HDL: 48. HDL_3 analysis: ↑apo CIV, ↑PON1, ↑complement C3, ↑apo AIV, ↑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 CIII, ↓apo CI ↑apo AI 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 AI, ↑apo CI, ↑apo CIII, ↑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 MS	380 peaks ↑ two forms of apo CIII	Mazur et al., 2010
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 and 10 healthy control subjects	KBr sequential ultracentrifugation 1D LC-MS/MS WB ELISA	Proteins identified: 67 ↓ apo AIV ↑SAA ↑complement C3	Alwaili et al., 2011

↑, increase. ↓, decrease.

2 AIM OF THE STUDY

Lipoproteins have attracted a great deal of interest because of their implication in the development of cardiovascular diseases, such as atherosclerosis. It is well known that high levels of LDL cholesterol and low levels of HDL cholesterol strongly associate with an increased risk of developing atherosclerosis. Moreover, clinical studies suggest that levels of apo B100 and apo AI, may be better predictors (Walldius et al., 2001). Since the protein component of these particles is largely responsible for carrying out their various functions, obtaining detailed information about the apolipoprotein composition and structure may contribute to reveal their role in atherogenesis and to identify new biomarkers of the pathology. The identification of new biomarkers could be useful in diagnosis and could improve the therapeutic approaches adopted 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, in the last decade several proteomic approaches have been applied to the study of lipoprotein-associated proteins showing that lipoproteins carry an array of proteins previously unsuspected. The majority of these studies have been performed on healthy subjects, while only few studies have tried to shed light on the apolipoprotein composition in relation to atherosclerosis.

Therefore, the aim of this study was to characterize the apolipoprotein component of plasma lipoproteins isolated from atherosclerotic patients undergoing carotid endarterectomy and to identify differentially expressed apolipoproteins between patients and controls.

3 MATERIALS AND METHODS

3.1 SAMPLE COLLECTION

Analyses were performed on 4 pooled plasma samples from 57 healthy volunteers and 79 patients undergoing carotid endarterectomy (stenosis ≥ 70%). All patients were under pharmacological treatment for both hypertension and dyslipidemia. The main clinical parameters of patients are reported in table 3. Blood samples were collected after an overnight fast into Vacutainer tubes containing EDTA and immediately processed. After centrifugation at 2000 x *g* for 10 min at 4°C, plasma was separated and stored at -80°C until analysis.

Table 3. Main clinical parameters of the patients undergoing carotid endarterectomy analysed in this study.

Clinical parameters	Patients (79)
Age, y	69.2 ± 7.2
Sex ratio, m/f	48/31
Body mass index, kg/m^2	26.3 ± 3.9
Symptomatic, %	40.0
Transient ischemic attack, %	28.6
Ictus, %	71.4
Diabetes, %	30.6
Hypertension, %	87.8
Dyslipidemic, %	83.7
Triglycerides, mg/dL	123.9 ± 53.2
Total cholesterol, mg/dL	172.1 ± 43.4
LDL cholesterol, mg/dL	97.4 ± 37.8
HDL cholesterol, mg/dL	47.3 ± 13.4

Values are mean ± SD

3.2 LIPOPROTEINS PURIFICATION

Lipoproteins were isolated by isopycnic ultracentrifugation according to the methods described by Himber et al. (Himber et al, 1995) and McDowell et al. (McDowell et al, 1995) with slight modifications (figure 3). Briefly, 3.9 mL of pooled plasma samples was added with NaBr (d=1.3 g/mL) in centrifuge tubes (Beckman, Ultraclear 14 x 95 mm), overlaid with 8.1 mL of 150 mM NaCl and centrifuged at 285000 x *g* for 48 h at 4°C in a

Optima L90 series ultracentrifuge equipped with a SW40 Ti rotor (Beckman Coulter). To avoid contamination, lipoprotein fractions were then further purified by a second centrifugation step. VLDL, LDL and HDL fractions were floated in saline solutions at density 1.006, 1.063 and 1.21 g/mL, respectively, and were centrifuged under the same conditions as described above but for 24 h.

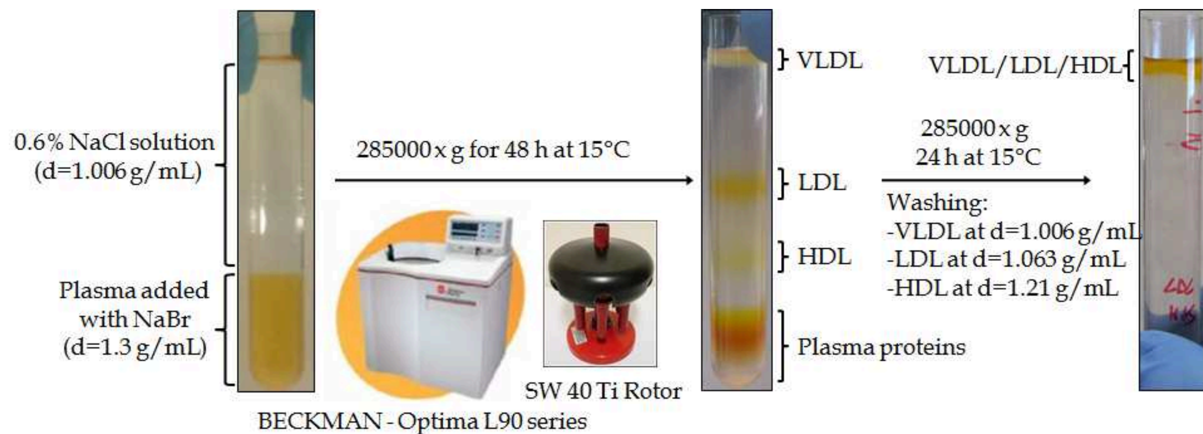


Figure 3. Lipoproteins purification method adopted in this study.

Lipoprotein fractions were then collected, desalted and concentrated using centrifugal filter units (10 KDa MWCO, Millipore). Protein concentrations were determined with a DC Protein Assay Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions, using bovine serum albumin as a standard. Aliquots of 500 µg of LDL and 300 µg of VLDL were delipidated by adding ice-cold tri-n-butylphosphate:acetone:methanol (1:12:1).

Precipitates were subjected to repeated boiling and sonication passages in 4% CHAPS, 10 mM TRIS, 1% DTT solution, cooled to room temperature, and added with 8M UREA, 4% CHAPS, 1% DTT, 0.4% CA solution for further solubilization followed by two-dimensional electrophoresis analysis. 50 µg of non-delipidated HDL were treated as LDL and VLDL precipitates.

3.3 2-DE ANALYSIS

Two-dimensional electrophoresis was conducted as previously described (Lepedda et al, 2009). Briefly, samples were applied to 70 mm IPG strips (pH 4–7, Bio-Rad, Hercules, CA, USA) by overnight rehydration loading at 20°C and subsequently focused at 50 µA/IPG strip for 22 kVh at 20°C.

After focusing, proteins were in-gel reduced (1% (w/v) DTT) and alkylated (2.5% (w/v) iodoacetamide), under continuous shaking, prior to the second dimension. The IPG strips were then sealed with 0.5% low melting point agarose in SDS running buffer at the top of slab gels (8 cm x 7 cm x 0.1 cm). SDS–PAGE was carried out, using 15%T, 3%C polyacrylamide gels, at 50V for 15 minutes and subsequently at 150V for about 90 minutes. Gels were stained with Mass Compatible Super Blue Stain Kit (Nurex srl, Sassari) and scanned at 36.3 µm resolution using a GS-800 densitometer (Bio-Rad, Hercules, CA, USA). Image analyses were performed using the PD Quest 2-D analysis software V 8.0.1 (Bio-Rad, Hercules, CA, USA).

3.4 IN-GEL DIGESTION AND MALDI-TOF MS ANALYSIS

Spots of interest were excised with sterile pipette tips and destained with 100 µl of 5 mM NH_4HCO_3/50% acetonitrile. Gel pieces were then dehydrated with acetonitrile, dried at room temperature and reswollen with 5 mM NH_4HCO_3 solution containing 10 ng/µl trypsin for 30 minutes on ice. Tryptic digestion was conducted overnight at 37°C. Then, the obtained peptides were mixed with an equal volume of α-cyano-4-hydroxycinnamic acid saturated solution (40% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid) and applied as a microcrystalline thin film onto a 96-spot MALDI target. Mass analyses were performed using a MALDI micro MX Mass Spectrometer (Waters). Peptide masses (plus one H^+) were submitted to database (NCBI and Swiss-Prot) search by using Mascot or Profound as search engines. Restrictions were Homo sapiens, 50–100 ppm mass deviation, maximum one missed cleavage by trypsin, and cysteine modification by carbamidomethylation.

3.5 WESTERN BLOTTING ANALYSIS

Western blotting was applied to compare expression of selected HDL proteins between patients and controls. Proteins were resolved by both mono-dimensional and bi-dimensional electrophoresis. After SDS–PAGE in 15%T, 3%C gels, electroblotting was performed at 350 mA for 1 hour 30 minutes onto PVDF membranes (GE Healthcare, UK). After transfer, membranes were blocked for 1 hour at room temperature with PBS-Tween-20 (0.1% Tween-20 in PBS) containing 3% non-fat dry milk and incubated overnight at 4 °C with the primary antibodies diluted in the same blocking buffer. The primary antibodies used were the following: goat polyclonal anti-apo AI antibody (Millipore), goat polyclonal anti-apo AII antibody (Millipore), mouse monoclonal anti-apo CI antibody (Abnova), rabbit polyclonal anti-apo CII antibody (Calbiochem), goat polyclonal anti-apo CIII antibody (Millipore), rabbit polyclonal anti-apo D antibody (Sigma-Aldrich), goat polyclonal anti-apo E antibody (Millipore), mouse monoclonal anti-SAA antibody (Abcam), goat polyclonal anti-apo B100 antibody (Chemicon). Then, membranes were washed with PBS-Tween-20 and incubated for 1 hour at room temperature with the (horseradish peroxidase) HRP-conjugated secondary antibodies diluted 1:5000 in blocking buffer (goat anti-mouse-HRP conjugate, Bio Rad; goat anti-rabbit-HRP conjugate, Bio Rad; rabbit anti-goat-HRP conjugate, Millipore). After membrane washing, proteins were visualized by ECL (Immun-Star western Chemiluminescent kit) followed by Q-One 4.6.3 software densitometric analysis (Bio-Rad Hercules, CA, USA).

A schematic workflow of the proteomic approach adopted is reported in figure 4.

Purified lipoprotein fractions

Fill Spin Recover Collect

Dialysis and concentration
Amicon centrifugal filters
(10,000 MWCO)

HDL 50 µg
LDL 500 µg
VLDL 300 µg

Delipidation:
Tri-n-butylphosphate:acetone:methanol 1:12:1
(90 minutes on ice)

Protein solubilization
- 4% CHAPS, 10 mM TRIS, 1% DTT
(boiling and sonication)
- 8M UREA, 4% CHAPS, 1% DTT, 0.4% CA
(vortexing and sonication)

1D SDS PAGE

Western blotting

2D electrophoresis

Detection

Electrotransfer
gel
filter paper
membrane
blotting pads

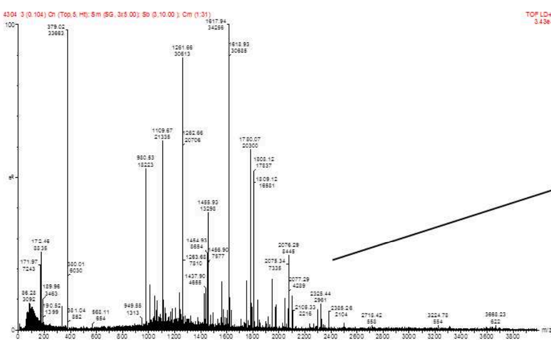
HDL

Mass compatible CBB staining

Image analysis
(PD Quest software, Bio-Rad)

SPOT EXCISION
DESTAINING: 50% ACN, 2.5 mM NH_4HCO_3
DEHYDRATION: 100% ACN
DIGESTION: 10ng/ml Trypsin in 5 mM NH_4HCO_3

Peptide mass fingerprinting analysis



MALDI TOF MS analysis
(MALDI Micro MX Micromass)

MASCOT Peptide Mass Fingerprint

http://www.matrixscience.com

Data base search

Figure 4. Schematic workflow of the proteomic approach adopted.

4 RESULTS

4.1 2-DE ANALYSIS

Two-dimensional electrophoresis combined with mass spectrometry was applied to identify VLDL, LDL and HDL apolipoproteins from patients undergoing carotid endarterectomy and healthy normolipidemic control subjects. Since the apo B100 is the major apolipoprotein of both LDL and VLDL, large amounts of LDL and VLDL were processed in order to maximize detection and identification of less abundant apolipoproteins.

Reproducible 2-DE maps were obtained from VLDL, LDL and HDL (figure 5, panels a-c). Moreover, by single spin and an additional washing step, we obtained a high degree of purification of all lipoprotein fractions (figure 5, panels a-c). This is evident considering that serum albumin, the most abundant plasma protein (about 50-60% of total) (fig. 5, panel d), is not detectable in any of 2-D gels after coomassie brilliant blue G-250 staining. Moreover, the lipoproteins purification method adopted allowed us to obtain HDL fractions without contaminants of VLDL/LDL origin, as shown by 1-DE western blotting using anti apo B100 antibody (figure 6).

By peptide mass fingerprinting (PMF), 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 were identified. All the identified proteins, their theoretical isoelectric point (pI) and molecular weight (Mr), the percentage of sequence coverage and some of known functions are listed in table 4. The sequence coverage ranged from 22% for Apo D to 61% for SAA.

The identified spots in both VLDL and LDL correspond to 2 isoforms of Apo J, 2 isoforms of Apo AIV, 5 isoforms of Apo E, 6 of Apo AI, 3 of Apo D, 2 of SAA, 1 of Apo CII and 2 of Apo CIII. Regarding to HDL, 3 isoforms of Apo E, 6 of Apo AI, 3 of Apo D, 2 of SAA, 1 of Apo CII and 2 of Apo CIII were identified.

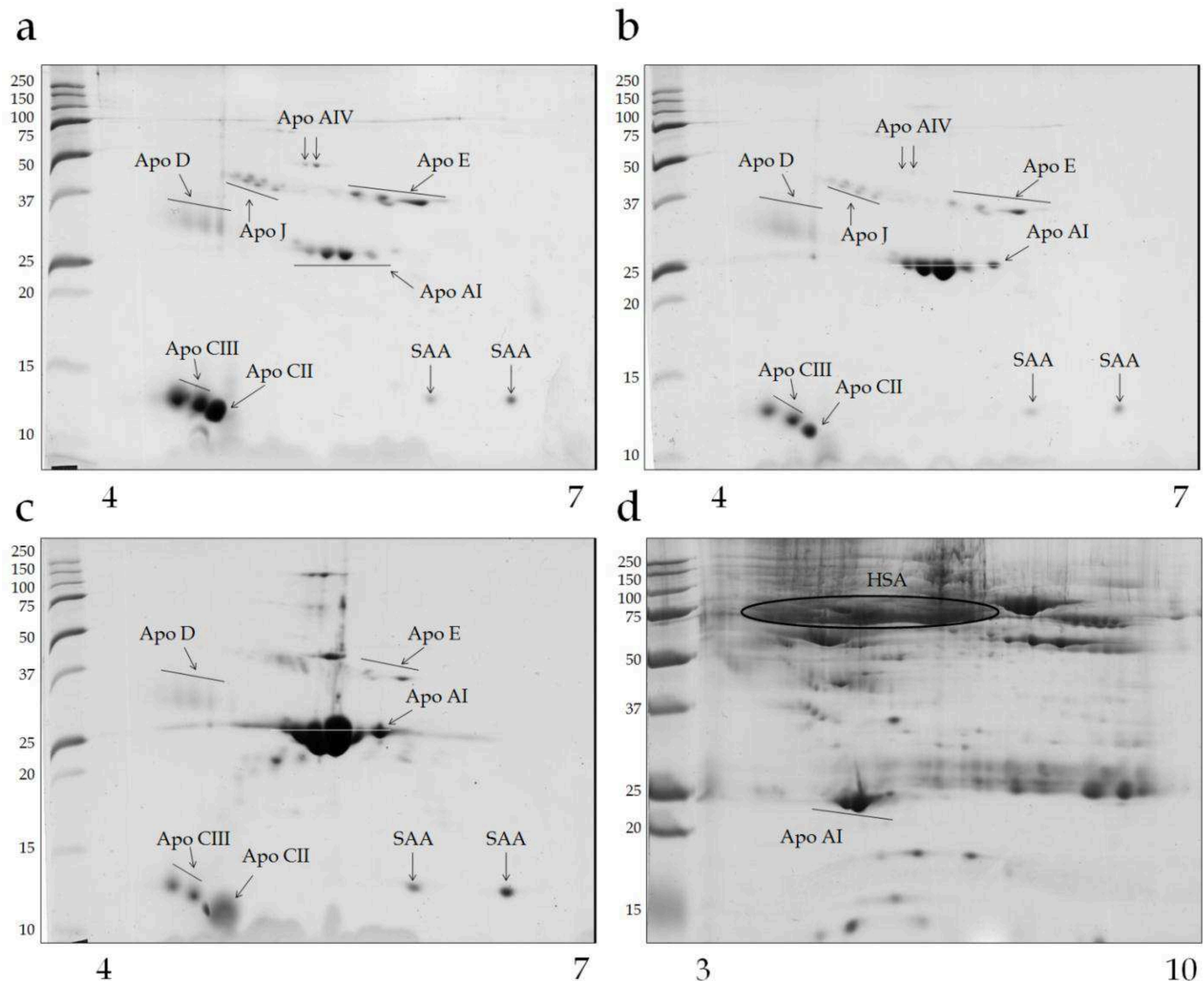


Figure 5. 2-DE representative maps of VLDL (panel a), LDL (panel b), HDL (panel c) and plasma (panel d).

Table 4. All the identified proteins, their theoretical isoelectric point (pI) and molecular weight (Mr), the percentage of sequence coverage and the differences in relative abundances obtained following image analysis are reported.

Isoforms	Apolipoproteins	Accession n°	Theo. pI	Theo. Mr (Da)	Seq. Cov. (%)	HDL	LDL	VLDL
2	APO J	gi|178855|	6,3	48772	26			
2	APO AIV	gi|178759|	5,2	45335	33			↓
5	APO E	gi|178853|	5,6	36185	31		↑	
6	APO AI	gi|90108664|	5,3	28061	33		↓	↓
3	APO D	gi|4502163|	5,1	21275	22		↑	↓
2	SAA	gi|225986|	5,9	11682	61		↑	↑
1	APO CII	gi|32130518|	4,7	11284	29			
2	APO CIII	gi|4557323|	5,2	10852	36	↓		

↑ higher levels in patients
↓ lower levels in patients

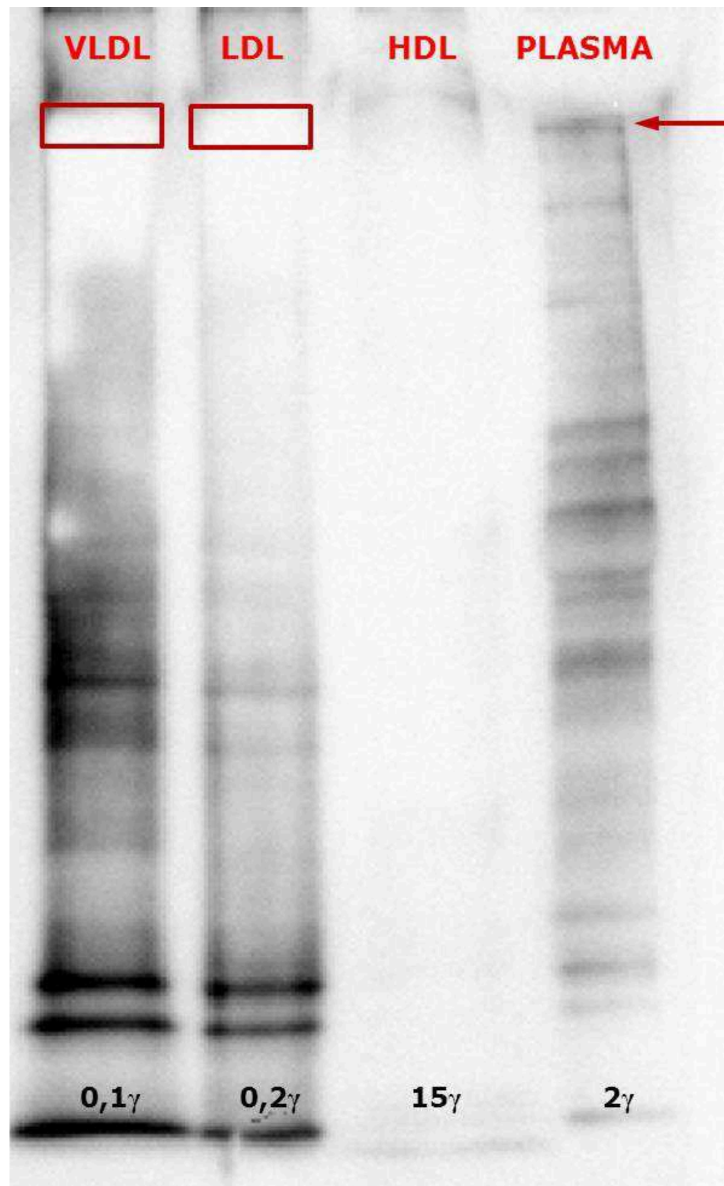


Figure 6. 1-DE western blotting of VLDL, LDL, HDL and plasma probed with anti- apo B100 antibody showing the purity of HDL fractions.

Image analysis revealed differences in the relative abundance of some identified apolipoproteins between patients having a carotid plaque and healthy subjects (table 4, figures 7-9). Relative abundances higher than two fold or lower than 0.5 fold, in patients compared to controls, were considered to be relevant. In particular, VLDL from patients presented higher levels of SAA and apo E, and lower levels of Apo AIV, Apo AI and Apo D while LDL displayed higher levels of Apo E, Apo D and SAA. Conversely, HDL showed quite similar apolipoprotein profiles between patients and controls apart from lower levels of Apo CIII in the former.

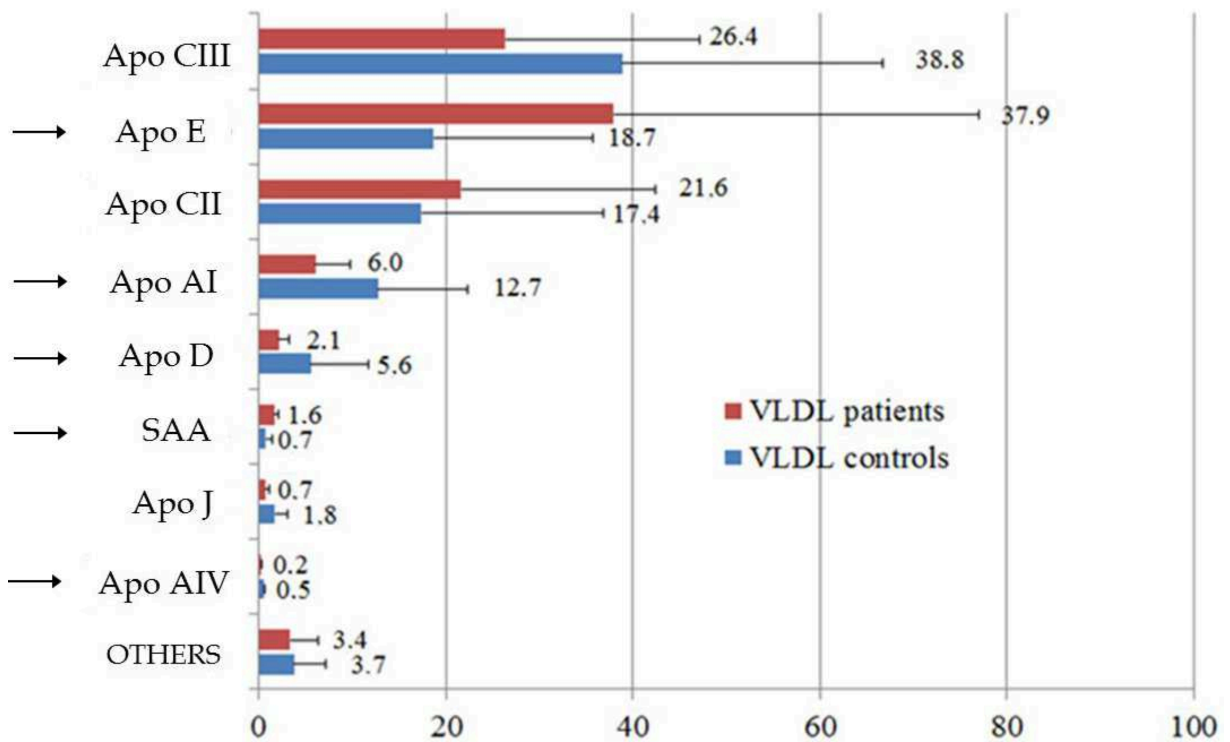


Figure 7. Relative abundances of the identified apolipoproteins in VLDL from both patients and controls. Differentially expressed proteins are evidenced by the arrows.

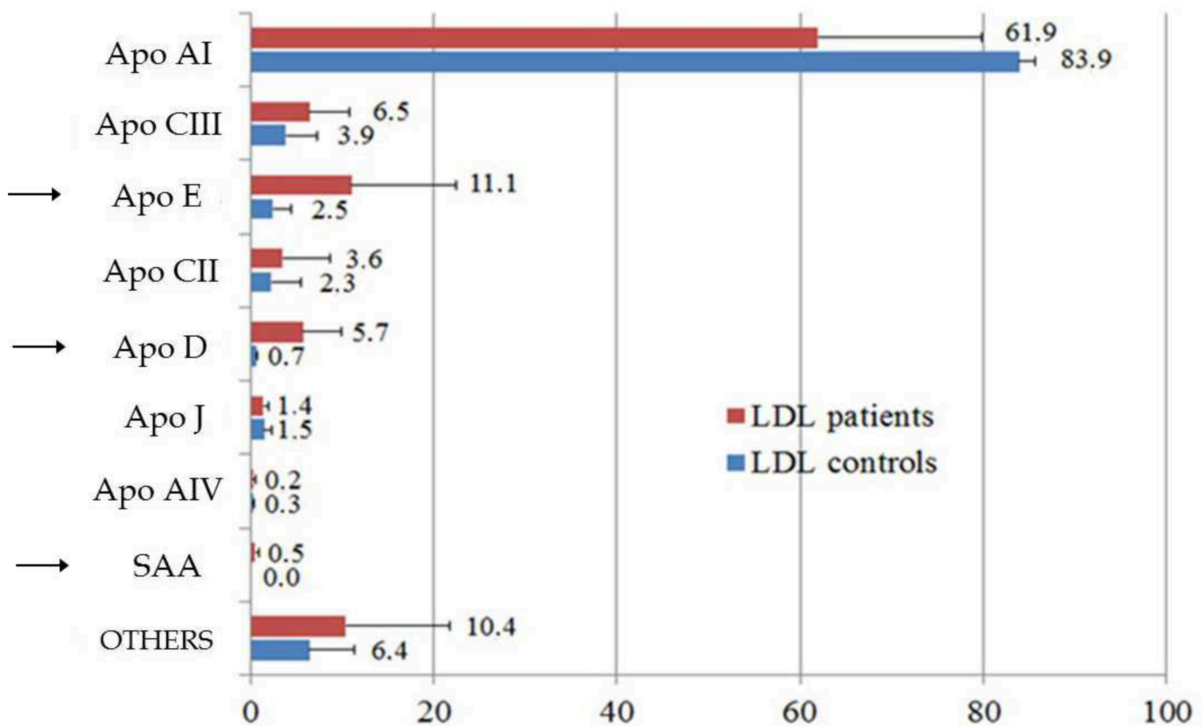


Figure 8. Relative abundances of the identified apolipoproteins in LDL from both patients and controls. Differentially expressed proteins are evidenced by the arrows.

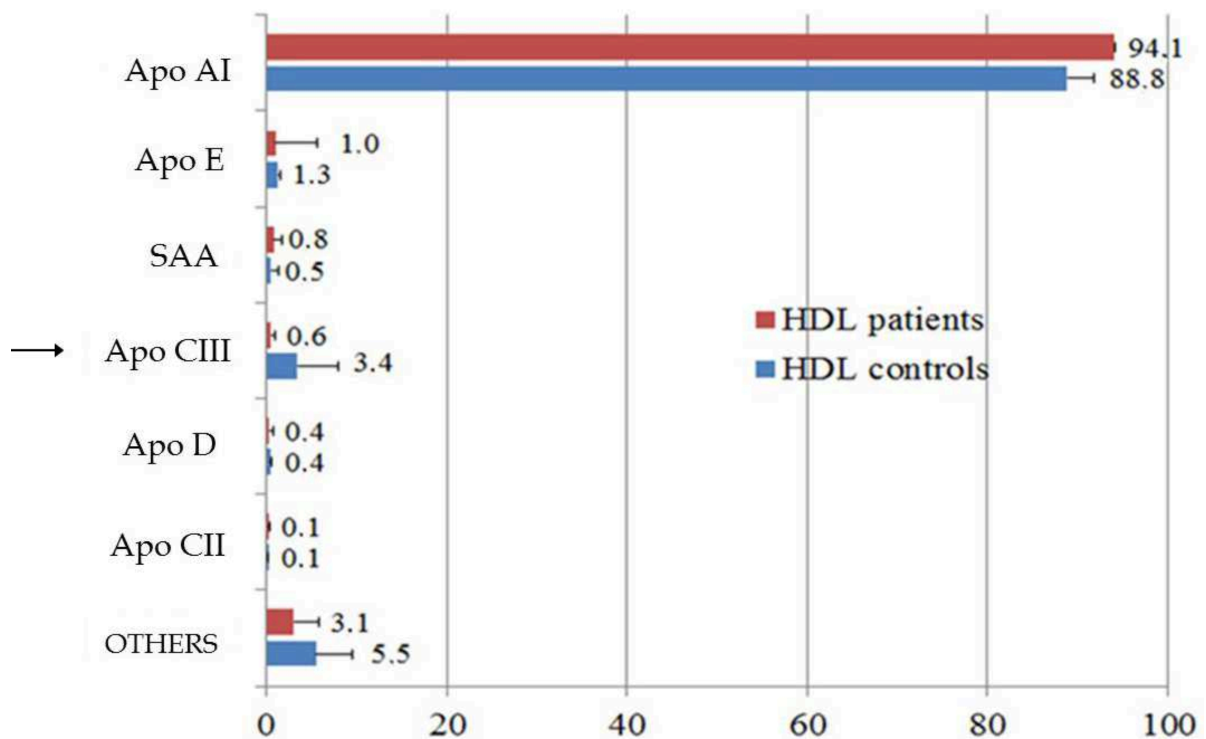


Figure 9. Relative abundances of the identified apolipoproteins in HDL from both patients and controls. Differentially expressed proteins are evidenced by the arrows.

4.2 WESTERN BLOTTING ANALYSIS

4.2.1 1-DE

1-DE western blotting analysis was performed on HDL fractions purified from 4 pools of patients who underwent carotid endarterectomy and from 4 pools of controls (figure 10). The image analysis revealed some differences (figure 11). Levels higher than 2 fold or lower than 0.5 fold in patients compared to controls were considered to be relevant. In particular, apo CII and SAA levels were higher (3 and 2.5 fold, respectively) while apo CI levels were lower (0.5 fold) in patients. It is worth noting that apo AI levels were superimposable in the two groups of sample. This is in agreement with the differential proteomic analysis that showed no differences in HDL apo AI between patients and controls.

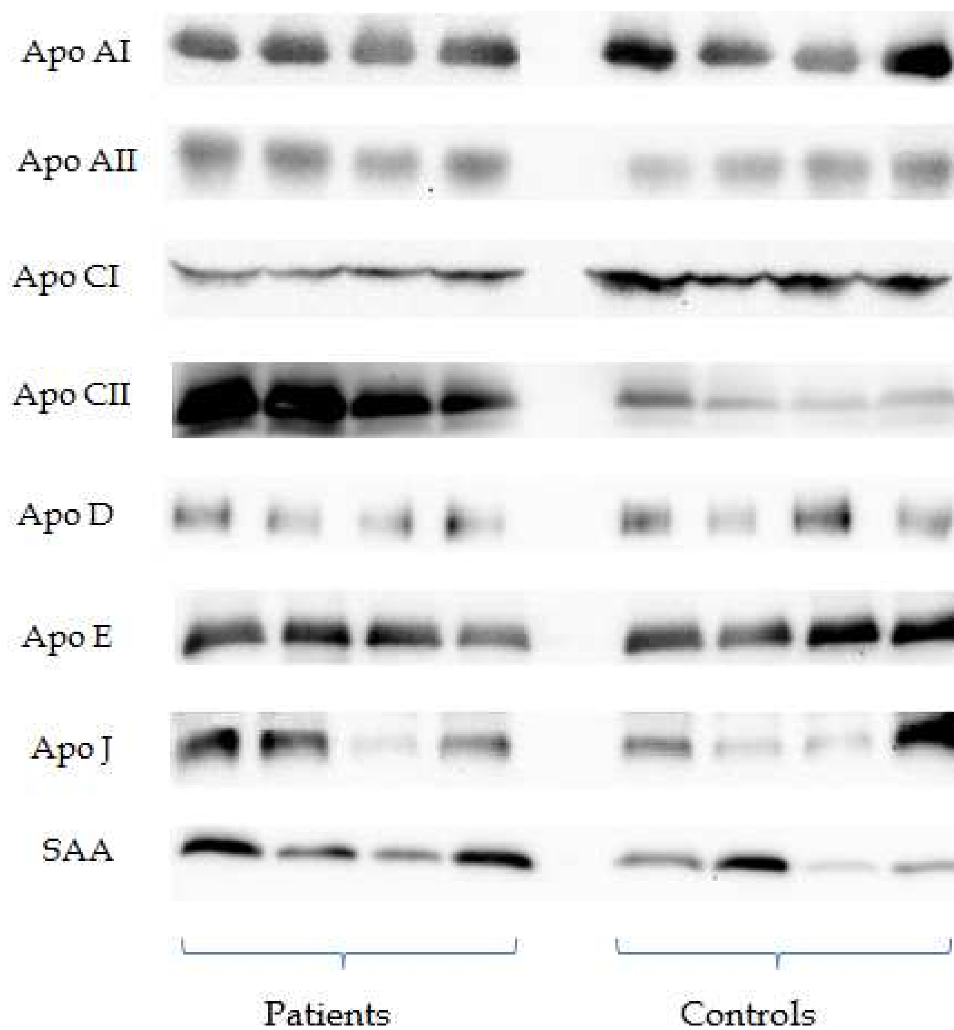


Figure 10. 1-DE western blotting of purified HDL probed with several anti-apolipoproteins antibody. The quantity of HDL loaded in the gel differed for each antibody depending on their sensitivity (0.3 µg for apo AI, 0.5 µg for apo AII, 4.5 µg for apo CI, 5.3 µg for apo CII, 1.8 µg for apo D, 2.2 µg for apo E and 4.5 µg for apo J and SAA).

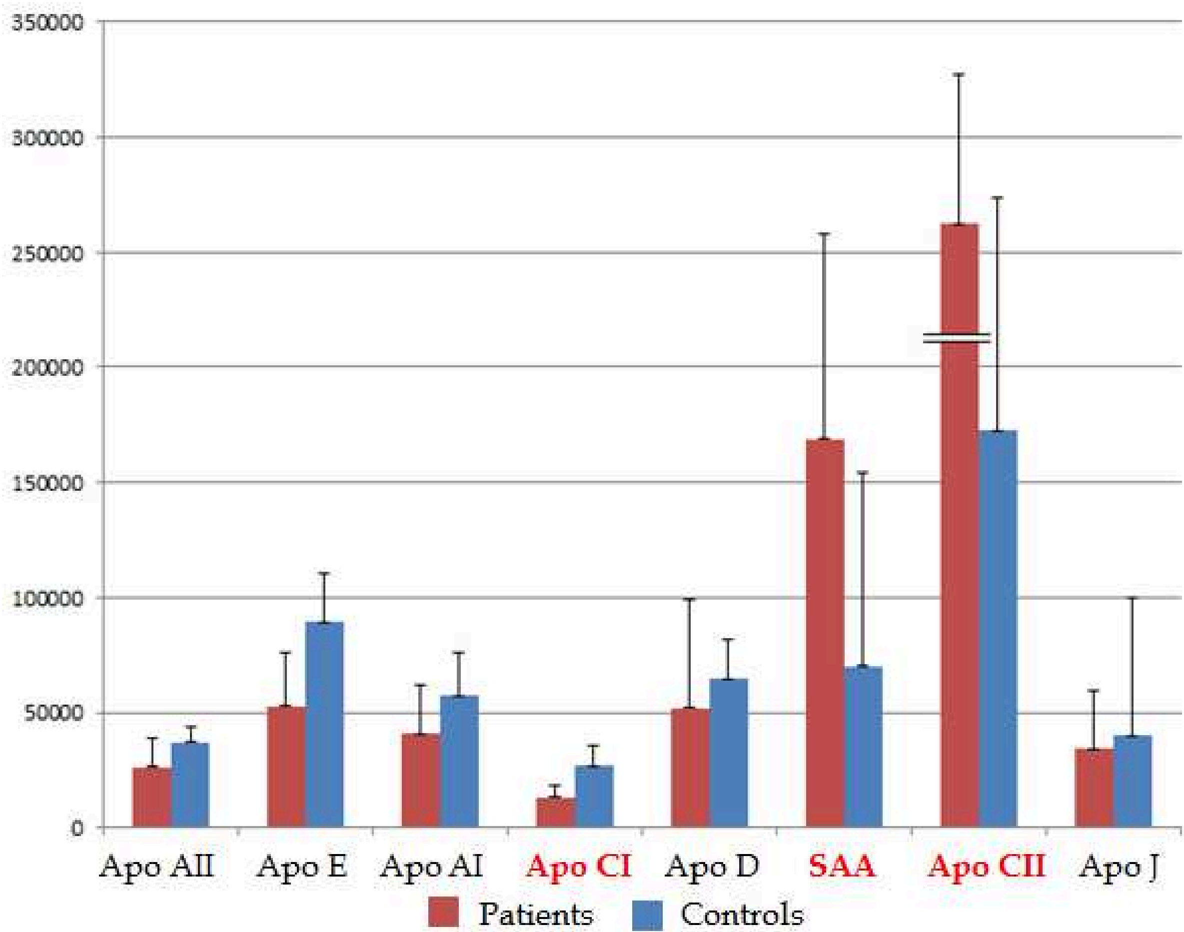


Figure 11. 1-DE western blotting image analysis showing different apolipoproteins profiles in patients. Differentially expressed proteins are reported in red.

4.2.2 2-DE

Preliminary results by 2-DE western blotting analysis performed on one HDL pool from patients and one HDL pool from controls showed that SAA and apo CII levels were higher in patients (figure 12), so partially confirming 1-DE western blotting results. The images regarding the 2-DE western blotting of non-differentially expressed HDL associated proteins are reported in figure 13.

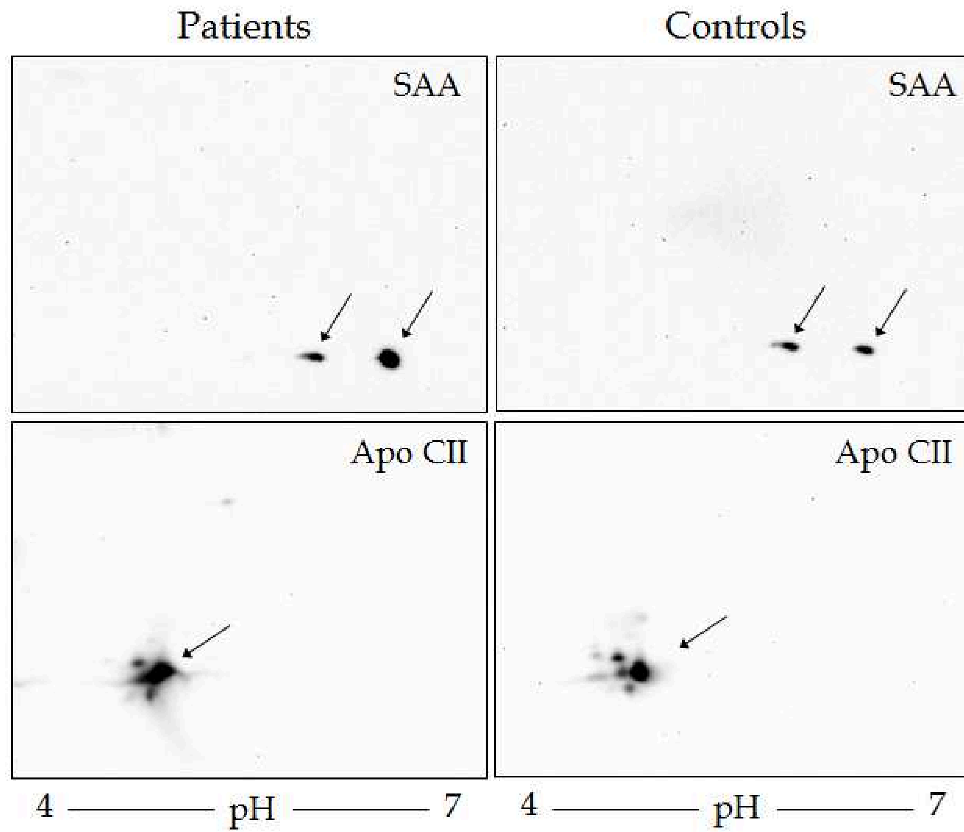


Figure 12. 2-DE western blotting of differentially expressed proteins in patients compared to controls. 25 μg of total HDL proteins for both SAA and CII detection were loaded.

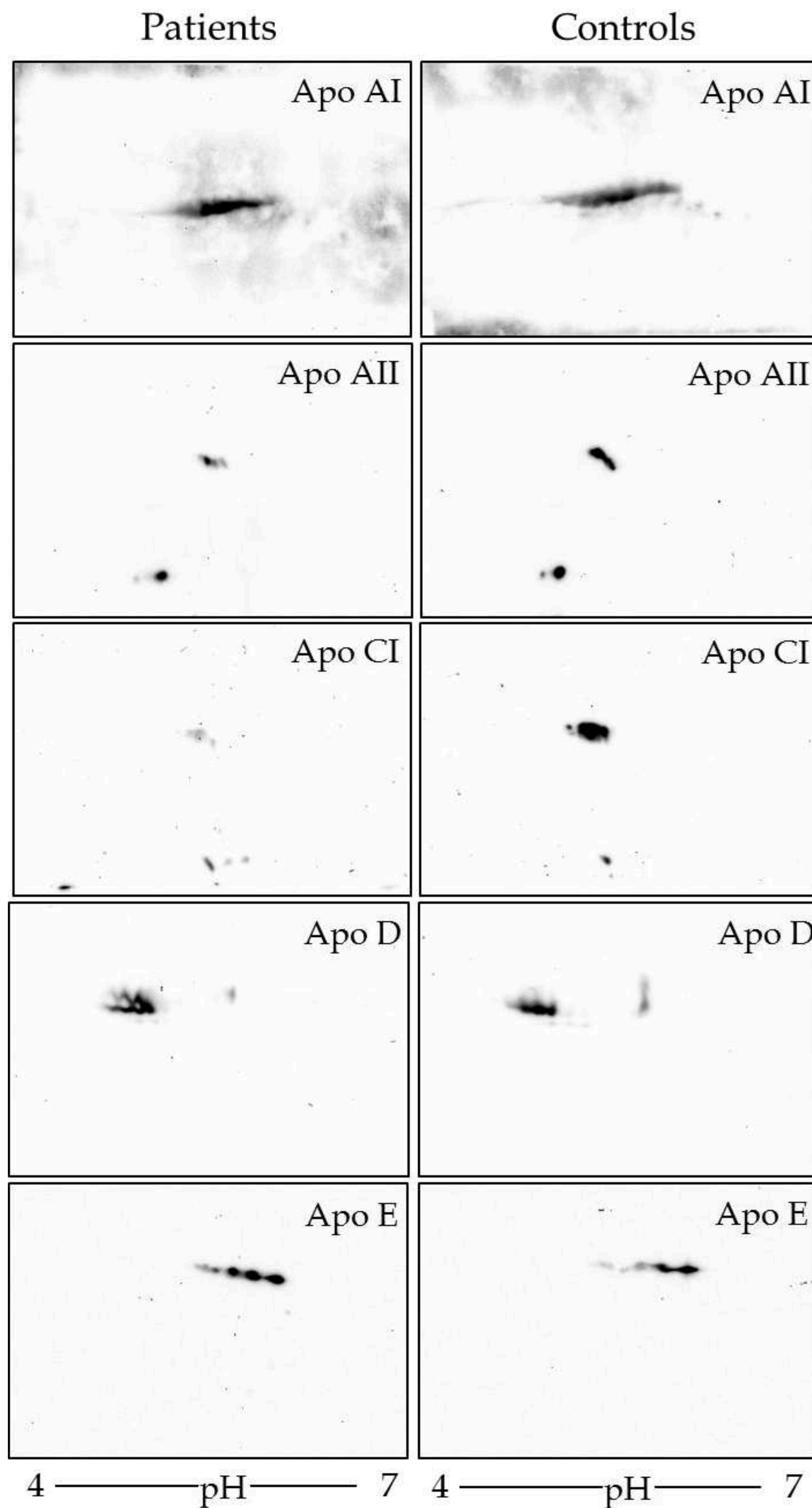


Figure 13. 2-DE western blotting of non-differentially expressed apolipoproteins. 10 µg of total HDL proteins were loaded for apo AI, 20 µg for apo AII, apo E and apo D and 50 µg for apo CI.

5 DISCUSSION

Atherosclerosis is a progressive disease of multifactorial etiology, which involves the interaction between genetic and environmental factors. Extensive epidemiological studies have shown that LDL cholesterol levels are directly related to the risk of developing atherosclerosis, whereas HDL cholesterol levels are inversely related to this risk. Since the protein component of lipoproteins is largely responsible for carrying out their various functions, obtaining detailed information about the apolipoprotein composition and structure may contribute to reveal their role in atherogenesis. For this purpose we purified plasma VLDL, LDL, and HDL fractions from 4 pooled plasma samples from 79 patients undergoing carotid endarterectomy and from 57 healthy volunteers by single isopycnic salt density gradient ultracentrifugation, followed by a second step of ultracentrifugation. This purification method allowed us to obtain highly purified lipoprotein fractions free of the major plasma contaminants such as albumin. Contamination of HDL by the other lipoprotein fractions was also excluded by western blotting analysis using a polyclonal anti apo B100 antibody.

By means of 2-DE followed by PMF several lipoprotein associated proteins were identified in VLDL, LDL, and HDL. All of them were already known to associate with lipoproteins. Image analysis has revealed that some of the identified proteins differed in relative abundance between patients and healthy subjects. In particular, VLDL from patients presented higher levels of SAA and apo E, and lower levels of apo AIV, apo AI and apo D. LDL displayed higher levels of apo E, apo D and SAA in patients.

As regard to HDL, 2-DE followed by PMF analysis showed quite similar apolipoprotein profiles between patients and controls apart from lower levels of Apo CIII in the former. Conversely, 1-DE and 2-DE western blotting analyses, performed only on HDL fraction, revealed some other differences. In particular, 1-DE analysis showed higher levels of apo CII and SAA and lower levels of apo CI in patients, while 2-DE confirmed 1-DE results for apo CII and SAA. It is worth noting that some of the above mentioned differences were in common to the various lipoprotein fractions analysed. In particular, higher levels of SAA in VLDL, LDL and HDL of patients were observed.

SAA is known as a positive acute-phase protein transported in plasma mainly associated to HDL even though different studies describe an association with VLDL and LDL as well (King et al, 2011). During an acute-phase reaction SAA plasma concentration can increase by up to 100- 1000-fold over the basal level (Uhlar & Whitehead, 1999) and becomes the major apolipoprotein on HDL displacing apo A-I (Coetzee et al, 1986). The presence of SAA on HDL reduces the affinity of this lipoprotein for hepatocytes but increases the affinity for macrophages (Kisilevsky & Subrahmanyan) redirecting the clearing of HDL from the liver towards macrophages, which has been postulated to lead to a functional change from anti-atherogenic/anti-inflammatory HDL particles, to pro-atherogenic HDL. Several epidemiological data associate CVD risk with elevated levels of SAA (Johnson et al, 2004; Schillinger et al, 2005) raising the question whether elevated plasma level of SAA is a consequence of inflammation or it has a direct effect on atherogenesis. In this regard SAA has been shown to have pro-atherogenic effects promoting monocytes and neutrophils chemotaxis and stimulating pro-inflammatory cytokines expression (Furlaneto & Campa, 2000; Badolato et al., 1994) thus contributing to the progression of atherosclerosis. Moreover it has been shown that overexpression of SAA could stimulate the synthesis of vascular proteoglycan with increased LDL-binding affinity (Wilson et al., 2008). Anyway, further studies are needed to answer the question whether elevated levels of SAA could play a causal role in atherosclerosis.

Another interesting result is the difference observed in the relative abundance of apo E between patients and controls in both VLDL and LDL. Apo E is involved in normal lipoprotein metabolism, especially in targeting remnant lipoproteins for removal from circulation being a ligand of LDL receptor and LDL receptor-related protein (Mahley & Rall, 2000). Apo E also influences the metabolism of lipoproteins in ways unrelated to its role as a ligand for receptors. The accumulation of apo E on the surface of lipoproteins slows the rate of lipolysis of lipoprotein triglycerides (Huang et al, 1998a; Huang et al, 1998b). Apo E can also affect plasma VLDL levels directly, regulating its synthesis and secretion (Huang et al, 1999). Generally, it is considered that apo E protects against the risk of developing atherosclerosis due to its role in targeting

remnant lipoproteins for removal from circulation. In contrast, apo E can, under certain conditions, increase atherosclerosis risk due to its role in inhibiting lipolysis or increasing VLDL production. Indeed, there is a strong body of evidence indicating that hypertriglyceridemia is an independent risk factor for atherosclerosis (Boullart et al., 2011; Sarwar et al., 2007).

Moreover, several studies linked the apo E4 isoform with greater risk for cardiovascular disease, even though in this regard results are often conflicting (Kolovou et al, 2003; Eichner et al, 2002). The higher apo E levels observed in both VLDL and LDL from patients could be related to the association of apo E with cardiovascular risk and raise the question about the isoform involved. Therefore it could be interesting to further investigate about this finding.

Overall, in this study different apolipoprotein profiles have been observed in VLDL, LDL and HDL of atherosclerotic patients undergoing carotid endarterectomy compared to healthy controls. Since only few proteomic studies on the apolipoprotein composition in relation to atherosclerosis have been performed, this study provides useful information on this topic. In the future it would be auspicable to analyze also atherosclerotic specimens of these patients in order to evaluate differences, if any, in apolipoprotein profiles between circulating and plaque retained lipoproteins. Indeed, improving the knowledge on apolipoprotein composition and structure may help in revealing the role of lipoproteins in atherogenesis. Moreover the identification of new biomarkers could be useful in diagnosis and could improve the therapeutic approaches adopted for the treatment of atherosclerosis.

6 SUMMARY

Atherosclerosis is a chronic inflammatory condition characterized by the accumulation of lipids and fibrous elements in the large arteries. The earliest and presumably initiating event in atherogenesis is the selective retention of circulating apo B100 containing lipoproteins, particularly LDL and Lp(a), in the subendothelial space, by means of specific interactions with artery wall proteoglycans.

Once retained, lipoproteins are more susceptible to oxidative and enzymatic modifications and induce a series of biological responses that lead to atherosclerotic lesion formation. These responses are: endothelial alterations, recruitment of macrophages and lymphocytes to the intima which in turn induces an inflammatory state in the arterial wall, accumulation of cholesteryl fatty acyl esters in macrophages (so-called 'foam cell' formation) and migration of smooth muscle cells that produce a fibrous cap of extracellular matrix over the lesion. The etiology of atherosclerosis is very complex and includes several important environmental and genetic risk factors. 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.

Lipoproteins are sopramolecular complexes consisting of hydrophobic molecules, particularly triacylglycerol and cholesteryl esters, stabilized with a coat of amphipathic compounds: phospholipids, unesterified cholesterol and proteins, referred to as apolipoproteins. The common function of all apolipoproteins is to help solubilize neutral lipids in the circulation but they have also roles in receptor recognition and hence in targeting of lipoproteins to specific destinations. Moreover several apolipoproteins are known to participate in lipoprotein metabolism modulating enzymatic activity. Therefore, obtaining detailed information about their composition and structure may contribute to reveal their role in atherogenesis and to identify new biomarkers of the pathology that could be useful in diagnosis and in improving the therapeutic approaches adopted for the treatment of atherosclerosis. The knowledge of lipoprotein protein composition has been improved in the last years especially with the improvement of proteomics technologies. Anyway, the majority of the lipoproteomic studies existing in literature are directed to the characterization of the lipoprotein

protein composition in healthy subjects, while few studies exist in relation to atherosclerosis. In this regard the aim of this study was to characterize the apolipoprotein component of plasma lipoproteins isolated from atherosclerotic patients undergoing carotid endarterectomy and to identify differentially expressed apolipoproteins between patients and controls. By means of 2-DE followed by peptide mass fingerprinting several lipoprotein associated proteins were identified in VLDL, LDL, and HDL. The analysis of both 2-DE maps and 1-DE and 2-DE western blotting has revealed different apolipoprotein profiles in the three lipoprotein fractions. In particular higher levels of SAA in VLDL, LDL and HDL from patients and higher levels of apo E in both VLDL and LDL from patients were observed. Several epidemiological data associate elevated levels of SAA with the risk of developing cardiovascular diseases. Moreover, it has been shown that SAA could have pro-atherogenic effects promoting both LDL retention and inflammation. Anyway, further studies are needed to answer the question whether elevated levels of SAA could play a causal role. As regard to apo E, it is considered protective against the risk of developing atherosclerosis due to its role in targeting remnant lipoproteins for removal from circulation being a ligand of LDL receptor and LDL receptor-related protein. Anyway, it has been shown that, under certain conditions, apo E can increase the risk of atherosclerosis due to its role in inhibiting lipolysis or increasing VLDL production. Moreover, several studies linked the apo E4 isoform with greater risk for cardiovascular disease. Overall, in this study different apolipoprotein profiles have been observed in VLDL, LDL and HDL of atherosclerotic patients compared to controls. More studies have to be done in the future not only to confirm the results obtained for apolipoproteins profiles of circulating lipoproteins, but also to analyse the atherosclerotic specimens in order to evaluate differences, if any, in apolipoprotein profiles between circulating lipoproteins and lipoproteins filtered in plaque.

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COLLABORATION TO OTHER RESEARCH ACTIVITIES

In parallel with the research activity described above, I took also part in other studies that have been published in various international journals. Some of these researches focused on several aspects of the atherosclerotic process, such as:

- Evaluation of intra-plaque low molecular weight (LMW) thiols (page II):
  - association between LMW thiols levels in plaque and their corresponding plasma levels;
  - evaluation of the equilibrium between LMW thiols levels and intra-plaque protein thiolation.

- New method for the quantification of LMW thiols bound to human albumin purified from different sources (plasma samples and atherosclerotic plaque extracts) by SDS-PAGE (page III).

- Relationship between plasma chondroitin sulfate level/structure and the presence/typology of the carotid atherosclerotic lesion (page IV).

- Moreover, I participated as co-author to the preparation of a book chapter on an overview of latest advances in proteomic studies on atherosclerosis and some related diseases, with particular emphasis on vascular tissue proteomics and lipoproteomics (page V).

Other researches focused on diverse topics such as:

- Elucidating the complex relationships between inflammation markers and reproductive axis by analyzing the possible changes of circulating inflammatory plasma indicators such as C-reactive protein (CRP), leptin, and glycosaminoglycans (GAGs) in women with either ovulatory or anovulatory menstrual cycles (page VII).

-Glycosaminoglycans content, distribution, and structure in porcine aortic and pulmonary valves and in pericardium before and after a detergent-based decellularization procedure utilized as xenograft in valvular replacement operations (page VIII).

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Evaluation of low molecular mass thiols content in carotid atherosclerotic plaques

Angelo Zinellu [a,b,*], Antonio Lepedda Jr. [c], Salvatore Sotgia [a], Elisabetta Zinellu [c], Bastianina Scanu [a], Franco Turrini [d], Rita Spirito [e], Luca Deiana [a], Marilena Formato [c], Ciriaco Carru [a,b,*]

[a] *Dipartimento di Scienze Biomediche, Cattedra di Biochimica Clinica, Università di Sassari, Italy*
[b] *Istituto Nazionale Biostrutture e Biosistemi, Osilo, Italy*
[c] *Dipartimento di Scienze Fisiologiche, Biochimiche e Cellulari, Università di Sassari, Italy*
[d] *Dipartimento di Genetica, Biologia, Biochimica, Università di Torino, Italy*
[e] *Centro Cardiologico "F. Monzino", IRCCS, Milano, Italy*

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Abstract

Objectives: Despite the evidence that both homocysteine and cysteine are important risk factors for vascular disease and atherosclerosis no information are reported about their effective amount in plaque and on the relationship with the other low molecular weight thiols.

Design and methods: We used capillary electrophoresis to measure thiols in both carotid plaque specimens and plasma samples from 37 patients undergoing carotid endarterectomy.

Results: Pearson's correlation shows that intraplaque homocysteine, cysteine and cysteinylglycine levels are related to their plasma concentrations. The distribution of intraplaque GSH and Glu-Cys was higher than that of the same thiols in plasma, whereas the other thiols were significantly less prevalent in plaque than in plasma. Intraplaque haemoglobin and GSH levels were correlated, thus suggesting their common origin from erythrocytes lysis.

Conclusion: Data suggest that increased levels of intraplaque glutathione may induce important effects on plaque fate by perturbing the normal LMW thiol redox state.
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Keywords: Thiols; Plaques; Glutathione; Plaque haemorrhage

Angelo Zinellu[1,2,3]
Antonio Lepedda, Jr.[4]
Salvatore Sotgia[1]
Elisabetta Zinellu[4]
Giommaria Marongiu[5]
Maria Franca Usai[4]
Leonardo Gaspa[1]
Pierina De Muro[4]
Marilena Formato[4]
Luca Deiana[1]
Ciriaco Carru[1,2]*

[1]Dept. Biomedical Sciences, University of Sassari, Sassari, Italy
[2]Centre of Excellence for Biotechnology Development and Biodiversity Research, University of Sassari, Sassari, Italy
[3]National Laboratory of the National Institute of Biostructures and Biosystems, Osilo, Italy
[4]Dept. Physiological, Biochemical and Cellular Science, University of Sassari, Sassari, Italy
[5]Institute of Vascular Surgery, University of Sassari, Sassari, Italy

Research Article

Albumin-bound low molecular weight thiols analysis in plasma and carotid plaques by CE

We describe a new method for the quantification of low molecular weight thiols, as homocysteine, cysteine, cysteinylglycine, glutamylcysteine and glutathione bound to human plasma albumin. After albumin isolation and purification by SDS-PAGE, thiols were freed from protein with tri-*n*-butylphosphine and successively derivatized with 5-iodoacetamidofluorescein. Samples were then injected and quantified in about 18 min by CE with laser induced fluorescence detection. Precision tests indicate a good repeatability of the method both for migration times (RSD < 0.63%) and areas (RSD < 2.98%). The method allows to measure all five low molecular weight thiols released from just 3 μg of albumin thus improving the other described methods in which only three or four thiols were detected. Due to the elevated sensitivity (LOD of 0.3 pM for all thiols), also low molecular weight thiols bound to albumin filtered in tissues could be quantified.

Keywords: Albumin / CE / Cysteine / Homocysteine / SDS-PAGE
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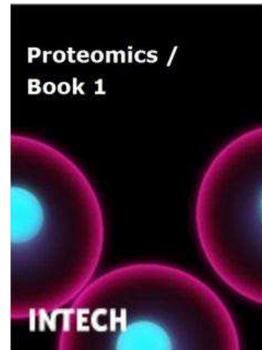
Association between human plasma chondroitin sulfate isomers and carotid atherosclerotic plaques

Zinellu Elisabetta[1], Lepedda Antonio Junior[1], Cigliano Antonio[1], Pisanu Salvatore[1], Zinellu Angelo[2], Carru Ciriaco[2], Bacciu Pietro Paolo[3], Piredda Franco[3], Guarino Anna[4], Spirito Rita[4], Formato Marilena[1].
[1]Dipartimento di Scienze Fisiologiche, Biochimiche e Cellulari, University of Sassari, Italy
[2]Dipartimento di Scienze Biomediche, University of Sassari, Italy
[3]Servizio di Chirurgia Vascolare-Clinica Chirurgica Generale, University of Sassari, Italy
[4]Centro Cardiologico "F. Monzino" IRCCS, University of Milano, Italy

Abstract

Several studies have evidenced variations in plasma glycosaminoglycans content in physiological and pathological conditions. In normal human plasma GAGs are present mainly as undersulfated chondroitin sulfate (CS). The aim of the present study was to evaluate possible correlations between plasma CS level/structure and the presence/typology of carotid atherosclerotic lesion. Plasma CS was purified from 46 control subjects and 47 patients undergoing carotid endarterectomy showing either a soft or a hard plaque. The concentration and structural characteristics of plasma CS were assessed by capillary electrophoresis of constituent unsatured fluorophore-labeled disaccharides. Results showed that the concentration of total CS isomers was increased of 21.4% ($p<0.01$) in plasma of patients, due to a significant increase of undersulfated CS. Consequently, in patients the plasma CS charge density was significantly reduced respect to that of controls. After sorting for plaque typology, we found that patients with soft plaques and those with hard ones differently contribute to the observed changes. In plasma from patients with soft plaques, the increase in CS content was not associated with modifications of its sulfation pattern. On the contrary, the presence of hard plaques was associated to CS sulfation pattern modifications in presence of quite normal total CS isomers levels. These results suggest that the plasma CS content and structure could be related to the presence and the typology of atherosclerotic plaque and could provide a useful diagnostic tool, as well as information on the molecular mechanisms responsible for plaque instability.



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Department of Pediatrics, Section of Hematology-Oncology, Baylor College of Medicine, Houston, USA
Co-editor: Dr. Ricardo J. Flores
Texas Children's Hospital, Baylor College of Medicine, USA

Overview of current proteomic approaches for discovery of vascular biomarkers of atherosclerosis

Lepedda Antonio Junior, Zinellu Elisabetta, Formato Marilena
University of Sassari/Dipartimento di Scienze Fisiologiche, Biochimiche e Cellulari, Italy

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.

Glycosaminoglycan and transforming growth factor β1 changes in human plasma and urine during the menstrual cycle, in vitro fertilization treatment, and pregnancy

Pierina De Muro, M.D.,[a] *Giampiero Capobianco, M.D., Ph.D.,*[b] *Marilena Formato, M.D.,*[a] *Antonio Junior Lepedda, M.D.,*[a] *Gian Mario Cherchi, M.D.,*[a] *Laila Gordini, M.D.,*[b] *and Salvatore Dessole, M.D.*[b]

[a] Department of Physiological, Biochemical and Cellular Sciences, University of Sassari, Sassari, Italy; and [b] Department of Pharmacology, Gynecology and Obstetrics University of Sassari, Sassari, Italy

Objective: To evaluate transforming growth factor β1 (TGF-β1) and glycosaminoglycans (GAG) changes in human plasma and urine during the menstrual cycle, IVF-ET, and pregnancy.
Design: Prospective clinical study.
Setting: University hospital.
Patient(s): Thirteen women with apparently normal menstrual cycle (group 1); 18 women undergoing IVF-ET (group 2); and 14 low-risk pregnant women (group 3).
Intervention(s): We assayed plasma and urine concentrations of TGF-β1, urine content, and distribution of GAG. Blood and urine samples were collected during days 2 to 3, 12 to 13, and 23 to 24 in group 1; in group 2, samples were obtained at menstrual phase, oocyte pick-up day, and 15 days after ET; in group 3, samples were obtained during gestational weeks 10–12, 22–24, and 30–32 and 1 month after delivery.
Main Outcome Measure(s): Changes in TGF-β1 and GAG content.
Result(s): The mean value of total urinary trypsin inhibitor/chondroitin sulfate (UTI/CS) showed a distinct peak at day 12 of the menstrual cycle in the fertile women in whom we monitored the ovulatory period. In the IVF-ET group, GAG distribution and TGF-β1 levels showed significant differences during the cycle. We observed increased levels of plasma TGF-β1 15 days after ET. A significant increase of total UTI/CS value with increasing gestation was detected.
Conclusion(s): Transforming growth factor β1 and GAG levels could represent an additional tool to monitor reproductive events and could be useful, noninvasive markers of ovulation and ongoing pregnancy. (Fertil Steril® 2009;92:320–7. ©2009 by American Society for Reproductive Medicine.)

Key Words: Transforming growth factor β1, glycosaminoglycans, urinary trypsin inhibitor, women

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Fine structure of glycosaminoglycans from fresh and decellularized porcine cardiac valves and pericardium

Cigliano Antonio[1§], Gandaglia Alessandro[2§], Lepedda Antonio Junior[1], Zinellu Elisabetta[1], Naso Filippo[2], Gastaldello Alessandra[2], Aguiari Paola[2], De Muro Pierina[1], Gerosa Gino[2],Spina Michele[3] and Formato Marilena[1].
[1]Dipartimento di Scienze Fisiologiche, Biochimiche e Cellulari, Università di Sassari, Via Muroni 25, 07100 Sassari, Italy.
[2]Dipartimento di Scienze Cardiache, Toraciche e Vascolari, Università di Padova, Via Giustiniani 2, 35128 Padova, Italy
[3]Dipartimento di Scienze Biomediche Sperimentali, Università di Padova, Viale G. Colombo 3, 35131 Padova, Italy.

Abstract

Cardiac valves are dynamic structures, undergoing cyclic mechanical stress, exhibiting a highly specialized architecture consisting of cells and extracellular matrix (ECM) with a relevant proteoglycan and glycosaminoglycan content, collagen and elastic fibers. Biological valve substitutes are obtained from xenogenic cardiac and pericardial tissues. To overcome the limits of such non-viable substitutes, tissue engineering approaches emerged to create cell repopulated decellularized ECM scaffolds. The present study was performed to determine the glycosaminoglycans content, distribution, and disaccharides composition in porcine aortic and pulmonary valves and in pericardium before and after a detergent-based decellularization procedure. The fine structural characteristics of galactosaminoglycans chondroitin sulfate (CS) and dermatan sulfate (DS) were examined by fluorophore-assisted carbohydrate electrophoresis. Furthermore, the mechanical properties of decellularized pericardium and its propensity to be repopulated by in vitro seeded fibroblasts were investigated.
Results show that galactosaminoglycans (GalAGs) and hyaluronan (HA) are differently distributed between pericardium and valves and within heart valves themselves before and after decellularization. The distribution of glycosaminoglycans is also dependent

from the vascular district and topographic localization. In native leaflets and sinuses of both cardiac and pericardial valves the Δdi-mono6S and Δdi-mono4S represent the major disaccharide types of GalAGs, followed by Δdi-nonS, whereas lower proportions of Δdi-diS were determined. In native pericardium, the vast majority of disaccharides, Δdi-mono4S and Δdi-diS, was resistant to degradation with chondroitinase AC II. The decellularization protocol adopted resulted in a relevant but not selective depletion of galactosaminoglycans. As a whole, data suggest that both decellularized porcine heart valves and bovine pericardium represent promising materials bearing the potential for future development of tissue engineered heart valve scaffolds.