Atherogenic modifications of LDL particles in diabetic patients

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Abstract
Clinical manifestations derived from atherosclerosis are the leading complications in patients with diabetes mellitus, being responsible of 50-70% of deaths in these individuals. The precise link between accelerated development and atherosclerosis and diabetes is not completely understood, although mechanisms related with hyperglycemia and diabetic dyslipidemia play an important role. Both mechanisms are closely related and frequently are two faces of the same coin. There are well-recognized qualitative modifications of low-density lipoproteins, such as an increased percentage of small, dense LDL particles with higher electronegative charge. These modifications disturbing physicochemical and biological characteristics of LDL particles are involved in the common mechanisms by which dyslipidemia and hyperglycemia promote increased atherosclerosis in diabetic patients.

Keywords: diabetes mellitus, atherosclerosis, modified lipoproteins, electronegative LDL particles.

Hyperglycemia and atherosclerosis
At present, it is broadly accepted that the atherosclerosis pathology starts with the onset of a dysfunction in the vascular endothelium, in response to an external aggres-
Matory chronic condition promotes a migration of elements of the immune system whose activity at long term leads into the characteristic lipid accumulation in the intima layer of the arterial wall. An important increase of the cell proliferation follows this phase and, consequently, the thickening of the arterial wall. Finally, the cytotoxicity, the apoptosis and the activation of thrombosis processes arise leading to the complication of the atherosclerotic injury and the onset of clinical events. Therefore, the endothelial dysfunction and the associated inflammatory process are the key initial events in the development of the atherosclerotic injury.

The clinical manifestations of the atherosclerotic disease constitute the main complication in the population with diabetes mellitus, as they are responsible of over 50% of the mortality, and most of the patients with T2D without previous cardiovascular disease have the risk of suffering a coronary event similar to the patients without diabetes who had already shown a myocardial infarction. This risk increase can be explained in part by the higher prevalence and atherogenic effect of the classic cardiovascular risk factors. However, the prognosis of cardiovascular events from these risk factors does not exceed the 50% of the cases, stating clearly that the responsible mechanisms of the accelerated development of the atherosclerosis in diabetes is unknown. These patients have also hypercoagulability and there is an increased systemic inflammatory condition, as it can be observed from the major plasma concentration of reactive C-protein and, as it will be discussed below, other processes derived from the hyperglycemia and dislipidemia situation that play an important role in the atherosclerosis development.

In spite of the advances carried out during the last decade about the molecular mechanisms involved in the diabetes atherosclerosis, it is still a reason of discussion if the glucose and the lipids exert independent effects on the development of the atherosclerotic injury. In fact, the glucose and lipids effects on the activation of different metabolic routes in the endothelial cell are surprisingly similar. The simple presence of a high concentration of glucose can cause endothelial dysfunction, basically due...
to the limited endothelial capacity of controlling the intracellular glucose levels. Several mechanisms have been suggested, as an increased consumption of NAPDH, the formation of advanced glycation end products (AGE), the activation of protein kinase C or specific transcription factors. The common link of these mechanisms is that the intracellular oxidative stress increase activating an inflammatory response by the vascular endothelium.6,7

Dyslypidemia and diabetes

Besides the direct effect of the hyperglycemia on the endothelial dysfunction, other authors have suggested that the dyslipidemia associated to the diabetes is the main cause of atherosclerosis, especially in the T2D.8 The so-called diabetic or atherogenic dyslipidemia is characterized by the presence of hypertriglyceridemia, reduced concentration of high-density lipoproteins (HDL) and predominance of small and dense low-density lipoproteins (LDL).9-12 It is important to point out that these lipid alterations are not usually present in the patients with T1D and are neither exclusive of the T2D, but its association is indeed constant with the insulin-resistance or hyperinsulinemia condition, in which the plasma concentration of non-esterified fatty acids (NEFA) is high.13 The cause of these alterations is mainly due to the high NEFAs in the plasma promote the hepatic triglyceride synthesis.14 Simultaneously, the insulin resistance increases the expression of apoprotein B (ApoB) in the liver. The result of both phenomena is an hepatic hypersecretion of very low-density lipoproteins (VLDL) enriched in triglycerides.15,16 To this point, we have to add the fact that the insulin-resistance reduces the expression of the endothelial lipoprotein lipase, causing a reduced catabolism of VLDL and favoring the hypertriglyceridemia.17 On the other hand, the reduced concentration of HDL is due to the reduction of the VLDL catabolism, as the lipolytic action of the endothelial lipoprotein lipase favors the formation of HDL from the surface material of the VLDLs.18 Finally, the T2D is characterized by a higher expression of the hepatic lipase as well as by an increase of the triglycerides interchange and the mediated cholesterol esters by the proteins that transfer them.19,20 Both enzymes take part in the maturation process of the VLDL, and its principal activity gives place to the formation of small and dense LDL particles7 (figure 2). On the other hand, the deficient clearance of the VLDLs by the previously mentioned mechanisms, gives place to the formation of residual lipoprotein particles that have an intermediate density (IDL) and, as the small and dense LDL, have an increased atherogenic potential as regards to the normal size LDL.

Several studies carried out in non-diabetic patients stated that the hypertriglyceridemia and the reduced HDL are cardiovascular risk factors (CVR). However, these alterations do not explain more than 25% of the CVR excess that the patients with T2D show, and much less in the case of T1D, which in general the lipid plasma concentrations are practically normal.22-24 On the other hand, the cholesterol bound to the LDLs (cLDL) is considered by the National Cholesterol Education Program/Adult Treatment Panel III of the United States (NCEP-ATP-III) as the main risk factor and the first therapeutic objective.25 The cLDL is usually normal even in patients with T2D and diabetic dyslipidemia, but it is the most potent prognostic factor of coronary disease in patients with or without diabetes.26 This apparent paradox is attributable in the diabetes mostly to the qualitative characteristics of the LDL than the plasma concentration may be most important. According to this hypothesis, there are several processes that converge in the diabetes and contribute to a series of modifications that the LDL suffers and that might cause it to be a more atherogenic particle.

Small/dense LDL (sDLLD)

It has already been mentioned that the presence of LDL particles of smaller size and higher density, due to an increase of the protein/lipid, takes place frequently in situations of hypertriglyceridemia and cholesterol bound to the reduced HDL (cHDL).27 Since in general these abnormalities come together, it is difficult to determine which proportion of the increase of CVR corresponds to each of them. Some studies have indicated that the CVR is increased in subjects with sdLDL predominance regardless of the triglycerides concentration or the LDL/HDL ratio,28,29 though other studies have not found this association.30 On this regard, several authors are posing that a better measure than the cLDL to determine the CVR might be the number of LDL particles by means of magnetic resonance imaging, or even the ApoB concentration or the total ApoB / cholesterol ratio.31,32 These parameters might reflect the presence of sdLDL particles, which is ignored by the simple cLDL measure. In this sense, assuming that the cLDL underestimates the cholesterol from the atherogenic lipoproteins (VLDL, IDL, LDL and sdLDL) --which is specially important in
hypertriglyceridemia situations—than the non HDL and ApoB cholesterol reflect better the cholesterol and the total mass of atherogenic particles, and there are more and more data that support that the non HDL cholesterol and the ApoB are better predictors of cardiovascular events than the cLDL; these parameters are included in the recommendations of the NCEP-ATP-III, the American Diabetes Association and the American Heart Association (AHA).

Beyond its association with the CVR, the sdLDL present a series of physicochemical characteristics that make it potentially atherogenic (figure 2). Its smaller size makes it cross the vascular endothelium more easily than the bigger sized LDL,\(^{33}\) favoring the cholesterol subendothelial accumulation.\(^{34}\) Moreover, the sdLDL is more susceptible to the oxidation, increasing its atherogenic potential,\(^ {35}\) as the oxidative modification of the LDL is a key factor in the triggering of the vascular inflammatory response.\(^ {36}\) The sdLDL has less affinity for the LDL receptor,\(^ {37}\) reducing its plasma clearance rate and increasing its time of permanence in blood. In a hyperglycemia condition, the LDL that remains more time in circulation has more possibilities of suffering non-enzymatic glycation process. As it will be mentioned below, the glycated LDL has several proatherogenic characteristics. Other characteristics of the sdLDL are a higher negative electric load and an increased content in proteins different from the ApoB.\(^ {38}\) All these factors make that a series of characteristics that increase its atherogenicity converge in sdLDL.

**Modifications of the LDL in the arterial wall**

**Oxidative stress**

Though several factors have been described that might trigger the inflammatory process associated to the atherogenesis, probably one has the most determining role is the oxidative modification of the LDL in the intima layer of the arterial wall.\(^ {1,3,36}\) (figure 3). This is a pro-oxidative environment compared to the plasma, taking in account that the concentration of anti-oxidative molecules is lower and the release of free radicals produced by the metabolism of the cells that surround this micro-environment, mainly endothelial, smooth muscular cells and macrophages.\(^ {36,37}\) The peroxidation cascade induced by free radicals in LDL generates multiple lipid products with inflammatory potential, among which the following ones are included: lysophosphatidylcholine, oxidized phospholipids, cholesterol oxides, aldehydes, and ketones.\(^ {40,41}\) All these compounds are able to induce, by different routes of signalizing mediated by kinases, the activation of transcription factors that might start an inflammatory response, as the nuclear factor kappa B, the activator protein 1, or the peroxisome proliferators-activated receptor alpha and gamma.\(^ {42-44}\) Thus, oxidized LDL might induce the expression and the release of most of the inflammatory mediators that have a relevant role in the atherosclerosis,\(^ {30-44}\) adhesion molecules,\(^ {45}\) chemokines,\(^ {46}\) cytokines,\(^ {47}\) growth factors,\(^ {48-49}\) matrix metalloproteinases\(^ {50}\) and receptors for modified lipoproteins.\(^ {51}\) Moreover, the oxidized LDL inhibits the production of nitric oxide\(^ {23}\) and alters the balance in the synthesis routes of prostaglandins, reducing the production of prostacyclin I\(_2\) and increasing the thromboxane A\(_2\).\(^ {53}\) Another characteristic of oxidized LDL is that accumulates with no control in the cytoplasm of macrophages and smooth muscle cells, inducing the formation of foam cells.\(^ {54}\) This accumulation takes place as they
are recognized and internalized through scavengers, as the LOX1, the SRA and the CD36, inducing cytotoxicity and apoptosis. Therefore, the oxidized LDL intervenes in all evolving phases of atherosclerosis, from the endothelial dysfunction, the recruitment of leukocytes, the cellular proliferation, the accumulation of lipids or the apoptosis up to the final phases of breakage of the atherosclerotic plate and the onset of thrombosis events.

In diabetes, this outlook might be worse given the chronic hyperglycemia. The atherogenic characteristics have been commented previously regarding to sdLDL predominating in the diabetic dyslipidemia, specially its high susceptibility to oxidation. On the other hand, hyperglycemia increases the intracellular oxidative stress through many mechanisms. It reduces the availability of NADPH, cofactor necessary for the activity of several anti-oxidative enzymes of the glutathione cycle. Other antioxidants, as Vitamin E, superoxide dismutase or catalase, have lower tissue concentrations in diabetic subjects. It also stimulates protein kinase C, that activates the pathway of cyclooxygenase and other peroxides, generating free radicals. The result is an increase of the sub-endothelium oxidative stress that favors the modification of the LDL retained in the arterial wall.

**Non-enzymatic glycosylation**

Another aspect that should be taken into account is the non-enzymatic glycosylation process that might affect both the LDL and other proteins of the arterial wall. The non-enzymatic glycosylation of proteins have some reversible initial phases (Schiff base, Amadori products) that derive into irreversible products, denominated AGE, some of which require an oxidative environment for its formation. These products are very frequent in diabetic patients, especially in structural proteins that have a long life period. The proteoglycans and the collagen of the arterial wall in diabetic patients are more glycated than in the normoglycemic individuals. This has a great importance in the atherosclerosis associated to the diabetes, as the lipoproteins are bound more greedily to the proteins of the extracellular glycated matrix, favoring its sub-endothelial retention, its later modification though different mechanisms and the starting of the inflammatory process. This process is also inverse, it means, the glycated LDL bounds with more affinity to the sub-endothelial proteoglycans.

As regards to the LDL, it is frequent that in a situation combined of hyperglycemia and oxidative stress glycoxidation phenomena takes place, and modified LDL have been detected with AGE (AGE-LDL, or LDL glycosylated) in injuries of diabetic patients. Moreover, glycemia stimulates the lypoperoxidative processes in LDL. In general, glycosylated LDL presents proatherogenic effects similar to those that the oxidized LDL show, though its intensity is usually higher. It has been described that the glycosylated LDL induces endothelial dysfunction and favors the production of chemokines, and chemokine receptors, adhesion molecules and receptors for modified lipoproteins, inducing cytotoxicity and apoptosis. It is likely that the products of lipid oxidation, also present in the oxidative LDL, induce part of the atherogenic effects of the glycosylated LDL, but other molecules intervene also. The AGE play a determining role, as several experiments show in which the inflammatory effect and the progression of the atherosclerosis induced by the glycosylated LDL are partially inhibited by specific antibodies that block the binding to the receptor AGE (RAGE), implying this receptor in the inflammatory action. In this sense, it has to be pointed out that the glycosylated LDL is not only recognized by the RAGE, but also by other scavenger receptors, expressing which is, at the same time, increased by glycosylated LDL itself. In this way, the lipid accumulation process in the atherosclerotic injury is strengthened in diabetes. On the other hand, it has also been described an atherosclerotic potential in glycosylated lipids, mainly in the glycerol phosphatidyletanolamine, which is the main lipid product of glycosylation. The presence of glycerol phosphatidyletanolamine in the LDL favors the oxidative modification and its uptake through the scavenger receptors.

**LDL modified in plasma circulation**

Different forms of LDL modified in plasma circulation have been detected. The oxidized LDL, the glycosylated LDL and the electronegative LDL are the principal modified forms that have been identified in blood.

**Oxidized LDL**

The ELISA methods are used in most of the studies in order to detect oxidized LDL in plasma. The differences in the specificity of the used antibodies (that recognize oxidized phospholipids or specific epitopes of the
oxidized ApoB), as well as the great heterogeneity that has oxidized LDL (the extensive oxidized particles are very different from the minimally oxidized), make that the observed values are very different, with concentration ranging between 0.001% of total LDL and 1-2%. In spite of the methodological differences that make its standardization difficult, the results obtained by different authors are quite consistent. Increased concentrations of oxidized LDL have been described in patients with family hypercholesterolemia and combined family hyperlipidemia. In patients with coronary and periphery vascular disease have been related with the thickness of the arterial wall and are considered as a prognosis factor of clinical cardiovascular event and the onset of metabolic syndrome. Regarding to diabetes, several studies have shown that the concentration of oxidized LDL is higher in patients with T2D and in prediabetic condition, and that is related to the glycemic control and the presence of vascular complications. Another related parameter is the susceptibility to the ex vivo oxidation of the total isolated LDL of patients with T2D, that coincides fully with these results and reinforces the idea that the LDL in T2D is more oxidized and is more oxidizable, especially when there is an inadequate glycemic control. This is due to the fact that in this situation there is a higher oxidative stress, more concentration of NEFA in plasma, a higher production of VLDL and predominance of sdLDL.

However, the results in patients with T1D are not so clear, not even in situations of inadequate glycemic control, with results clearly contradictory among different groups. Results have been published that show higher, lower or equal oxidizability in LDL in patients with T1D compared to a normoglycemic population. As a whole, it can be asserted that in the T1D the LDL is not more oxidizable than that of the normoglycemic individuals, and only in individuals with a long evolution diabetes time and a scarce metabolic control the LDL has been observed more susceptible to oxidation. Regarding to the presence of oxidized LDL, few studies have been done and they have found differences related to the normoglycemic individuals.

**Glycosylated LDL**

As it is logic, high concentrations of glycosylated LDL have been detected in patients with T1D and T2D and they are related directly to the glycemic control and with the presence of the microalbuminuria. The initial products of glycosylation (Schiff base and Amadori products) are bond to Lys of the proteins altering its physico-chemical characteristics, in this case of the ApoB of the LDL. This is the majority form of glycosylated LDL in plasma, as the half-life time of the LDL is relatively short (3-5 days). The glycosylated LDL shows a reduced plasmatic catabolism and a higher susceptibility to the oxidation. Inflammatory properties have also been described as it activates the route of the MAP-kinase and the STAT5 transcription factor, increasing monocytes chemotaxis and the migration of smooth muscular cells. However, these effects have a lower intensity than those presented by the glycosidase LDL and oxidized LDL, leading some authors to set out if the inflammatory effects of the glycosylated LDL is not due to the coexistence of lipoperoxidative processes provided its higher oxidizability. In this sense, it has also been described that the sdLDL, besides being more oxidizable, is glycated more easily.

LDL has also been detected in plasma that contains AGE. Since the formation of AGE requires higher formation time than the LDL plasmatic half-life, it has been suggested that these AGE-LDL have been generated in the arterial wall and have come out to plasmatic circulation after the formation. As it has been stated previously, AGE-LDL or glycosylated LDL shows a higher inflammatory potential than the glycosylated LDL; however, it remains to establish its relevance in the plasmatic circulation.

**Electronegative LDL**

The LDL is a set of heterogeneous particles that might differ in the lipid / protein ratio, density, size and electric load. Based on this last characteristic and using ion exchange chromatography, agarose electrophoresis or capillary electrophoresis, several authors have detected a minority form of LDL with a higher negative electric load. A specific ELISA has also been developed. The modifications described previously have in common that they generate an increase of the negative load in the LDL particle. In this way, it can be considered that the electronegative LDL (LDL[-]) comprises several modified LDL, included the oxidized LDL, the glycosylated LDL and the sdLDL. However, the proportion of LDL(-) is of approximately 5% in healthy individuals, but it can exceed the 10-20% in individuals with dislipidemia or diabetes. Considering that the amount of oxidized or glycosylated LDL in
plasma is quite lower, this implies that the LDL(-) also includes LDL particles with other types of modifications. Among those that have been described, several ones present atherogenic characteristics, as a higher content of NEFA117,118 (associated to insulin-resistance and diabetes conditions), lipolyzed particles by phospholipases118,119 (associated to underlying atherosclerosis conditions and systemic inflammation) and, paradoxically, LDL particles of big size and low density similar to the LDL ones116 (associated to dysfunctions in VLDL catabolism).

In vitro studies have demonstrated that the LDL(-) induces the release of cytokines,120 chemokines121 and growth factors,122 and activates the inflammatory transcription factors in endothelial cells123 and in circulating leukocytes.124 Moreover, it induces the cytotoxicity and apoptosis, specially potent effects in isolated lipoproteins in diabetic patients.125,126 On the other hand, LDL(-) shows a high aggregation degree,127 which might favor its sub-endothelial retention and its binding to proteoglycans. It binds with a low affinity to the LDL receptor, but it has also a low affinity to scavenger receptors, implying an increased time of permanence in blood.128 Another important characteristic is an abnormal increase of proteins different from the ApoB, as ApoE or ApoC-III, which explains in part its higher density and also some of its atherogenic characteristics.129,130 There is no clear agreement regarding to the mechanism responsible of the inflammatory activity of LDL(-). Some authors indicate that this is due to the presence of oxidized lipids,111,115,122,124,130 but others have pointed out increased contents in lysophosphatidylcholine and NEFA as responsible for the inflammatory action118,119,127,128. In fact, Gaubatz et al.129 asserted that most of the part of the negative electric load present in the LDL(-) is due to the content in NEFA, stressing the important role that these compounds have in the LDL(-) atherogenic characteristics. The increase in NEFA might be related to an insulin-resistance situation, which is frequent in diabetes, but also with a content of acetyldihydrocholine of the platelet activator factor (PAF-AH, also named phospholipase associated to lipoproteins [Lp-PLA]) in the LDL(-) 5-10 times higher than the non-modified LDL,131 as lysophosphatidylcholine and NEFA are the degradation products of this enzyme. The observation that the reduction of the content in PAF-Ah after the treatment with insulin in patients with T1D is associated to a lower capacity of inducing the release of chemokines in endothelial cells supporting the possible inflammatory role of the PAF-AH132 associated to LDL(-). Towards the same direction points the observation that the content in lysophosphatidylcholine of LDL in patients with T2D is increased and is related with its contents in PAF-AH.133

The proportion of LDL(-) is increased in pathologies that show a high CVR, as familial hypercholesterolemia,129 hypertriglyceridemia,116 and renal dysfunction,134, T1D,97,132,135, T2D94,112,136,137 and insulin-resistance,138 and the presence of atherosclerosis pathology has been associated.139,140 The treatment with statins reduces this proportion in hyperglycemic patients.113,129,141 Moreover, the improvement of the glycemic control with insulin reduces the LDL(-) in patients with T1D,97,132 though this might not happen in patients with T2D.94,136 This might imply that the non enzymatic glycation processes have a more relative importance in the T1D, while the oxidative modification would play a more relevant role in the T2D. On the other hand, it has also been suggested that the presence of the underlying atherosclerosis can increase the proportion of LDL(-), rising the systematic inflammation level that increases the phospholipase expression that might generate LDL(-).135 Precisely the PAF-AH is considered a CVR factor142 and its expression is increased in situations of oxidative stress or inflammation.143 The fact that the concentration of PAF-AH is increased in patients with diabetes,133,144 might be directly related to the higher proportion of LDL(-) and, in contrast, it might be one of the causes of the CVR increase in the diabetes. Likewise, other lipolytic enzymes that might intervene in the qualitative modification of the li-
Poproteins have an increased expression in situations of systemic inflammation. Since in patients with diabetes the systemic inflammation level is higher than in the healthy population, such inflammation might have a relevant role in the accelerated development of the atherosclerosis that such patients show.

Conclusions
The qualitative modifications of the LDL that alter its functionality are frequent in diabetic patients. In a pathological situation as the T2D, in which the hyperglycemia, the oxidative stress, the systemic inflammation and the increase of NEFA in plasma appear simultaneously, LDL might suffer multiple modifications that increase its atherogenicity. All these processes are intimately linked among them, so the increase of the NEFA is due to the insulin-resistance that will lead to hyperglycemia, the glycation and lipoperoxidation strengthen mutually and favor an inflammatory condition that stimulates the production of lipolytic enzymes that also modify the lipoproteins and increase its contents in NEFA that increase the oxidability at the same time.

The potential usefulness of the LDL modifications in diabetic patients is stated, first, in its contribution to clarify the physiopathology of the early onset and the aggressive development of atherosclerosis. Second, though there are no epidemiologic studies and clinical trials addressed specifically to demonstrate that the modifications of the LDL might explain the percentage of vascular events that are not detected by the classic risk factors, it is not difficult to consider that the qualitative modification of the LDL plays a relevant role in the high CVR of the diabetic patients. The treatments with antioxidants have not shown to be efficient in the reduction of atherosclerosis events, but some therapies well determined in the reduction of the CVR as the treatment with statins, besides reducing the LDL cholesterol, reduces the proportion of modified LDL particles in plasma.

At present, the measures addressed to reduce the atherogenicity have to be based on the reduction of the LDL cholesterol, the non-HDL cholesterol and the ApoB.


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