

Modified low-density lipoproteins as biomarkers in diabetes and metabolic syndrome

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1. ABSTRACT

Cardiovascular disease of atherosclerotic origin is the main cause of death in diabetes mellitus and metabolic syndrome. One of the mechanisms involved in such increased risk is the high incidence of lipoprotein modification in these pathologies. Increased glycosylation, oxidative stress or high non-esterified fatty acid levels in blood, among other factors, promote the modification and subsequent alteration of the properties of lipoproteins. Since the modification of low-density lipoprotein (LDL) is the triggering factor in the development of atherosclerosis, considerable research has been focused on the quantification of modified LDLs in blood to be used as biomarkers of cardiovascular risk. The present review deals with the main molecular mechanisms involved in the modification of LDL in diabetes and metabolic syndrome and briefly describe the atherogenic effects

that these modified LDLs exert on the arterial wall. The possibility of using the high levels of modified LDLs or their immunocomplexes as a predictive tool for cardiovascular risk in diabetes-related pathologies is also discussed.

2. INTRODUCTION

Metabolic syndrome and type 2 diabetes mellitus confer an increased risk of cardiovascular disease (CVD). Compared with non-diabetic individuals, diabetic patients have 2 to 4 times increased risk for stroke and death from heart disease (1). Glucose intolerance and type 2 diabetes are core components of metabolic syndrome. A major underlying cause of CVD in patients with MS or diabetes is the presence of a characteristic form of atherogenic dyslipidemia

(2), but other characteristics of this disease contribute synergistically to the increase of the cardiovascular risk (CVR). Among these characteristics, two phenomena, non-enzymatic glycosylation and oxidative stress, are exacerbated in diabetes and affect the function of a number of macromolecules including lipoproteins. Both phenomena are closely interconnected and play a relevant role in the development of atherosclerosis in patients with diabetes (3). Lipoproteins modified by non-enzymatic glycosylation and/or oxidation change their native properties. Thus, high-density lipoproteins (HDL) lose their antiatherogenic potential whereas low-density lipoproteins (LDL) acquire proinflammatory, proapoptotic and proatherogenic characteristics. Besides these modifications, lipoproteins can also be affected by other chemical processes, described in detail below, which lead to the formation of modified LDL particles. The involvement of modified LDL in the development of the atheromatous plaque suggests that its quantification in plasma could reflect the evolution of atherosclerotic lesions, representing a valuable tool for the prediction and stratification of CVR (4).

3. NON-ENZYMATIC GLYCOSYLATION

As a result of hyperglycemia, proteins, lipids and nucleic acids are glycosylated by non-enzymatic processes. Regarding proteins, glucose reacts with the amino groups of lysine and arginine residues, forming an unstable by-product (Schiff base) and, later, the stable Amadori product. Structural proteins with a long half-life, such as collagen, are the most affected by non-enzymatic glycosylation processes but other proteins in blood, such as albumin or immunoglobulins, are also glycosylated and their quantification (fructosamine, i.e. glycosylated proteins in blood) is used as an indicator of glycemic control. Of course, the protein moiety of all lipoproteins can be also glycosylated during their lifetime in circulation and, as a consequence, the normal function of lipoproteins is compromised (5).

Non-enzymatic glycosylation also induces the formation of oxygen free radicals, a phenomenon known as glycoxidation (6). This process generates a rearrangement of molecular bonds and leads to the formation of advanced glycation end-products (AGE), which irreversibly change the function of proteins. The formation of AGE requires longer period of time than the formation of Amadori products and it is generally assumed that it affects mainly to structural proteins. However, AGEs associated to proteins with relatively short mean life, such as apolipoprotein B (apoB) in LDL, have been detected in blood circulation (7).

The modification by methylglyoxal (MG) or other highly reactive aldehydes is another type of modification related to hyperglycemia, that does not directly involve glucose (8). MG is a glucose metabolite of the dicarbonyl type with a high reducing

power. These metabolites rapidly react with arginine residues of proteins forming a heterocyclic compound (hydroimidazolone) which is part of the heterogeneous family of AGE compounds. Thornalley and coworkers have demonstrated the existence of MG-modified LDL (MG-LDL) in blood and have observed that their concentration is increased in patients with diabetes and decreases after treatment with metformin (9, 10).

4. OXIDATIVE STRESS

Increasing evidence from experimental and clinical studies suggests that systemic oxidative stress plays a major role in the pathogenesis of diabetes mellitus and atherosclerosis (7). Besides the glycosylation-associated oxidation (glycoxidation) of proteins, described above, another major cause of increased oxidative stress in diabetes is that, as a result of hyperglycemia, there is an increase in mitochondrial activity that favors the production of reactive oxygen species (ROS), such as the superoxide anion (O_2^-) or the hydroxyl radical ($\cdot OH$) (11). Therefore, alterations of the oxidative stress-related parameters are frequent in the plasma of these individuals. Oxidative stress is particularly relevant in the intima layer of the arterial wall, a microenvironment surrounded by metabolically active cells (smooth muscle cells, macrophages, endothelial cells) that generate ROS and that does not have the abundant antioxidant defenses present in blood. The primary cellular damage resulting from this free radical reactivity, which mainly affects cellular membranes, is a process known as lipid peroxidation. Oxidative modification can damage all macromolecules in the subendothelial space, but lipoproteins are especially affected by oxidation due to their high content of lipids (12). ROS mainly oxidize the unsaturated fatty acids of the phospholipids located on the surface of the lipoproteins, being LDL highly sensitive to this modification. As a result, a number of oxidized lipids (lipoperoxides, oxidized phospholipids, oxidized fatty acids, oxidized cholesterol) and derived products (lysophosphatidylcholine, aldehydes, ketones) are formed in lipoproteins, having most of them proinflammatory, proliferative and apoptotic properties (13).

5. EFFECTS OF OXIDATION AND NON-ENZYMATIC GLYCOSYLATION ON LIPOPROTEIN FUNCTION

The knowledge gathered from three decades of research has shown that the modification of LDL is a key event in the development of atherosclerosis (13). By far, oxidative modification is the most studied mechanism, but alternative modifications are gaining strength as putative mechanisms involved in the development of atherosclerosis. Figure 1 shows different mechanisms of modification that could affect LDL in diabetes and metabolic syndrome. The

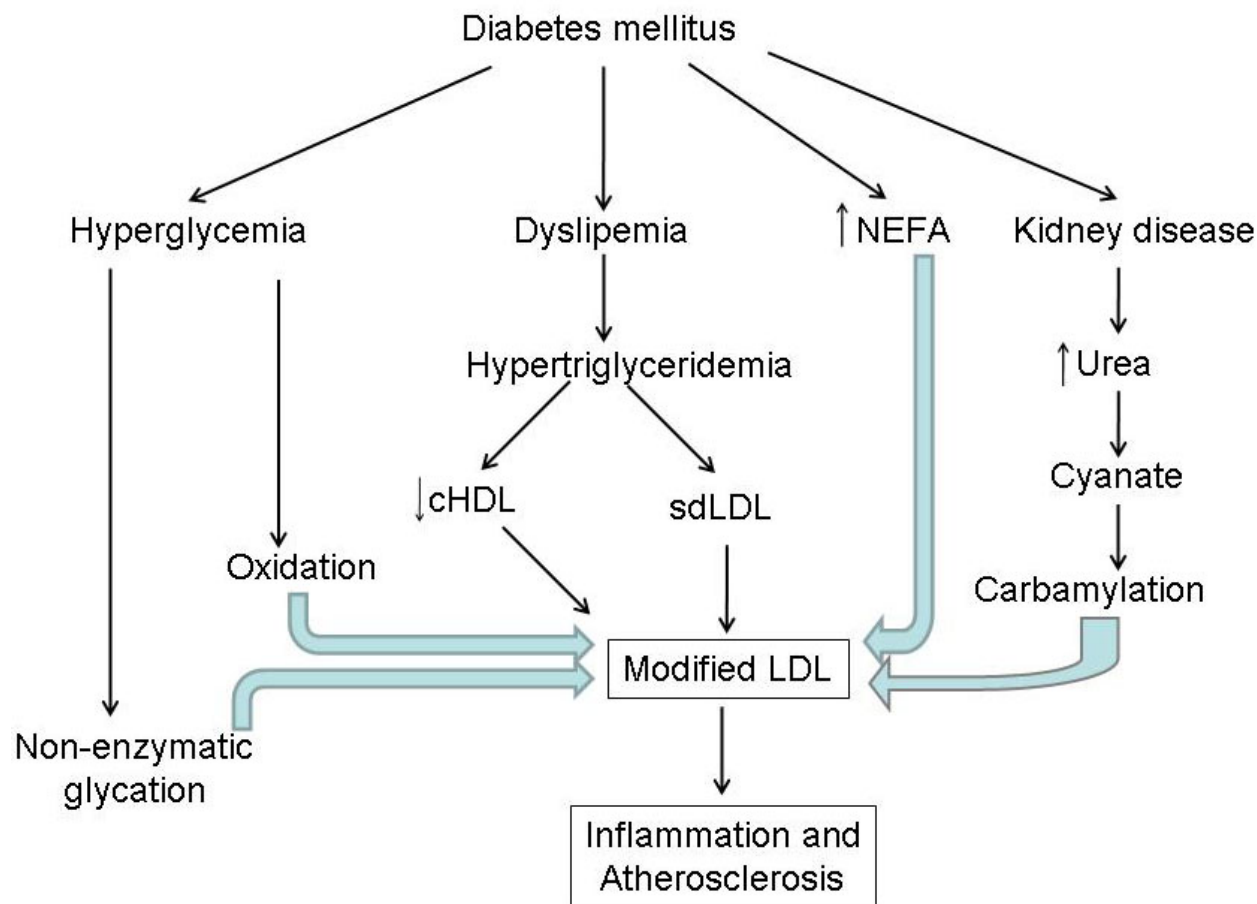


Figure 1. Different features of diabetes promote LDL modification. High levels of glucose stimulate non-enzymatic glycosylation and oxidation, thereby generating different forms of glycated LDL and oxidized LDL. These processes of modification are favored by the presence of atherogenic dyslipipemia, which promotes the formation of small, dense LDL particles that are prone to oxidation and glycosylation. In addition, atherogenic dyslipipemia also generates dysfunctional HDL with lower capacity to prevent LDL modification. Another consequence of dyslipipemia is the elevation non-esterified fatty acids (NEFA) concentration which stimulates the overloading of LDL particles with NEFA. Finally, when kidney disease is present the generation of thiocyanate from urea favors the carbamylation of LDL.

oxidatively-modified form of low density lipoprotein (oxLDL) is a proinflammatory and proatherogenic particle containing protein adducts and inflammatory lipids that promotes atherosclerosis by different mechanisms (14, 15). First, oxidation generates lipid-derived molecules, such as malondialdehyde (MDA), which promotes the derivatization of lysine and arginine residues in apolipoprotein B. This provokes the loss of affinity for the LDL receptor, and the increased binding to scavenger receptors (SR). As a consequence, oxLDL is able to induce massive intracellular accumulation of cholesterol esters by macrophages (16, 17). In addition, the oxidation-derived lipid products generated in oxLDL induce the different cell types in the arterial wall to express cytokines, chemokines and growth factors. In this way, oxLDL promotes the chronic inflammatory and cell proliferation processes which are characteristic of atherosclerosis (18-22). Ox-LDL is also cytotoxic

and apoptotic, favoring the formation of the necrotic nucleus of advanced atheromatous lesions. In fact, ox-LDL is a mixture of particles with different degrees of oxidation whose atherogenic properties change depending on the oxidative stage of LDL (23). Thus, minimally oxidized LDL is much more inflammatory than extensively oxidized particles but it has less capacity to induce foam cell formation.

Regarding glycosylation, it must be distinguished between glycosylated LDL (gl-LDL), LDL-modified with AGE (AGE-LDL) and LDL modified with MG (MG-LDL) (3, 24). The main atherogenic property of gl-LDL is a loss of its affinity for the LDL receptor (25) but it is not very inflammatory, in contrast with AGE-LDL that, since is originated from oxidative processes, is inflammatory, apoptotic and induces foam cell formation (5, 6). Concerning MG-LDL, it has been reported that has smaller particle size, greater

Table 1. Modifications affecting LDL and the most probable location of modification

| Pathological cause | Mechanism | Modified LDL | Location | Increase of electronegativity |
|-----------------------|--|--|--|-------------------------------|
| Hyperglycemia | Non-enzymatic glycosylation | Glycosylated LDL AGE-LDL MG-LDL | Arterial wall Arterial wall Plasma | + |
| Oxidative stress | Lipid peroxidation Desialylation Nitration | Oxidized LDL Desialylated LDL Nitrated LDL | Arterial wall/ Plasma | + |
| Diabetic dyslipidemia | Elevated NEFAs | NEFA-LDL | Plasma | + |
| Nephropathy | Elevated urea | Carbamylated LDL | Plasma | + |
| Arterial lesion | Enzymatic (proteases and lipases) | Proteolyzed LDL Lipolyzed LDL | Arterial wall Arterial wall | - |

susceptibility to aggregation and greater affinity for binding to proteoglycans of the arterial wall (26). Of note, the short half-life of circulating LDL (2.5–3.5 days) has been an argument against the formation of gl-LDL or AGE-LDL in blood since, in absence of reducing agents, 6–7 days are necessary for glucose to modify proteins. Therefore, it has been implicitly assumed that the formation of these modified particles would occur mainly in LDL retained in injured areas of the arterial wall for a period longer than its plasma lifetime and their presence in the blood would be a reflection of the development of arteriosclerotic lesions (24). In contrast, the modification by MG does not directly involve glucose but this metabolite with a high reducing power rapidly reacts with arginine residues. Thus, the relevance of MG-LDL, within the AGE-LDL family, is that it could be formed during LDL plasma lifetime.

6. OTHER MODIFICATIONS AFFECTING LDL

Besides oxidation and glycosylation, other modifications of LDL have been described to occur *in vivo* (27). Some of them would occur mainly in the intima layer of the arterial wall while others are probably more relevant during blood circulation. Table 1 summarizes the different modifications that could affect lipoproteins and the most probable environment where this modification occurs.

6.1. Enzymatic modifications

Regarding modifications occurring in the arterial wall, during the development of atherosclerotic lesions there is a hyperexpression of proteolytic and lipolytic enzymes such as phospholipase A2 (PLA2), sphingomyelinase (SMase), cholesterol esterase (CEase), metalloproteinases (MMPs) or cathepsins (28–32). Therefore, it is presumed that LDL retained in the arterial wall is affected not only by oxidative or glycosylative processes but also by proteases and

lipases. As a consequence, lipoproteins isolated from the arterial wall show protein fragmentation and a high content of enzyme-mediated lipid degradation products such as lysophosphatidylcholine, ceramide or free cholesterol (33). Nowadays, although it is still accepted that lipid peroxidation has a role in atherogenesis, it is considered that other enzyme-related mechanisms of LDL modification, such as degradation by lipases or proteases, could have an even more predominant role in the generation of modified LDL in the arterial wall (34, 35). The main atherogenic effect of enzymatic modifications is that these processes trigger LDL aggregation and fusion, favoring its subendothelial retention (36). Hence, retained LDL is exposed to undergo further modifications by other mechanisms such as oxidation or glycosylation. Other effect of enzyme-mediated lipolysis is the formation of lipids, such as lysophosphatidylcholine or ceramide, which would not be formed by oxidation or glycosylation and display apoptotic and inflammatory properties.

6.2. Carbamylated LDL

Enzymatic processes preferentially occur in the artery wall, but LDL can also be modified by other mechanisms in the blood circulation. Recently, the presence of carbamylated LDL in plasma has been reported (37). The carbamylation of LDL occurs due to spontaneous, non-enzymatic chemical modification of the amine-containing residues in apoB by urea-derived cyanate (38). This modification is especially relevant to smokers, since tobacco smoke favors the formation of thiocyanate, and also in patients with chronic uremia due to severe renal insufficiency (39). Then, carbamylation of LDL could have a role in the development of atherosclerosis in patients with diabetes that also present kidney disease. Among the atherogenic properties of carbamylated LDL, it has been reported that is immunogenic, prothrombotic, proliferative and that induces endothelial dysfunction (40–42).

6.3. Nitrated LDL

Although less studied than ROS-mediated modification, LDL can also be modified by reactive nitrogen species (RNS), a process known as nitration. This phenomenon is closely related to oxidative modification because the main reactive molecule is peroxynitrite (ONOO⁻), which derives from nitric oxide (·NO) and superoxide anion O₂⁻. Besides promoting lipoperoxidation, nitration of apoB in LDL results in the derivatization of tyrosine and oxidation of cysteine, which alters apoB structure (43). As occurs with carbamylation, nitration of LDL is a process that occurs in plasma (44) and is potentiated in smokers and in patients with severe kidney disease (45, 46).

6.4. Desialylated LDL

Other form of modified LDL detected in plasma is desialylated LDL, which has a reduced content in sialic acid, the final carbohydrate in the apoB-enzymatic glycosylation chains. Desialylated LDL is increased in patients with diabetes and it has the capacity to induce the formation of foam cells being, therefore, potentially atherogenic (47). The desialylation process has been attributed to oxidative processes since these favor the non-enzymatic hydrolysis of sialic acid bound to apoB (48). Desialylated LDL shares some properties with oxidized LDL; thus, it could be a reflection of oxidative stress (49).

6.5. NEFA-loaded LDL

Although it cannot be strictly considered a chemical modification, overloading of LDL with non-esterified fatty acids (NEFA) confer some atherogenic properties to LDL (50, 51). NEFA are usually transported in circulation by albumin, however, when NEFA concentration rises and the capacity of albumin to bind NEFA is exceeded, these lipids bind to other macromolecules, mainly lipoproteins. This phenomenon could have especial relevance *in situations* of insulin resistance such as metabolic syndrome or diabetes and it has been reported that LDL from diabetic patients has a high NEFA content (52). LDL with an increased NEFA content is inflammatory and its structure is altered favoring its aggregation (53-56). This could explain the observations that diabetic LDL is more inflammatory than LDL from subjects without diabetes, despite not having increased rates of lipoperoxidation (57).

7. ELECTRONEGATIVE LDL: A POOL OF MODIFIED FORMS OF LDL IN BLOOD

A common property of the different forms of modified LDL is an increase of the electric charge of these particles (58, 59). According to this property, total LDL can be subfractionated by anion exchange chromatography into two populations, a major

subfraction of non-modified native LDL and a minor subfraction of electronegative LDL (LDL(-)). This minor subfraction accounts for 2-10% of total LDL in normolipidemic subjects. LDL(-) is heterogeneous in terms of size, density, lipid and protein content (60-62). The most widely accepted idea is that LDL(-) is a pool of LDL particles modified by several mechanisms. However, only a small part of LDL(-) would consist of oxidized, glycosylated, nitrated, desialylated or carbamylated LDL because LDL(-) proportion is much higher than that described for these modified LDLs (0.1-1%) (63).

Besides the chemical modifications previously described, other alterations in the composition of LDL also contribute to the presence of LDL(-). It has been reported that NEFA content is a major contributor to the electronegativity of LDL particles (56). In addition, both small dense and very large LDL particles also present an increased electronegative charge (64). The same occurs with LDL particles that contains other apolipoproteins different than apoB (65). Indeed, LDL(-) is characterized by abnormal size (small or large) and increased content of NEFA and minor apolipoproteins that include, among others, apoA-I, apoE, apoC-III, apoD, apoJ or apoF (56, 60, 65, 66). Therefore, an increased proportion of LDL(-) in blood would reflect a range of metabolic abnormalities, which are associated with high CVR and systemic inflammation. Accordingly, the proportion of LDL(-) is increased in a number of metabolic diseases with increased CVR, such as familial hypercholesterolemia, hypertriglyceridemia, diabetes, metabolic syndrome, severe renal disease, non-alcoholic fatty liver disease and also in patients with angiographically-established coronary disease (58, 67, 68). Moreover, LDL(-) proportion dramatically increases during the early phase of acute myocardial infarction and after cerebral ischemia (69, 70).

Since LDL(-) is a mixture of modified LDL particles and has an abnormal composition, it shows inflammatory, apoptotic and proliferative properties (58, 68, 71, 72). These atherogenic characteristics displayed by LDL(-) isolated from normolipemic and normoglycemic subjects are exacerbated in LDL(-) isolated from diabetic subjects, especially when these subjects are in poor glycemic control (73). Then, LDL(-) from diabetics would be more atherogenic than LDL(-) from normoglycemic subjects. This could be due to the confluence in poorly-controlled diabetic patients LDL of multiple factors including, increased oxidation/nitration, increased glycosylation, increased NEFA content, smaller size and, if kidney disease is also present, increased carbamylation.

8. WHY DOES DIABETIC DYSLIPIDEMIA STIMULATE LIPOPROTEIN MODIFICATION?

A common metabolic abnormality associated with diabetes is a specific dyslipidemia that includes

a spectrum of quantitative and qualitative changes in lipids and lipoproteins (74). This anomalous lipid profile, known as diabetic or atherogenic dyslipidemia, is characterized by high levels of triglycerides and apoB, low concentration of high density lipoprotein (HDL) cholesterol, and increased postprandial lipidemia. This abnormal lipid profile is typical of diabetes but it is also present in pre-diabetic situations such as insulin resistance and metabolic syndrome (1, 75, 76).

The origin of diabetic dyslipemia comes from an increased hepatic production of very low density lipoprotein (VLDL) due to the high plasma concentration of NEFA. In this situation, VLDL particles are very large due to a very high content of triglycerides (77, 78). Hypertriglyceridemia alters some enzymatic activities related to VLDL catabolism, specifically the enzymes cholesteryl ester transfer protein (CETP) and hepatic lipase (HL). Hypertriglyceridemia stimulates the enzymatic activity of CETP, which facilitates the transfer of triglycerides from triglyceride-rich lipoproteins (i.e. VLDL) to HDL and LDL in exchange for cholesteryl esters (79). This leads to an increase in the triglyceride content of HDL and LDL (80). Triglyceride-enriched HDL particles are subjected to increased catabolism; consequently, they have a short plasma half-life. In addition, triglyceride-enriched LDL particles undergo subsequent hydrolysis via HL, thereby reducing the LDL particle size (81).

In contrast to HDL, which has atheroprotective properties, LDL and VLDL are considered atherogenic, being apoB their main protein component. Despite that 80-90% of apoB is associated with LDL and that apoB concentration is high in diabetes, the LDL cholesterol levels are usually normal in these patients (82). This peculiarity is explained by the prevalence of LDL particles of small size (small, dense LDL, sdLDL). sdLDL have lower relative cholesterol content and higher relative apoB and triglyceride content than normal LDL particles (81). Thus, an increase in triglyceride-rich lipoproteins is commonly associated with a reduction in HDL concentration and an increase in sdLDL levels. This means that at a given LDL cholesterol concentration, diabetic patients have a greater number of LDL particles (83, 84).

sdLDL particles are more atherogenic than large buoyant LDL due to several characteristics that facilitate their modification (81, 84, 85). First, sdLDL has a lower affinity for the LDL receptor, which implies a lower rate of plasma clearance and longer time in the circulation; this would expedite LDL modification by different mechanisms such as oxidation, glycosylation, desialylation or carbamylation. Second, sdLDL crosses the endothelial barrier easier than native LDL since this is a process mainly dependent on the size of the lipoprotein particle. In addition, sdLDL also binds with greater affinity to the proteoglycans that constitute the

intima layer of the arterial wall, favoring subendothelial retention of lipoproteins. Third, sdLDL has a greater susceptibility to be modified by oxidative mechanisms and also by non-enzymatic glycosylation(86).

To these intrinsic pro-atherogenic properties of sdLDL, it must be added the qualitative alterations in HDL function in a diabetic dyslipidemia situation. The antiatherogenic role of HDL goes beyond that its classic role in the reverse transport of cholesterol. HDL has a determinant action in the protection of LDL against modifications, some enzymes and apolipoproteins associated to HDL, such as apoA-I, apoJ, paraoxonase, platelet-activating factor acetylhydrolase (PAF-AH) or lecithin-cholesterol acyl transferase (LCAT) act synergistically preventing the oxidation of LDL (87, 88). However, the glycosylation and oxidation of these proteins also affect their functionality, compromising the antioxidant and anti-inflammatory capacity of HDL. Therefore, the concentration of HDL is not only diminished in patients with diabetes, but it is also dysfunctional. In this way, the impairment of the HDL anti-atherogenic properties in diabetes favors the formation of modified LDL.

9. sdLDL AS A BIOMARKER OF CVR

It is well documented that small dense LDL (sdLDL) levels are elevated in conditions linked to atherosclerosis, such as metabolic syndrome, disease in which sdLDL has been reported to be an independent predictive factor for cardiovascular events (89, 90). Other studies concur with the concept that sdLDL cholesterol (sdLDL-C) is a better marker for predicting CVR than total LDL cholesterol (91, 92). However, not all the studies agree; these discrepancies could depend on the methods used to measure sdLDL. The recent use of homogeneous assays has allowed to evaluate sdLDL in large clinical trials (90). In a large prospective study using these assays it was found that sdLDL-C is associated with coronary heart disease even in patients with low CVR based on their LDLc levels (93). The value of sdLDL-C as an independent CVR factor has also been suggested by comparing with intima media thickness measurement (94). sdLDL has been associated with poor outcome after angioplasty in peripheral artery disease (95). Several atherogenic properties have been ascribed to sdLDL, which can be further modified in plasma by several mechanisms, such as desialylation, glycation, and oxidation (90), as described above. These modifications would confer more atherogenic properties to this LDL and, consequently, a closer relation with CV events.

10. ASSOCIATION OF MODIFIED LDLs WITH CVR

Owing to its known role in atherosclerosis, different forms of modified LDLs have been proposed as

biomarkers for CVR and for detecting the vulnerability of atherosclerotic plaques. Most studies using modified LDLs as biomarkers have been conducted with oxLDL (96-99). Holvoet *et al.* developed the first immunoassay to detect the presence of oxLDL in plasma, which was reported to be increased in atherosclerotic patients (100). Since then, a multitude of studies have associated oxLDL concentration with different expressions of vascular disease, and its concentration is increased in pathologies with increased CVR. Thus, oxLDL is increased in patients with atherosclerosis and correlates with the severity of coronary disease (4, 101-105). Moreover, oxLDL increases after acute myocardial infarction and is associated with plaque instability (90). oxLDL also acutely increases after percutaneous coronary intervention (93, 106, 107). It has been recently reported that oxLDL also increases in ischemic stroke (108). In stroke, particularly in large artery atherosclerosis subtype, an increased oxLDL concentration in acute phase was associated with higher mortality or worse outcome. These observations suggest that oxLDL in blood comes from the arterial wall and could be a biomarker of atherosclerotic plaque vulnerability.

Regarding its predictive value as a CVR marker, numerous studies have described increased concentrations of oxLDL in diseases with high vascular risk, such as hypercholesterolemia, hypertension, chronic heart failure, peripheral arterial disease, diabetes, metabolic syndrome, obesity and renal disease (4, 109-117). In spite of this, some studies cast doubts on the usefulness of oxLDL as an independent predictive biomarker of future cardiovascular events (118, 119). The main concern comes from the fact that oxLDL values strongly correlate with other known lipid risk factors, including total and LDL cholesterol (120). Thus, even though the involvement of oxLDL in atherosclerosis has been clearly established, its value as an independent biomarker of CVR is moderate. This is due to different factors. On the one hand, some conflicting results have been reported; for instance, not all studies have found the association of oxLDL levels with the burden of atherosclerotic lesions (121, 122). Choi *et al.* also reported that statin therapy increased the titers of oxLDL measured by two independent ELISAs but found no quantitative changes in coronary angiography (123). Several factors could underlie these conflicting results. First, there is no international standardization in oxLDL determination, due to the use of different antibodies that recognize epitopes of different stages of LDL oxidation (120). Second, at least three commercial ELISAs have been widely used, but each antibody recognizes different epitopes that could reflect different processes, thereby preventing an adequate comparison of the results obtained by different groups. On the other hand, oxLDL represents only a part of the modified LDL particles that can be found in circulation. In specific pathologies other forms

of modified LDL could be more relevant than oxLDL. For instance, carbamylated LDL possibly plays an important role in patients with severe renal disease. Unfortunately, there are no established guidelines for modified LDL evaluation that allow its application to the clinical practice and its usefulness as a predictive biomarker. Regarding diabetes, the usefulness of AGE-LDL as biomarker is discussed in detail below.

11. LDL(-) AS A BIOMARKER OF CVR

An alternative to oxLDL as a biomarker could be LDL(-). Modified lipoproteins such as oxLDL and AGE-LDL are considered biomarkers for active atherosclerotic lesions, as they are supposed to be generated in the arterial wall. But, as discussed above, LDL(-) represents not only a pool of modified LDLs but also reflects the existence of metabolic abnormalities leading to alterations in the composition of the lipoprotein. Therefore, the quantification of LDL(-) would be especially useful in asymptomatic patients (58, 63). LDL(-) proportion is high in several groups of subjects with enhanced CVR, such as familial hypercholesterolemia, hypertriglyceridemia, diabetes, renal disease, and non-alcoholic fatty liver disease (68, 90). Furthermore, LDL(-) proportion highly increases after stroke (70) and myocardial infarction (69), being its concentration higher in acute than in chronic coronary disease (124). Moreover, LDL(-) levels are associated with the severity of coronary disease angiographically-determined (125) and with the carotid intima-media thickness (126).

Specifically in diabetes, several studies have confirmed by different methods an increased proportion of LDL(-) compared to healthy subjects (57, 72, 127-131). Interestingly, the elevated proportion of LDL(-) decreases after insulin therapy in type 1 but not in type 2 diabetes, which suggests that non-enzymatic glycosylation has a more relevant role in LDL(-) generation in type 1 than in type 2 diabetics. In the same context, it was observed that the oral antihyperglycemic agent pioglitazone decreases the negative charge of LDL (130). Also in prediabetic insulin-resistant subjects the proportion of LDL(-) is increased (132). The clinical importance of LDL(-) in the metabolic syndrome has been studied by Chen and co-workers. These authors found a correlation between L5, the most electronegative form of LDL, plasma levels and the number of metabolic syndrome criteria (according to the Framingham score) in asymptomatic patients (133).

Unfortunately, there are no standardized methods for LDL(-) quantification in large-population studies. The usual methods are size exclusion chromatography combined with ultracentrifugation or capillary electrophoresis. But these methodologies are not applicable to a large number of subjects

Table 2. Main clinical studies investigating OxLDL, LDL(-), AGE-LDL and their immunocomplexes in Diabetes, Metabolic Syndrome, Obesity and related diseases

| | References |
|--|-------------------------|
| Oxidized LDL and its immunocomplexes | |
| Increased levels found in diabetic subjects with small dense LDL | 130,131 |
| Increased levels found in diabetic subjects with poor glycemic control | 123,132,124 |
| Elevated concentration is a predisposing factor in diabetes for atherosclerosis | 133,134,135,156,157,158 |
| Elevated concentration is a predisposing factor in diabetes for nephropathy | 135,149,159 |
| Increased levels found in subjects with MS in comparison to those without MS | 112,114,115 |
| Associated with obesity | 90,91,92,93,107 |
| High levels were associated with a cardiometabolic risk score in obese children | 108,109,110 |
| LDL(-) | |
| Increased proportion in patients with type 2 diabetes in comparison to non-diabetics | 53,68,121,123,130,131 |
| Increased proportion or mobility in type 1 diabetic compared to non-diabetics | 120,122,123,124 |
| LDL (-) levels are significantly higher in MS subjects than in control subjects | 126 |
| Insulin resistance is associated with high levels of LDL(-) | 125 |
| AGE-LDL and its immunocomplexes | |
| High levels in patients with type 1 diabetes | 156,157,159 |
| High levels in patients with type 2 diabetes | 158,161,162 |
| Increased levels are associated with retinopathy in patients with type 1 diabetes | 160 |
| Elevated concentration is a predisposing factor in diabetes for nephropathy and macroalbuminuria | 148,159,163 |

and are not accessible to most of clinical chemistry laboratories. Abdalla and coworkers have developed monoclonal antibodies to perform immunoassays for LDL(-) measurement in population studies (134, 135). However, these methods are not commercially available and it is unknown if all modifications affecting LDL(-) could be detected.

Measuring the presence of auto-antibodies to LDL(-) could be an alternative approach for LDL(-) quantification. Similarly to oxLDL or AGE-LDL, LDL(-) shows immunogenic properties, and it has been proposed that LDL(-) is internalized by macrophages in the subendothelial space, thereby promoting anti-LDL(-) production by B cells. Auto-antibodies for LDL(-) and immunocomplexes (IC), consisting of LDL(-) and anti-LDL(-) antibodies, could be released to the lumen space. The presence of these auto-antibodies and IC has been detected in plasma. Moreover, the proportion of auto-antibodies for LDL(-) is increased in coronary disease, particularly in the acute phase of unstable angina (124, 134, 135). However, the role of these auto-antibodies is controversial since it has been described that administration of anti-LDL(-) protects from atherosclerosis development in mice (136). Further studies are necessary to elucidate the possible role of auto-antibodies anti LDL(-) in the evolution of atherosclerotic plaques and during acute vascular events.

12. MODIFIED LDL AS A BIOMARKER OF CV RISK IN DM AND METABOLIC SYNDROME

As discussed throughout this review, lipoproteins from diabetic patients are subjected to

a number of modifications. Accordingly, diabetics have increased concentrations in blood of several modified LDLs, including oxLDL, glycLDL, AGE-LDL, MG-LDL and LDL(-). Table 2 summarizes the main studies on the relation of modified LDL and CVR in subjects with diabetes, metabolic syndrome or related diseases. Diabetic patients with phenotype B of LDL subfraction (predominance of sdLDL) have even higher plasma levels of oxLDL and glycLDL than those with phenotype A (predominance of large LDL) (137, 138). It has also been described that patients with poor glycemic control have increased concentration of different types of modified LDL (130, 139), and that glycemic optimization decreases these levels (131). Hypolipemic treatment also decreases the concentration of modified LDL (70); this suggests that the determination of these modified forms of LDL as independent CVR biomarkers is rather controversial, particularly in type 2 diabetic patients, who usually have an altered lipid profile. Thus, some studies suggested that in the context of diabetes, oxLDL is a factor that predicts CV events, whereas others did not find this association when corrected by lipid profile (101, 140). However, an independent association of oxLDL with atherosclerosis progression is found in some studies in which intima-media thickness (IMT) or nephropathy are evaluated (141, 142).

In diabetic patients, besides the presence of modified LDL, autoantibodies anti-modified LDL (anti-oxLDL, anti-AGE-LDL, anti-MDA-LDL) forming immuno-complexes (IC) with modified LDLs are detected in blood. Recently, anti-ribosylated-glycated-LDL has also been found in type 1 and type 2 diabetic patients (143). Although data from the

studies of ICs are sometimes difficult to interpret, it is generally accepted that IgM antibodies would have a protective effect, whereas IgG antibodies would be directly related with atherosclerosis (144). Thus, IgM anti-AGE-LDL concentration has been reported to protect from CVR both in diabetic and non-diabetic subjects (145, 146). In contrast, some studies show a positive relationship of IgG autoantibodies titers with the development of atherosclerosis (124, 147, 148). Other authors, however, disagree with this direct relation; thus, Ascitto and coworkers reported that low levels of IgG anti-MDA-LDL correlates with high risk of postoperative death after carotid endarterectomy (149), whereas high levels of these antibodies are associated with decreased plaque inflammation (150). Moreover, studies performed in diabetic mice shows that treatments with anti-oxLDL IgG (151) or AGE-LDL immunization (152) protect against atherosclerosis, thereby suggesting a protective role for IgG autoantibodies. Regarding LDL(-) autoantibodies and diabetes, there is a higher concentration of auto-antibodies anti-LDL(-) in type 1 and type 2 diabetic patients, as well as in those with impaired glucose tolerance, than in control subjects (153).

The above described discrepancies could come from the difficulty of measuring modified LDL and autoantibodies, which could be due to the fact that both molecules are strongly associated in IC (154). The evaluation of IC is technically difficult, since it requires a previous precipitation of IC before the quantification of modified LDL or autoantibodies by immunoassay. IC-oxLDL and IC-AGE-LDL have been detected mainly in type 1 diabetes (155). In this regard, are noteworthy the studies of Virella and Lopes-Virella *et al.* describing that in these subjects the major part of oxLDL and AGE-LDL is associated to antibodies as IC (156). IC-LDLs have a higher atherogenic effect than modified LDL alone and seem to be a solid predictor of CVR (157-160). Sobenin *et al.* reported that, even in the absence of clinical manifestations, elevated levels of IC-LDL are increased in early carotid atherosclerosis, measured as IMT (161). Orekhov *et al.* suggested that IC-LDL can be considered biomarkers for macrovascular disease in type 1 diabetes (162). Several studies performed in large populations of type 1 diabetic subjects (DCCT/EDIC cohort) have shown that IC-oxLDL and IC-AGE-LDL concentrations are associated, independently of other risk factors, with IMT and atherosclerosis progression (163, 164), coronary calcification (165), risk of nephropathy (166) and progression of retinopathy (167). Similar studies conducted in type 2 diabetic patients (VADT cohort) have shown that high levels of IC-MDA-LDL are associated with myocardial infarction and acute CV events (168), retinopathy (169), and macroalbuminuria (170).

13. SUMMARY AND PERSPECTIVES

Diabetic patients have increased plasma concentrations of LDLs modified by different mechanisms. In general, this concentration correlates with other lipid risk factors attributed to diabetic dyslipemia, which somehow prevents its use as an independent biomarker of cardiovascular risk. However, some studies show that modified LDL and, more specifically immunocomplexes of modified LDLs, could be independent risk factors, being its plasma concentration associated with atherosclerosis progression in type 1 and type 2 diabetic patients. Therefore, the detection of modified LDL and immunocomplexes could help to better predict cardiovascular risk in diabetes and probably in other pathologies related to cardiovascular disease. It would be very useful to develop a specific profile of modified LDL that, combined to genetics of patients with diabetes or metabolic disease, could predict the risk of cardiovascular disease and to personalize therapy. However, more mechanistic studies are warranted to gain insight into the molecular processes leading to LDL modification and their consequences for atherosclerosis development in patients with diabetes.

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