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DIRETTORE: PROF. BRUNO MASALA

EFFECT OF LIPID LOWERING THERAPY ON LDL-S-HOMOCYSTEINILATION STATUS IN CHRONIC KIDNEY DISEASE PATIENTS

RELATORE:
PROF. CIRIACO CARRU

CORRELATORE:
DR. ANGELO ZINELLU

TESI DI DOTTORATO DI:
DR. ELISABETTA PISANU

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1 INTRODUCTION

1.1 Lipoprotein description

A lipoprotein is a molecule that has a globular shape and is a biochemical combination of lipid and protein. Lipoprotein metabolic pathways are a part of the overall body fuel metabolism. The lipoprotein particle core is composed of lipid, primarily triacylglycerols and cholesteryl ester. Lipids are an important source of energy and are essential components of cell structure. Fatty acids are high-energy metabolic fuels, whereas cholesterol and phospholipids are essential components of cell membranes. The storage form of fatty acids are their glycerol esters, triacylglycerols (triglycerides, TG) and phospholipids. The main sites of fatty acid metabolism are the liver and muscle, while the main storage depot is the adipose tissue. They are essentially insoluble in water, yet must be moved from the tissue of origin to the tissues in which they will be stored or consumed as components of lipoprotein particles. Lipoproteins are classified on the basis of their hydrated density. It is important to remember that such classification is somewhat arbitrary, and implies the existence of clearly separate structures in what are population of constantly interchanging particles. Lipids are carried in the blood plasma as plasma lipoproteins, macromolecular complexes of specific carrier proteins, apolipoproteins ("apo" means "detached" or "separate," designating the

protein in its lipid-free form [Otvos et, al 2002]. The core of these spherical particles contains primarily cholesteryl ester and triglyceride. These insoluble molecules are surrounded by a coating of proteins and phospholipids that are amphipathic; that is, they have both polar and nonpolar regions. Apolipoproteins have four major roles: first, they are involved in the assembly and the secretion of the lipoprotein (apo B-100 and B-48); second, they provide structural integrity to the lipoprotein (apo B, E, A-I, A-II); third, they act as co-activators of enzymes (apo A-I, C-II, C-III); finally, they bind or dock to specific receptors and proteins for cellular uptake (apo A-I, B-100, E). The role of several apolipoproteins (A-IV,A-V, D and J) is still incompletely understood [Genest J, 2003].

Different combinations of lipids and proteins produce particles of different densities, ranging from chylomicrons to high-density lipoproteins [Kwiterovich PO, 2002] and their nomenclature is purely a function of the density of each particle. Lipoprotein particles are characterized by their size, density, flotation constant, and elecrtophoretic mobility. These particles can be separated by ultracentrifugation. The main lipoprotein classes present in plasma are chylomicrons, very-low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), low-density lipoprotein (LDL), and high-density lipoprtotein (HDL) [Havel RJ et al, 1955]. Further subspecies are present within these main classes. Particle density increases

from chylomicrons to HDL, and particle size decreases from chylomicrons to HDL. Moreover each of these particles performs a different functions by binding to specific receptors or acting as cofactors for enzymes, signals, targeting lipoproteins to specific tissues or activating enzymes that act on the lipoproteins, and that can be detrimental (VLDL, IDL, LDL) or beneficial (HDL) to your heart health.

Chylomicrons are the largest lipoproteins, the least dense and are rich in triacylglycerols. Chylomicrons are synthesized in the ER of epithelial cells in the intestinal lumen following the absorption of digested fat, then move through the lymphatic system and enter the bloodstream via the left subclavian vein. The apolipoproteins that predominate before the chylomicrons enter the circulation include apoB-48 and apoA-I, apoA-II and apoA-IV. Chylomicrons are transported in the blood to tissues such as skeletal muscle, fat, and the liver. The capillary beds of these tissues contain high concentrations of lipoprotein lipase (LPL). LPL hydrolyzes TG in the chylomicrons into free-fatty acids that are either oxidized by the muscle cells to generate energy, stored in adipose tissue, oxidized in the liver, or used in hepatic VLDL synthesis [Gotto A et al, 1999 (a)]. Once the chylomicrons have been processed by LPL, the TG-depleted chylomicron is called a remnant particle, which is then transported to the liver for further processing. When the diet contains more fatty acids than are needed

immediately as fuel, they are converted to triacylglycerols in the liver and packaged with specific apolipoproteins into very-low-density lipoprotein (VLDL). Excess carbohydrate in the diet can also be converted to triacylglycerols in the liver and exported as VLDLs. In addition to triacylglycerols, VLDLs contain some cholesterol and cholesteryl esters, as well as apoB-100, apoC-I, apoC-II, apoC-III, and apo-E. These lipoproteins are transported in the blood from the liver to muscle and adipose tissue, where activation of LPL by apoC-II causes the release of free fatty acids from the VLDL triacylglycerols. Adipocytes take up these fatty acids, reconvert them to triacylglycerols, and store the products in intracellular lipid droplets; myocytes, in contrast, primarily oxidize the fatty acids to supply energy. Most VLDL remnants are removed from the circulation by hepatocytes. The uptake, like that for chylomicrons, is receptor-mediated and depends on the presence of apoE in the VLDL remnants. The loss of triacylglycerol converts some VLDL to VLDL remnants (also called intermediate density lipoprotein, IDL); further removal of triacylglycerol from VLDL produces low-density lipoprotein (LDL) but only a small portion of VLDL is involved in this transformation. Very rich in cholesterol and cholesteryl esters and containing apoB-100 as their major apolipoprotein, LDLs carry cholesterol to extrahepatic tissues that have specific plasma membrane receptors that recognize apoB-100. LDL

receptors in peripheral cells or the liver bind with LDL and clear it from the blood. LDL particles carry the majority of the cholesterol in the blood, supplying cholesterol to the cells. Peripheral cells utilize LDL cholesterol for cell membrane structure and also the production of hormones. LDL is an atherogenic lipoprotein particle, and it is established that higher levels of LDL are associated with increased cardiovascular disease risk [Rifai N et al, 1997]. Normally, LDL particles contain only 4-8% of their mass as triglycerides. Under certain circumstances, especially in conditions of elevated triglyceride, LDL particles can be enriched in triglycerides and depleted in core cholesteryl esters. As a result, the LDL particles are smaller and have greater density. LDL particle size, therefore, is due to changes in core constituents, with an increase in triglycerides and a relative decrease in cholesterol leading to smaller, denser LDL particles. LDL particles in most higher mammals, including humans and non-human primates, are the main carriers of cholesterol [Genest J, 2003]. In addition, the heterogeneity of LDL particle composition, due to differences in the amount of cholesterol per particle, suggests that particle size is an important consideration in the atherogenic potential of the LDL. Although the exact mechanism is not fully appreciated, small, dense LDL particles containing more cholesterol ester (phenotype B) are considered to be more atherogenic than buoyant LDL (phenotype A) particles. Small, dense LDL

is thought to be more susceptible to modification and may therefore be more toxic to the vascular endothelium. A sequence of immunologic and inflammatory events in the arterial wall contributes to atherogenesis and the development of atherosclerotic lesions. These advanced lesions occlude coronary artery blood flow and contribute to clinical presentations such as unstable angina or myocardial infarction [Gotto A et al, 1999 (b)]. The fourth major lipoprotein type, high-density lipoprotein (HDL), originates in the liver and small intestine as small, protein-rich particles that contain relatively little cholesterol and no cholesteryl esters. It is well established that increased HDL levels are associated with decreased risk of coronary heart disease, whereas reduced HDL levels increase this risk. HDLs contain apoA-I, apoC-I, apoC-II, and other apolipoproteins, as well as the enzyme lecithin-cholesterol acyl transferase (LCAT), which catalyzes the formation of cholesteryl esters from lecithin (phosphatidylcholine) and cholesterol. LCAT on the surface of nascent (newly forming) HDL particles converts the cholesterol and phosphatidylcholine of chylomicron and VLDL remnants to cholesteryl esters, which begin to form a core, transforming the disk-shaped nascent HDL to a mature, spherical HDL particle. This cholesterol-rich lipoprotein then returns to the liver, where the cholesterol is unloaded; some of this cholesterol is converted to bile salts. HDL may be taken up in the liver by receptormediated endocytosis, but at least some of

the cholesterol in HDL is delivered to other tissues by a novel mechanism. HDL can bind to plasma membrane receptor proteins called SR-BI in hepatic and steroidogenic tissues such as the adrenal gland. These receptors mediate not endocytosis but a partial and selective transfer of cholesterol and other lipids in HDL into the cell. Depleted HDL then dissociates to recirculate in the bloodstream and extract more lipids from chylomicron and VLDL remnants. Depleted HDL can also pick up cholesterol stored in extrahepatic tissues and carry it to the liver, in reverse cholesterol transport pathways. In one reverse transport path, interaction of nascent HDL with SR-BI receptors in cholesterol-rich cells triggers passive movement of cholesterol from the cell surface into HDL, which then carries it back to the liver. In a second pathway, apoA-I in depleted HDL interacts with an active transporter, the ABC1 protein, in a cholesterol-rich cell. The apoA-I (and presumably the HDL) is taken up by endocytosis, then resecreted with a load of cholesterol, which it transports to the liver. The ABC1 protein is a member of a large family of multidrug transporters, sometimes called ABC transporters because they all have *A*TP-*b*inding *c*assettes; they also have two transmembrane domains with six transmembrane helices. These proteins actively transport a variety of ions, amino acids, vitamins, steroid hormones, and bile salts across plasma membranes.

1.2 Alterations in lipoprotein levels

Lipoprotein disorders result from abnormal synthesis, processing, or catabolism of plasma lipoprotein particles, and they lead to the onset of several diseases known as dyslipidemia. In western societies and in emerging economies, lifestyle contributes to the expression of lipoprotein disorders. Many dyslipoproteinaemias have a genetic aetiology. Four types of lipoprotein abnormalities are observed: elevated LDL cholesterol; reduced HDL cholesterol, usually with increased triglycerides and VLDL cholesterol; elevated levels of chylomicron remnants and IDL; and elevated levels of lipoprotein (a) [Lp(a)] particles [Breslow JL, 1991]. Lipoprotein transport genes have been implicated in each of these abnormal lipoprotein phenotypes.

Elevated total cholesterol primarily reflects elevated LDL cholesterol, which constitutes 70% of plasma cholesterol. A number of genetic conditions have been identified that affect LDL cholesterol levels. Familial hypercholesterolemia (FH) is a relatively common cause of elevated LDL cholesterol and is present in ≈5% of patients with MI [Brown MS et al, 1991]. FH homozygotes typically have sixfold elevations in LDL cholesterol, with total cholesterol levels of 650 to 1000 mg/dL, and they can be identified at birth by markedly elevated cholesterol in umbilical cord

blood [Goldstein JL et al, 1991]. FH heterozygotes have LDL cholesterol levels twice as high as normal, or approximately 140 mg/dL higher than family members with two normal genes.

Reduced HDL cholesterol (<35 mg/dL) has been found, in a large number of studies, to be a potent independent risk factor for CAD. Low levels of HDL, also known as hypoalphalipoproteinemia (HA), includes a variety of conditions, ranging from mild to severe. The etiology of HDL deficiency ranges from secondary causes, such as smoking, to primary or familial causes including specific genetic mutations, such as Tangier disease and fish-eye disease. Approximately half the population variance in HDL cholesterol is probably attributable to genetic factors [Breslow JL, 1989]. Hypoalphalipoproteinemia has no clear-cut definition. An arbitrary cutoff used frequently in the old literature includes a HDL level that falls within the 10th percentile of HDL levels. A more practical definition derives from the theoretical cardioprotective role of HDL. The US National Cholesterol Education Program (NCEP) Adult Treatment Panel III (ATP III) redefined the HDL level that constitutes a formal CHD risk factor. The minimum desirable level was raised from 35 to 40 mg/dL for both men and women. For people with metabolic syndrome, however, the designated HDL levels that contribute to the syndrome are gender-specific. For men, a desirable HDL level is still one that is above 40 mg/dL, but for women, the desirable

level is greater than 50 mg/dL to satisfy the definition of metabolic syndrome. Low HDL is also frequently associated with high triglycerides. It has been described that the patient with a desirable LDL level (100 mg/dL) in association with low HDL (25 mg/dL) level would have a CHD risk equivalent to a patient with elevated LDL (220 mg/dL) but a higher, desirable HDL level (45 mg/dL) [Castelli WP, 1988].

Hypertriglyceridemia is defined as an abnormal concentration of triglyceride in the blood. Hypertriglyceridemia may be primary or secondary in nature. Primary hypertriglyceridemia is the result of various genetic defects leading to disordered triglyceride metabolism. Secondary causes are acquired causes, such as, high fat diet, obesity, diabetes, hypothyroidism, and certain medications. Hypertriglyceridemia is typically not an isolated abnormality: it is frequently associated with other lipid abnormalities and the metabolic syndrome (abdominal obesity, insulin resistance, low HDL, high triglyceride, and hypertension), which are linked to coronary artery disease [Ford ES et al, 2002]. Hyperchylomicronaemia is a rare disorder of severe hypertriglyceridaemia associated with elevations in fasting plasma triglycerides >11.3mmol/L (>1000 mg/dl). The clinical presentation is characterized by recurrent bouts of pancreatitis and eruptive xanthomas. It is caused by a markedly reduced or absent lipoprotein lipase (LPL) activity or, more rarely, by the absence of its activator, apo C-II

[Feoli-Fonseca JC et al, 1998; Santamarina-Fojo S 1998]. This leads to a lack of hydrolysis of chylomicrons and VLDL and their accumulation in plasma, especially in the postprandial phase. This can lead to extreme elevations of plasma triglycerides of 113 mmol/L (>10 000mg/dl). The plasma from a patient with very high triglycerides is milky white and, after it has been standing overnight in a refrigerator, a clear band of chylomicrons can be seen on top of the plasma.

In case-control studies, elevated plasma levels of Lp(a) have been found to be an independent risk factor for CAD [Berg K, 1992]. Plasma Lp(a) concentrations >20 mg/dL have been reported to confer increased risk of CAD, as well as cerebrovascular and peripheral vascular disease. Lp(a) >39 mg/dL was the most common familial dyslipidemia in a study of patients with confirmed CAD before age 60 years, accounting for 19% of patients with a familial dyslipidemia [Genest JJ Jr et al, 1992]. Approximately 90% of the variability in Lp(a) levels in the population is attributable to the apo(a) gene [Boerwinkle E et al, 1992]. Lp(a) levels are inversely correlated with apo(a) size, and individuals with smaller apo(a) isoforms are therefore presumed to be at greater risk of CAD [Berg K, 1992]. Lp(a) may participate in both thrombogenic and atherogenic processes because of the plasminogen-like properties of apo(a) and the LDL-like properties of Lp(a) [Scanu AM et al, 1990]. Fibrinolysis by plasmin requires the

conversion of plasminogen to plasmin by a tissue-type plasminogen activator. In vitro studies suggest that Lp(a) may interfere with this process. In addition, apo(a) can compete with plasminogen for binding to fibrin and to plasminogen receptors on cultured endothelial cells, preventing assembly of the fibrinolytic system on cell surfaces [Berg K, 1992].

In the organism the levels of lipoprotein can change because of the normal physiological processes. Moreover, numerous demographic factors within the population are correlated with differences in lipid and lipoprotein concentration. In newborns, total cholesterol, triglycerides and most lipoprotein and apolipoprotein concentrations rise rapidly from the low values in cord blood to 80% of adult values by 4 days of age. In children, lipid concentrations of both boys and girls remain stable until just before puberty, when a transient fall in triglycerides, total cholesterol (TC) and LDL-cholesterol occurs [Strobl W et al, 1985]. In all individuals, TC concentrations gradually increase with age, although the magnitude of increase is not as great in more recents studies as it was 20 or more years ago [Johnson CL et al, 1993].

The major behavioral factors that affect the lipid, lipoprotein, and apolipoprotein serum concentrations are diet, obesity, cigarette smoking, alcohol and caffeine intake, exercise, and stress. The effect of diet on lipid and lipoprotein concentrations is well established. The extent of this effect,

however, varies among individuals. It has been shown that an increase in dietary intake of cholesterol can cause serum TC concentration to rise over 5% in only 30% of the studied population [Cooper GR et al, 1988]. Various fatty acids appear to have different effects on the lipoprotein profile and cardiovascular risk. In general, diet rich in mono- and polyunsatured fatty acid cause serum TC, LDL-C, apo B, and TG to decrease, while diet rich in satured fat, mainly palmitic acid, does not appear to increase [Kloer HU, 1989; Grundy SM et al, 1990].

1.3 LDL structure modifications

Low density lipoprotein (LDL) modification in the vascular wall seems to be a key factor in atherosclerosis development. LDL may play a role at several stages in atherogenesis; however, the precise mechanisms by which LDL promotes the development of lipid-laden foam cells in the fatty streak lesion remain to be elucidated. Uptake of cholesterol by way of the classic LDL receptor pathway cannot result in appreciable cholesterol accumulation because the LDL receptor is subject to feedback inhibition by cholesterol. However, certain modified forms of LDL are taken up by way of the scavenger receptor mechanism. This results in substantial cholesterol accumulation and foam cell formation in macrophages, because the

scavenger receptor is not regulated by the cellular cholesterol content [Brown MS et al, 1983].

In a metal-catalyzed reaction, the fatty acid hydroperoxides subsequently form aldehydes such as malondialdehyde, 4-hydroxynonenal and hexanal [Esterbauer H et al, 1987]. These aldehydic products then react with the ε-amino of lysine residues on apo B [Lecomte E et al, 1993; Steinbrecher UP, 1987], forming Schiff bases and increasing the negative charge on LDL. This prevents recognition by the LDL receptor. However, oxidized LDL (Ox-LDL) is processed by the scavenger receptor pathway, leading to cholesterol accumulation and foam cell formation.

1.3.1 LDL oxidation

The most plausible and biologically relevant modification of the LDL particle appears to be due to oxidation. One of the initial events in LDL oxidation is the free radical peroxidation of polyunsaturated fatty acids in LDL. Initially, there is conjugated diene formation due to hydrogen abstraction and molecular rearrangement. After oxygen uptake, peroxy radicals form, which in turn abstract hydrogen from another fatty acid, initiating an autocatalytic reaction that leads to the formation of hydroperoxides. Also, the major protein of LDL, apo B-100, undergoes

fragmentation due to oxidative scission. Many lines of evidence suggest that oxidation of low-density lipoprotein (LDL) plays an important role in the pathogenesis of atherosclerosis particularly in animals, and oxidized LDL in circulation is a well-known risk marker of human cardiovascular diseases, the principal cause of which is atherosclerosis [Steinberg D et al, 1989; Steinberg D et al, 1997; Berliner JA et al, 1995; Fraley AE et al, 2006; Tsimikas S, 2008; Parthasarathy S et al, 2008; Steinberg D, 2009]. Under conditions of oxidative stress, lipid molecules containing polyunsaturated fatty acids (PUFA) in LDL are easily oxidized. A variety of lipid oxidation products are formed, and subsequently, apoB protein is covalently modified by these oxidized lipids [Witztum JL et al, 1991; Itabe H, 1998]. Reactive oxygen species induce fragmentation of apoB, producing peptides ranging from 14 kDa–500 kDa. The polyunsaturated fatty acids in cholesteryl esters, phospholipids and triglycerides are also subjected to free radical-initiated oxidation to yield a broad array of smaller fragments, including aldehydes and ketones that can become conjugated to amino lipids or to apoB. The fragments further participate in chain reactions that propagate and amplify the damage. OxLDL has been shown to be present in atherosclerotic lesions of laboratory animals and humans [Witztum JL et al, 2001]. Incubating LDL with a monolayer of arterial endothelial cells in the presence of transition metals converted the

lipoprotein to a form that was taken up much more rapidly by macrophages than native LDL. The avid uptake of oxLDL by macrophage scavenger receptors leads to lipid-laden foam cells and fatty streak development in the arterial wall, which is one of the earliest steps in the progression of the atherosclerotic plaque. On intensive absorption of oxy-LDL, macrophages transform to lipid-overloaded "foam cells" and secrete a factor stimulating formation of their colonies. This results in clustering of cells with lipid inclusions and the arising of primary pre-atherogenic damage to the blood vessel wall as fatty streaks [Lankin VZ, 2003; Steinberg D et al 1989; Witztum JL et al, 1991; Yla-Herttuala S, 1994; Mackness M et al, 1995; Goldstein JL et al, 1979]. Thus, oxidative stress accompanied by intensification of free radical oxidation of lipids in biomembranes and LDL of blood plasma promotes the arising and progression of atherosclerosis [Lankin V, 1992]. The oxidation of polyunsaturated fatty acids can lead to the formation of aldehydes that modify lysine residues in apo B-100 [Esterbauer H et al, 1992]. Adducts of lysine residues with malondialdehyde and 4-hydroxynonenal have been characterized extensively and antibodies raised against these species. These antibodies avidly stain atherosclerotic lesions in LDL receptor-deficient rabbits [Boyd HC et al, 1989; Harberland ME et al, 1988], apo E deficient mice [Palinski W et al, 1994], and humans [Palinski W et al, 1989; YIa-Herttuala

S et al, 1989] with no demonstrable staining in normal arteries. As the oxidative modification hypothesis would predict, these epitopes largely co-localize in macrophages, although one might argue that they are not specific for LDL and could represent modification of other proteins in the atherosclerotic lesion as well. Consistent with this assertion is a study showing that LDL isolated from atherosclerotic lesions possesses properties that resemble those of Ox-LDL formed in vitro [YIa-Herttuala S et al, 1989], indicating that LDL oxidatively modified is accumulated in vascular walls. The diversity of LDL oxidation provides different biological effects to vascular cells [Yoshida H et al, 1998; Lee H et al, 2000; Glass CK et al, 2001; Boullier A et al, 2006]. For example, the tissue factor expression in endothelial cells is induced by mildly oxidized LDL but not by highly oxidized LDL. The lipids in highly oxidized LDL are cytotoxic and pro-apoptotic, whereas the mildly oxidized LDL is not, and stimulates the proliferation of smooth muscle cells and may contribute to prolong of macrophage foam cells via a PI3 kinase/Akt-dependent mechanisms. The plasma antioxidants provide effective protection against oxidation of LDL [Frei B et al, 1988]. This means that the major site of LDL oxidation is the subendothelial space. The transit of LDL across this space may yield a small amount of circulating LDL that is oxidized. Consistent with these findings, human plasma shows immunoreactivity

towards epitopes generated on Ox-LDL [Holvoet P et al, 1995; Palinski W et al, 1996]. However, the existence of oxidized LDL in the circulation remains controversial on the basis of artifacts that may occur during ex vivo handling of plasma and isolation of LDL.

1.3.2 LDL glycation

LDL glycation is considered to be a proatherogenic modification contributory to the increased susceptibility of patients with diabetes to atherosclerotic disease. It was first described by Schleicher et al in 1981 [Schleicher E et al, 1981]. They showed that the extent of LDL glycation varied as a function both of the duration of LDL incubation and the concentration of glucose in the incubation mixture. These reserchers were also the first to demonstrate that people with diabetes undergo increased in vivo glycation of apolipoprotein B (Apo B, the surface protein of LDL); this gave rise to the hypothesis that lipoprotein glycation contributes to the accelerated atherosclerosis of diabetes. Glycation is the non enzymatic reaction of glucose with susceptible amino groups in the side chains of amino acid residues (usually lysine) in proteins to form stable covalent adducts leading to structural alterations and, consequently, to functional abnormalities. Although the initial reaction, the formation of fructose-

lysine, is reversible, further rearrangement of the protein side chains can lead to more stable products. Under physiological conditions, glucose in solution exists in a stable pyranose ring structure in equilibrium with the open-chain aldehyde form [Bunn HF et al, 1981]. The aldehyde (or ketone) group of reducing sugars or low-mass compounds derived from glucose, such as glycoaldehyde or methylglyoxal, reversibly condense with the amino groups on proteins, forming a labile Schiff's base (or aldimine), which irreversibly undergoes a rearrangement to a more stable ketamine that is known as the Amadori product. This Amadori product can undergo further cycles of condensation between amino and aldehyde groups which may result in oxidative fragmentation to yield heterogeneous compounds referred to as advanced glycation end products (AGEs) [Thornalley PJ et al, 1999; Voziyan PA et al, 2003; Brown BE et al, 2006; Basta G et al, 2004]. These irreversible reactions are collectively termed "browning" or the "Maillard" reaction [Maillard LC, 1912]. Glycated LDL has been shown to induce functional changes in various cell types including enhancement of chemotactic properties in monocytes, [Millican SA et al, 1998; Zoltowska M et al, 2004] stimulating migration and proliferation of smooth muscle cells, and increasing platelet aggregation, NO production, and Ca^{2+}ATPase activity. [Makita T et al, 1999; Taguchi S et al, 2000; Ferretti G et al, 2002] Activation of the MAPK pathway with increased

ERK phosphorylation and PKC activity and of STAT5 with increased src kinase activity and p21waf expression has been invoked in the mediation of glycated LDL effects in smooth muscle and endothelial cells [Velarde V et al, 2001; Brizzi MF et al, 2002]. Additionally, glycation of LDL increases its interaction with arterial proteoglycans, a property considered to have atherogenic importance. [Edwards IJ et al, 1999]. Further to direct proatherogenic biologic effects, the capacity of glycated LDL for vascular damage is accentuated by its propensity to promote oxidation of the apolipoproteins and the lipids in the particle core, yielding glycoxidized LDL.

1.3.3 LDL methylation

Modification of native LDL by reductive methylation (MeLDL) abolishes the binding of the lipoprotein to LDL receptors, and causes the cellular uptake of these derivatized molecules to be mediated by apparently nonspecific mechanisms [Mahley RW et al, 1977; Weisgraber KH et al, 1978]. It has been shown recently that the initial uptake of native and reductively methylated LDL is not significantly different in normal rabbit aorta [Wiklund O et al, 1985]. Fischman et al show by en face autoradiography that LDL and MeLDL were both focally sequestered by

the healing rabbit abdominal aorta at the edges of regenerating endothelial islands and it seem that there was a significant difference in lipoprotein accumulation between healing abdominal and intact thoracic aorta, with healing aorta accumulating between three and five times more lipoprotein than intact aorta [Fischman AJ et al, 1987].

1.3.4 LDL carbamylation

Carbamylation of a protein is usually associated with a partial loss of function [Park KD et al, 2004; Pieniazek A et al, 2003]. Usually, carbamylated proteins have no positive effect on the human or animal body and are not needed in normal metabolism. The resulting in vivo carbamylation changes the structure of proteins and modifies the activity of enzymes, cofactors, hormones, and antibodies [Steinbrecher UP et al, 1984; Kraus LM et al, 2001; Weisgraber KH, 1978]. Carbamylated low-density lipoprotein (cLDL) is a recently identified type of modified low-density lipoprotein (LDL) that seems to be important in atherosclerosis in humans [Kraus LM et al, 2001; Ok E et al, 2005; Apostolov EO et al, 2005]. It is generated by irreversible chemical modification of the protein component of the LDL particle, apolipoprotein B, and by urea-derived cyanate present in human blood plasma. Several studies have revealed that the protein

component of the LDL particle, apolipoprotein B (ApoB), may be carbamylated at lysine or the terminal protein amino acids [Kraus LM et al, 2001; Canal J et al, 1973; Weisgraber KH et al, 1978; Gonen B et al, 1983]. Under physiological conditions it is likely that the molar concentration of cyanate is approximately 0.8% of the molar concentration of urea [Marier JR et al, 1964]. The reaction of cyanate with NH_2-terminal reactive amino groups occurs approximately 100-fold faster than the reaction with epsilon-amino groups, but significant carbamylation of lysine epsilon-amino groups may still occur [Stark GH et al, 1960], leading to the formation of stable adducts. Cyanate can also react with protein sulphydryl groups, leading to the formation of thio-carbamates which are unstable on acid hydrolysis [Qin W et al, 1993]. The formation of thiocarbamates seems to occur to a lesser degree than the formation of lysine derivatives. The elevation of blood urea in uremic patients causes a proportional increase in plasma cLDL [Apostolov EO et al, 2005] because the transformation of urea to cyanate is a spontaneous, nonenzymatic reaction which causes the carbamylation [Shah SV et al, 2008]. The LDL of chronic renal failure patients on dialysis has been shown to induce greater monocyte–endothelial cell adhesion [O'Byrne D et al, 2001]. Carbamylated LDL has strong atherogenic effects on endothelial cells by impacting the cell cycle causing cell injury [Apostolov EO et al,

2007; Ok E et al, 2005] and promoting monocyte adhesion through the overexpression of ICAM-1 and VCAM-1 molecules [Apostolov EO et al, 2007]. The initial mechanisms of these cLDL-induced events are not known. In particular, there is no information regarding whether cLDL uses any specific receptors or messengers. Horkko and coauthors proposed that cLDL lacks the ability to bind the LDL receptor (LDLR) on the endothelial surface, and so cLDL uptake is slow compared to native LDL (nLDL) [Horkko S et al, 1994]. With an increasing degree of carbamylation, cLDL binding switches from LDLR to scavenger receptors, which leads to faster uptake of cLDL from blood relative to nLDL [Horkko S et al, 1994; Gonen B et al, 1985].

1.3.5 LDL acetylation

Mouse peritoneal macrophages and other macrophages take up and degrade LDL that has been modified by chemical acetylation (acetyl-LDL) at rates that are 20-fold greater than those for the uptake and degradation of native LDL [Goldstein JL et al, 1979]. The receptor for acetylated low density lipoprotein particles (acetyl-LDL) was first detected on cultured murine peritoneal macrophages [Goldstein JL et al, 1979]. When these cells accumulate cholesterol ester via acetyl- LDL through the scavenger

receptor pathway, they adopt a foamy appearance due to cytoplasmic lipid droplet formation. Acetylated LDLs has been used as an endothelial marker for over twenty years [Voyta JC et al, 1984]. However, Acetylated- LDL uptake is not specific when used *in vitro*, because other cells such as macrophages that are equipped with scavenger receptors also endocytose the compound. Xiao-Miao et al have showns that, when flushed bone marrow cells are exposed to DiI-Ac-LDL, a large proportion of the cells endocytosed DiI-Ac- LDL as demonstrated by flow cytometric analysis [Xiao-Miao Li et al, 2009].

Haller et al could not find a difference in the response of VCAM surface expression between native LDL and acLDL [Haller H et al, 1995]. Although acLDL is not abundant in humans, acetylation alters the biological properties of the lipoprotein similar to oxidation [Steinberg D et al, 1989]. Both oxLDL and acLDL bind to a so-called "scavenger" receptor, which differs from the native LDL receptor [Matsumoto A et al, and 1990; Kodama T et al, 1990; Nagelkerke JF et al, 1983].

1.3.6 LDL thiolation

Low molecular weight (LMW) aminothiols such as homocysteine, cysteine, cysteinylglycine, glutathione and glutamylcysteine are able to

react with a number of thiol groups, many of which are present in proteins and other biological significant molecules. The importance of protein-mixed disulphide formation as a functional response to oxidative modification in living organisms has been known for a long time. This process can take place in all biological compartments as a consequence of oxidative events in pathological or physiological conditions. Plasma *S*-thiolated proteins have been detected in healthy humans, in patients with cardiovascular diseases and in several cell types after oxidant exposure [Di Simplicio P et al, 2003]. Thiols in plasma are mainly linked to albumin but interactions between homocysteine with ceruloplasmin, fibrin, annexin II, and transthyretin have also been reported [Sengupta S et al, 2001 (b); Majors AK et al, 2002; Lim A et al, 2003]. These post-translational modifications may have important functional consequences on proteins. As is true for any other chemical reaction, the formation extent of thiol protein derivatives is dependent on time and concentration. The longer the duration of exposure and the higher the concentrations of LMW aminothiols, the greater the biochemical damage inflicted. Furthermore, if the molecules attacked are long-lived and the derivation reactions irreversible, the harmful effects will be cumulative and the clinical consequences progressive. Recent studies have demonstrated that also plasma lipoproteins are susceptible to thiolation [Ferguson E et al, 1999]. The

detection of all thiols linked to apoB may be important to understand the mechanism by which thiols interact with lipoproteins and to evaluate the reactions involved in lipoprotein thiolation both *in vivo* and *in vitro*. The amount of Hcy bound to LDL may be related not only to its plasma concentration, but also to the availability of -SH free sites in the apoprotein primary sequence that depend on the concentration of other thiols as well. Thus the interaction between physiological plasma thiols and apoprotein may be connected to the balance among the different concentrations of all thiols.

1.3.6.1 LDL homocysteinilation

In 1932 Burtz and du Vigneaud discovered a new amino acid by treating methionine with sulfuric acid at the University of Illinois [DuVigneaud V, 1952]. Because this amino acid was similar in structure to cysteine and contained an extra carbon atom, they named it homocysteine. Homocysteine (Hcy) is a non-protein sulfur-containing amino acid which is an intermediate product of methionine metabolism. Adenosine is transferred from ATP to methionine to form S-adenosylmethionine (SAM) in a reaction catalyzed by methionine adenosyltransferase (SAM synthetase). SAM is a major donor of methyl groups for various

methylation reactions. When a –CH3 group is transferred by methyltransferases to the respective acceptor, SAM is converted to S-adenosylhomocysteine (SAH), which is subsequently hydrolyzed by SAH hydrolase to adenosine and homocysteine.

Homocysteine is metabolized by two alternative pathways. First, it may be remethylated to methionine by methionine synthase, which uses 5-methyltetrahydrofolate and vitamin B12 as cofactors (the remethylation pathway). Second, in the trans-sulfuration pathway catalyzed by cystathionine B-synthase (CBS), Hcy undergoes condensation with serine to form cystathionine. Although Hcy is a normal metabolite, its excess can be extremely toxic to human, animal, yeast, and bacterial cells. The non-protein amino acid homocysteine, owing to its structural similarity to the protein amino acids methionine, isoleucine, and leucine, enters the first steps of protein synthesis and is activated by methionyl-, isoleucyl-, and leucyl-tRNA synthetases *in vivo* [Jakubowski H, 2006]. Homocysteine and cysteine are sulfhydryl compounds (R-SH); compounds that contain a free sulfhydryl group are known as thiols. A general chemical property of thiols is their ability to oxidize in the presence of an electron acceptor such as molecular oxygen to form disulfides. The homocysteine will autooxidize to form homocystine. Homocysteine can oxidize with other thiols such as cysteine and glutathione to form mixed disulfides, and these compounds

are referred to as homocysteine cysteine mixed disulfide and homocysteine-glutathione mixed disulfide. Peptides and proteins may also contain free cysteine residues that can autooxidize or react with low-molecular weight thiols to form stable disulfide bond complexes [Yilmaz N, 2012].

Du Vigneaud investigated the role of homocysteine in metabolism and the ability of homocysteine and choline to replace methionine as an essential nutrient for growth of animals. However, little else was known about the importance of homocysteine in medicine or vascular disease in the 1950s. Later, it was demonstred in monkeys a relation between arteriosclerosis and sulfur amino acid metabolism [McCully KS, 2007]. Homocysteine has now become a rather famous amino acid. In 1962 children with mental retardation, dislocated ocular lenses, accelerated growth, osteoporosis, and a tendency to thrombosis of arteries and veins were discovered to excrete the amino acid homocystine in their urine. These children had a rare inherited enzymatic defect in homocysteine metabolism that was caused by deficiency of cystathionine synthase, an enzyme requiring pyridoxal phosphate (vitamin B6) for normal activity [Carson NAJ et al, 1962; Gerritsen T et al, 1962; Mudd SH et al, 1964].

Homocysteine circulates in the blood at a concentration of ~10 μM. In plasma, about 2/3 of total Hcy is bound to protein cysteine residues by

disulfide bridges (Hcy-protein disulfides) and most of the remaining 1/3 circulates in oxidized form as low-molecular- weight disulfides, homocystine (Hcy-S-S-Hcy), or Hcy-S-S-cysteine. Only about 1% of total Hcy circulates in free reduced form [Medina M et al, 2001]. Hyperhomocysteinemia has been established as an independent, modifiable risk factor for cardiovascular disease [Nygard O et al, 1997; Al-Obaidi MK et al, 2000; AronowWS et al, 2000 (a); Aronow WS et al, 2000 (b)]. However, the mechanism of homocysteine pathogenesis is still not clear. The possible mechanisms through which increased Hcy levels exert a toxic effect are as follows: oxidation, nitrosylation and DNA and protein hypomethylation [Loscalzo J, 1996; Perna AF et al, 2003; Ingrosso D et al, 2003]. Another potential mechanism of Hcy toxicity is protein homocysteinylation. In general, protein homocysteinylation, that is, the binding of Hcy to proteins, can occur in the following ways:

(i) the acylation of free amino groups, termed protein-*N*-homocysteinylation, in particular, the binding of Hcy to the ε-amino group of lysine residues and the terminal amino group of proteins mediated by Hcy thiolactone (HcyT), an Hcy derivative [Jakubowski H, 2002];

(ii) oxidation of thiol groups, mediated by Hcy in its free form, termed protein-*S*-homocysteinylation and directed towards proteine cysteine

residues [Sengupta S et al, 2001] (Fig.1). In particular, some previous studies have demonstrated that plasma lipoproteins are susceptible to *N*-homocysteinylation [Jakubowski H, 1999; Ferguson E et al, 1999; Ferretti G et al, 2003 (b)]. Many potentially harmful effects of homocysteine have been characterized *in vitro*, such as stimulation of oxidative stress, scavenging of nitric oxide, enhanced coagulation, and apoptosis. However, most of these effects were observed at high Hcy concentrations, far exceeding values observed under physiological or even pathological conditions. It is now well established that homocysteine is an accepted independent risk factor for several major pathologies including cardiovascular disease, birth defects, osteoporosis, Alzheimer's disease, and renal failure. Interestingly, many of the pathologies associated with homocysteine are also linked to oxidative stress. Reactive oxygen species (ROS) can in turn elevate tension of redox stress, and cause damage to cells. Oxidative stress is also linked to declines in pulmonary, brain, circulatory, and reproductive function [Suszynska J et al, 2010]. The mechanisms by which hyperhomocysteinaemia may be involved in the development of atherogenesis are only partially understood, but induction of oxidative damage and endothelial injury have been suggested [McDowell IFM et al, 2000]. However, it is not clear whether homocysteine is a marker or a causative agent [Jacobsen DW, 2000].

Post-translational modifications by Hcy may have important functional consequences on proteins. As is true for any other chemical reaction, the formation extent of Hcy protein derivatives is dependent on time and concentration. The mechanisms by which hyperhomocysteinaemia may be involved in the development of atherogenesis are only partially understood, but induction of oxidative damage and endothelial injury have been suggested [McDowell IFM et al, 2000].

1.3.6.2 LDL N-Homocysteinylation

Homocysteine thiolactone is an intramolecular thioester of homocysteine. Although Hcy-thiolactone was obtained by chemical synthesis in 1934, the first indication of its biological significance came almost 50 years later with the discovery of the enzymatic conversion of Hcy to Hcy-thiolactone in error editing reactions of some aminoacyl-tRNA synthases. Homocysteine thiolactone is the five-membered condensed ring form of homocysteine. Thiolactone formation is unique to homocysteine owing to the extra carbon atom within the side chain (cysteine has only a single carbon within its side chain, whereas homocysteine has two) [Necat Yilmaz 2012]. Homocysteine thiolactone is thus formed from an intramolecular condensation reaction between thiol and carboxylic acid, and it may also

occur in plasma in submicromolar concentrations. Many researchers, and especially Jakubowski, suggest that metabolic conversion of Hcy to Hcy-thiolactone followed by subsequent spontaneous protein N-homocysteinylation by Hcy-thiolactone may contribute to Hcy toxicity in humans [Jakubowski H, 2002]. This hypothesis suggests that the conversion to Hcy-thiolactone is linked to atherosclerosis in humans. The formation of Hcy-thiolactone can be detrimental for two reasons. First, its reaction requires ATP and thus causes non-productive consumption of cellular energy [Jakubowski H, 2000 (a)]. Second, Hcy-thiolactone is a reactive intermediate that causes protein N-homocysteinylation through the formation of amide bonds with ε-amino groups of protein lysine residues. Resulting protein damage necessitates the removal of N-homocysteinylated proteins by proteolytic degradation, which would further deplete cellular energy and limit cell growth. Homocysteine-containing proteins are also toxic and induce an autoimmune response, which is associated with atherosclerosis in humans [Jakubowski H, 2003; Jakubowski H, 2000 (b)].

Protein targets for the modification by Hcy-thiolactone include fibrinogen, low-density lipoprotein, highdensity lipoprotein, albumin, hemoglobin, and ferritin. Pathophysiological consequences of protein N-homocysteinylation include protein and cell damage, activation of an adaptive immune response and synthesis of auto-antibodies against *N*-Hcy-proteins, and

enhanced thrombosis caused by *N*-Hcy-fibrinogen [Jakubowski H, 2008]. The interaction between LDL and Hcy-thiolactone induces the formation of homocystamide-LDL adducts (Hcy-LDL). The synthesis of HcyT is directly proportional to the plasma Hcy/methionine ratio [Jakubowski H, 2000 (b)]. Because HcyT binds protein lysyl residues by amide linkage, individual proteins are homocysteinylated at rates proportional to their lysine contents. Moreover, the reaction between lysine apoprotein and Hcy-thiolactone yields an increase in the number of the sulfhydryl groups of apoprotein, thus increasing the number of sites that may be bound by plasma aminothiols [Ferretti G et al, 2004]. Jakubowski demonstrated that Hcy-induced vascular damage could be related to homocysteine-thiolactone (Hcy-thiolactone), an Hcy reactive product formed in human cells from the enzymatic conversion of homocysteine to the corresponding thioester [Jakubowski H, 1997]. *N-H*omocysteinylation occurs even at HcyT concentrations as low as 10 nmol/L, which is a physiological concentration [Jakubowski H, 1999]. *N*-homocysteinylation leads to protein damage consisting of multimerization and precipitation of extensively modified proteins. Homocysteinylation of lipoproteins is accompanied by structural and functional alterations [Ferretti G et al, 2004; Vignini A et al, 2004; Ferretti G et al, 2003 (a)] and it has been suggested that homocysteinylation could increase the atherogenicity of lipoproteins and contribute to abnormal

interactions with cells. Ferretti et al. [Ferretti G et al, 2004] theorized that *N*-homocysteinylated LDLs are internalized by membrane receptors with intracellular release of Hcy after hydrolytic degradation. N-homocysteinylated LDL, in which 10% or 25% of lysine residues have been modified (i.e., containing 36 mol and 89 mol N-linked Hcy/mol LDL), is taken up and degraded by human monocyte-derived macrophages significantly faster than native LDL. The interaction between HcyT and LDL causes LDL aggregation and higher uptake of *N*-homocysteinylated LDL (Hcy-LDL) by cultured macrophages [McCully KS et al, 1975]. The mechanism underlying N-Hcy-LDL toxicity may involve a decrease in endothelial Na+,K+-ATPase activity, leading to an overload with sodium and, subsequently, with calcium. This in turn causes reduced production of nitric oxide and generation of peroxynitrate, a highly reactive nitrogen metabolite. Taken together, these observations suggest that protein N-homocysteinylation may contribute to endothelial dysfunction, a key event initiating the development of atherosclerotic plaque.

1.3.6.3 LDL S-Homocysteinylation

Apart from *N*-homocysteinylation of LDL, which is due to the interaction of LDL with Hcy thiolactone, apolipoprotein B of LDL, which has several

thiol groups, could bind to Hcy to form homocysteine-*S*-LDL (Hcy-*S*-LDL). However, this modification of LDL has not been studied in detail. Since a majority of Hcy binds to the protein cysteine residues, we have hypothesized that the deleterious effects of elevated Hcy levels might be due to its ability to bind critical, accessible cysteine residues in the protein, thereby modulating its structure and/or function. Cysteine residues in proteins play important roles in protein folding and influence the quaternary structure through the formation of intra/intermolecular disulfide bonds. Cysteine residues are also a part of the catalytic domain of several proteins, bind to metals, are redox active and have conserved motifs in the active site regions of various proteins that are involved in cell signaling, transcriptional regulation and trafficking [Barford D, 2004]. In fact, cysteine rich proteins with exposed unsaturated cysteine residues have a higher propensity to react with circulating homocysteine. In plasma Hcy is mainly linked to cysteine residue at Cys34 of albumin, but interactions between Hcy and ceruloplasmin, fibrin, and transthyretin have also been reported [Sengupta S et al, 2001 (a); Majors AK et al, 2002; Lim A et al, 2003]. Hcy-mediated posttranslationalmodifications may have important functional consequences. In the primary structure of LDL apoB-100 there are at least 9 free sulfhydryl groups (–SH) that could potentially bind plasma free aminothiols [Yang C et al, 1994]. However, only a few of these

–SH sites are *S*-thiolated (~0.5 nmol/nmol apoB), thus suggesting that free –SH sites for *S*-thiolation may have a limited accessibility [Zinellu A et al, 2006 (b)] and not all the cysteines bind to homocysteine or to the other low molecular weight thiols such as cysteine, cysteinylglycine, glutathione and glutamylcysteine that are common in plasma. Zinellu et al. reported that increasing concentrations of plasma Hcy lead to higher *S*-homocysteinylation of lipoprotein in vivo. These data, calculated with Pearson correlation analysis, were confirmed by multiple regression analysis, by which they also found that plasma Cys and CysGly both seem to inhibit interaction between Hcy and apoprotein. By multiple regression analysis, they also found that LDL cholesterol concentrations may influence the quantity of Hcy bound to LDL. So, in hypercholesterolemic individuals, increased amounts of homocysteinilated LDL could accumulate in the intima. There, modified LDL could be avidly taken up from macrophages by membrane receptor or by phagocytosis, leading to intracellular cholesterol accumulation and foam cell formation [Naruszewicz M et al, 1994]. Finally, the increase of LDL Hcy suggests that LDL atherogenicity may be enhanced by the modification of its chemical and biological properties, leading to endothelial vascular injury and deposition of cholesterol, lipids, and Hcy in the intima space and the development of atherosclerotic plaques.

1.4 Chronic kidney desease

Chronic kidney disease is a worldwide public health problem. In the United States, there is a rising incidence and prevalence of kidney failure, with poor outcomes and high cost. It refers to a condition related to irreversible kidney damage that can further progress to endstage renal disease (ESRD).

In the normal person, the kidneys, weighing about 4 ounces each, process about 200 liters of blood daily to remove waste products and excess water. They metabolize 25-hydroxyvitamin D to active 1,25 dihydroxy-vitamin D (calcitriol), which regulates absorption of calcium from foods and affects bone formation. Kidneys are critical to erythropoietin formation, which stimulates red blood cell production. They also regulate renin, which regulates blood volume and blood pressure. The kidneys function as excretory, biosynthetic, and metabolic organs, vital for maintaining normal physiology. Although dialysis can replace some kidney functions, it cannot replicate the biosynthetic and metabolic activities of the normal kidney.

There is an even higher prevalence of earlier stages of chronic kidney disease. Increasing evidence, accrued in the past decades, indicates that the adverse outcomes of chronic kidney disease, such as kidney failure, cardiovascular disease, and premature death, can be prevented or delayed. Patients at higher risk for CKD include patients with diabetes,

hypertension, or a family history of hypertension, diabetes, or CKD. With regard to risk stratification for adverse outcomes from chronic kidney disease, patients with chronic kidney disease would be included in the ‘‘very high risk’’ group. Individuals without chronic kidney disease, but with risk factors for chronic kidney disease (‘‘CKD risk factors’’), would constitute the ‘‘high risk’’ group. Individuals without chronic kidney disease or CKD risk factors would constitute the ‘‘low risk’’ group. CKD appears more often in minority groups: black American, Native American, Hispanic, Asian, and Pacific Islander populations are at higher risk of developing CKD than are white Americans. In these populations, diabetes and hypertension, which are the predominant causes of ESRD, are more common and tend to be familial [Coresh J et al, 2003]. CKD is usually silent until its late stages, and without aggressive screening, detection may not occur until immediately before symptomatic kidney failure develops. At this point in the disease process, few opportunities exist to prevent adverse outcomes, such as further decline in kidney function necessitating dialysis, cardiovascular complications, shortened lifespan, and poor quality of life. Earlier stages of chronic kidney disease can be detected through laboratory testing. Screening asymptomatic individuals at increased risk could allow earlier detection of chronic kidney disease. Initiation of treatment for cardiovascular risk factors at earlier stages of chronic kidney

disease should be effective in reducing cardiovascular disease events both before and after the onset of kidney failure. Unfortunately, chronic kidney disease is often ‘‘under-diagnosed’’ and ‘‘under-treated’’ resulting in lost opportunities for prevention.

The National Kidney Foundation defined ‘‘chronic kidney disease’’ as including conditions that affect the kidney, with the potential to cause either progressive loss of kidney function or complications resulting from decreased kidney function. Chronic kidney disease was thus defined by the presence of the following clinical evidence:

I: Kidney damage for ≥ 3 months, is defined by structural or functional abnormalities of the kidney, with or without decreased GFR, manifest by either:

- Pathological abnormalities
- Markers of kidney damage, including abnormalities in the composition of the blood or urine, or abnormalities in imaging test (eg. urine albumin:creatinine ratio ≥30 mg/g [3.4 mg/mmol])

II: GFR <60 mL/min/1.73 m^2 for ≥3 months, with or without kidney damage.

Kidney damage is defined as pathologic abnormalities or markers of damage, including abnormalities in blood or urine tests or imaging studies. Chronic Kidney Disease is defined as either a low GFR rate of less than 60

mL/min/1.73 m^2, or normal GFR plus evidence of kidney damage (most commonly albuminuria, hematuria or abnormal renal ultrasound for three or more consecutive months) regardless of age [Nation Kidney Foundation, 2002]. The severity of CKD is classified according to the level of GFR, regardless of the etiology, into five stages:

Stage	Description	GFR (mL/min/1.73 m^2)	Action plans (Includes actions for preceding stages)
-	At increased risk for CKD	=90 (with risk factors for CKD)	Screening, reduction of risk factors for CKD
1	Kidney damage with normal or elevated GFR	=90	Diagnosis and treatment of comorbid conditions, interventions to slow disease progression, reduction of risk factors for CKD
2	Kidney damage with midly decreased GFR	60 to 89	Extimation of disease progression
3	Moderately decreased GFR	30 to 59	Evaluation and treatment of disease complications
4	Severely decreased GFR	15 to 29	Preparation for kidney replacement therapy (dialysis, transplantation)
5	Kidney failure	<15 (or dialysis)	Kidney replacement therapy if uremia is present

- Stage one: kidney damage with a normal or increased GFR (≥ 90 mL/min/1.73 m^2);

- Stage two: kidney damage with a mild decrease in in GFR (60-89 mL/min/1.73 m^2);
- Stage three: a moderate decrease in GFR (30-59 mL/min/1.73 m^2);
- Stage four: a severe decrease in GFR (15-29 mL/min/1.73 m^2);
- Stage five: kidney failure (<15 mL/min/1.73 m^2).

Estimated GFR can be calculated by formulas that are based on the serum creatinine concentration, age, and gender, for specific ethnic groups. GFR is central to the definition and classification of CKD because it is an overall measure of kidney function. However, it has also been shown that most patients with a reduced GFR have fairly stable renal function during follow-up [Foster MC et al, 2077]. Therefore, CKD prognosis is dependent on associated cardiovascular risk factors or evidence of vascular damage (e.g. microalbuminuria) rather than a measure of kidney function by GFR [Ahmed AK et al, 2010]. It is likely that age-related diffuse atherosclerosis is responsible for renal function decline in certain patients with associated cardiovascular co-morbidities. Concordance between reduced GFR, microalbuminuria and associated cardiovascular and renal risk is not clear. Microalbuminuria increases the risk for cardiovascular events similar to, and independent of, an impaired GFR [John R et al, 2004]. The appearance of pathological albuminuria often precedes the functional deterioration that

is evidenced by a decline in GFR. Importantly, albuminuria has also been shown to be a potent independent marker of cardiovascular (CV) risk in both diabetic and nondiabetic persons. Similar to GFR, the link between albuminuria and adverse CV events was first recognized in the more overt situations of macroalbuminuria (urine albumin/creatinine ratio [ACR] >300 mg/g) [Kannel WB et al, 1985; Grimm RH et al, 1997], and then this link was extended to more modest elevations such as microalbuminuria (ACR, 30 to 300 mg/g) [Keane WF et al, 1999]. Microalbuminuria also correlates with adverse CV events. The ability of microalbuminuria to predict adverse CV events is not restricted to a high-risk population. In fact, Hillege et al demonstrated the ability of microalbuminuria to predict CV and non-CV mortality in the general population [Hillege HL et al, 2002]. More recently, it has become increasingly recognized that CV risk begins to rise within currently defined normal levels of albuminuria (ACR<30 mg/g). It also appears that microalbuminuria is a determinant factor for developing a renal event (need to start renal replacement therapy) regardless of GFR. In another study, the risk of developing a renal event was 12-fold in patients with Stage one CKD with microalbuminuria as compared to patients with no CKD. The risk was only 2.4-fold in Stage three patients with microalbuminuria [Ishani A et al, 2006]. Macroalbuminuria is also a risk marker in identifying individuals at risk for accelerated GFR loss. The

decline in kidney function was also defined as an increase in serum creatinine equal to or greater than 35 μmol/L over five years of follow-up. Clinical abnormalities in CKD are influenced by the rapidity and the extent of nephron obliteration, independent of the specific etiology. Several biomarkers of oxidative stress have been also found at elevated concentration in patients with CKD. Oxidative stress is defined as tissue damage caused by the disequilibrium between pro- and antioxidant factors. It is present in a large variety of pathological conditions and it is believed that it functions as a pathogenetic agent in many of these conditions. One of the main effects of oxidative stress is the decrease in the biological activity of nitric oxide (NO) [Widlansky ME et al, 2003]. This effect is expressed through the endothelial dysfunction, which is considered a precursor of atherosclerosis [Bonetti PO et al, 2003]. Among these biomarkers of oxidative stress is malondialdehyde (MDA). MDA, a lipid hydroperoxide, is formed by β-scission of peroxidized polyunsaturated fatty acids. Increased plasma MDA in patients with CKD compared with healthy controls suggests increased systemic oxidative stress [Agarwal R, 2003]. CKD patients show increased levels of both MDA and All/UA ratio, indicating a more marked oxidative stress, compared to healthy subjects. Some authors suggest that oxidative stress in chronic kidney might be a consequence of higher ROS production, since total antioxidant

capacity has been found similar to that in healthy subjects [Cachofeiro V et al, 2008]. Not all patients with CKD progress to the later stages of CKD or to ESRD because they die prematurely from other causes [The United State Renal Data System, 2007]. The risk of death is especially high in late-stage kidney disease; a 30-year-old patient with end-stage renal disease faces an equivalent risk of death to a 90-year-old without CKD [Foley RN et al, 1998], due to the increased occurrence of comorbidity in these subjects. CKD comorbidities, such as cardiovascular disease (CVD), contribute to the high death rate in CKD. The patients who progress and survive to reach ESRD might be considered lucky, although they rarely feel that way. A large cohort study comprising >130 000 elderly subjects showed that increased incidence of cardiovascular events could be in part related to the fact that persons with renal insufficiency are less likely to receive appropriate cardioprotective treatments [Shlipak MG et al, 2002]. However, beyond the effects of lack of appropriate therapy, it is clear that accelerated CVD is prevalent in subjects with CKD. ESRD is associated with several specific complications caused by the uremic state per se, which can contribute to the development and progression of CVD through volume overload with consequent hypertension, anemia, uremic pericarditis, and cardiomyopathy. Evidence for the relationship between renal dysfunction and adverse CV events was perhaps first recognized in

the dialysis population in whom the incidence of CV death is strikingly high. Approximately 50% of individuals with ESRD die from a cardiovascular cause [Tonelli M et al, 2006; Foley RN et al, 1998; Herzog CA et al, 1998] and the cardiovascular mortality is 15 to 30 times higher than the age-adjusted cardiovascular mortality in the general population [Foley RN et al, 1998; Parfrey PS et al, 1999]. This disparity is present across all ages, but it is most marked in the younger age group (25 to 34 years old), where the cardiovascular mortality is 500-fold greater in ESRD patients compared with age-matched controls with normal renal function [Sarnak MJ et al, 2003]. A growing number of studies have demonstrated that the relationship between renal dysfunction and increased cardiovascular morbidity and mortality extends across the spectrum of renal dysfunction to encompass the mildest degrees of renal impairment. Sarnak et al. [Sarnak MJ et al, 2003; Tonelli M et al, 2006] listed the candidates of traditional and non-traditional risk factors that may explain the increased risk of CVD in CKD. Traditional risk factor are older age, male sex, hypertension, higher LDL-C, lower HDL-C, diabetes mellitus, smoking, physical inactivity, menopause, family history of CVD, and left ventricular hypertrophy. They also proposed nontraditional risk factors such as albuminuria, homocysteine, lipoprotein(a), apolipoprotein(a) isoforms, lipoprotein remants, anemia, abnormal calcium/phosphate

metabolism, extracellular fluid overload, electrolyte imbalance, oxidative stress, inflammation (elevated CRP), malnutrition, thrombogenic factors, sleep disturbances, and altered nitric oxide/endotheli balance [Tetsuo S et al, 2012]. Hypertension in and of itself represents a powerful risk factor for CVD in CKD and is almost invariably present in patients with renal failure. Also, endothelial dysfunction [Wever R et al, 1999; Vaziri ND et al, 2002; Stehouwer CDA et al, 2004; Endemann DH et al, 2004; Passauer J et al, 2005] and remodeling of blood vessels [Foley RN et al, 1996] may participate not only in vascular complications in patients with kidney disease but also in the maintenance of elevated blood pressure. CKD is known to complicate both the procedural results and the longterm management of cardiovascular risk factors. It is important to recognize that CKD does impact the efficacy of antihypertensive medications, so with respect to blood pressure, it makes this risk factor more difficult to control [Lubanski MS et al, 2009].

1.5 Chronic Kidney Disease and Dyslipidemia

In 1836, Richard Bright commented on the "milky serum" of patients with (what today would be called) end‑stage renal disease, almost certainly the first recognition of hyperlipidemia. CKD is characterized by specific

metabolic abnormalities of plasma lipoproteins [Tsimihodimos V et al, 2008; Kaysen GA, 2009]. These abnormalities involve all lipoprotein classes and shows variations depending on the degree of renal impairment, the etiology of primary disease, the presence of nephrotic syndrome (NS) and the method of dialysis (hemodialysis or peritoneal dialysis) for patients undergoing renal replacement therapy. A significant factor which determines the levels of plasma cholesterol-rich lipoproteins, except of the deterioration in renal function, is the degree of proteinuria. Dyslipidemia associated with CKD [Kaysen GA et al, 2004; Trevisan R et al, 2006] contributes to the inflammatory response in renal failure. In patients with kidney disease, however, the relation of dyslipidemia to cardiovascular risk is confounded and the underlying pathomechanisms are complex. The causes of dyslipidemia are undoubtedly complex. An interesting new hypothesis states that prenatal programming [Barker DJ et al, 2006], e.g. the result of intrauterine malnutrition, causes obesity, dyslipidemia, type 2 diabetes, and a deficit in nephron numbers, thus increasing in parallel cardiovascular and renal risks [Vikse BE et al, 2008]. Hypertriglyceridemia is one of the most common quantitative lipid abnormalities in patients with CKD [Attman PO et al, 2009; Vaziri ND et al, 2006 (a); Kwan BC et al, 2007]. The concentrations of triglyceride-rich lipoproteins (VLDL, chylomicrons, and their remnants) start to increase in

early stages of CKD and show the highest values in nephrotic syndrome and in dialysis patients, especially those who are treated with peritoneal dialysis. High-density lipoprotein (HDL) cholesterol is subnormal in the majority of patients. Low HDL cholesterol has been ascribed to low activity of lecithin‑cholesterol acyltransferase, which normally increases the uptake of esterified cholesterol by HDL. The change in HDL concentration is favored by the common presence of microinflammation in uremic patients [Chmielewski M et al, 2008]. This impairment can, at least in part, be attributed to the reduction in the activities of HDL-associated enzymes, such as paraoxonase (an enzyme that inhibits the LDL oxidation) [Dirican M et al, 2004; Liberopoulos EN et al, 2004]. Even more marked changes are seen in the lipoprotein profile, the result of changes in lipid catabolism and to some extent lipogenesis. Decreased lipolysis is primarily the result of diminished activity of lipoprotein‑lipase and hepatic lipase. Because of decreased catabolism of lipoproteins [Ikewaki K et al, 2005] and chylomicrons [Weintraub M et al, 1992], intermediates such as chylomicron remnants and VLDL remnants as well as intermediate density lipoproteins accumulate and their concentrations are elevated. Because of their prolonged half‑life, this is particularly true for the atherogenic small dense LDL [Quaschning T et al, 1999; Rajman I et al, 1998], LDL particles

tend to be smaller, denser and more atherogenic [O'Neal D et al, 1996]. It is important that the plasma concentration of lipid subfractions may not fully reflect the cardiovascular risk because the turnover (and by implication the residence time of lipoproteins in the circulation) are significantly altered [Ikewaki K et al, 2005]; this exposes the lipoproteins to the risk of post‑ribosomal modification. Post-translational modifications acquired during the prolonged residence time in the circulation include glycation [Fishbane S et al, 1997], oxidation [Karabina SA et al, 2003], carbamylation [Horkko S et al, 1992], methylation [Mahley RW et al, 1977; Weisgraber KH et al, 1978], acetylation [Steinberg D et al, 1989], S-homocysteinylation and N-homocysteinylation [Jacobsen DW et al, 2005]. As a result, the affinity for the classic LDL receptor is diminished and such modified lipoproteins are taken up by the scavenger receptor [Koniger M et al, 1999] which is increased in uremia [Ando M et al, 1996] and, among others things, favors the development of foam cells in atherosclerotic plaques. In 2001, the National Cholesterol Education Program (NCEP) Adult Treatment Panel (ATP) III published guidelines that recommend decreasing LDL-C below 100/mgdL in adults with ischemic heart disease and those with equivalent risk [Expert Panel on Detection, Evaluation, And Treatment of High Blood Cholesterol In Adults, 2001]. Following the NCEP ATP III guidelines, European Renal Association-European Dialysis

Transplantation (ERA-EDTA) published guidelines on the management of dyslipidemia in patients with CKD [European best practice guidelines Expert Group on Haemodialysis. European Renal Association, 2002] in 2002. In the following year, the National Kidney Foundation Kidney Disease Outcomes Quality Initiative (NFK K/DOQI) released guidelines for dyslipidemia in CKD [Kidney Disease Outcomes Quality Initiative (K/DOQI) Group, 2003]. These guidelines specific for patients with CKD recommend controlling LDL-C below 100 mg/dL because of the very high risk for CVD in these patients, particularly patients on dialysis.

Several studies have shown that lipid-lowering treatment with statins is effective in reducing the risk for CVD in |early stages of CKD, whereas the benefit of such medication may be limited to those with an elevated level of LDL-C in patients with CKD stage 5. Statins have proven to be highly effective in patients with initial stages of chronic kidney disease. In view of excessive cardiovascular risk statins should be administered in patients with advanced CKD as well [Grzegorz P et al, 2009]. It was also report that statins treatment appears more efficacious in patients with CKD than in those without it.

2. AIM OF THIS STUDY

CKD is currently recognized as an important global population health problem. In developed countries, the progressive increase in numbers of CKD patients and those requiring renal replacement therapy (RRT) is reaching epidemic levels, growing by 5–8% annually [Meguid El Nahas A et al, 2005; The United States Renal Data System, 2004]. CKD includes conditions that affect the kidney, with the potential to cause either progressive loss of kidney function or complications resulting from decreased kidney function. Chronic kidney disease (CKD) is defined as structural and/or functional damage to the kidney or a glomerular filtration rate (GFR) of <60 mL/min/1.73 m^2, for three months or more, irrespective of cause [National Kidney Foundation, 2002]. The prevalence of end-stage renal disease (ESRD) is increasing all over the world, and the main cause of death in this population is cardiovascular disease [National Kidney Foundation, 2005; Varela AM et al, 2006]. The main risk factors for cardiovascular disease are arterial hypertension, diabetes mellitus, age, male gender, family history, menopause and dyslipidemia [elevated total cholesterol and low-density lipoprotein (LDL), as well as decreased high-density lipoprotein (HDL) and hypertriglyceridemia] [Sarnak MJ et al, 2000]. Patients with chronic kidney disease usually have an elevated ratio

of low-density lipoprotein cholesterol to high-density lipoprotein cholesterol [Appel GB et al, 2004; Prichard S, 2003; McCullough PA, 2004; Kalantar-Zadeh K et al, 2003; Sahadevan M et al, 2002]. Dyslipidemia is a primary risk factor in the development of a number of disease multitudes ranging from atherosclerosis to stroke [Kannel WB, 2005; Kannel WB et al, 2004]. Disturbances of lipid transport and metabolism are common complications of chronic renal failure, regardless of the cause of renal disease, which may persist or deteriorate during renal replacement therapy [Ma KW et al, 1992; Attman PO et al, 2003;Appel GB et al, 2004]. We have also recently reported that LDL in CKD patients was characterized by an elevated degree of S-homocysteinilation [Zinellu A, 2010]. Since it has been amply reported that dyslipidemia contributes not only to CVD but also to the progression of CKD [Romayne Kurukulasuriya L et al, 2007], through lipid deposition that elicits pro-inflammatory and pro-fibrotic reactions in the kidney, the control of dyslipidemia is one of the targets for the treatment of CKD [Sever PS et al, 2000]. Experimental and clinical evidences show that statin, as well as improving lipid profile, may have specific renoprotective properties [Zoja C et al, 2010]. Moreover, preliminary data suggest that the combination of statin with ezetimibe (EZE), a cholesterol absorption inhibitor, provides

complementary effects on lipids over that achieved with statin monotherapy [Dembowski E et al, 2009 Statin].

The aim of this study is to evaluate the effect of hypolipidemic drugs on the LDL thiolation in proteinuric nefropatic patients during lipid lowering therapy. We enrolled thirty CKD patients who were treated with three different hypolipidemic regimens: simvastatin alone (40 mg/day) or ezetimibe/ simvastatin combined therapy (10/20 or 10/40 mg/day). Considering that proteins are thiolated in response to oxidative stress (OS), evaluation of free malondialdehyde plasma levels, allantoin/uric acid ratio (All/UA) was also performed to monitor OS in patients during drug treatment.

3. MATERIALS AND METHODS

3.1 Study participants

30 CKD patients were selected at the Istituto di Patologia Medica - Azienda Ospedaliero Universitaria, with the following inclusion criteria: age >18; LDL-cholesterol > 100 mg/dl (without concomitant hypolipidemic drugs) presence of proteinuric chronic nephropathy defined as creatinine clearance > 20 ml/min/1,73 m2 combined to a urinary protein excretion rate > 0.3 g/24h, without evidence of urinary tract infection or overt heart failure (New York Heart Association class III or more). Exclusion criteria are represented by: previous or concomitant treatment with steroids, anti-inflammatory and immunosuppressive agents, vitamin B6, B12, folate or statin; evidence or suspicion of renovascular disease, obstructive uropathy, type I diabetes mellitus, vasculitides. All patients were in stable treatment with RAS inhibitor therapy (ACE inhibition by benazepril plus angiotensin II antagonism by valsartan) for at least six months. Enrolled patients were randomized to receive 40 mg/day simvastatin (group 1, n=10), or ezetimibe/simvastatin 10/20 mg/day (group 2, n=10), or ezetimibe/simvastatin 10/40 mg/day (group 3, n=10). Patients were treated for 12 months and were evaluated at baseline and at 4, 8 and 12 months of therapy.

Informed consent was obtained from each patient and control, and the study was approved by our Institution's Ethics Committee. The study complied with the principles of the Helsinki Declaration. This study has been registered with clinicaltrials.gov (NCT00861731).

3.2 Lipoprotein isolation

Blood was collected in sampling vacutainer vials containing EDTA. Plasma was prepared by centrifugation at 2000x*g* for 10 min at 47C. LDLs were isolated by ultracentrifugation according to Himber *et al.* [Himber J et al, 1995] and McDowell *et al.* [McDowell IF et al, 1995]. Briefly, 0.9mL plasma was added to a centrifugation tube containing KBr (0.4451 g) adjusting the density of plasma to 1.300 g/mL. This was then overlaid with 2.1mL of 150 mmol/L NaCl and centrifuged at 541000x*g* for 2 h at 47°C. LDL orange colored band was recovered and mixed (ratio 1:1) with a solution containing KBr and EDTA 1% (density 1.063 g/mL), and centrifuged at 541000x*g* for 2 h at 47C.

3.3 Apo-B bound thiols preparation and quantification

We precipitated 200 mg of LDL apoprotein with SSA (final concentration, 7.5%) and then centrifuged it at 2000*g* for 5 min. The protein pellet was

washed twice with 500 mL SSA 5% and once with 1 mL of acetonitrile/water (70/30) to remove SSA residue that could interfere with subsequent steps. After centrifugation, we discarded 950 mL of supernatant and dried the apoprotein under decreased pressure in a Concentrator 5301 for 30 min at 60 °C. After dissolving apoprotein in 200 mL of 50 mmol/L NaOH at 60 °C for 30 min, we reduced disulfide bonds by incubation with 20 mL of 100 mL/L tri-*n*-butylphosphine in *N,N*-dymethilphormamide for 10 min, added 900 mL of acetonitrile, vortex-mixed and centrifuged the solution at 2000*g* for 5 min, and then dried 1 mL of supernatant under decreased pressure at 60 °C for 4 h. Dry samples were resuspended with 100 mL of derivatization medium (0.08 mmol/L 5-iodoacetamidofluorescein, 25 mmol/L sodium phosphate buffer, pH 12.5). After 15 min at room temperature, derivatized samples were diluted 40 times in water and analyzed by capillary electrophoresis as previously described by Zinellu et al [Zinellu A et al, 2006 (a)]. Briefly, we used a 75-mm i.d. and 57-cm-long (50 cm to the detection window) uncoated fused-silica capillary and performed analysis by applying 30 nL of sample under nitrogen pressure (0.5 psi for 5 s) in a mixture of 30 mmol/L sodium phosphate, 33 mmol/L boric acid, and 75 mmol/L *N*-methyl-d-glucamine, pH 11.3. The separating conditions (18 kV, 165 mA at normal polarity) were reached in 20 s and were held at a constant voltage for 15 min.

Separations were carried out at 40 °C and monitored at 488 nm excitation and 520 nm emission wavelength.

3.4 Total plasma thiols measurement

We measured total plasma thiols by capillary electrophoresis laser-induced detection as described by Zinellu et al [Zinellu A et al, 2003]. Briefly, 100 µL of standard or plasma sample with 10 µL of TBP (10%) were mixed, vortexed for 30 s and subsequently incubated at 4°C for 10 min. At the end of incubation 100 µL of 10% TCA were added, vortexed for 10 s and then centrifuged for 10 min at 3000 x *g*. 100 µL of supernatant were mixed with 100 µL of 300 mmol/L Na3PO4 at pH 12.5 and with 25 µL of 5-IAF (4.1 mmol/L), and subsequently incubated at room temperature for 10 min. The mix was diluted 1/100 before injected in CE-LIF.

3.5 LMW thiol redox status evaluation

LMW thiol redox status were measured by capillary electrophoresis laser induced detection, as previously reported by Carru et al [Carru C et al, 2004]. 200 mL of plasma sample were deproteinized by adding 50 mL of 15% SSA and centrifuged at 20006*g* for 5 min. To 150 mL of supernatant was added 30 mL of 1 mmol/L NaOH. Then 50 mL of sample was mixed

with 100 mL of 100 mmol/L sodium phosphate buffer, pH 12.5, and 15 mL of 0.8 mmol/L 5-IAF. After vortex-mixing, samples were incubated for 15 min at room temperature. Derivatized samples were diluted 100-fold in water and analyzed by capillary electrophoresis.

3.6 MDA and All/UA ratio determination

MDA and All/UA ratio were determined by capillary electrophoresis UV detection as previously described by Zinellu et al [Zinellu A et al, 2011]. Briefly, then, 200 μl of obtained plasma was filtered in Vivaspin 500 microconcentrators by centrifugation at 3,000×g for 10 min to remove proteins. The filtered sample was directly injected into the capillary.

3.7 Protein and cholesterol analysis

ApoB-100 protein content of LDL fractions was measured by Lowry's method. Total cholesterol, HDL and tryglicerides were measured by enzymatic methods using commercial kits (Boehringer-Mannheim, Mannheim, Germany).

3.8 Statistical analysis

All results are expressed as mean values (mean ± SD) or median values (median and range). The distribution of variables in the study group was assessed by the Kolmogorov-Simirnov test. Effect of drug treatments was evaluated by one way repeated measures ANOVA. Correlation analysis between variables was performed by Pearson's correlation. Calculations were performed using the Statgraphics plus 5.1 package for Windows (Rockville, MD, USA).

4 RESULTS

Results of clinical characteristics of all CKD patients, before starting the drug treatment, are shown in Table 1. We have previously reported [Zinellu A et al, 2010] that nephropatic patients show typical dyslipidemia, with elevated levels of plasma triglycerides, LDL and plasma thiols. In particular, as expected, more than 50% of CKD subjects were hyperhomocysteinemic (Hcy > 15 μmol/L), versus 10% in the healthy population, and apoB-Hcy levels in nephropatic patients (27.5 nmol/lmol apoB) were twofold the mean values of healthy subjects (13–14 nmol/lmol apoB) [Zinellu A et al, 2010]. We found, by Pearson's correlation, that both Hcy plasma concentration and apoB-Hcy levels were inversely related to GFR ($r = -0.41$, $p = 0.02$ and $r = -0.47$, $p = 0.008$ respectively).

As regards the lipid profile, after randomization, we found no significant differences in the clinical characteristics analyzed among the three groups. As expected after 4 months' therapy, a significant improvement in lipid profile was already observed for all groups, in particular in those subjects under the concomitant administration of ezetimibe/simvastatin 10/40 mg/day (Table 2). In this group we observed (after 12 months' treatment) a decrease of 39% in total cholesterol, 61% in LDL-C, 32% in triglycerides,

and an important reduction in LDL/HDL ratio (3.26 ± 1.6 at baseline vs 1.14 ± 0.5 after 12 months, p = 0.001), with a 5% increase in HDL-C.

The effect of drugs on LMW thiols was also evaluated. The total level of LMW plasma thiols remained unchanged during treatment for all patients, as shown in Table 3. However the reduced forms of thiols (rHcy, rCys, rGSH and rGlu-Cys) increased significantly (Table 4). The trend was also evident for rCys-Gly but data fell short of statistical significance (p = 0.055). The linear trend indicates that the reduced forms were significantly affected by treatment with elevated doses of simvastatin both alone (for rCys and rGlu-Cys) or in combination with ezetimibe (for rHcy, rCys and rGSH). After 1 year's therapy, in the group with the concomitant administration of ezetimibe/simvastatin 10/40 mg/day a greater increase of r-thiols was found (+34.1%). The quantity of thiols bound to LDL apoprotein is strictly affected by drug treatment (Table 5), and is greater in patients treated with ezetimibe/simvastatin 10/40 mg/day (-31%) compared with simvastatin alone (-14%). In particular, as reported in Fig. 2, treatment with simvastatin alone resulted in a greater reduction from baseline after four months compared with combined treatment 10/40 mg/day (-42% vs -39% respectively) but for longer therapy times the combined treatment ensured a better efficacy in reducing apoB-Hcy (-37% vs -42% after 12 months).

Finally, oxidative stress was evaluated by measuring MDA and All/UA ratio in plasma. Baseline levels of both MDA and All/UA were inversely related to GFR ($r = -0.42$, $p = 0.02$ and $r = -0.44$, $p = 0.015$ respectively). Moreover, a positive relationship between apoB bound thiols and MDA ($r = 0.49$, $p = 0.007$) and All/UA ratio ($r = 0.40$, $p = 0.03$) was found at baseline. All/UA ratio was also correlated with apoB-Hcy levels ($r = 0.38$, $p = 0.04$). As reported in Table 6, a significant decrease of both MDA and All/UA ratio was observed, during therapy, in all patients (-19% for both MDA and All/UA ratio after 12 months) with a more pronounced effect in patients of group 3 (-26% for MDA and -28% for All/UA ratio after 12 months). Fig. 3 shows a comparison between the decrease of All/UA levels and apoB bound thiols during drug treatment: the decrease in LDL thiolation was significantly associated with the decrease of All/UA ratio during lipid lowering therapy ($r = 0.37$, $p = 0.04$ at 8th month and $r = 0.43$, $p = 0.02$ at 12th month).

5 DISCUSSION AND CONCLUDING REMARKS

Low molecular weight (LMW) aminothiols such as homocysteine, cysteine, cysteinylglycine, glutathione and glutamylcysteine are found in biological fluids both in reduced and oxidized form. Able to react with a number of thiol-combining groups, they interact via redox disulfide exchange reactions, and reduced, free-oxidized, and protein-bound forms of these species compose a dynamic system referred to as thiols redox status. Increased thiolation of plasma proteins thiol groups is a typical manifestation of oxidative stress [Barford D, 2004]. We have recently described that CKD patients have higher levels of Hcy bound to LDL than the healthy population [Zinellu A et al, 2010]. The damage to endothelial cells induced by modified LDL plays an important role in the development of atherosclerosis. In CKD the elevated levels of LDL, which are more homocysteinylated than in the general population, may also be responsible for an increment in the transport of both Hcy and Cys in the subendothelial space. Hcy has been reported to cause remodeling of extracellular matrix in the arterial wall and to activate latent elastolytic metalloproteinase pro-MMP-2 by disulfide bond formation with the propeptide [Bescond A et al, 1999] . On the other hand, Cys has been reported to auto-oxidize very fast and to dramatically accelerate Hcy oxidation, and this could lead to the formation of ROS in the subendothelial space [Hogg N, 1999] which may

promote the oxidation of retained LDL, transforming them into highly atherogenic molecules that promote the inflammatory process in the vessel [Libby P, 2002]. Renal dysfunction is frequently associated with oxidative stress, probably as a consequence of ROS production and this may drive a reduced form of thiols and in particular Hcy and Cys (which were elevated in nephropathic subjects) to react with apoprotein –SH. Therefore, the increase in renal damage induces a rise in oxidative stress, thus increasing the quantity of these proatherogenic thiols linked to LDL. This is consistent with recent findings which have described that the plasma levels of the reduced form of albumin (albumin- Cys 34 -SH) decreased progressively with a decrease in renal function of CKD patients, leading to an increase in the oxidized form of protein (albumin-Cys ^{34} -S-S-X) [Matsuyama Y et al, 2009]. The elevated levels of LDL-S-Hcy found in CKD may contribute, at least in part, to a major risk of CVD typical of this pathology. In patients with CKD the risk of CVD is three to five fold higher than in the general population and patients who progress to the end stage of renal disease have a mortality ten to twenty higher than age-, gender-, and race- matched healthy controls, with more than 50% of this excess burden due to cardiovascular risk [Go AS et al, 2004]. The majority of subjects with CKD do not die from kidney failure but rather succumb to CVD. People with CKD are at risk from CVD due to both traditional factors (eg, smoking,

diabetes, dyslipidemia, HTN), as well as CKD-related factors (eg, reduced renal filtration, microalbuminuria, anemia, hyperparathyroidism, oxidative stress, etc.) [McCullough PA et al, 2011] .

In proteinuric patients, dyslipidemia has a highly atherogenic profile, with increased total and LDL cholesterol, triglyceride, and a decreased high-density lipoprotein (HDL) cholesterol [Vaziri ND, 2006 (b); Vaziri ND, 2003]. Atherosclerosis is a spread arterial disease triggered in response to various insults such as dyslipidemia, which is prevalent in CKD patients, and is the main cause of cardiovascular disease. Considering the elevated levels of LDL in these patients, the noxious effect of modified LDL may be consistent. Lowering levels of low-density lipoprotein cholesterol play an important role in cardiovascular disease prevention and comprise the main target of hypolipidemic therapy [Heart Protection Study Collaborative Group, 2002]. Statins are considered the most effective drug in terms of improving serum lipid profile [Ong HT, 2005]. Treatment with HMG-CoA reductase inhibitors (statins) has now definitively been shown to reduce the likelihood of cardiovascular events (myocardial infarction, ischemic stroke, and the need for coronary revascularization) in people without kidney disease [Heart Protection Study Collaborative Group, 2002; Sever PS et al, 2004]. Given the marked function, it is of major relevance and importance to identify whether CKD patients might also benefit from alteration of lipid

fractions, and how this might best be achieved. Bearing in mind that the animal model and preclinical evidence suggests dyslipidemia might also be a factor promoting worsening renal function, it could be legitimately asked whether treating it may also therefore have a nephroprotective effect [Kassimatis TI et al, 2009]. Statin treatment brings benefits that can be attributed not only to the lipid-lowering potency, but also to various pleiotropic anti-atherosclerotic properties of these drugs (namely anti-inflammatory, anti-oxidative and anti-thrombotic) [Kostapanos MS et al, 2008]. Nevertheless, not all patients tolerate high-dose statins and the incidence of abnormalities in liver function or myopathy may increase in a dose-dependent manner with this class of drugs [Conard S et al, 2010]; and some patients still do not meet any treatment goal. Statin therapy combined with a lipid-lowering therapy which has a different mechanism of action, such as ezetimibe, may provide complementary effects on lipids that surpass those of high-dose statins. Ezetimibe selectively inhibits the intestinal absorption of cholesterol and related phytosterols. Its molecular target is the sterol transporter, Niemann-Pick C1-like 1 (NPC1L1), which is responsible for cholesterol absorption in the intestine [Sudhop T et al, 2002; Dujovne CA et al,; Knopp RH et al, 2003; Davis HR et al, 2004; Garcia-Calvo M et al, 2005; Lammert F et al, 2005; Merck/Schering-Plough Pharmaceuticals, 2008]. It has a mechanism of action that is

complementary to that of statins and has been shown to reduce LDL-C levels significantly more than placebo. Ezetimibe also improves other lipids such as high-density lipoprotein cholesterol (HDL-C), triglycerides, and Apo B [Sudhop T et al, 2002; Dujovne CA et al, 2002; Knopp RH et al, 2003; Davis HR et al, 2004; Garcia-Calvo M et al, 2005; Lammert F et al, 2005; Merck/Schering-Plough Pharmaceuticals, 2008].

Through this work on the effect of lipid lowering therapy on the levels of homocysteine linked to LDL (and more in general on the levels of all thiols bound to LDL) has been investigated. We found more elevated levels of total plasma Hcy, Cys, GSH and Glu-Cys in patients than in controls and also found that Hcy and Cys bound to LDL significantly increased in nephropathic subjects [Zinellu A et al, 2010]. As expected, the treatment determines a significantly improvement on the lipid profile in all patients. The most obvious results were observed for subjects treated with ezetimibe/simvastatin 10/40 mg/day, belonging of the third group. In all treated patients a decrease in LDL thiolation was found, with a greater efficacy attained from combined therapy with higher simvastatin dose, by which a decrease of S-bound thiols of 31% was reached after 1 year's therapy. In particular in this group, after 12 months' treatment, the reduction of apoB-Hcy was greater than 40%, thus approaching the normal LDL-S-Hcy levels of the healthy population (13–14 nmol/lmol apoprotein).

The level of total homocysteine and other thiols during treatment remains unchanged: this data accord with what has been previously reported by Milionis et al., [Milionis, HJ et al, 2003] and Dierkes et al [Dierkes J et al, 2004]. Moreover we found, for the first time, a significant increase of reduced forms of thiols during therapy administration. We hypothesize that decreased levels of protein bound thiols and increased levels of reduced thiols may be a consequence of oxidative stress improvement during drug treatment. Several biomarkers of oxidative stress have been found at elevated concentrations in patients with CKD. These include products of lipid oxidation (lipid peroxides, malondialdehyde, and thiobarbituric acid reactive substances) and oxidized LDL [Agarwal R, 2004 ; Diepeveen SH et al, 2004], advanced oxidation protein products (AOPP) [Witko-Sarsat V et al, 1998], F2 isoprostanes [Ikizler TA et al, 2002], and 8-hydroxyl 2-deoxyguanosine (marker of oxidative DNA damage) [Puchades Montesa MJ et al, 2009]. The nature of oxidative stress in chronic kidney disease still remains unclear. In general, impaired oxidative balance may result from a combination of increased ROS production and reduced clearance, as well as an ineffective antioxidant defense mechanism, though some authors have found no differences in total antioxidant capacity between CKD patients and healthy people [Zinellu A et al, 2012]. Moreover there is little information about the anti-oxidant effects on CKD

patients undergoing lipid lowering therapy. [Cachofeiro V et al, 2008]. To investigate the oxidative stress in our patient cohort, we measured plasma MDA and All/UA ratio. As previously reported [Dounousi E et al2006; Witko-Sarsat V et al, 1998; Yilmaz MI et al, 2006], we found at baseline a significant inverse relationship between GFR and OS parameters.

Moreover, we found that both MDA and ALL/UA ratio were positively related to the concentration of LDL bound thiols, thus confirming that the oxidative stress moves forward to mixed disulphide formation between free thiols and apoprotein. We also found a significant reduction in both MDA and All/UA ratio in all CKD patients during therapy, with a great improvement in patients treated with combined therapy (ezetimibe/simvastatin 10/40 mg/day). The decrease of LDL bound thiols and the level reduction of All/UA ratio were significantly correlated, thus suggesting that an improvement of oxidative stress during treatment may induce a reduction of LDL thiolation degree.

Finally, the data obtained by this pilot study indicate that the elevated levels of LDL-S-homocysteinylation (and more in general S-thiolation) found in CKD patients may depend, not only on the elevated levels of total thiols of these individuals, but principally on the excess of oxidative stress typical of this pathology. A lipid lowering therapy with simvastatin and ezetimibe reduces the oxidative stress thus yielding a decrease in LDL

linked thiols. A greater effect is guaranteed by combined therapy with higher simvastatin doses. Therefore, among the several beneficial effects described for lipid lowering drugs we also propose their ability to reduce the quantity of LDL linked homocysteine thus decreasing not only LDL levels but also LDL atherogenicity.

7 TABLES AND FIGURES

Table 1: Clinical and demographic characteristics of study participants

	All (n=30)	Group 1	Group 2	Group 3 (n=10) Eze/Sim 10/40 mg/day
	Mean ± SD or Median (range)	Mean ± SD or Median (range)	Mean ± SD or Median (range)	Mean ± SD or Median (range)
Sex, F/M(%F)	11/19 (37%)	2/8 (20%)	4/6 (40%)	5/5 (50%)
Age, Years	60±11	63±11	58±12	59±9
Etiology of CKD				
Diabetic nephropaty	7/30	3/10	2/10	2/10
Chronic gomerulonephritis	11/30	3/10	5/10	3/10
Other	12/30	4/10	3/10	5/10
Kidney profile				
Creatinine, mg/dL	1.75 ± 0.77	1.92 ± 0.98	1.63 ± 0.62	1.70 ± 0.71
GFR, ml/min per 1.73 m^2	55 ± 30	61 ± 48	52 ± 19	53 ± 8
Proteinuri, g/24h	0.99 ± 1.27	0.91 ± 0.63	0.81 ± 0.81	1.25 ± 2.00
Lipid profile				
Total cholesterol, mg/dL	239 ± 43	232 ± 34	230 ± 41	254 ± 53
LDL-C, mg/dL	160 ± 37	164 ± 34	156 ± 32	165 ± 47
HDL-C, mg/dL	49 ±15	44 ± 8	47 ± 12	57 ± 19
LDL/HDL ratio	3.50 ±1.28	3.75 ± 1.00	3.50 ± 1.12	3.26 ± 1.71
Triglycerides, mg/dL	143 ± 69	141 ± 70	136 ± 62	151 ± 80
Blood pressure				
Systolic BP, mmHg	130 ± 9	131 ± 9	127 ± 11	132 ± 8
Diastolic BP, mmHg	80 (60-95)	80 (70-85)	80 (60-95)	80 (70-90)
Reduced thiols				
rCysGly, µmol/L	4.64 ± 1.49	4.96 ± 1.28	4.89 ± 1.90	4.06 ± 1.15
rHcy, µmol/L	0.44 ± 0.31	0.56 ± 0.48	0.41 ± 0.18	0.36 ± 0.15
rCys, µmol/L	22.3 ± 1.5	23.4 ± 1.3	21.5 ± 1.9	22.0 ± 1.2
rGSH, µmol/L	1.03 ± 0.47	1.10 ± 0.58	0.96 ± 0.29	1.03 ± 0.52
rGluCys, µmol/L	0.61 ± 0.25	0.68 ± 0.24	0.67 ± 0.29	0.47 ± 0.19
rThiols, µmol/L	29.0 ± 6.0	30.7 ± 6.5	28.4 ± 6.3	27.9 ± 5.3
Total thiols				
tCysGly, µmol/L	35.6 ± 8.9	38.8 ± 9.1	34.4 ± 8.3	33.6 ± 9.2
tHcy, µmol/L	18.4 ± 11.2	20.7 ± 15.0	18.5 ± 11.6	16.0 ± 5.9
tCys, µmol/L	299 ± 67	296 ± 62	281 ± 51	319 ± 85
tGSH, µmol/L	6.46 ± 2.91	6.43 ± 2.69	5.50 ± 1.66	7.45 ± 3.89
tGluCys, µmol/L	4.30 ±1.10	4.41 ± 1.06	3.94 ± 1.03	4.53 ± 1.23
tThiols, µmol/L	364 ± 75	367 ± 63	344 ± 60	381 ± 99
ApoB-thiols				
ApoB-CysGly, nmol/µmol_apoB	60.3 ± 10.9	61.1 ± 10.0	60.1 ± 10.6	59.8 ± 13.0
ApoB-Hcy, nmol/µmol_apoB	27.5 ± 12.5	26.4 ± 15.5	27.5 ± 12.9	28.7 ± 10.9
ApoB-Cys, nmol/µmol_apoB	397 ± 133	396 ± 171	379 ± 65	417 ± 150
ApoB-GSH, nmol/µmol_apoB	7.46 ± 1.86	7.44 ± 2.64	7.55 ± 1.32	7.39 ± 1.56
ApoB-GluCys, nmol/µmol_apoB	1.38 ± 0.63	1.36 ± 0.61	1.37 ± 0.50	1.40 ± 0.81
ApoB-Total thiols, nmol/µmol_apoB	494 ± 142	493 ± 186	476 ± 65	514 ± 158

Table 2: Drug's effect on lipid profile (values expressed as mg/dL)

		Baseline				
Total cholesterol	All patients	239±43	147±26***	148±20***	148±29***	p<0.0001
	Sim 40	232±34	164±24**	156±18**	154±35**	p=0.0002
	E/S 10/20	230±41	137±19***	142±17**	138±21**	p<0.0001
	E/S 10/40	254±53	141±26***	150±23**	152±28**	p=0.0001
LDL-C	All patients	160±37	74±23***	75±19***	73±24***	p<0.0001
	Sim 40	160±34	91±24**	87±16**	86±31**	p=0.0003
	E/S 10/20	156±32	73±11***	76±15***	70±16***	p<0.0001
	E/S 10/40	165±47	59±21***	63±20**	63±17**	p<0.0001
HDL-C	All patients	49±15	50±15	51±17	52±16	p=0.087
	Sim 40	44±8	45±10	45±9	44±8	p=0.900
	E/S 10/20	47±12	45±11	49±16	49±15	p=0.181
	E/S 10/40	57±19	60±19	60±22	62±18	p=0.163
LDL/HDL ratio	All patients	3.5±1.3	1.6±0.7***	1.6±0.6***	1.6±0.7***	p<0.0001
	Sim 40	3.8±1.0	2.1±0.7**	2.0±0.6**	2.0±0.7**	p=0.0004
	E/S 10/20	3.5±1.1	1.7±0.7**	1.7±0.4**	1.5±0.5**	p<0.0001
	E/S 10/40	3.3±1.7	1.1±0.6**	1.2±0.6**	1.1±0.5**	p=0.0006
Triglycerides	All patients	143±69	116±61**	115±55*	109±51**	p=0.002
	Sim 40	141±70	140±63	122±69	114±41	p=0.183
	E/S 10/20	136±62	96±45**	89±26	93±23	p=0.032
	E/S 10/40	151±80	113±68	134±57	118±75	p=0.034

ANOVA with Bonferroni correction

*= p<0.05; ** p< 0.01; *** p<0.001. vs Baseline.

Table 3: Drug's effect on reduced LMW thiols (values expressed as µmol/L)

		Baseline				
rCys	All patients	4.64 ±1.49	4.92 ± 1.70	5.10 ±1.97	5.07 ± 1.79	NS
	Sim 40	4.96±1.27	5.40±1.81	5.15±1.43	5.56±1.63	NS
	E/S 10/20	4.89±1.90	4.70±1.73	5.03±2.40	4.93±2.21	NS
	E/S 10/40	4.06±1.14	4.70±1.67	5.12±2.16	4.72±1.52	NS
rHcy	ALL	0.44±0.31	0.48±0.37	0.52±0.42	0.52±038*	p=0.007
	Sim 40	0.56±0.48	0.59±0.55	0.63±0.66	0.66±0.60	NS
	E/S 10/20	0.41±0.18	0.43±0.20	0.47±0.18	0.44±0.16	NS
	E/S 10/40	0.36±0.15	0.40±0.29	0.45±0.30	0.46±0.23	p=0.041
rCys	All patients	22.31±4.57	24.40±7.35	26.01±7.96**	27.05±7.51***	p<0.0001
	Sim 40	23.42±4.85	25.49±6.38	25.98±5.78	28.26±6.18*	p=0.005
	E/S 10/20	21.49±4.57	21.97±6.82	25.05±9.28	22.90±6.29	NS
	E/S 10/40	22.02±4.56	25.86±6.67	26.98±9.08	30.01±8.58*	p=0.003
rGSH	All patients	1.03±0.47	1.17±0.63	1.43±0.84*	1.30±0.68*	p=0.0007
	Sim 40	1.10±0.58	1.30±0.88	1.29±0.51	1.40±0.76	NS
	E/S 10/20	0.96±0.29	0.96±0.33	1.42±0.87	1.04±0.39	NS
	E/S 10/40	1.03±0.52	1.26±0.59	1.57±1.11	1.45±0.81	p=0.018
rGlu-Cys	All patients	0.61±0.25	0.70±0.40	0.81±0.49*	0.85±0.63	p=0.008
	Sim 40	0.68±0.24	0.88±0.44	0.85±0.43	0.99±0.53	p=0.043
	E/S 10/20	0.67±0.29	0.56±0.21	0.84±0.55	0.73±0.41	NS
	E/S 10/40	0.47±0.19	0.68±0.49	0.74±0.52	0.82±0.88	NS
r-thiols sum	All patients	28.97±6.00	31.56±9.62	33.80±10.53*	34.72±9.74***	p<0.0001
	Sim 40	30.66±6.50	33.48±9.38	33.84±7.61	36.79±8.45*	p=0.002
	E/S 10/20	28.39±6.31	28.57±8.83	32.77±12.73	29.98±9.03	NS
	E/S 10/40	27.86±5.39	32.81±10.78	34.79±11.62	37.37±10.71*	p=0.002

ANOVA with Bonferroni correction

*= p<0.05; ** p< 0.01; *** p<0.001. vs Baseline

Table 4: Drug's effect on LMW linked to LDL (values expressed as µmol/L)

		Baseline				
	All patients	60.31±10.88	50.06±9.87***	50.80±9.80***	53.46±13.90*	p=0.013
	Sim 40	61.07±9.99	52.70±10.21	52.73±7.21*	53.66±11.47	NS
	E/S 10/20	60.11±10.56	49.08±7.46	50.07±9.09	56.31±13.27	NS
	E/S 10/40	59.75±13.01	48.41±11.93	49.61±12.99	50.3917.18	NS
ApoB-Hcy	All patients	27.51±12.80	18.42±11.17***	18.16±11.25***	18.28±10.17***	p=0.0001
	Sim 40	26.36±15.50	17.28±14.22***	17.24±13.20**	16.74±11.19**	p=0.002
	E/S 10/20	27.48±12.91	18.90±9.30	18.58±9.94	19.73±10.00	p=0.003
	E/S 10/40	28.70±10.87	19.08±10.54**	18.67±11.54*	18.37±10.14*	p=0.011
ApoB-Cys	All patients	397.2±132.9	324.1±116.2***	315.1±142.0**	321.2±127.5**	p=0.003
	Sim 40	396.4±171.1	326.9±160.4	328.4±176.6	348.6±155.5	p=0.014
	E/S 10/20	378.6±64.8	324.8±72.7	325.7±137.8	333.5±126.4	NS
	E/S 10/40	416.5±150.3	320.6±111.5	291.2±117.6	281.5±97.4*	p=0.012
ApoB-GSH	All patients	7.46±1.86	6.04±1.89***	6.18±2.01*	6.13±1.74*	p=0.008
	Sim 40	7.44±2.64	6.06±1.83	6.09±1.66	5.79±1.79	p=0.010
	E/S 10/20	7.55±1.32	6.51±1.48	6.74±2.18	7.11±1.81	NS
	E/S 10/40	7.39±1.56	5.55±2.34	5.70±2.21	5.49±1.27	p=0.046
ApoB-GluCys	All patients	1.38±0.63	1.11±0.63***	0.99±0.59***	1.11±0.59***	p=0.0003
	Sim 40	1.36±0.61	0.99±0.47	0.88±0.47	1.06±0.59	NS
	E/S 10/20	1.37±0.50	1.19±0.45	0.95±0.51	1.18±0.58	p=0.013
	E/S 10/40	1.40±0.81	1.14±0.92	1.15±0.77	1.10±0.64	p=0.035
ApoB-thiols sum	All patients	493.9±141.8	399.7±130.4***	391.2±153.2**	400.2±137.3**	p=0.0009
	Sim 40	492.7±186.1	403.9±181.7*	405.3±189.7*	425.8±164.3*	p=0.006
	E/S 10/20	475.2±65.2	400.4±82.7	402.0±147.8	417.9±136.7	NS
	E/S 10/40	513.8±158.2	394.8±122.0*	366.3±129.3	356.9±109.3*	p=0.010

ANOVA with Bonferroni correction

*= p<0.05; ** p< 0.01; *** p<0.001. vs Baseline.

Table 5: Drug's effect on oxidative stress

		Baseline				
	All patients	217.7±142.7	195.1±129.2	183.4±130.7	175.6±122.5*	p=0.004
	Sim 40	247.8±95.4	223.8±112.9	227.1±136.4	219.9±129.1	p=0.250
	E/S 10/20	174.4±163.3	155.6±143.5	145.9±140.2	134.7±118.9	p=0.162
	E/S 10/40	230.9±162.8	205.9±132.9	177.0±114.1	172.3±116.0	p=0.03
All/UA ratio	All patients	0.0147±0.0072	0.0133±0.0068	0.0123±0.0056	0.0119±0.0051	p=0.002
	Sim 40	0.0170±0.0097	0.0166±0.0095	0.0152±0.0075	0.0153±0.0063	p=0.231
	E/S 10/20	0.0114±0.0035	0.0103±0.0021	0.0102±0.0023	0.0092±0.0030	p=0.056
	E/S 10/40	0.0156±0.0064	0.0129±0.0059***	0.0116±0.0048*	0.0113±0.0039	p=0.03

ANOVA with Bonferroni correction

*= p<0.05; ** p< 0.01; *** p<0.001. vs Baseline.

Fig 1: Post-translational modification of plasma proteins by homocysteine. (A) Reaction between a protein lysine ε-amino group and homocysteine thiolactone. (B) Reaction between a protein cysteine thiolate anion and homocysteine–cysteine mixed disulfide.

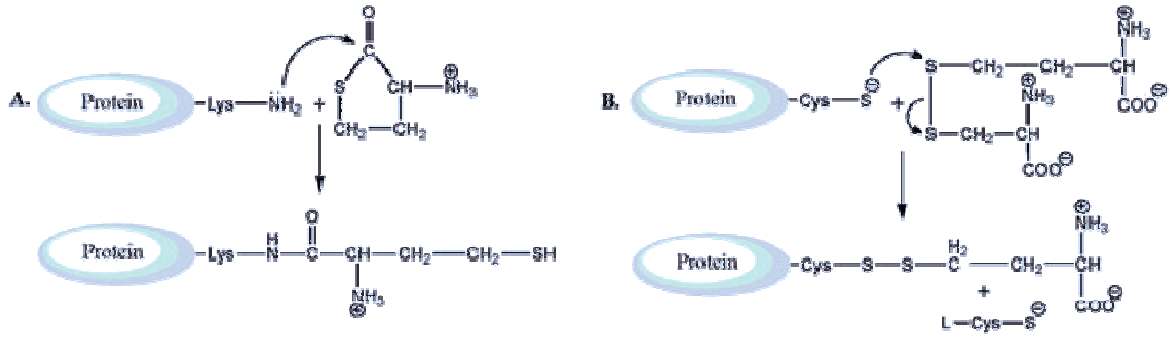


Figure 2: Drug's effect on Homocysteine linked to LDL

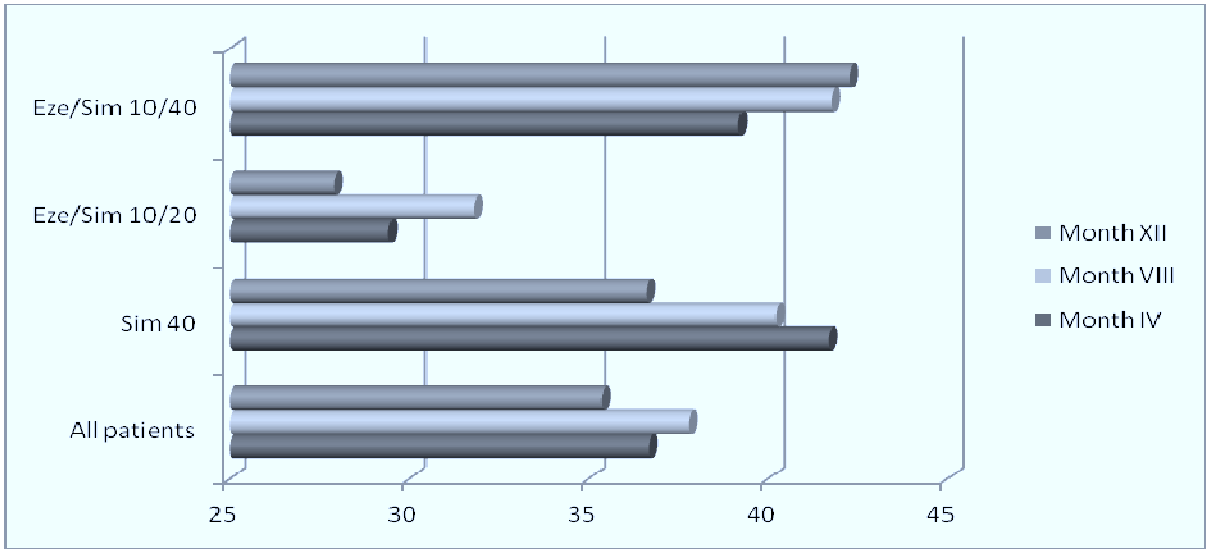
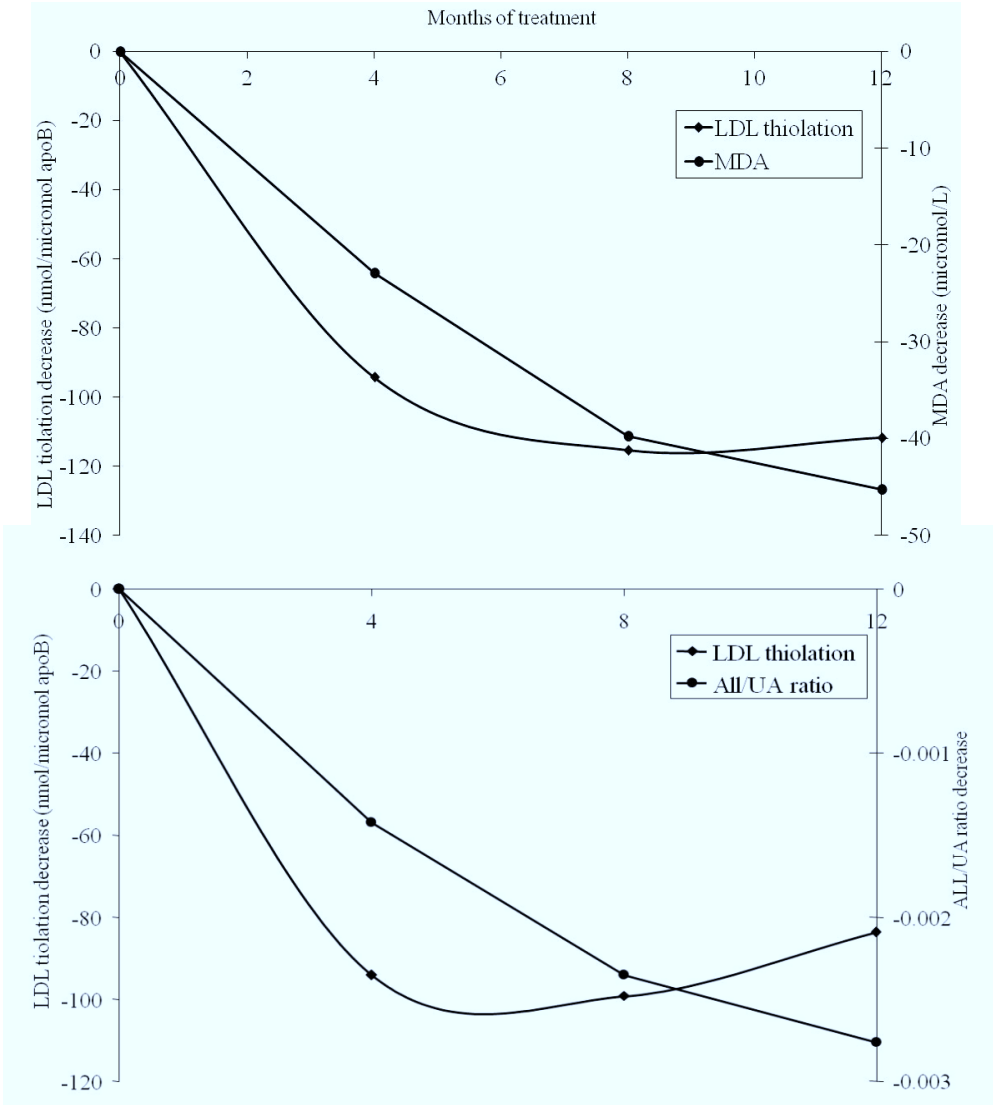


Figure 3: Comparaison between the decrease of plasmatic MDA levels and All/UA ratio vs homocysteine linked to LDL



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