GRADUATE SCHOOL IN BIOCHEMICAL, NUTRITIONAL AND METABOLIC SCIENCES
PH.D. IN EXPERIMENTAL AND CLINICAL NUTRITION

UNIVERSITY OF MILAN
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METABOLIC SYNDROME, OXIDATIVE STRESS AND LIPOPROTEINS: WHAT ARE THE BIOCHEMICAL EFFECTS OF WEIGHT LOSS?

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2012/2013

“El sueño de la razón produce monstruos”

I would like to thank my PhD supervisor, Professor Benvenuto Cestaro, for supporting me during these three years. I am also very grateful to Doctor Roberta Cazzola for her scientific advice and many insightful discussions. A heartfelt thank goes to Doctor Carla Camerotto and Doctor Francesca Deriu that worked with me. My acknowledgment goes to all people who have made this work possible and in particular to Doctor Flavio Muzio, Doctor Anna Boggio and Doctor Erica Cassani for the recruitment of patients.

Table of contents

Abbreviations

AAPH	2,2'-azobis-2-methyl-propanimidamide-dihydrochloride-induced
ABC	ATP-Binding/Cassette
ALT	Alanine Aminotransferase
ApoA-I	Apolipoprotein A-I
ApoA-II	Apolipoprotein A-II
ApoA-IV	Apolipoprotein A-IV
ApoB-100	Apolipoprotein B-100
ApoB-48	Apolipoprotein B-48
ApoC-I	Apolipoprotein C-I
ApoC-II	Apolipoprotein C-II
ApoC-III	Apolipoprotein C-III
ApoD	Apolipoprotein D
ApoE	Apolipoprotein E
AST	Aspartate Aminotransferase
BMI	Body Mass Index
CE	Cholesteryl Esters
CRP	C-Reactive Protein
CVD	Cardiovascular Disease
EGIR	European Group for the Study of Insulin Resistance
HDL	High Density Lipoprotein
HOMA-IR	Homeostatic Model Assessment - Insulin Resistance
IDF	International Diabetes Federation
LCAT	Lecithin-Cholesterol Acyl Transferase
LDL	Low Density Lipoprotein
MDA	Malonyldialdehyde

NCEP-ATP III	National Cholesterol Education Program's Adult Treatment Panel III
PAF-AH	Platelet-Activating Factor-Acetyl Hydrolase
PL	Phospholipids
PON1	Paraoxonase 1
PyrCE	Cholesteryl (pyren-1-yl) hexanoate
PyrPC	β-(Pyren-1-yl) decanoyl γ-palmitoyl L-α phosphatidylcholine
ROS	Reactive Oxygen Species
TAG	Triacylglycerols
TLR	Toll-like receptors
VLDL	Very Low Density Lipoprotein
WHO	World Health Organization

Abstract

Aim. To examine and compare the composition and the oxidisability of the hydrophobic core and amphipathic surface of lipoproteins in a group of adult males with metabolic syndrome before and after weight loss of at least 5% of their initial weight following a hypocaloric diet.

Methods. The concentration of cholesterol, triglycerides, phospholipids, and proteins was studied to analyse the chemical composition of lipoproteins. Pyrenyl-cholesteryl ester and pyrenyl-phosphatidylcholine, incorporated into the hydrophobic core or amphipathic surface of LDL and HDL, respectively, were used for measuring 2,2'-azobis-2-methyl-propanimidamide-dihydrochloride-induced peroxidation kinetics in these regions.

Results. In comparison with the initial time, there was an improvement of HDL oxidisability with an increase of lag-time and a reduction of the velocity of propagation of peroxidation in the core of HDL. Parallel but opposite, there was a worsening in the susceptibility to peroxidation of LDL with a reduction in lag-time and an increased propagation rate of peroxidation in the LDL core. After weight loss, LDL showed a higher content of triglycerides whereas HDL showed reduced triglycerides content. In both LDL and HDL, the increase in the percentage of triglycerides was connected with an increased propagation rate of peroxidation kinetic and with a reduction of the lag-time in the hydrophobic core.

Conclusions. A more detailed knowledge of the chemical composition and oxidisability of both the surface and core of lipoproteins could be a useful additional means for better understanding the mechanisms that link the changes in lipoproteins metabolism to the risk of cardiovascular disease in patients with metabolic syndrome after weight loss.

Introduction

Obesity and metabolic syndrome receive more attention every day. The increase in the number of people with metabolic syndrome now represents a serious problem for national health systems.

In subjects with metabolic syndrome, there is a clear increase of general low grade inflammation and consequently an increase of oxidative stress. Inflammation and oxidative stress are closely linked and influence each other. At the same time inflammation and oxidative stress affect the properties and functions of lipoproteins. The alteration in lipids metabolism, inflammation and oxidative stress increase the risk of metabolic disorders, such as type 2 diabetes, the risk of cardiovascular disease, and so on.

A thorough study of the metabolic syndrome requires a multidisciplinary approach. The study of lipoproteins and lipid metabolism offers an unusual and privileged perspective with which to analyse metabolic syndrome. Lipoproteins are a bridge between the biochemical and metabolic aspects of "syndrome X" and the clinical aspects related to arteriosclerosis, cardiovascular risk and heart disease. If it is true that the study of lipoproteins provides an inherently multidisciplinary overview, studying and using them in the laboratory requires a solid basis of biochemistry for understanding lipoproteins metabolism and the oxidative alterations of their components. Also, with regard to weight loss, it is important to deepen the argument on different theoretical levels and start understanding clinical aspects prior to changes in lipoproteins metabolism. Lipoproteins again offer a privileged although complex view of the problem.

Obesity, metabolic syndrome and inflammation

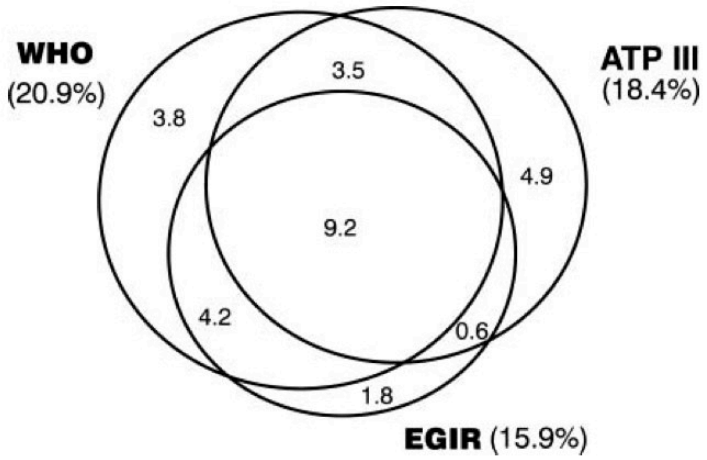
In the 1980s, Reaven proposed that patients with high blood pressure, hyperinsulinaemia, impaired glucose tolerance, increased plasma triglycerides concentrations and decreased high density lipoproteins cholesterol concentrations had a plurimetabolic syndrome which, at the time, was described as syndrome X, and is commonly referred to as the metabolic syndrome [1].

Several different sets of criteria have been proposed during the past decades for the diagnosis of the metabolic syndrome [2]. The original World Health Organization (WHO) recommendations were not designed to be an exact definition, but were formulated as a working guideline to be improved in the future. The recommendations were part of a WHO report on the definition, diagnosis and classification of diabetes. The WHO definition is based on the assumption that insulin resistance is one of the major underlying contributors to the metabolic syndrome. Following the publication of the WHO definition in 1999, the European Group for the Study of Insulin Resistance (EGIR) proposed a modified version to be used in non-diabetic subjects only, which is simpler to use in epidemiological studies since it does not require a euglycaemic clamp to measure insulin sensitivity.

The National Cholesterol Education Program's Adult Treatment Panel III (NCEP-ATP III) definition was presented in 2001 as part of an educational program for the prevention of coronary heart disease [3]. This definition was designed to facilitate diagnosis in clinical practice and differed in two major ways from other definitions. First, it did not include a measure of insulin resistance as a component, and second, it was not glucose-centric, and treated glucose abnormalities as being of equal importance to other components in making the diagnosis. The NCEP-ATP III guidelines state that the metabolic syndrome may be diagnosed when a person has three or more of the five diagnostic components. These components are: central obesity, an elevated TG level, a reduced HDL-cholesterol level, elevated blood pressure and an elevated fasting glucose concentration. Importantly, the NCEP-ATP III definition includes waist circumference as the measure of obesity. The NCEP-ATP III criteria for the metabolic syndrome have been widely used in both clinical practice and epidemiological studies. Alberti et al. [4] studied the difference

in prevalence rates for the metabolic syndrome using these three definitions, starting with a large Australian work of lifestyle and glucose intolerance. Only 9% of the individuals studied met the criteria for all three definitions, although each of the three definitions identified approximately 16-21% of the Australian population as having metabolic syndrome. Figure 1 shows the overlaps of the prevalence data obtained with the three different sets of criteria.

Figure 1 - This graph shows the prevalence of the metabolic syndrome observed in the Australian population using WHO, EGIR, and NCEP-ATP III definitions and their overlaps [4].



In 2005, the International Diabetes Federation (IDF) proposed a new classification that included central obesity as an essential element for diagnosis, changing the parameters of waist circumference [5]. The NCEP-ATP III definition and the IDF definition of the metabolic syndrome do not identify the same patients. The metabolic syndrome defined by NCEP-ATP III criteria confers a higher risk of vascular events than the metabolic syndrome defined by IDF criteria. The IDF wanted its new definition to encourage the identification of patients at increased risk of cardiovascular events. Therefore, there are important differences between the NCEP-ATP III and IDF definitions of the metabolic syndrome that may explain the weaker association of the IDF metabolic syndrome with vascular events. First, the lowering of the cut-off value for waist circumference leads to the inclusion of patients with a relatively lower level of this risk factor in the IDF category of the metabolic syndrome. Second, the mandatory status of the waist criterion in the new definition results in a relatively lower prevalence of other (potentially stronger) metabolic syndrome risk factors in patients with the metabolic syndrome. In particular, the high triglycerides and low HDL cholesterol metabolic syndrome components are relatively underrepresented in patients with IDF-defined metabolic syndrome. However, the greatest vascular risk in our investigation was conferred by these lipid traits [2].

Table 1 - Diagnostic criteria for metabolic syndrome according to NCEP-ATP III [3] *and according to IDF* [5].

NCEP-ATP III (2001)	IDF (2005)
Waist circumference • ≥ 102cm in men • ≥ 88cm in women Triglycerides ≥ 150 mg/dl or drug treatment HDL cholesterol • < 40 mg/dl in men • < 50 mg/dl in women Blood pressure • ≥ 130 mm Hg systolic or • ≥ 85 mm Hg diastolic Or drug treatment for hypertension Fasting glucose ≥ 110 mg/dl or previous diagnosis of type 2 diabetes	Waist circumference • ≥ 94 cm in men • ≥ 80 cm in women Triglycerides ≥ 150 mg/dl or drug treatment HDL cholesterol • < 40 mg/dl in men • < 50 mg/dl in women Blood pressure • ≥ 130 mm Hg systolic or • ≥ 85 mm Hg diastolic Or drug treatment for hypertension Fasting glucose ≥ 110 mg/dl or previous diagnosis of type 2 diabetes

Prospective population studies show that the metabolic syndrome confers a 2-fold increase in relative risk for atherosclerotic events, and in individuals without established type 2 diabetes mellitus, a 5-fold increased risk for developing diabetes when compared with people without this syndrome. This implies that the metabolic syndrome imparts a relatively high long-term risk for both atherosclerotic cardiovascular disease and diabetes [3].

At present, it is not clear whether the metabolic syndrome has a single cause, and it appears that it can be precipitated by multiple underlying risk factors. The most important of these underlying risk factors are abdominal obesity and insulin resistance. The abdominal visceral adipose tissue is a source of cytokines and adipokines, which influence interactions between the immune system and the vascular wall, inducing a state of chronic inflammation and increased oxidative stress [6]. When the picture is complicated by changes in carbohydrate and lipid metabolism, the risk factors for cardiovascular and cerebrovascular disease, which are already high in a state of uncomplicated obesity, are increased. It is still not clear what the alteration is, but it seems increasingly clear that the single alteration should be able to influence others, defining a complex inflammatory state, as that of the metabolic syndrome. Atherogenic dyslipidaemia, described as the combination of raised triglycerides and small dense LDL and HDL particles, is an independent and important risk factor for coronary heart disease. In metabolic syndrome, the lipid perturbations are amplified by immune system activation and insulin resistance, indicating a sort of vicious circle with important and complex interactions that promote the development of atherosclerosis [7]. Atherosclerosis is an inflammatory disease characterised by vascular wall infiltration by macrophages and T cells associated with lipid infiltration [8]. Implication of the immune system in atherosclerosis is still incompletely understood, but recent works have highlighted the

role of the innate immune system in generating a response in the presence of tissue aggression, which would subsequently lead to the activation of inflammatory pathways [9]. Therefore, the metabolic perturbations that mediate important and complex interactions with the immune system and the vascular wall in relation with atherosclerosis are reviewed, with particular emphasis on the mechanisms that mediate immune activation. The immunological paradigm has been challenged in the 1990s with the danger model of immunity, in which the immune system responds to damaged cells rather than to foreign ones [10]. This model of immunity has allowed expansion of the scope of immunological implication in diseases with an inflammatory component that might be detrimental to the host. The discovery at the end of the 20th century of the Toll-like receptors (TLR) in mammalian innate immune cells including macrophages and dendritic cells has reinforced the view that the innate immune system plays a key role in inflammatory response [11]. Furthermore, the discovery that, beside bacterial products, endogenous substances such as oxidised LDL and heat shock proteins mediate the activation of TLR has reinforced the view that the innate immune system plays a key role in the genesis of atherosclerosis [12]. Therefore, immune cells are the gatekeepers that detect cellular damage and initiate a response allowing our body to defend against 'offending' insults.

Endogenous danger signals are from intracellular or secreted extracellular products. Some are constitutive, whereas others are inducible and require either neosynthesis or modifications before they can activate the innate immune system. Atherosclerosis is characterised by a chronic inflammatory state in which interplay between metabolic factors and cytokines leads to stimulation of the innate immune system when these signals are detected as a danger. Therefore, signals from different sources including modified lipid products, endogenous inducible factors, and cytokines are implicated in a complex inflammatory response that relies on tissue damage as the primary stimulating event, leading to immune activation.

There is also an evolutionary perspective that could explain why metabolic disorders are linked to inflammation. The ability to extract and store energy from food and the possibility to protect themselves from infection are indispensable for the survival of any form of life. It is highly likely that metabolic and immune functions have evolved from a common ancestral structure. For example, the Drosophila fat body performs functions that can be compared to the liver and the haematopoietic and immune systems in humans. It is possible to imagine a situation in which common or overlapping pathways regulate both metabolic and immune functions through common key regulatory molecules and signalling systems [13].

Lipoproteins metabolism

Cholesterol, cholesteryl esters, triglycerides and phospholipids are essentially insoluble in water. These lipids must be transported from the tissues where they are produced to those in which they are stored or used. They are transported through the plasma in macromolecular aggregates, called lipoproteins, consisting of specific transporter proteins, called apolipoproteins, which combine different proportions of phospholipids, cholesterol, cholesterol esters and triglycerides [14]. Lipoproteins have a spherical structure containing a hydrophobic core of lipids with the hydrophilic side chains of the amino acids of apolipoproteins on the outside and a surface layer composed of phospholipids with the fatty acids oriented toward the core of the particle. The different combinations of lipids and proteins generate particles with densities ranging from that of chylomicrons, the lowest, to that of high density lipoproteins, the highest. Based on these differences in density, the particles can be separated by ultracentrifugation. The classification of serum lipoproteins has evolved historically through several phases corresponding with the development of different laboratory methodologies. With the arrival of the analytical ultracentrifugation in the 1940s, lipoproteins were classically separated into four major classes designated as chylomicrons (exogenous triglycerides rich particles of d <0.94 g/ml), very low density lipoproteins (VLDL, endogenous triglycerides rich particles of d=0.94-1.006 g/ml), low density lipoproteins (LDL cholesteryl ester rich particles of d=1.006-1.063 g/ml), and high density lipoproteins (HDL particles containing approximately 50% protein of d=1.063-1.21 g/ml). With subsequent improvements in ultracentrifugation techniques, further heterogeneity was detected within each of those major lipoprotein classes; this resulted in the need for further subdivision into several density subclasses such as HDL2a (d=1.10-1.125 g/ml), HDL2b (d=1.063-1.10 g/ml), and HDL3 (d=1.125-1.21 g/ml). There is no doubt that the separation of lipoproteins by ultracentrifugation has been essential for the advances in this field [15].

Figure 2 - (a) Structure of a low-density lipoprotein. (b) The four classes of lipoproteins viewed in the electron microscope after negative staining. Chylomicrons 50-200 nm, VLDL 28-70 nm; LDL 20-25 nm, HDL 8-11 nm [14].

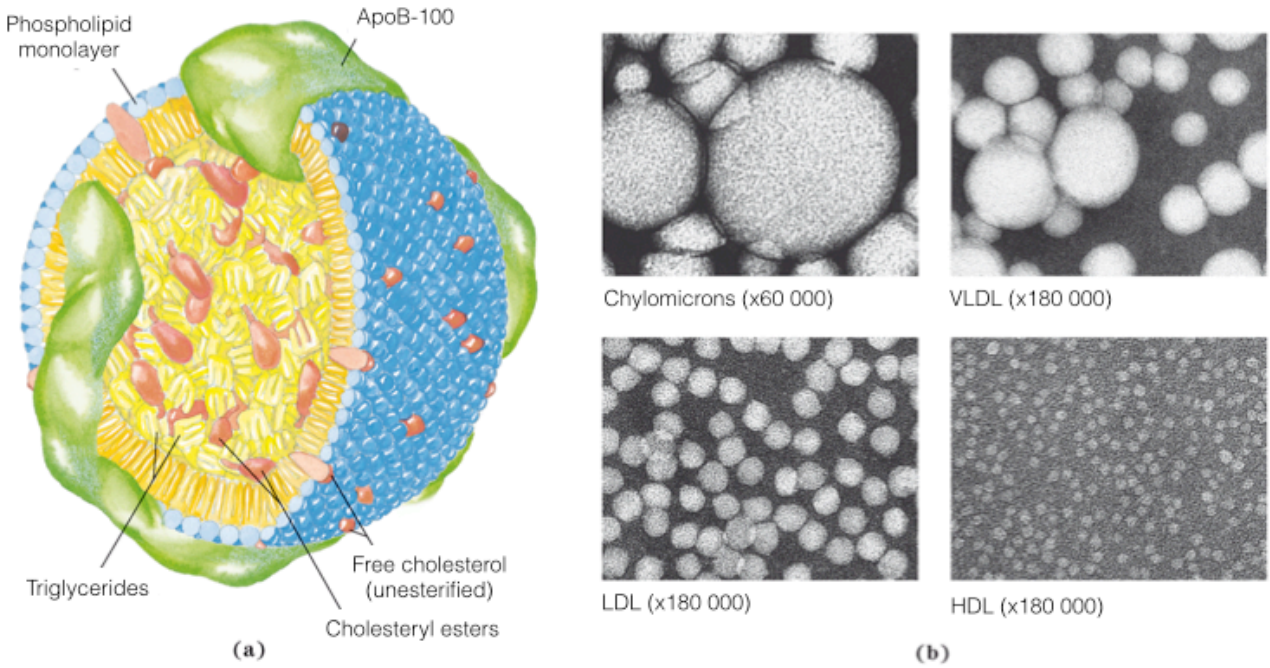


Table 2 - Apolipoproteins of human plasma lipoproteins [16].

Apolipoproteins	Molecular mass	Association with lipoproteins	Function
ApoA-I	28 331	HDL	Activates LCAT; interacts with the ABC transporter.
ApoA-II	17 380	HDL	Inhibits LCAT.
ApoA-IV	44 000	Chylomicrons, HDL	Activates LCAT; transport/removal of cholesterol.
ApoB-48	240 000	Chylomicrons	Transport/removal of cholesterol.
ApoB-100	513 000	VLDL, LDL	Binds to the receptor for LDL.
ApoC-I	7 000	VLDL, HDL	
ApoC-II	8 837	Chylomicrons, VLDL, HDL	Activates lipoprotein lipase.
ApoC-III	8 751	Chylomicrons, VLDL, HDL	Inhibits lipoprotein lipase.
ApoD	32 500	HDL	
ApoE	34 145	Chylomicrons, VLDL, HDL	Promotes the removal of the VLDL and chylomicrons remnant.

LCAT = Lecithin-Cholesterol Acyl Transferase, VLDL = Very Low Density Lipoprotein, LDL = Low Density Lipoprotein, HDL = High Density Lipoprotein. ABC = ATP-Binding/Cassette.

Each class of lipoprotein has a specific function determined by the site in which it was synthesised and by its composition in lipids and apolipoproteins. In human plasma, at least nine different types of apolipoproteins were detected, which were distinguishable by size, interactions with specific antibodies and a characteristic distribution in the different lipoprotein classes. The protein components act as signals that direct lipoproteins to specific tissues or activate enzymes which act on lipoproteins themselves.

Chylomicrons are the largest and the less dense lipoproteins, as they contain a high proportion of triacylglycerol. They are synthesised in the smooth endoplasmic reticulum of epithelial cells lining the small intestine, and by moving through the lymphatic system, they enter the bloodstream through the thoracic duct and the left subclavian vein. The apolipoproteins of chylomicrons are ApoB-48 (specific for this class of lipoproteins), ApoE, and ApoC-II. ApoC-II activates the lipoprotein lipase of the capillaries of adipose tissue, heart, and skeletal muscle tissue of the mammary gland during lactation, allowing the release of fatty acids to the tissues. Chylomicrons carry fatty acids ingested in the diet to those tissues in which these compounds are degraded to produce energy or to be deposited as an energy reserve. The remnant chylomicrons are now deprived of triacylglycerol, but still contain cholesterol, ApoE and ApoB-48. The remnant chylomicrons, which are linked by specific receptors for ApoE, arrived at the liver through the bloodstream, and mediate uptake through endocytosis. Within the liver, the remnant chylomicrons release cholesterol and are degraded in lysosomes.

When the diet contains more fat than is immediately necessary, lipids are converted into triglycerides in the liver and transferred to specific lipoproteins, forming very low density lipoproteins (VLDL). Even the carbohydrates can be transformed into triacylglycerol in the liver and exported through the VLDL. Besides triglycerides, VLDL contain cholesterol, cholesteryl esters and the apolipoproteins ApoB-100, ApoC-I, ApoC-II, ApoC-III and ApoE. These lipoproteins are transferred from the liver into the blood, to the muscle and to adipose tissue, where the activation of lipoprotein lipase operated by ApoC-II determines the release of free fatty acids from the triacylglycerols of VLDL. Most of the remnant VLDL is removed from the bloodstream by hepatocytes. As in the case of chylomicrons, their intake is mediated by receptors and is dependent on the presence of ApoE in remnant VLDL.

With the loss of triglycerides, VLDL are converted in remnant VLDL (also called intermediate density lipoproteins, IDL) that, with the further removal of triacylglycerols, are converted into low density lipoproteins (LDL). The LDL are very rich in cholesterol and cholesteryl esters and ApoB-100 is their main apolipoprotein. They carry cholesterol to extrahepatic peripheral tissues that possess a specific receptor to recognise ApoB-100. The apolipoprotein B-100 is a single polypeptide chain, among the longest of which are

known to be formed by a sequence of 4636 amino acids and a molecular weight of 513 000 Da. An LDL particle contains a core of about 1500 molecules of cholesteryl ester, surrounded by an envelope of approximately 500 molecules of cholesterol and 800 phospholipid molecules, as well as a single molecule of ApoB-100. Apo B-100 is recognised by specific protein receptors, called LDL receptors, on the surface of cells that need to take-in cholesterol. The binding of LDL to their receptor triggers the process of endocytosis that transfers LDL and its receptor within the cell, inside an endosome. The endosome then merges with a lysosome containing enzymes that hydrolyse cholesteryl esters, releasing cholesterol and fatty acids into the cytosol. Even ApoB-100 of LDL is degraded into amino acids that are released into the cytosol, while the LDL receptor is not degraded and returns to the surface of the cell, thereby starting again the endocytosis process of LDL. ApoB-100 is also present in VLDL, but its LDL receptor binding domain is not available and the conversion of VLDL in LDL exposes the binding domain of the receptor for apoB-100.

The fourth type of lipoprotein, called high-density lipoprotein (HDL), has its origin in the liver and small intestine in the form of small particles that are rich in protein and contain limited quantities of cholesterol, but no cholesteryl esters. The HDL contain, among others, the apolipoproteins ApoA-I, ApoC-I, ApoC-II and also the lecithin-cholesterol acyltransferase (LCAT) enzyme that catalyses the formation of cholesteryl ester using lecithin and cholesterol. LCAT is present on the surface of HDL and converts the cholesterol of chylomicrons and remnant VLDL into cholesteryl ester, which lies within the HDL. In this way, the HDL particles undergo a change in shape from a flat disc to a ball, which is the typical structure of mature HDL. The HDL rich in cholesterol return to the liver where the cholesterol is unloaded. HDL can be picked up by the liver by receptor-mediated endocytosis, but at least part of the cholesterol contained in HDL is released into other tissues through different mechanisms. HDL can bind to a membrane receptor called SR-BI in hepatic tissue and in steroidogenic tissues such as the adrenal gland. This type of receptor endocytosis is not common, but provides a partial and selective transfer of cholesterol and other lipids from HDL to the cells. The HDL, so drained, dissociates from the receptor and goes back into circulation to extract other lipids from the remnant chylomicrons and VLDL remnants. Once emptied, HDL can remove cholesterol stored in extrahepatic tissues and transfer it to the liver through the various mechanisms of reverse cholesterol transport. Through one of these pathways, the interaction of an empty HDL with the SR-BI receptor of a cell that is rich in cholesterol determines the passive transport of cholesterol to HDL from the cell surface. Via a second mechanism, ApoA-I in the HDL interacts with the active transporter ATP-Binding/Cassette 1 (ABC1) that is present in cells that are rich in cholesterol. The ApoA-I and presumably the entire HDL is taken up by endocytosis and then secreted full of cholesterol to be transported to the liver.

HDL in subjects with metabolic syndrome

HDL have been shown to exert a wide spectrum of anti-atherogenic activities, including the promotion of reverse cholesterol transport, anti-inflammatory actions in vasculature [17] and the protection of LDL against oxidation [18]. Moreover, the capacity of HDL to accumulate [19,20] and neutralise [21] or transport oxidised lipids to the liver [22] suggests a role for HDL in the detoxification of these molecules *in vivo*. Recent evidence suggests that structural modifications and composition alterations of HDL due to chronic inflammation and acute phase responses may result in the loss of the normal biological function of these lipoproteins [17,23]. Furthermore, changes in lipid composition associated with metabolic syndrome, such as hypercholesterolaemia and hypertriacylglycerolaemia, have been shown to influence the anti-atherogenic capacity of HDL [17].

Oxidative modification of HDL has been suggested to be another pathway that is involved in the generation of less functional HDL [24]; however, while LDL oxidation has been widely investigated [25,26], information on HDL oxidation and its role in atherogenesis is scarce. The oxidation of HDL lipids leads to the formation of a complex mixture of chemical compounds [27] such as conjugated dienes, arachidonic acid-derived isoprostanes, lipid peroxides, and aldehydes. Some of these aldehydes, such as malondialdehyde (MDA), can easily react with lipids and proteins, giving rise to the formation of cross-links between lipid tails and amino acids residues, which decrease the fluidity of the compartment in which these alterations have taken place. Such alterations may impair the activity of apolipoproteins and HDL-associated proteins and, therefore, the anti-atherogenic capacity of HDL.HDL is known to undergo dramatic modifications in both structure and composition as a result of the concerted actions of the acute-phase response and inflammation. The close association between inflammation, oxidative stress, dyslipidaemia, and atherosclerosis suggests that such HDL alterations play a significant role in disease progression. As a result, HDL particles progressively lose normal biological activities and acquire altered properties. Such altered HDL particles have been termed “dysfunctional HDL” and it is essential to emphasise that the degree of loss of normal HDL function compared with the absence of this function depends on the assay used to characterise HDL functionality [28]. Both the plasma levels and apolipoprotein content of HDL can be significantly altered during the acute phase as well as during acute and chronic inflammation; levels of ApoA-I and ApoA-II decrease, whereas those of ApoA-IV and ApoE increase. The decrease in HDL ApoA-I levels in inflammatory states is related to both decreased ApoA-I synthesis in the liver and ApoA-I replacement in HDL particles by serum amyloid A. In the circulation, serum amyloid A does not exist in a free form; it is usually associated with HDL. Elevated plasma levels of serum amyloid A are accompanied by elevated levels of lipid-free ApoA-I, probably due to the dissociation of ApoA-I from HDL [29,30].

Chronic inflammation that is characteristic of metabolic disease, such as metabolic syndrome and type 2 diabetes, is associated with elevated plasma levels of IL-6. As a result, the liver produces serum amyloid A, which replaces ApoA-I and paraoxonase 1 in HDL. Oxidative stress, hyperglycaemia, and the elevated activity of CETP are other important modulators of HDL function. Oxidative stress modifies specific amino acids in ApoA-I, whereas hyperglycaemia results in ApoA-I glycation. CETP exchanges cholesteryl esters and TG between HDL and triglycerides-rich lipoproteins, such as VLDL and LDL; as a result, HDL become enriched in triglycerides. Such enrichment in triglycerides induces conformational changes in ApoA-I, which becomes less accessible for interaction with other lipoproteins, including LDL, and prevents the elimination of oxidised lipids from LDL. Subsequent HDL hydrolysis by hepatic lipase produces small, dense HDL that are enriched in triglycerides and in serum amyloid A and contain ApoA-I in an incorrect conformation; such HDL possess deficient functionality compared with normal HDL particles.

Apart from its replacement by serum amyloid A, ApoA-I can undergo other modifications in circulation. Amino acid residues in ApoA-I, such as methionine, cysteine, tyrosine, and lysine residues, can be selectively modified under the action of pro-oxidants that are secreted by arterial wall cells and non-enzymatically glycosylated in the presence of high levels of glucose. Oxidised amino acid residues, including chlorotyrosines, nitrotyrosines and oxidised lysine and methionine residues, are present in ApoA-I isolated from plasma and from human atherosclerotic lesions; furthermore, the ApoA-I content of chloro- and nitrotyrosines is increased in the plasma of patients with cardiovascular disease. Myeloperoxidase was recently shown to bind to HDL within human atherosclerotic lesions, and biophysical studies revealed that myeloperoxidase binding occurs via specific interactions with ApoA-I. This likely facilitates the observed selective targeting of ApoA-I for site-specific chlorination and nitration by myeloperoxidase generated reactive oxidants *in vivo*. One apparent consequence of myeloperoxidase catalysed ApoA-I oxidation includes the functional impairment of the ability of HDL to promote cellular cholesterol efflux via the ABCA1 system. Myeloperoxidase-mediated loss of the atheroprotective functional properties of HDL may thus provide a novel mechanism linking inflammation and oxidative stress to the pathogenesis of atherosclerosis [31].

HDL-associated enzymes, including platelet-activating factor-acetyl hydrolase (PAF-AH), paraoxonase 1 (PON1), and lecithin–cholesterol acyltransferase (LCAT), can become dysfunctional and/or depleted under inflammatory conditions, in metabolic diseases involving low HDL levels (type 2 diabetes, metabolic syndrome), and in premature coronary heart disease. HDL provides an amphipathic environment, where PON1 finds an optimal location to exert its activity. The complex HDL-PON1 is a repository for potentially toxic, hydrophobic components of plasma, notably oxidised lipids. Induction of the acute-

phase response is associated with decreased PON1 activity, probably due to the replacement of PON1 by serum amyloid A [32,33]. Furthermore, decreased PON1 activity may be caused by enzyme inactivation as a result of oxidation and/or homocysteinylation and/or glycation. Same authors found decreased serum concentrations of PON1 in subjects with metabolic syndrome and in patients with type 1 and type 2 diabetes, who feature elevated levels of inflammation and oxidative stress. Serum PON1 activity decreases with age and is lower in subjects with metabolic syndrome and low HDL, as well as in patients with type 2 diabetes and familial hypercholesterolaemia, compared with age-matched healthy control subjects. Other authors have reported alterations in oxidative stress in patients with metabolic syndrome, who did not have diabetes, and even in obese subjects; at the same time, no changes were detected in the activity of the enzyme paraoxonase. Moreover, low PON1 activity toward paraoxon has been reported to represent an independent risk factor for coronary events in men at high cardiovascular risk [34]. HDL-associated PAF-AH activity, expressed as a percentage of total serum PAF-AH activity, has been shown to be lower in hypercholesterolaemic patients than in control subjects. In the same subjects, in contrast, LDL-associated PAF-AH activity was elevated, suggesting a major redistribution of PAF-AH activity in the plasma of dislipidaemic individuals from ApoA-I- to ApoB-containing lipoproteins [35]. Although apolipoproteins and enzymes are major determinants of altered HDL function, it is considerably influenced by changes in lipid content. HDL core enrichment in triglycerides with cholesteryl esters depletion is the most frequent abnormality of HDL lipid composition and occurs in hypertriglyceridaemic states, associated with the decreased activity of lipoproteins lipase, the decreased activity of hepatic lipase, and/or the decreased activity of LCAT. All of these metabolic alterations are frequently observed in the acute phase and during inflammation (obesity and metabolic syndrome are characterised by a low grade inflammation). In addition, HDL triglycerides content can be raised as a consequence of elevated CETP-mediated triglycerides transfer from VLDL to HDL. Under such conditions, triglycerides typically replace cholesteryl esters in the HDL core, resulting in a low cholesteryl esters/triglycerides ratio and in a decrease in plasma HDL cholesterol levels; this is another feature of the acute phase response [36]. Interestingly, a similar elevation in HDL triglycerides, decrease in HDL cholesterol and increase in inflammatory markers is observed in the postprandial phase. Human acute phase HDL obtained from patients undergoing bypass surgery are enriched in triglycerides and depleted of cholesteryl esters. Acute phase HDL also contain elevated levels of non-esterified fatty acids, lysophosphatidylcholines and isoprostanes compared with normal HDL; in addition, cholesteryl esters levels are decreased. Similarly, HDL3 from subjects with myocardial infarction are enriched in triacylglycerols (TAG) and depleted of phospholipids (PL). As a consequence of decreased LCAT activity, increased HDL concentrations of free cholesterol are frequently observed in inflammatory states; in addition, HDL free cholesterol is elevated in genetic LCAT deficiency [36].

HDL metabolism is substantially altered in dyslipidaemic states, including hypertriglyceridaemia, hypercholesterolaemia, mixed dyslipidaemia and hypo- and hyperalphalipoproteinaemia, and also during infection and inflammation. As discussed above, hypertriglyceridaemia is characterised by decreased levels of HDL cholesterol and increased HDL triglycerides content due to the action of CETP. Such low HDL cholesterol dyslipidaemias associated with hypertriglyceridaemia are characteristic of metabolic diseases associated with elevated cardiovascular risk, such as type 2 diabetes and metabolic syndrome. Mechanisms leading to reduced plasma HDL cholesterol levels and HDL particle numbers in hypertriglyceridaemic states are as follows:

1. small HDL particles, which result from the intravascular lipolysis of triglycerides-enriched HDL, are cleared more rapidly from the circulation;
2. triglycerides-enriched HDL are intrinsically more unstable in the circulation, with ApoA-I being loosely bound;
3. lipolysis of triglycerides-enriched HDL lower HDL particle numbers by causing ApoA-I to be shed from HDL particles and cleared from the circulation;
4. dysfunctional lipoprotein lipase or reduced lipoprotein lipase activity contributes to the lowering of HDL levels by reducing the availability of surface constituents of triglycerides-rich lipoproteins that sequester to the plasma pool of nascent HDL particles [37].

The cholesteryl esters/triglycerides ratio therefore represents a critical factor in determining HDL particle stability and plasma residence time; HDL possessing decreased cholesteryl esters/triglycerides ratios are less stable than normal particles [38]. Importantly, a decrease in circulating HDL cholesterol levels and an increase in triglycerides levels are typical components of the acute phase reaction [29]. HDL metabolism critically depends on the activity of CETP. In metabolic diseases such as type 2 diabetes and metabolic syndrome, elevated CETP activity results in the increased transfer of cholesteryl esters from HDL to triglycerides-rich lipoproteins and in reciprocal triglycerides transfer, producing triglycerides-enriched HDL and decreasing HDL cholesterol levels. Conversely, CETP deficiency reduces the exchange of triglycerides and cholesteryl esters between HDL and triglycerides rich lipoproteins and elevates HDL cholesterol due to cholesteryl esters retention. As a consequence, increased CETP activity is thought to be proatherogenic in humans [39].

In hypercholesterolaemia, abnormalities of HDL metabolism include moderate decreases in plasma ApoA-I and HDL cholesterol levels. HDL heterogeneity and particle profile largely reflect abnormalities in HDL metabolism. In the atherogenic dyslipidaemias of metabolic syndrome and type 2 diabetes, circulating levels of large, cholesterol-rich HDL decrease in parallel with a decrease in HDL cholesterol. By contrast, levels of small, dense, cholesterol poor HDL particles and their content of ApoA-I are rarely reduced in

low HDL cholesterol dyslipidaemia [40]. In obesity and insulin resistance (frequent features of both metabolic syndrome and type 2 diabetes), plasma levels of large HDL decrease in parallel with those of HDL cholesterol, whereas levels of small HDL do not. As a result, metabolic syndrome, type 2 diabetes, obesity, and insulin resistance are all characterised by the prevalence of small, dense HDL [41].

Small, dense HDL also prevail in coronary heart disease patients. In male participants in the Framingham Offspring Study, subjects with coronary heart disease displayed higher levels of small particles [32]. Similarly, subjects with new cardiovascular events possessed higher levels of small HDL and lower levels of large HDL than subjects without such events in the Veterans Affairs HDL Intervention Trial (VA-HIT) study [42]. Coronary artery disease patients also displayed elevated levels of lipid-poor ApoA-I . The increase in small HDL and decrease in HDL of intermediate size, as measured by nuclear magnetic resonance, are associated with coronary artery disease severity in men admitted for diagnostic coronary arteriography [43,44]. Small HDL also prevail in peripheral arterial disease [45].

Alterations in HDL composition and metabolism, as occurring in dyslipidaemia and inflammation, are intimately associated with impaired biological activities. However, data on HDL cholesterol efflux capacity in atherogenic dyslipidaemia are conflicting. Some authors reported a diminished HDL capacity to deliver cholesteryl esters to hepatic cells through interaction with SR-BI as a result of HDL enrichment in triglycerides [46]. In contrast, others have reported normal cholesterol efflux capacity of serum from hypertriglyceridaemic subjects, which is an observation that can be related to normal contents of HDL phospholipids, a key determinant of HDL mediated efflux. Furthermore, HDL from hypertriglyceridaemic patients with low HDL cholesterol levels possess a normal capacity to extract cholesterol from smooth muscle cells. Consistent with these results, triglycerides-enriched HDL are not deficient in cholesterol efflux properties from cholesterol-loaded macrophages [47].

The intrinsic cholesterol efflux capacity of HDL is considerably impaired during inflammation. Cellular cholesterol efflux is largely mediated by ApoA-I-containing HDL particles; ApoA-I replacement by serum amyloid A can therefore have a significant impact on efflux. Enrichment of HDL with serum amyloid A (up to high serum amyloid A contents of 86% of total HDL protein) results in increased HDL binding to, decreased cholesterol efflux capacity from, and increased selective cholesteryl esters uptake by macrophages. Importantly, serum amyloid A selectively impairs the cholesterol efflux properties of small, dense HDL3 particles. Less pronounced enrichment of HDL with serum amyloid A *in vivo* (up to 27% of total HDL protein) does not influence cholesterol efflux but enhances HDL binding to macrophages [48].

The presence of serum amyloid A increases both HDL affinity to and selective cholesteryl esters uptake by macrophages but reduces affinity to and cholesteryl esters uptake by hepatocytes. Decreased phospholipids contents in inflammatory HDL constitute another factor that contributes to deficient HDL cholesterol efflux properties. Together, these changes lead to a significant shift in the HDL-mediated cholesterol transport from hepatocytes toward macrophages under acute phase conditions. Biologically, such alterations may serve to redirect cholesterol to immune cells and to sites of injury and inflammation [49]. Abnormal lipid composition may also impair cholesterol efflux properties of HDL particles, as demonstrated by the diminished capacity of large cholesteryl esters-enriched HDL2 isolated from subjects with homozygous CETP deficiency to accept cholesterol from lipid-loaded mouse peritoneal macrophages. Normalisation of the lipid composition of such HDL, as a result of the transfer of excess cholesteryl esters to SR-BI overexpressing cells, improves HDL cholesterol efflux capacity [49].

Oxidative modification represents another factor involved in the impairment of HDL cholesterol efflux capacity. *In vitro* oxidation of ApoA-I by myeloperoxidase results in the selective inhibition of ABCA1 dependent cholesterol efflux from macrophages [50]. The cholesterol efflux capacity of ApoA-I may be also impaired as a consequence of non-enzymatic glycosylation [28].

The central role of ApoA-I in HDL mediated cholesterol efflux is consistent with the deleterious role of ApoA-I mutations. However, not all mutations in ApoA-I lead to decreased cholesterol efflux capacity. ApoA-I Milano, a molecular variant of ApoA-I, displays potent capacity for cholesterol efflux. Carriers of ApoA-I Milano exhibit severe hypoalphalipoproteinaemia but are not at increased risk for premature CHD [51]. Finally, the capacity of HDL particles to extract cholesterol from peripheral cells may be impaired as a result of alterations in cellular HDL receptors, primarily ABCA1 [52].

Oxidative stress and metabolic syndrome

Accumulating evidence also demonstrates that damage to cellular components from reactive oxygen species (ROS) plays an integral role in the age-related deterioration of biochemical and physiologic processes and in the incidence of age-related disease. Oxidative stress, which is an excessive production of ROS outstripping endogenous antioxidant defence capacity, is implicated in oxidative damage to nucleic acids, proteins, carbohydrates, and lipids. From the molecular point of view, all of these alterations are derived directly or indirectly from the increase in low grade inflammation which involves, as a first effect, an increase in oxidative stress [6]. In recent years it has been assumed, with increasing accuracy, that the increase in levels of inflammation and oxidative stress represents the common thread of many seemingly different diseases such as diabetes and hyperglycaemia, hypertension, metabolic syndrome and obesity, depression, psychosis and attention-deficit disorder [53].

It is well known that mitochondria are the primary site for ROS generation as a by-product of aerobic metabolism, and that the accumulation of mitochondrial oxidative damage over time diminishes the cellular efficiency in energy production. ROS-induced damage is complex and frequently irreversible, and further impairs mitochondrial function, rendering them prone to further ROS generation. The cellular membranes are probably one of the first structures in a cell that can be been damaged by oxidative stress. The membrane fluidity is a consequence of the different molecules in the lipid bilayers. For example, the excessive presence of cholesterol and more saturated fat acids than unsaturated fat acids in phospholipids of membranes stiffens the bilayer structure of the membrane. Free radical can damage all cellular macromolecules, such as proteins, nucleic acids, and glycosaminoglycans, but above all affects polyunsaturated fat acids of the membrane phospholipids. This can give rise to two secondary types of reaction [54,55]: the formation of a cyclic lipid peroxide that can fragment during successive reactions and give rise to aliphatic chains containing two functional carbonyl groups, creating compounds such as malonyldialdehyde (MDA); or interaction between the lipid peroxyl radical and another lipid molecules with the formation of a stable lipid peroxide and a new lipid radical that can propagate further peroxidation reactions. Compounds of the MDA type are highly toxic because their carbonyl groups can react spontaneously with

the amino group of the membrane proteins and phospholipids forming stable covalent bounds. The formation of these bonds can cause serious damage to membranes and in general with regard to cellular function [56]. The cellular membrane holds 70% of all proteins in a cell and proteins represent the major holders of biological function. The peroxidation process reduces the freedom and possibility of movement of all proteins with a consequent loss of fluidity and hardening of the membrane. The diminished membrane fluidity encountered in these degenerative processes is the cause of secondary metabolic damage to cells. All of this results in the reduced capacity of the proteins to collide with the ligands with which they must interact in order to carry out their own functional and biological roles, such as enzymatic proteins interacting with their own specific substrate, the receptor proteins and their own specific hormone or neurotransmitter, the ion channels that permit electrolyte flow and the transmembrane carrier proteins for amino acids and glucose.

One of most important examples of all this process involves the insulin receptor. This receptor is made like cone-shaped with the lower extremities outside the cell. With the hardening of the membrane, this receptor is pushed towards the interior of the cell. Usually, the insulin receptor protrudes out of the membrane of 1 Å, while, with the loss of fluidity of the membrane, the possibility to interact with insulin is reduced compared to the normal situation. In this way, oxidative stress and hardening of the membrane promotes insulin resistance. It is well-known that insulin acts as an important hormone controlling the production of energy and the synthesis of macromolecules such as proteins. A greater capacity to use energy is closely linked to the possibility of reducing oxidative damage in the cell. Accumulating evidence has suggested that insulin exerts important redox-regulating actions in various insulin-sensitive target organs, implying the systematic anti-oxidative role of insulin as a hormone [57]. These aspects are likely to demonstrate how the interaction between oxidative stress and metabolic syndrome is more profound and multifaceted than may appear at first glance.

The role of oxidised LDL in atherogenesis

LDL lipoproteins are composed of a core enriched in hydrophobic triglycerides and cholesterol ester molecules, enclosed by an envelope of phospholipids and unesterified cholesterol and apolipoprotein ApoB-100 [58]. The LDL lipoproteins are generally divided into three main subclasses based on density: LDL1 and LDL2 (density 1018-1030 g/mL), LDL3 (1030-1040 g/mL), LDL4 and LDL5 (1040-1065 g/mL). The small, dense LDL can more easily penetrate into the sub-endothelial space of the vascular wall and are more susceptible to oxidation [59]. A leading current hypothesis of the pathogenesis of atherosclerosis is that the release of oxygen free radicals from arterial endothelial cells and smooth muscle cells leads to the oxidation of LDL. Oxidative modification of LDL enhances its atherogenicity [60]. It has been demonstrated that the class of scavenger

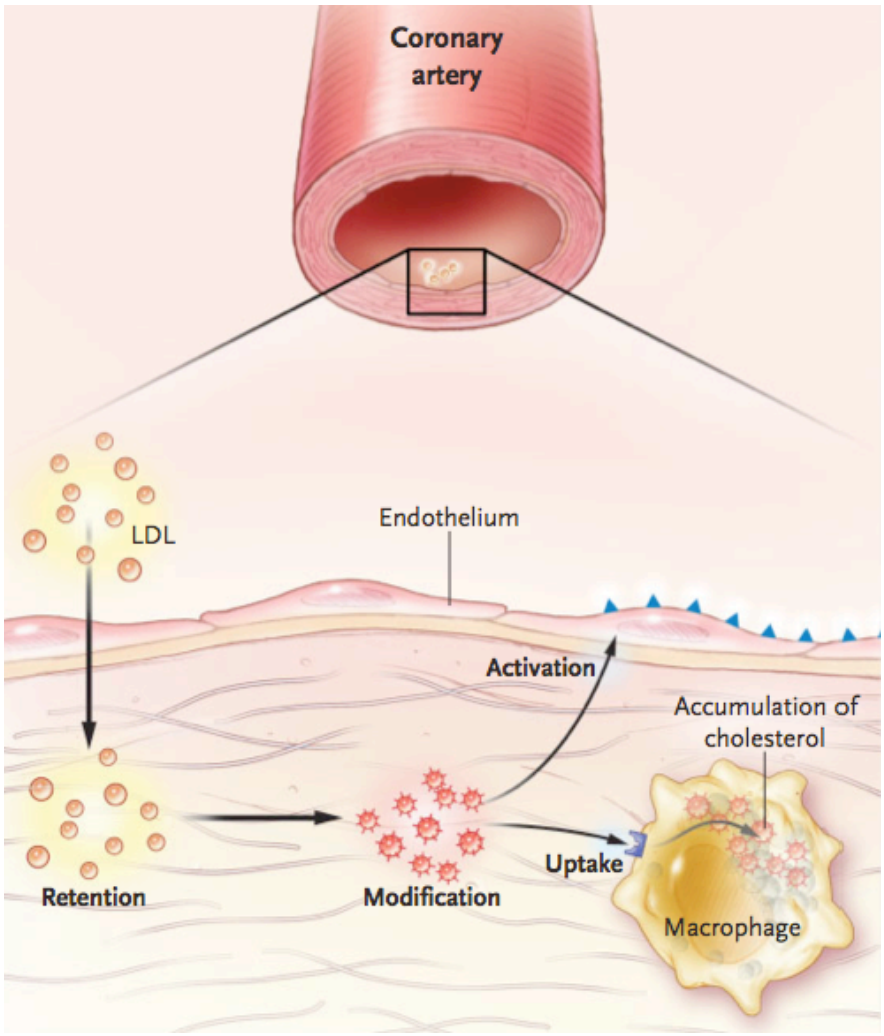
receptors on macrophages takes up oxidised LDL, leading to the formation of foamy macrophages. Oxidised LDL also is chemotactic for monocytes and can cause endothelial damage. Oxidative damage of LDL is believed to occur primarily in the sub-endothelial space of the vascular wall. During circulation, LDL enters and re-emerges from the sub-endothelial space. Increasing the time needed to remove fixed amounts of LDL from the circulation creates conditions that favour an increase in the number of LDL exposed to oxidative stress and the duration of exposure. Several components of LDL, including ApoB-100, phospholipids, cholesterol and unsaturated fatty acids, may be subject to oxidative stress [61]. Vitamin E is the principal fat soluble antioxidant that protects lipoproteins, but when it is consumed, the chain reaction continues and develops degradation products such as malondialdehyde [62].

Aldehydes can form Schiff bases with amino groups of lysine residues, creating cross-links between lipids and proteins or between lipid molecules. These changes alter the protein fraction and reduce the affinity of LDL for its receptor, but increase the affinity for the scavenger receptor of macrophages, through which oxidised LDL exert their atherogenic action. Macrophages incorporate oxidised LDL, contributing to foam cell formation and playing a key role in cellular events that lead to the development of atherosclerotic lesions. The oxidation of lipoproteins in the plasma seems to occur with difficulty due to the presence of high concentrations of antioxidants and proteins that chelate metal [63].

It has been suggested that oxidised LDL are generated in the vessel wall and spread into the bloodstream after plaque rupture, increasing the permeability of plaques and leading to ischemic and inflammatory damage [64]. Several studies have found wide variation in the susceptibility of LDL from different individuals to oxidation *ex vivo* [58], although it has not yet been demonstrated convincingly that such variations are associated with the risk of atherosclerosis [63]. Vitamin E, the major antioxidant carried in LDL, may be one factor that influences the susceptibility of LDL to oxidation. Increasing the vitamin E content of lipoproteins *in vitro* or *in vivo* (by dietary supplementation) increases the lag phase for the initiation of LDL oxidation under certain conditions [63].

Other lipoprotein characteristics such as size and density also influence the extent of oxidation. Small-dense LDL is more susceptible to oxidation than large buoyant LDL, and increased levels of small-dense LDL are seen in various conditions that increase the risk of vascular disease, including diabetes, combined hyperlipidaemia, and familial dyslipidaemic hypertension. The increased susceptibility to oxidation may relate to these particles' increased content of triglycerides, polyunsaturated fatty acids, and/or decreased vitamin E content [63].

Figure 3- Macrophages incorporate oxidised LDL. In patients with hypercholesterolaemia, excess LDL infiltrates the artery and is retained in the intima. Oxidative and enzymatic modifications lead to the release of inflammatory lipids that induce endothelial cells to express leukocyte adhesion molecules. The modified LDL particles are taken up by scavenger receptors of macrophages, which evolve into foam cells. [8]



The presence of an increased number of small, dense LDL particles is a constant feature of the dyslipidaemia of abdominal adiposity, as they are associated with insulin resistance, intra-abdominal fat, and hypertension [65-67]. LDL comprises a spectrum of particles that vary in size, density, chemical composition, and atherogenic potential. In conditions of elevated triglycerides, LDL particles become enriched in triglycerides and depleted in core cholesteryl esters. Hepatic lipase then acts to hydrolyse these triglyceride-rich LDL, forming smaller, denser LDL particles. The presence of small, dense cholesterol-depleted LDL particles is associated with an increased risk of myocardial infarction and worsened severity of coronary artery disease [68]. The Familial Atherosclerosis Treatment Study showed that the strongest predictor of coronary artery stenosis regression, induced by aggressive lipid lowering, was the increase in LDL buoyancy, not the change in LDL cholesterol level [69].

Although the mechanisms underlying the association of small, dense LDL with increased risk of coronary artery disease are not clear, several hypotheses have been proposed. One explanation is that the presence of small, dense LDL particles is a marker of an atherogenic lipoprotein phenotype comprised of elevated triglycerides, reduced HDL, and elevated ApoB-100, which together increase coronary artery disease risk [70].

The protective role of HDL in atherogenesis

The apparent importance of oxidative modification of low density lipoprotein in atherogenesis raises the question of how the oxidative modification of HDL might affect its cardioprotective actions [24]. HDL particles are the mainstay of atheroprotective defence mechanisms in humans. Reverse cholesterol transport from the peripheral tissues is the best known and salient activity. Moreover, such diverse mechanisms as antioxidant and anti-inflammatory functions via paraoxonase or apo A-I and A-II have been demonstrated. High-density lipoproteins also inhibit cytokine-induced expression of endothelial cell adhesion proteins, reduce superoxide production, and neutralise C-reactive protein pro-inflammatory activity. HDL further possess inhibitory effects on thrombosis and apoptosis. Table 3 summarises the potential cardioprotective actions of HDL. However, under circumstances which are incompletely understood, atheroprotective activities of HDL may become deficient, via a process designated HDL dysfunctionality.

Table 3 - Potential cardioprotective actions of HDL [24].

Actions	References
Reverse cholesterol transport	[71]
Inhibiting the formation and neutralising the effects of oxidised LDL	[72-74]
Inhibition of endothelial cell adhesion molecule expression	[75]
Binding bacterial endotoxin	[76]
Inhibiting LDL/oxidised LDL retention in the artery wall	[77]
Inhibiting platelet aggregation	[78]
Removal of lipid hydroperoxides	[19]

The level of high density lipoprotein (HDL) in plasma is an equally strong or stronger - but inverse - predictor of atherosclerotic vascular disease risk than LDL. It is held that each 1 mg/dL increase in HDL cholesterol level is associated with a 2% to 3% decrease in the multiadjusted risk of coronary heart disease. Consequently, it was stated in the NCEP-ATP III that HDL cholesterol concentrations in excess of 60 mg/dL counteract 1 risk factor [3]. The universality of this knowledge was recently challenged in some epidemiological

studies. In evaluating the significance for cardiovascular risk of HDL cholesterol levels, some aspects such as the size of HDL, the concentration of some apolipoproteins as ApoA-I, oxidation and glycation of HDL proteins and lipids have become highly important [23]. In patients with diabetes, glycation of HDL-associated enzymes and especially of ApoA-I depends on glucose concentration and is augmented in the presence of phospholipids. Deficient anti-inflammatory properties of HDL in type 2 diabetes mellitus have been ascribed to (a) HDL enrichment with conformational alterations of ApoA-I; (b) glycation of apolipoproteins and/or HDL-associated enzymes; and (c) oxidative modification of HDL lipids, apolipoproteins and/or enzymes [23,79].

Most studies looking at the relative susceptibility of lipoproteins to oxidation have found HDL to be more susceptible than LDL, with few exceptions. Despite their lower intrinsic antioxidant content, smaller HDL3 particles have been reported to protect LDL from oxidation more efficiently than HDL2 particles, possibly due to the preferential association of paraoxonase with HDL3. In addition, HDL containing ApoA-I as their only apolipoprotein may be more protective against LDL oxidation than HDL containing both ApoA-I and ApoA-II [80,81].

Weight loss and metabolic syndrome

Obesity and metabolic syndrome are important risk factors for coronary heart disease, ventricular dysfunction, congestive heart failure, stroke, and cardiac arrhythmias. Weight loss is probably one of the most important weapons against obesity, metabolic syndrome and an increased cardiovascular risk [82]. Intentional weight loss can improve or prevent many of the obesity-related risk factors for coronary heart disease (i.e., insulin resistance and type 2 diabetes mellitus, dyslipidaemia, hypertension and inflammation). Moreover, these metabolic benefits are often found after modest weight loss (5% of initial weight) and continue to improve in a monotonic fashion with increasing weight loss [83]. Fat loss can improve all features of the metabolic syndrome:

1. Weight loss can prevent the development of new diabetes in high-risk persons who are overweight or obese. Lifestyle dietary and activity modifications, which resulted in modest (5%) weight loss, decrease from 4- to 6-years the cumulative incidence of diabetes by 50% in men and women who were overweight or obese and had impaired glucose tolerance [84].
2. Fat loss could in some cases decrease serum LDL cholesterol and triglyceride concentrations, whereas increases in serum HDL cholesterol typically are seen when weight loss is sustained. The beneficial effects on serum lipids are related to the percentage of weight lost, and regaining the lost weight leads to a relapse in serum concentrations. A sustained weight loss of 5% is needed to maintain a decrease in serum triglyceride concentrations [85].
3. Weight loss decreases both systolic and diastolic blood pressure in a dose-dependent fashion; therefore, greater weight loss is generally associated with greater improvement in blood pressure [86].
4. Obesity is associated with an increase in circulating inflammatory markers, including C reactive protein (CRP) and cytokines (i.e., IL-6, IL-18 etc.). Adipose tissue itself is a likely source of these excess cytokines. The increase in inflammatory markers is associated with insulin resistance and is an important predictor of atherosclerotic events. Weight reduction decreases plasma CRP concentration. The decrease in CRP is directly related to the amount of weight loss, fat mass, and change in waist circumference [87].

Although fat loss could modify many cardiovascular disease (CVD) risk factors, it is not known whether weight reduction decreases CVD events or CVD mortality in obese people. Inflammation and oxidative stress are two of the main factors that must be considered in new studies about obesity and metabolic syndrome. One of the leading events that could connect vascular oxidative stress to atherosclerosis is the oxidative modification of lipoproteins. Oxidative damage to lipoproteins can not only make LDL atherogenic but can also reduce the anti-atherogenic properties of HDL [43,44]. While the effects on LDL have already been investigated the effects of oxidative stress on HDL have yet to be studied carefully. Moreover, the study of the different parts of lipoprotein, the core and the envelope, could give new information about the lipoproteins oxidisability and their oxidative damage.

Lipoproteins and weight loss

An interesting meta-analysis studied the effects of weight reduction by diets on plasma lipids and lipoproteins by analysing results from the 70 studies [85]. The importance of this meta-analysis is due to the fact that there is little clarity on the effectiveness of weight reduction on changes in plasma lipids and especially on the change of lipoproteins metabolism. Of primary interest is the fact that weight reduction is associated with a decrease of total cholesterol, LDL cholesterol, VLDL cholesterol and triglycerides when the levels of these lipids in blood are pathological. Overall correlations for the relationship between weight and change in various lipids and lipoproteins were also of primary interest. Weight reduction was associated with about 10% of the variance in change in total cholesterol. Each kilogram weight loss is associated with a 0.05 mmol/L decrease in total cholesterol. Mechanisms to explain how changes in body weight influence changes in lipids and lipoproteins results have not been clearly defined; however, body weight is the most important determinant of increased cholesterol synthesis that is often associated with obesity. Daily cholesterol production rate has been directly and significantly correlated with excess body weight [88]. An estimated cholesterol synthesis of about 20 mg/dl for each kilogram body fat has been reported [89]. In addition to obesity being associated with hypercholesterolaemia, obesity is often associated with hypertriglyceridaemia, which is thought to result from either an increase in triglycerides production rate and impaired removal. Results from these meta-analyses indicate that each kilogram of weight loss is associated with a 0.015 mmol/L decrease in triglycerides.

Lipoprotein lipase activity generally increases with weight loss, particularly once weight stabilises. However, during acute energy restriction, tissue concentrations of lipoprotein lipase have been reported to decrease by 50% to 80% [90]. Because of the decrease in lipoprotein lipase during active weight loss, triglycerides-rich lipoprotein synthesis in likely diminished and VLDL cholesterol catabolism impaired. Thus, the transfer of lipids to HDL cholesterol is limited, resulting in decreased HDL cholesterol during active weight loss.

When weight stabilised at a reduced level, lipoprotein lipase was reported to increase with an associated increased hydrolysis of VLDL cholesterol and transfer of lipids to HDL cholesterol. When subjects were at a reduced but stabilised weight, HDL cholesterol increased. Gender was significantly related to the change in HDL cholesterol and in triglycerides with weight loss; HDL cholesterol was expected to increase by approximately twice as much in males as in females. Triglycerides in males are expected to decrease by about 0.13 mmol/L more than in females. Males may show greater changes in triglycerides and in HDL cholesterol but the predicted changes for females were still beneficial. The age of the subjects is related to changes in LDL cholesterol with weight loss via other lipids or lipoproteins. There is an approximately four-fold difference in the predicted decrease in LDL cholesterol with weight reduction. Younger subjects are expected to decrease LDL cholesterol by 0.65 mmol/L, but older subjects are expected to decrease LDL cholesterol by only 0.21 mmol/L. Serum HDL cholesterol concentration have been reported in several population studies to be lower in cigarette smokers than in non-smokers; however, smoking has no effect on other lipids and lipoproteins. There is a negative association between cigarette smoking and body weight [91]. Alcohol is associated with increased triglycerides and HDL cholesterol concentration in the general population [92].

The weight reduction also affects the concentration of apolipoproteins and the concentration of enzymes connected to lipoproteins metabolism, consequently modifying the functions of the lipoproteins. In men with metabolic syndrome, weight loss of about 10% of their initial weight decreases the concentration of ApoC-III by about one third and this reduction is associated with a decrease of ApoB-48 and triglycerides [93]. ApoC-III is presented in chylomicrons, in VLDL and in HDL and inhibits the lipoproteins lipase; consequently, its reduction stimulates the removal of triglycerides from the core of the HDL and from the other classes of lipoproteins with ApoC-III.

Other works focus their attention on changes in HDL metabolism after weight loss. Previous studies have shown that hypercatabolism ApoA-I is the primary mechanism underlying the reduction of HDL cholesterol in obese people with insulin resistance [94]. In some cases, weight reduction in subjects with metabolic syndrome results in an increase in HDL cholesterol; this increase is attributed to a significant decrease of the catabolism rate of the ApoA-I with no change in the production rate of this apolipoprotein [95]. ApoA-I can be found only in HDL and activates LCAT, which catalyses the formation of cholesteryl ester using lecithin and cholesterol. This process results in the formation of HDL larger with a higher concentration of cholesteryl esters in the core and a reduction of free cholesterol on the amphipathic surface. HDL are divided into different subpopulations of particles with different size, density, electrophoretic mobility and composition also in lipids and apolipoproteins. This heterogeneity has important

implications for the function and properties of HDL. The infarction of cholesteryl esters in the core of HDL ends an increase in the size of HDL and their progressive instability. Studies with discoidal reconstituted high density lipoproteins suggest that HDL spontaneously increased the number of ApoA-I molecules from two to three [96] with a further stimulus to the action of LCAT.

Studies on obese female subjects undergoing laparoscopic gastric banding show that weight decrease is also associated with a reduction in the plasma concentration of CEPT [97]. This enzyme is responsible for the transfer of triglycerides from VLDL and LDL to HDL and the reduction in concentration stops the massive transfer of triglycerides to HDL that is typical of the metabolic syndrome and dyslipidaemia.

Aim of the study

A low-grade chronic inflammation can initiate and perpetuate an inflammatory cycle and pathophysiological signalling of inflammatory cells and adipocytes. Several adipokines have been suggested to act as a link between accumulated fat mass and insulin resistance [98]. The peripheral resistance to insulin is believed to play a central pathophysiological role in the metabolic syndrome, a condition that is characterised by dyslipidaemia (high triacylglycerols and low HDL-cholesterol), impaired carbohydrate metabolism and hypertension [99]. The anti-oxidative and anti-inflammatory action of HDL is now recognised as a major mechanism mediating its cardioprotective effect. One of the main events that could connect vascular oxidative stress to atherosclerosis is the oxidative modification of lipoproteins [100]. Oxidative damage to lipoproteins can not only make LDL atherogenic, but also can reduce the anti-atherogenic properties of HDL [43,44]. As previously described, metabolic syndrome can exacerbate lipoprotein oxidation and dysfunction [101]. Lipid peroxidation can occur both in the envelope and the core of plasma lipoproteins. The possibility of following the lipid peroxidation of the individual regions of LDL and HDL could lead to more detailed information on the modifications that are the basis of the increased risk of cardiovascular diseases observed in obesity and metabolic syndrome.

A previous study [102] that aimed to investigate the composition, oxidisability and fluidity of the hydrophobic core and surrounding amphipathic envelope of HDL in two adult male groups with the aim of providing more detailed information on the features of HDL in mixed dyslipidaemia patients, has already proven a change in the percentage composition of HDL that are enriched in triglycerides with a parallel increase oxidisability, with a reduction of the lag-time and an increase in the velocity of propagation of peroxidation.

Based on these data, I performed the following study, in which I investigated the change in susceptibility to peroxidation of the hydrophobic core and the surrounding envelope of LDL and HDL in obese males (BMI between 25 and 40 Kg/m^2) with metabolic syndrome [15]. The most ambitious aim of this study is to evaluate the change of these parameters

after weight decreases of at least 5% of initial weight following a hypocaloric diet. The lipid composition of all classes of lipoproteins was also measured as it represents an important area of study for understanding the biochemical basis of changes in oxidisability.

A study of the correlations between the state of susceptibility to peroxidation of lipoproteins, the chemical composition of the different lipoprotein classes and the most common clinical parameters has the aim of investigating the possible mechanisms of changes in these parameters and the possible correlations with medical practice.

Materials and methods

Materials

Analytical grade chemicals and solvents were used with double-distilled water. β-(Pyren-1-yl) decanoyl γ-palmitoyl L-α phosphatidylcholine (PyrPC), cholesteryl (pyren-1-yl) hexanoate (PyrCE), 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH), were from Sigma Aldrich (Milan, Italy). The kits for the determination of cholesterol and triglycerides were from Scalvo (Siena, Italy). Organic solvents were purchased from Merck (Merck Italy, Milan, Italy).

Subjects

The recruitment took place at *Istituti Clinici di Perfezionamento* and at *Ospedale Luigi Sacco*, Milan. To be included in the study, patients had to have the following characteristics: age between 18 and 55 years (but not undergoing menopause in women), BMI between 25 and 40 kg/m^2, absence of major diseases, lack of drug therapies in place that are able to influence lipid profile (e.g. oestroprogestinics, statins, oral hypoglycaemic agents, treatment with thyroid hormone synthesis), lack of use of antioxidant supplements, no smoking and alcohol consumption of less than 25 g/day.

The recruited subjects underwent a medical visit. Blood pressure and physical data (weight, height, waist circumference, and hip circumference) were determined during a complete clinical examination. Family history information was collected for overweight and obesity, dyslipidaemia, diabetes and cardiovascular disease. Physiological history was assessed, investigating the level of physical activity, the presence of cigarette smoking and consumption of alcoholic beverages and the characteristics of the menstrual cycle or the possible presence of menopause in women. Proximate and remote medical history were assessed, investigating the presence of any major diseases or the presence of ongoing drug therapy or antioxidant supplementation. Dietary food recall of one day and survey of food weekly frequency consumption was collected.

Clinical chemistry parameters (complete blood count, total cholesterol, HDL cholesterol, triglycerides, blood glucose, insulin, creatinine, AST, ALT, CRP, TSH, uric acid) were determined under strictly standardised conditions with dedicated commercial kits. To achieve the goal of weight loss of at least 5% of the initial weight, a second medical visit was performed. Blood pressure and physical data were determined on a second occasion, as were clinical chemistry parameters.

The study was approved by the ethics committee of the *Istituti Clinici di Perfezionamento* and *Ospedale Luigi Sacco* and was carried out in accordance with the principles of the Declaration of Helsinki as revised in 2000. Subjects gave their written consent to the study.

Diet intervention

The diet intervention was aimed at achieving weight loss of at least 5% by focusing on reduced caloric intake. All subjects followed the same type of diet. All specific intervention strategies included a calorie goal of 1400 to 2000 kcal per day (with <30% of calories from fat and >15% from protein) according to clinical evaluation. The program included both group and individual counselling sessions with a dietician. Table 1 shows the average nutritional analysis of diets.

Table 1 - Average nutritional analysis of diets administered to patients with the objective of reducing the weight of the 5%. Calorie goal of 1400 to 2000 kcal per day. Results were reported as mean ± Standard Error of the Mean (SEM).

Average nutritional analysis					
Proteins (% of total calories)	18 ± 0.3	Saturated fats (g)	13 ± 1.5	Ca (mg)	736 ± 84
Lipids (% of total calories)	28 ± 1.9	Polyunsaturated fats (g)	6 ± 0.9	Fe (mg)	15 ± 0.9
Carbohydrates (% of total calories)	54 ± 2.2	Fibres (g)	26 ± 2.5	Na (mg)	2193 ± 262
Alcohol (% of total calories)	0	Cholesterol (mg)	191 ± 18	K (mg)	3514 ± 226
		Vitamin E (mg)	10 ± 0.7	P (mg)	1051 ±133
		Folate (μg)	295 ± 15	Zn (mg)	10 ± 0.7

Blood collection and separation

Both at the initial time and to achieve the goal of weight loss, blood samples were obtained after overnight fasting by venipuncture and were collected in heparinised Vacutainer tubes. The sample was transported in a refrigerated box to our laboratories where it was processed for the separation of plasma. Each stage of the separation was carried out whilst maintaining the sample at a temperature of approximately 4°C using a bath of water and ice. Plasma was separated by centrifugation at 3000 rpm at 4°C before being aliquoted and stored at -80°C until use.

Plasma lipoproteins separation

Lipoproteins were isolated from plasma by ultracentrifugation using a multistep discontinuous density gradient of KBr, with an adaptation of procedure 16 in ref. [103] to "Optima Max" tabletop ultracentrifuge (Beckman Coulter). To remove albumin completely, the HDL fraction (density, 1.063–1.210 g/ml) was subjected to a second centrifugation (procedure 15 in ref. [103]). After separation, lipoproteins were dialysed against 10 mM phosphate buffered saline pH 7.4 (10 mM sodium phosphate buffer pH 7.4 containing 154 mM NaCl) at 4°C in the dark for 12 hours in order to eliminate KBr. Isolated and dialysed lipoproteins were stored.

Lipoprotein characterisation

The proteins concentration of each lipoprotein fraction was determined with the Lowry method using bovine serum albumin as standard [104]. Total lipids were extracted from each lipoprotein fraction following the Folch procedure [105]. The phospholipid content was determined according to Bartlett [106]; cholesterol and triglycerides were determined using a commercial reagent kit from Scalvo (Siena, Italy) [107,108].

Labelling of lipoproteins with fluorescent probes

We incorporated β-(pyren-1-yl) decanoyl γ-palmitoyl L-α phosphatidylcholine (PyrPC) or cholesteryl (pyren-1-yl) hexanoate (PyrCE) in their monomeric forms, as previously described [102,109], into HDL and LDL to measure the oxidisability of the hydrophobic core and surrounding amphipathic envelope. To label the hydrophobic core of this lipoproteins, plasma was incubated with PyrCE (10 nmol/mL) mixed with egg phosphatidylcholine (1:10 mol:mol). PyrPC (5 nmol/mL) was used to label the lipoproteins envelope. The probes were suspended with chloroform methanol (2:1), and then evaporated under a nitrogen stream. The probes were dissolved with a 10 mM phosphate

buffered saline pH 7.4 (10 mM sodium phosphate buffer pH 7.4 containing 154 mM NaCl) and then added to plasma. Plasma was incubated with probes for one hour at room temperature in the dark before the isolation of lipoproteins as described above.

Ohnishi et al. [109] showed that pyrene-labelled lipids are incorporated into lipoproteins in the positions where the corresponding non-pyrene lipids were located with respect to the distribution between the hydrophobic core and the amphipathic surface, without introducing drastic changes in the chemical compositions of lipoproteins.

Lipoprotein peroxidation

The two different pyrene derivatives were used to follow lipid peroxidation in low and high density lipoproteins. We used the fluorescence decrease of PyrCE to monitor the lipid peroxidation in the hydrophobic core of LDL and HDL, and that of the amphipathic probes PyrPC, to follow lipid peroxidation in the envelope of both lipoproteins. Labelled lipoproteins were resuspended (100 µg protein/mL) in phosphate-buffered saline pH 7.4, and peroxidised at 37°C by incubation with AAPH 1 mM. The AAPH is a water soluble compound which decomposes at 37°C generating a continuous flow of free radicals.

The oxidation of labelled LDL and HDL was monitored by the decrease in the pyrene fluorescence emission intensity at 378 nm (excitation λ=343 nm). This decrease was expressed as:

$$((F_o-F_t)/F_o) \times 100$$

where F_o represents the fluorescence intensity at zero time of peroxidation and F_t the residual fluorescence for ever experimental time. The kinetics is expressed by a sigmoidal curve that can be divided into an initial latency phase, and a second propagation phase followed by a termination phase. The kinetic profile of these curves allows the evaluation of at least two indices and offers a good description of the process of plasma peroxidation. These indices are: the maximal rate of oxidation, which can be calculated from the slope of the fluorescence curve during the propagation phase, and the lag-time, expressed in minutes and calculated from the intersection of the linear regression of the propagation phase tangent with that of the lag-phase. The length of the lag phase (lag-time) and the velocity of the reaction in the propagation phase (slope) of peroxidation kinetics were calculated and used as indices of lipoprotein oxidisability [110].

Statistics

Data are expressed as mean ± SEM. The average values in subjects after and before weight lost were compared. The differences between the two times were analysed statistically using the Wilcoxon test for matched pairs [111] and were considered significant for p values ≤ 0.05. Correlations between variables were calculated by Spearman Rank Correlation Coefficient [112]. All statistical analyses were performed using GraphPad Instat software (GraphPad Software, V 2.02).

Results

Subjects

For this study, we enrolled 106 patients, of which 44 were obese (males 27 females 17) and 62 were obese with metabolic syndrome (males 32 females 30), defined according to The National Cholesterol Education Program's Adult Treatment Panel III definition (ATP III) [3]. At the end of my PhD course, 23 subjects had lost more than 5% of their initial weight, of which 3 were obese (2 males and 1 female) and 20 were obese with metabolic syndrome (16 males and 4 females). The following results refer to 16 males obese with metabolic syndrome that lost more than 5% of their initial weight. Table 3 reports the features of the subjects at initial time and after weight loss. There were differences in blood pressure (both systolic and diastolic), waist and hip circumference. waist/hip ratio, glycaemia, insulin, HOMA-IR and ALT, regardless of whether there were differences in complete blood count, total cholesterol, HDL cholesterol, triglycerides, creatinine, AST, CRP, TSH and uric acid. There was a significant reduction of weight with an average percentage decrease of 8.28 ± 0.69%. There was a significant reduction in systolic and diastolic blood pressure after weight loss and a significant decrease in waist circumference, hip circumference and waist/hip ratio. Also, glucose metabolism bettered with a reduction of glycaemia, insulin and HOMA-IR after weight loss. Lipids concentration in blood did not change significantly although there was a trend of reduction of triglycerides which decreased from 135.1 mg/dl to 119.5 mg/dl. Total cholesterol, HDL cholesterol, total cholesterol/HDL cholesterol ratio, as well as HDL cholesterol/triglycerides ratio, did not change with weight reduction in this population. The diagnosis of metabolic syndrome according to NCEP-ATP III criteria was based on 5 standards and after weight loss, the subjects examined were improved in at least 3 criteria with better blood pressure, better glucose metabolism and a reduction of waist circumference. It is noteworthy that the lipaemia fell within the normal range even at the initial time of study. ALT is considered a surrogate marker of liver injury [113] and the significant decrease of ALT levels after weight loss was considered indicative of a reduction in hepatic inflammation. Finally, there was a reduction of CRP, with values that were halved from 0.40 mg/dl to 0.21 mg/dl, although this change did not quite reach statistical significance.

Table 3 - Anthropometric characteristics, blood pressure and clinical blood parameters of subjects before and after weight loss.

	Before weight lost		After weight lost		Wilcoxon test
	Mean	SEM	Mean	SEM	p
Weight (kg)	100.8	2.4	92.3	2.0	**< 0.0001**
BMI (kg/m^2)	33.83	0.93	30.99	0.78	**< 0.0001**
Systolic blood pressure (mmHg)	142	4	127	3	**0.0017**
Diastolic blood pressure (mmHg)	90	2	78	2	**0.0007**
Waist circumference (cm)	113.8	2.4	106.1	1.7	**< 0.0001**
Hip circumference (cm)	112.6	2.5	108.6	1.9	**0.0017**
Waist/hip ratio	1.01	0.01	0.98	0.01	**0.0001**
Glycaemia (mg/dl)	106.9	5.1	98.2	4.2	**0.0085**
Insulin (µU/l)	14.71	3.92	8.47	1.18	**0.0098**
HOMA-IR	3.83	1.04	2.03	0.32	**0.0049**
Total cholesterol (mg/dl)	195.0	6.6	189.8	7.3	0.2744
HDL cholesterol (mg/dl)	47.1	3.0	47.9	3.6	0.5830
LDL cholesterol (mg/dl)*	119.2	6.0	118.1	5.3	0.8999
Total cholesterol/ HDL cholesterol ratio	4.36	0.24	4.21	0.25	0.1439
Triglycerides (mg/dl)	135.1	15.6	119.5	12.9	0.1514
HDL cholesterol/triglycerides ratio	0.49	0.063	0.51	0.068	0.9152
Uric acid (mg/dl)	6.58	0.34	6.50	0.38	0.7615
Creatinine (mg/dl)	0.97	0.04	0.96	0.06	0.9102
AST (U/l)	29	2	29	2	0.8394
ALT (U/l)	43	4	32	3	**0.0007**
CRP (mg/l)	0.40	0.11	0.21	0.07	0.0781
Leukocytes (10^3/ml)	6.8	0.35	6.4	0.28	0.3203
Erythrocytes (10^6/ml)	5.2	0.16	5.1	0.20	0.0830

SEM = Standard Error of the Mean, BMI = Body Mass Index, HOMA-IR = Homeostatic Model Assessment - Insulin Resistance, AST = Aspartate Aminotransferase, ALT = Alanine Aminotransferase, CRP = C Reactive Protein.

** Calculated by Friedewald formula.*

Lipoproteins susceptibility to peroxidation

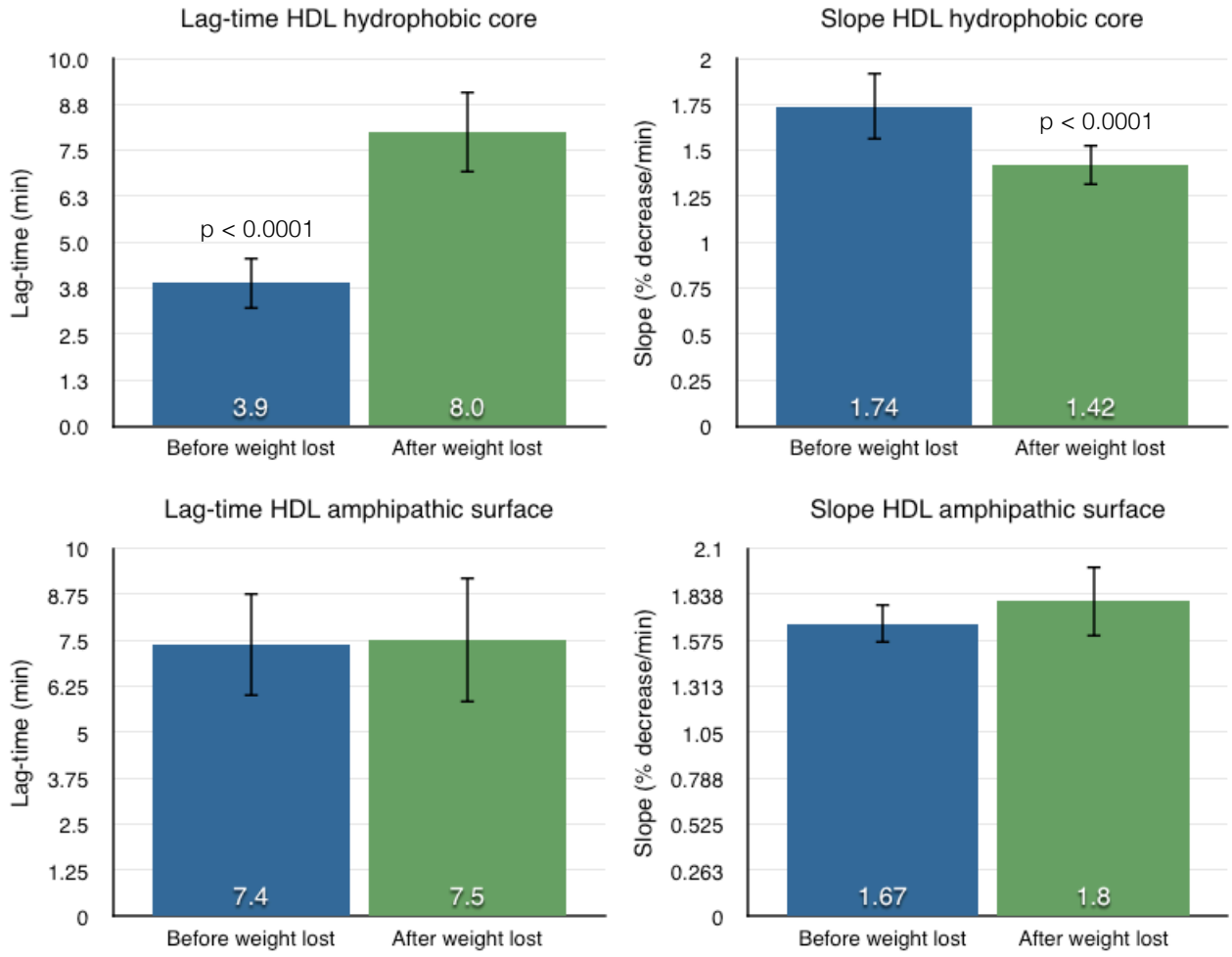
Table 4 shows the results for lipoproteins peroxidation: lag-time results are expressed in minutes while slope measures are expressed as percentage decrease for one minute. The hydrophobic core of LDL showed a reduction in lag-time after weight loss (from 17.8 minutes to 8.5 minutes) regardless of whether the same region of HDL showed a greater resistance to peroxidation after weight loss (lag-time from 3.9 minutes to 8 minutes). There were also differences in slope after weight reduction that increased in the hydrophobic core of LDL and decreased in the HDL core (from 0.82% decrease/min to 1.16% decrease/min and from 1.74% decrease/min to 1.42% decrease/min, respectively). The parameters indicative of susceptibility to peroxidation of the surrounding amphipathic envelope of lipoproteins did not statistically significantly differ after and before weight loss.

Table 4 – Oxidisability of the hydrophobic core and amphipathic surface of HDL and LDL.

	Before weight lost		**After weight lost**		**Wilcoxon test**
	Mean	**SEM**	**Mean**	**SEM**	**p**
HDL hydrophobic core lag-time (min)	3.9	0.7	8.0	1.1	**< 0.0001**
HDL hydrophobic core slope (% decrease/min)	1.74	0.18	1.42	0.11	**< 0.0001**
HDL amphipathic surface lag-time (min)	7.4	1.4	7.5	1.7	0.4637
HDL amphipathic surface slope (% decrease/min)	1.67	0.11	1.80	0.20	0.6685
LDL hydrophobic core lag-time (min)	17.8	1.8	8.5	1.3	**< 0.0001**
LDL hydrophobic core slope (% decrease/min)	0.82	0.06	1.16	0.09	**0.0006**
LDL amphipathic surface lag-time (min)	17.5	1.9	13.9	2.7	0.2769
LDL amphipathic surface slope (% decrease/min)	0.87	0.08	0.92	0.09	0.9799

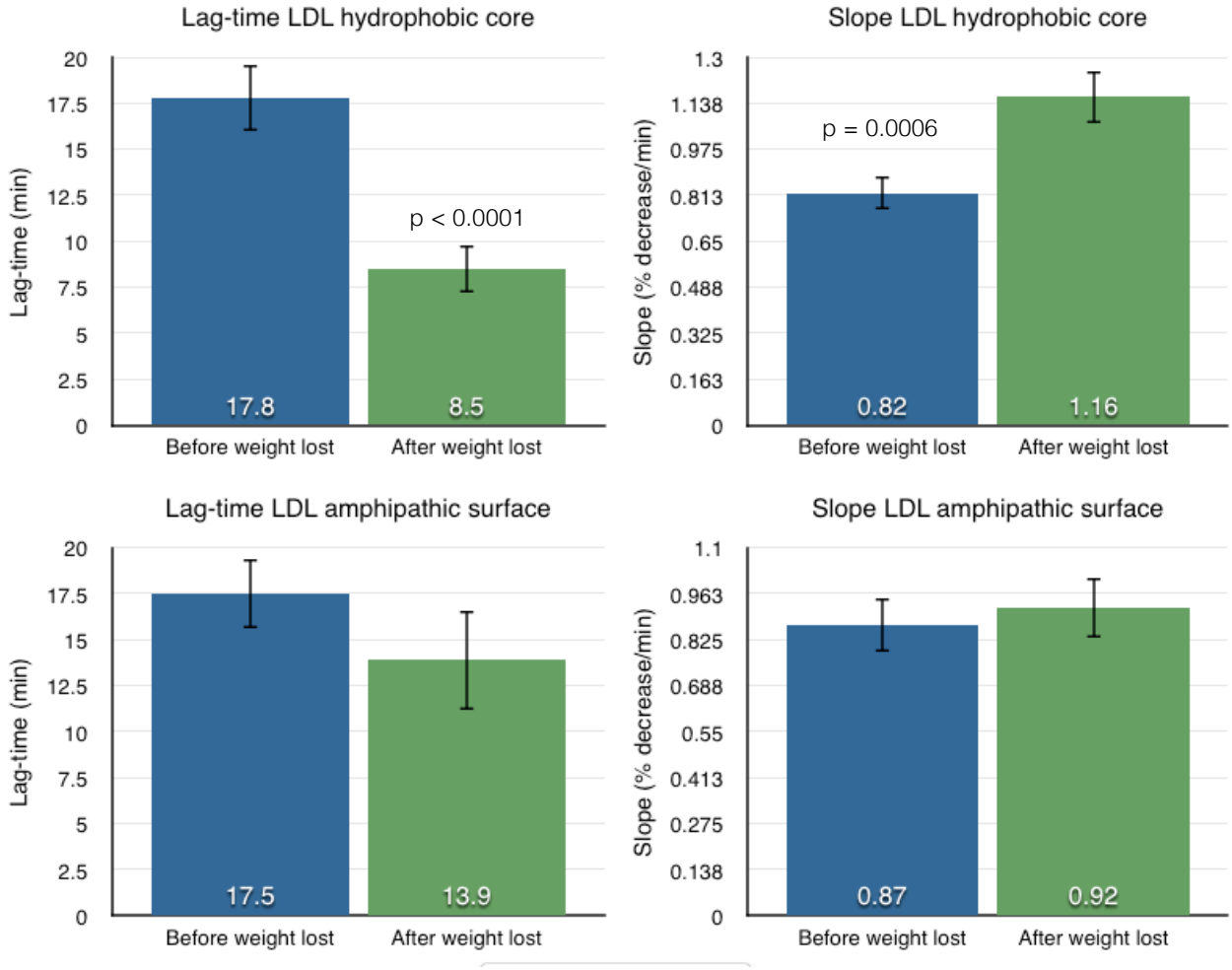
SEM = Standard Error of the Mean, VLDL = Very Low Density Lipoproteins, LDL = Low Density Lipoproteins, HDL = High Density Lipoproteins.

Figure 4 - Oxidisability of the hydrophobic core and amphipathic surface of HDL. These histograms graphically show the results for the lag-time and slope as indices of peroxidation of HDL. The top line shows the data relative to the hydrophobic core, while the lower line shows the data of the amphipathic surface.



In confirmation of the interdependence of data, there are correlations between the parameters of HDL and LDL peroxidation in different regions themselves, as well as between them and the data of chemical composition of lipoprotein. There is an inverse correlation between lag-time of the hydrophobic core of HDL and LDL and the velocity of propagation of the peroxidation (slope) in the same region of lipoproteins (R=-0.3399, p=0.0208, 95% CI -0.5795 to -0.0461; R=-0.3456, p=0.0186, 95% CI -0.5838 to 0.05262 respectively). There was a strong positive correlation between lag-time of core and lag-time of amphipathic envelope both in HDL and LDL (for HDL R=0.4437, p=0.002, 95% CI 0.1675 to 0.6554 and for LDL R= 0.752, p<0.0001, 95% CI 0.5849 to 0.8579). Interestingly, lag-time of the envelope of HDL also correlated positively with the lag-time of both regions of LDL (hydrophobic core R=0.3661, p=0.0123, 95% CI 0.07601 to 0.5991 and amphipathic envelope R=0.4318, p=0.0027, 95% CI 0.1531 to 0.6469).

Figure 5 - Oxidisability of the hydrophobic core and amphipathic surface of LDL. These histograms graphically show the results about the lag-time and slope as indices of peroxidation of LDL. The top line shows the data relative to the hydrophobic core, while the lower line shows the data of the amphipathic surface.



There were correlations between anthropometric and clinical blood parameters and indicators of peroxidation and chemical composition of lipoproteins, which will be summarised below. BMI was strictly inversely correlated with lag-time of the hydrophobic core and surrounding envelope of HDL (R=-0.5738, p<0.0001, 95%CI -0.7447 to -0.3323 and R=-0.5165, p=0.0002, 95% CI -0.7061 to -0.2578, respectively), whereas it was positively correlated with the slope of HDL core (R=0.3142, p=0.0335, 95% CI 0.1735 to 0.5601). BMI was also positively correlated with the concentration of proteins, both in VLDL (R=0.3417, p=0.0201, 95% CI 0.04815 to 0.5809) and in LDL (R=0.4275, p=0.003, 95% CI 0.1479 to 0.6438), and with the percentage of triglycerides in HDL (R=0.4653, p=0.0011, 95% CI 0.1938 to 0.6706), whereas it was inversely correlated with the percentage of cholesterol in HDL (R=-0.4397, p=0.0022. 95% CI -0.6525 to -0.1626).

The value of waist circumference as well as that of hip circumference were inversely correlated to lag-time both in the core and in the envelope of HDL (for waist circumference R=-0.3743, p=0.0104, 95% CI -0.6052 to -0.08543 and R=-0.3743, p=0.0104, 95% CI -0.6052 to -0.08543, respectively, and for hip circumference R=-0.3083, p=0.0371, 95% CI -0.5556 to -0.01083 and R=-0.4669, p=0.0011, 95% CI -0.6718 to -0.1958, respectively).

There were a positive correlation between concentration in plasma of HDL cholesterol and slope of HDL core (R=0.4606, p=0.0013, 95% CI 0.1879 to 0.6673) and an inversely correlation between plasma HDL cholesterol and slope of core and envelope of LDL (R=-0.292, p=0.0489, 95% CI -0.5431 to 0.007006 and R=-0.4214, p=0.0035, 95% CI -0.6394 to -0.1406 respectively).

The total cholesterol/HDL cholesterol ratio was inversely correlated to concentration of protein in HDL (R=-0.6127, p< 0.0001, 95% CI -0.7703 to -0.3846). The HDL cholesterol/triglycerides ratio was positively correlated to velocity of the reaction of peroxidation in core of HDL (R=0.3311, p=0.0246, 95% CI 0.03629 to 0.573) and inversely correlated to the slope of both the core and envelope of LDL (R=-0.3478, p=0.0179, 95% CI -0.5854 to -0.05504 and R=-0.3703, p=0.0113, 95% CI -0.6022 to -0.0808, respectively). There was also a negative correlation of this ratio with proteins concentration in VLDL (R=-0.6739, p< 0.0001, 95% CI -0.8095 and -0.47) and a positive correlation with proteins concentration in HDL (R=0.4189, p=0.0038, 95% CI 0.1376 to 0.6376).

Lipoproteins composition

Data show statistically significant differences before and after weight lost in lipoprotein compositions. Table 5 shows the chemical composition of lipoproteins: the results for lipids are expressed in ratio weight/weight and in nmoles/ml, while the results for proteins are expressed in µg/ml. Usually, the lipid composition of lipoproteins is expressed in weight/weight ratio to better understand the interaction between the different classes of lipids; generally, this is the common way to show results in the literature and in biochemical books. I think that relative values and absolute values have to be compared and although ratio weight/weight remains the standard way to express these data, the evaluation of changes in the number of moles offers a different perspective of reasoning. LDL show a higher percentage content of triacylglycerols (TAG) after weight loss (from an initial 20% to a 26% after weight reduction), whereas HDL reduce the percentage content of TAG (from 24% to 20%). Proteins concentration and the percentages of cholesterol and of phospholipids (PL) decrease after weight loss in LDL, regardless of an increase in HDL. There were no differences in VLDL composition before and after weight reduction.

Table 5 - Chemical composition of lipoproteins at initial time and after weight loss, divided into different classes. The results for lipids are expressed in weight/weight ratio of total lipids and in nmoles/ml, while the results for proteins are expressed in µg/ml. Molecular mass for cholesterol = 386.675 kg/moles. Average molecular mass for triglycerides = 869.6 kg/moles. The total phospholipid concentration of lipid extracts was determined by phosphorous assay. The phospholipids/proteins ratio is expressed in weight/weight ratio.

		Before weight lost		After weight lost		Wilcoxon test
		Mean	SEM	Mean	SEM	p
Proteins VLDL	(µg/ml)	853	109	798	99	0.6322
Cholesterol VLDL	(% w/w)	32	1.43	30	1.06	0.1040
	(nmoles/ml)	989	140	842	119	0.4332
Triglycerides VLDL	(% w/w)	52	1.40	53	0.96	0.9032
	(nmoles/ml)	700	99	671	98	0.7820
Phospholipids VLDL	(% w/w)	16	0.19	17	0.43	0.1016
	(nmoles/ml)	236	32	235	31	0.9515
Phospholipids/proteins ratio	(w/w)	24.8	3.74	24.9	3.05	0.8603
Proteins LDL	(µg/ml)	1281	88	1135	64	**0.0443**
Cholesterol LDL	(% w/w)	46,3	0.60	43	0.62	**0.0012**
	(nmoles/ml)	2617	155	2638	155	0.8469
Triglycerides LDL	(% w/w)	20,4	0.59	26	0.46	**< 0.0001**
	(nmoles/ml)	512	30	699	43	**0.0003**
Phospholipids LDL	(% w/w)	34,0	0.24	31	0.43	**0.0017**
	(nmoles/ml)	930	49	942	51	0.7820
Phospholipids/proteins ratio	(w/w)	59.3	4.64	66.2	4.38	0.4037
Proteins HDL	(µg/ml)	876	62	1044	59	**0.0010**
Cholesterol HDL	(% w/w)	37	0.49	39	0.46	**0.0005**
	(nmoles/ml)	1211	69	1151	89	0.8209
Triglycerides HDL	(% w/w)	24	0.35	20	0.34	**< 0.0001**
	(nmoles/ml)	332	22	262	21	**0.0125**
Phospholipids HDL	(% w/w)	39	0.45	41	0.42	**0.0046**
	(nmoles/ml)	609	38	608	45	0.8209
Phospholipids/proteins ratio	(w/w)	55.7	3.64	46.3	3.60	0,1754

SEM = Standard Error of the Mean, VLDL = Very Low Density Lipoprotein, LDL = Low Density Lipoprotein, HDL = High Density Lipoprotein.

Figure 6 - Lipid composition of the different classes of lipoprotein at initial time and after weight loss. The percentages of phospholipids are shown in yellow, the percentages of triglycerides are shown in green and the percentages of cholesterol are shown in blue. All data are expressed in weight/weight ratio on total lipids.

VLDL = Very Low Density Lipoproteins, LDL = Low Density Lipoproteins, HDL = High Density Lipoproteins.

To better investigate the molecular reasons for the changes in parameters of peroxidation in lipoproteins, the correlation between lag-time and slope in the different areas of HDL and LDL and the lipoprotein chemical composition was studied. The lag-time of the hydrophobic core of HDL was positively correlated with the percentage of cholesterol in HDL, and it was inversely correlated with the percentage of triglycerides in HDL (R=0.4805, p=0.0007, 95% CI 0.2126 to 0.6813 and R=-0.6378, p<0.0001, 95% CI -0.7865 to -0.4191 respectively). To confirm this, a positive correlation was identified between the change after weight loss in the lag-time of the HDL core and the variation of the percentage of cholesterol in HDL, along with a negative correlation between the change of lag-time in the same region of HDL and the change of percentage of triglycerides in HDL (R=0.6084, p=0.0021, 95% CI 0.2496 to 0.8203 and R=-0.4166, p=0.048, 95% CI -0.7138 to -0.007752 respectively). There was a positive correlation between the velocity of propagation of peroxidation in core of HDL (slope) and the percentage of triglycerides in HDL, irrespective of whether there was an inverse correlation between the slope of HDL core and percentage of phospholipids in HDL (respectively R=0.3659, p=0.0124, 95% CI 0.07572 to 0.5989 and R=-0.3339, p=0.0233, 95% CI -0.5750 to -0.03936). To complete the picture of the correlation there was a negative correlation between the lag time in the hydrophobic core of HDL and the phospholipids/proteins ratio in HDL (R=-0.4537, p=0.0091, 95% CI -0.6984 to -0.1140). With regard to LDL, lag-time of the hydrophobic core was positively correlated with the percentage of cholesterol in LDL and inversely correlated with the percentage of triglycerides in LDL (R=0.5241, p=0.0002, 95% CI 0.2675 to 0.7113 and R=-0.5074, p=0.0003, 95% CI -0.6999 to -0.2463, respectively). The slope in the core of LDL was inversely correlated with the percentage of cholesterol in LDL and positively correlated with the percentage of triglycerides in LDL (for cholesterol R=-0.3849, p=0.0083, 95% CI -0.6129 to 0.09762 and for triglycerides R= 0.5063, p=0.0003, 95%CI 0.2448 to 0.6991). There was a positive correlation between the lag-time of LDL amphipathic surface and the percentage of cholesterol in LDL and a negative correlation with the percentage of triglycerides in LDL (R=0.4086, p=0.0048, 95% CI 0.1254 to 0.6302 and R=-0.3318, p=0.0243, 95% CI -0.5735 to -0.03705, respectively). Finally, there was a negative correlation between the change in the percentage of triglycerides in LDL after weight loss and the variation of the percentage of triglycerides in HDL (R=-0.4536, p=0.0297, 95% CI -0.7355 to -0.03782).

Discussion

The susceptibility to peroxidation of the core of LDL and HDL appears to be especially influenced by their content of triglycerides. Consequently, the transfer of triglycerides from HDL to LDL results in an improvement of HDL and in a worsening of LDL in relation to the oxidisability. There was a strong reduction in the percentage of TAG in HDL from 24% to 20% (also supported by a reduction of absolute value of TAG expressed in nmol/ml). The change in chemical composition also justifies the change in susceptibility to peroxidation of HDL: resistance to the initial peroxidation increases while the propagation rate of peroxidation processes decreases in the core of HDL. This change in oxidisability is probably the result of the reduction of TAG in the core of HDL. This hypothesis is supported by an inverse correlation between the percentage of TAG in HDL and the lag-time in the core of HDL and also by a positive correlation between the percentage of cholesterol in HDL against lag-time in the HDL core. Further confirmation was provided by the fact that there is a positive correlation between the change in lag-time of the HDL core and the variation in the percentage of cholesterol in HDL and a negative correlation with the change of percentage of TAG. Also, the chemical composition of LDL changed dramatically and there is a strong increase of TAG from 20% to 26% (also supported by an increase of absolute value of TAG expressed in nmol/ml). Studying LDL after weight loss, a reduction in the percentage of cholesterol (from 46% to 43%) and phospholipids (from 33% to 31%) is also evident. The LDL enriched in TAG and with less cholesterol (probably due to less cholesteryl esters) are probably smaller and denser, increasing the cardiovascular risk [70]. Although the mechanisms underlying the association of small, dense LDL with increased risk of coronary artery disease are not clear, several hypotheses have been proposed. Mechanistically, small, dense LDL particles enter the arterial wall more easily [114], bind to arterial wall proteoglycans more avidly [115], and are highly susceptible to oxidative modification [116], leading to macrophage uptake, all of which may contribute to increased atherogenesis. The major oxidisability of LDL is shown by the dramatic reduction of lag-time and by the increase in the velocity of propagation of the reaction of lipid peroxidation in the core of LDL after weight loss. When studying lipoproteins out of plasma and without the protection of plasma antioxidant molecules, our experimental

model mimics the condition of lipoproteins in extravasal matrix. Similarly to *in vivo* expectations, lipoprotein oxidation in our *in vitro* model was induced by a flux of aqueous peroxyl radicals derived from the thermal decomposition of AAPH, which first comes into contact with different molecular species on the lipoproteins' surface like PL, cholesterol and proteins. Since the PL oxidisability to this radical species is known to be higher than that of cholesterol and apolipoproteins, it is reasonable to suppose that the polyunsaturated fatty acids of PL are the first substrates that start to be peroxidised, thus promoting the initial lipid peroxide formation in the lipoproteins surface region. The peroxidised lipids so formed on the surface can then propagate the peroxidation into the core of the lipoproteins. These peroxides probably propagate more easily in the core of lipoproteins because there is a higher fluidity, which is attributed to the fluidising effect arising from the increased presence of TAG. This effect is also probably due to the fact that TAG are a more oxidisable substrate than other lipid molecules in the core of lipoproteins such as cholesteryl esters (CE). This mechanism of peroxidation agrees well and is supported by our experimental evidence: lag-time in the core of LDL is negatively correlated with the percentage of TAG in LDL and positively correlated with the percentage of cholesterol in LDL, in parallel to that which occurs in HDL.

To explain the possible mechanisms underlying these findings, we refer to a study conducted previously in our laboratory that examined and compared the composition, fluidity and oxidisability of HDL hydrophobic core and amphipathic surface in two groups of adult males (25 kg/m^2 < BMI < 30 kg/m^2); the former were mixed dyslipidaemic patients and the latter age- and BMI-matched healthy controls. [102]. Mixed dyslipidaemia and metabolic syndrome have features in common, as well as potentially similar pathogenic mechanisms. In the current study, the levels of fat-soluble vitamins and the degree of unsaturation of the lipids in the core and in the surface of lipoproteins were not measured, because the levels of these parameters were not significantly different in the two groups investigated in a previous study and also did not show any significant correlations between these parameters.

Dyslipidaemia changes the chemical composition, the physical characteristics and the response to oxidative stress in HDL [102]. These alterations are determined by a higher free cholesterol to phospholipid ratio in surface and TAG to CE ratio in the core and from higher malondialdehyde (MDA) levels and lower alpha-tocopherol and beta-carotene to neutral lipid ratios that cause a more rigid surface and more fluid core in HDL. These chemical and physical changes are accompanied by a dramatic reduction in the lag-time and an increase in the propagation rate of the core of HDL that testify to an increased susceptibility to peroxidation. These results offer some opportunity to investigate the changes that affect HDL in subjects with or without dyslipidaemia and alterations in lipid metabolism. Cazzola et al. [102] described the modification of some lipoprotein-related

enzymes in subjects with dyslipidaemia. In dyslipidaemic patients, the cholesterol ester transfer protein (CETP) was shown to promote a massive transfer of TAG from VLDL to HDL [70]. In the same way, the HDL-enriched in TAG was found to be more efficient in promoting the transfer of free cholesterol from the other lipoprotein classes and cell membranes to their amphipathic surface, and the TAG enrichment of HDL was shown to be paralleled by a decrease in lecithin-cholesterol acyltransferase (LCAT) activity [117,118]. These changes could justify the modifications that affect HDL in subjects with dyslipidaemia.

Even if the concentrations of apolipoproteins and enzymes involved in lipoproteins metabolism such as apolipoproteins, LCAT and CEPT were not measured in the current study, the results of previous studies can explain the effect of changes in the composition of lipoproteins induced by diet and weight loss in our population. In fact, weight loss has been suggested to change the concentration and the function of different apolipoproteins and enzymes connected with lipoproteins. In particular, weight reduction: a) decreases the concentration of apolipoprotein C-III (ApoC-III) promoting the action of lipoprotein lipase with a reduction of TAG in HDL [93]; b) decreases the catabolism of apolipoprotein A-I (ApoA-I); and c) reduces CEPT levels [97,119]. ApoAI actives LCAT with a reduction in free cholesterol on the surface of HDL [95]. The LCAT activity promotes the formation of larger spherical HDL. These larger HDL are unstable and spontaneously shed a molecule of ApoA-I to form more stable particles, each of which now contains three molecules of ApoA-I instead of two molecules, further promoting this process [96]. This biochemical hypothesis is supported by the increased concentration of total proteins in HDL after weight loss. Changes in the concentration and in the activity of apolipoproteins and enzymes reduce the percentage of TAG in HDL core, as shown by the chemical analysis. Weight loss also produces a reduction in plasma levels of CEPT, interrupting the massive transfer of TAG from VLDL to HDL [97,119]. In this way, VLDL and LDL are enriched in TAG. In this study, the chemical composition of VLDL did not change after weight loss. Supposedly, the effects of CEPT reduction are offset by the contemporary reduction of Apo-CIII that promotes the action of lipoprotein lipase with a reduction of TAG in VLDL, as seen in HDL.

Oxidation makes HDL proinflammatory [24], while the changes described here might restore their powerful anti-inflammatory action. Dietary treatment also induces improvements that would most likely result in a reduction of proinflammatory factors and the reduction of weight modifies some fundamental features to define the cardiovascular risk in obese subjects: a) weight loss reduces abdominal fat (waist and hip circumference and waist to hip ratio decreased in subjects after weight loss); b) there is a significant improvement in insulin resistance with a reduction of glycaemia, of insulin concentration and of the HOMA-IR; c) there is a significant improvement in liver function (with a

reduction in ALT concentration from 43 U/l to 32 U/l); d) the concentration of triglycerides decreases (in these subjects there is a trend of reduction from 135.1 mg/dl to 119.5 mg/dl although not statistically significant); and e) there is a general reduction of inflammation levels (CRP from 0.40 mg/dl to 0.21 mg/dl although not statistically significant). These modifications could lead to a reduction of proinflammatory factors in order to compensate or even exceed the increased susceptibility of LDL to oxidation.

The changes described here result in a redistribution of plasma lipids in the different lipoproteins classes, tending towards a more physiological condition and reversing the alterations induced by metabolic syndrome. The lipoproteins in the blood form a dynamic system and the different lipoproteins interact with each other, changing their form and their function. The change in one of the different classes of lipoproteins causes a modification of all other types. HDL probably represent the initial point of change in lipid metabolism during weight loss. This hypothesis is supported from the evidence of a strong correlation between the lag-time of the envelope of HDL and the lag-time of the core of HDL, as well as with both of the regions of LDL, as if the resistance to the initial peroxidation of HDL and LDL was interdependent on one other. To sum up, after weight loss, the anti-atherogenic properties of HDL might appear enhanced, whereas LDL might seem more atherogenic and it is likely this corresponds to the recapture of a physiological order. On the other hand, weight loss is a process with an active phase and with a stabilisation phase and the adaptive response of lipoproteins may be different for each of these phases [85]. The process of weight loss is probably a period of greater fragility of obese patients, and these data should prompt researchers to consider the active phase of weight loss as an additional risk factor.

Although this study has provided new useful information on the oxidisability of the hydrophobic core and amphipathic surface of lipoproteins after weight loss, some limitations may also be discussed. First, the study population was relatively small and it was limited to male subjects. Therefore, the conclusions should not be generalised to all patients with metabolic syndrome. Second, although supported by other studies in our laboratory [102], this work lacks measurements of fat-soluble vitamins and malondialdehyde. Lastly, although it is accepted that the oxidisability of lipoproteins may play a role in the development of atherosclerosis, the validity of the assay performed *in vitro* to predict the *in vivo* progression of cardiovascular disease has yet to be proven.

In conclusion, the results of this study seem to indicate that more detailed knowledge of the composition, the chemical properties and the oxidisability of both the surface and core of lipoproteins could be a useful additional means for better understanding of the mechanisms that link the changes in lipoprotein metabolism to the risk of cardiovascular disease in patients with metabolic syndrome following weight loss.

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Francisco Goya
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