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Review

Electronegative low-density lipoprotein: Origin and impact on health and disease

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ABSTRACT

Oxidative modifications in lipoproteins (LP), especially in low-density lipoproteins (LDL), are associated with initiation and progression of atherosclerosis. The levels of a sub-fraction of LDL with oxidative characteristics, named electronegative LDL [LDL(−)], minimally oxidized LDL, and minus LDL, are known to be increased in subjects with familial hypercholesterolemia, hypertriglyceridemia, nonalcoholic steatohepatitis, diabetes mellitus, coronary artery disease, patients undergoing hemodialysis, and athletes after aerobic exercise. In addition to the oxidative profile, physical and biological characteristics of LDL(−) consist of nonenzymatic glycosylation, increased expression and activity of platelet-activating factor acetylhydrolase (PAF-AH) and phospholipase A_2 (PLA_2), enriched NEFA content, hemoglobin and ApoB-100 cross-linking, and increase in ApoC-III and ApoE in LDL. Herein, we summarize the state of the art of the up-to-date body of knowledge on the possible origin and impact of LDL(−) in health and disease. Further, the potential perspectives of using LDL(−) as a biomarker in conditions under metabolic stress are also discussed.

Contents

1. Introduction

Oxidative modifications in lipoproteins (LP), especially in low-density lipoproteins (LDL), are associated with atherosclerosis and many studies have detected oxidatively modified LDL particles in humans [1–6], monkeys [7], and rabbits [8].

Oxidized LDL (oxLDL) results from exposure of LDL to a number of oxidizing agents (such as superoxide anion and hydrogen peroxide present in cells, especially macrophages present in the arterial wall), enzymes (such as lipoxygenases), and products of myeloperoxidase. This exposure may lead to depletion of antioxidant compounds and later oxidation of the lipid and protein components of LDL particles [9]. Previously, Esterbauer et al. found that oxLDL is an important atherogenic factor occurring in plasma, arteries, and plaques of humans and experimental animals [10]. According to Toshima et al., lack of association with hypertension, serum cholesterol, smoking, and sex suggested that oxLDL is an independent risk factor for cardiovascular heart disease (CHD) [11].

Avogaro et al. described a sub-fraction of LDL with oxidized characteristics that was named electronegative LDL [LDL(−)] [1]. Later, this particle was denominated as minimally oxidized LDL

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Table 1
Behavior of LDL(−) in disease and health conditions.

Study design	Endpoint (LDL(−) content)	References
Familial hypercholesterolemia		
FH × NL	↑ FH	[13,14,16]
FH × FH plus SIM (2 months)	↑ FH, basal	[29]
FH × FH plus SIM (3 months)	↑ FH, basal	[16]
FH × FH plus SIM (6 months)	↑ FH, basal	[16]
FH × FH plus SIM or SIM + α-tocopherol	↑ FH, basal	[29]
Other dyslipidemias		
HTG × NL	↑ HTG	[13]
HC × NL	↑ HC	[30]
Diabetes mellitus		
DM2 × control	↑ DM	[18,20,22]
DM2 with MA × control	↑ DM, with MA	[19]
DM2 with MA × DM2 without MA	↑ DM, with MA	[19]
DM2 × DM2 with acarbose	↑ DM, basal	[31]
DM1 with poor GC × control	↑ DM	[21]
DM1 with good GC × control	↑ DM	[21]
DM1 with poor GC × DM1 with good GC	↑ DM, with poor GC	[21]
Exercise		
Athletes before AE × athletes after AE	↑ After	[28]
Renal disease		
HD × control	↑ HD	[25]
HD × PD	↑ HD	[26]
HD × HD plus α-tocopherol	↑ HD, basal	[32]
Other situations		
ACS × control	↑ ACS	[24]
SA × control	↑ SA	[24]
CAD × control	↑ CAD	[23]
Nonalcoholic SH × alcoholic SH	↑ Nonalcoholic SH	[17]

ACS: acute coronary syndrome; AE: aerobic exercise; CAD: coronary artery disease; DM: diabetes mellitus; GC: glycemic control; HC: hypercholesterolemic; HD: hemodialysis; HTG: hypertriglyceridemic; MA: microalbuminuria; NL: normolipidemic; PD: peritoneal dialysis; SA: stable angina; SH: steatohepatitis; SIM: simvastatin.

Table 2
Physicochemical properties, composition, and oxidative parameters of electronegative LDL in comparison with native LDL.

Characteristics	Electronegative LDL	References
Total cholesterol	Higher	[14–16,35]
Triglycerides	Higher	[14–16,21,22,35,36]
Phospholipids	Lower	[14]
Apo B	Lower	[14–16,21,22,35,36]
Apo E	Higher	[14,16,21,22,35,36]
Apo CIII	Higher	[14,16,21,22,35,36]
NEFA	Higher	[14–16,21,22,35,36]
PAF-AH activity	Higher	[15,21,22,36]
IL-8	Higher	[16,35]
IL-6	Higher	[27]
MCP-I	Higher	[16,35]
GM-CSF	Higher	[34]
GROβ	Higher	[34]
GROγ	Higher	[34]
Lysophosphatidylcholine	Higher	[35]
TBARS	Higher	[37]
Conjugated dienes	Higher	[7,37,38]
Alpha-tocopherol	Lower	[37]
Sialic acid	Higher	[14,38]
Lycopene	Lower	[22]
Lag phase	Higher	[14,16,21,22]
Affinity to LDLr	Lower	[16]
Electrophoretic mobility	Higher	[6]
Loss of secondary structure of Apo B	Higher	[37]
Total PUFA	Lower	[7]
Total cholesterol	Higher	[14–16,35]

GM-CSF: granulocyte/monocyte colony-stimulating factor; GROβ: growth-related oncogene β; GROγ: growth-related oncogene γ; IL-6: interleukin-6; IL-8: interleukin-8; LDLr: low-density lipoprotein receptor; MCP-I: monocyte chemotactic protein-1; NEFA: non-esterified fatty acids; PAF-AH: platelet-activating factor acetylhydrolase; PUFA: polyunsaturated fatty acids; TBARS: thiobarbituric acid-reactive substances.

and LDL minus based on its properties of electric mobility. Afterwards, Fabjan et al. showed that LDL modifications induced by oxidizing agents lead to the formation of LDL(−), as well as to particles with higher degree of oxidation identified as LDL(2−), which is more electronegative than LDL(−) [12]. Since then, more than one hundred of articles in the literature have assessed the role of LDL(−) in different chronic diseases and physiological conditions. Similar to oxLDL, the LDL(−) content is increased in subjects with familial hypercholesterolemia [13–16], hypertriglyceridemia [13], nonalcoholic steatohepatitis [17], diabetes mellitus [18–22], and coronary artery disease [23,24], in addition to patients undergoing hemodialysis (HD) [25,26] and athletes after aerobic exercise [27,28] (Table 1). Despite the relevance of these results, the origin and biochemical role of LDL(−) in health and disease is not yet completely clear.

In this review, our aim was to evaluate the "*state of the art*" of LDL(−). Therefore, retrospective information on physicochemical, inflammatory, and immune characteristics, such as effects of life style (including diet, exercise) and drugs on modulation of LDL(−) in different physiological and pathological processes, will be discussed.

2. Physicochemical characteristics of LDL(−)

LDL(−) is a modified LDL sub-fraction present *in vivo*, which was first isolated by ion-exchange chromatography [1]. Usually, the plasma concentration of this particle is lower than 10% of total LDL in healthy subjects, and greater than 10% of total LDL in patients with high cardiovascular risk [13,14,16,22]. The mechanisms that explain the increase of LDL(−) generation in individuals with CHD are not entirely clear. According to Yologlu et al., it is likely that the LDL particles in dyslipidemic subjects are more prone to oxidation than in normolipidemic individuals. Therefore, the presence of a large number of lipoprotein particles with pro-inflammatory capacity would contribute to endothelial dysfunction thus increasing the cardiovascular risk [33]. In this context, the interaction of LDL(−) with endothelial cells promotes the release of granulocyte/monocyte colony-stimulating factor (GM-CSF) that contributes to increase foam cells formation, thus amplifying the oxidative stress in the intima and changing the quality and composition of the extracellular matrix in the atheroma [34].

Differences in physicochemical properties, composition, and oxidative parameters between native and electronegative LDL could explain the atherogenic and inflammatory actions of this particle (Table 2). LDL(−) has low levels of antioxidant vitamins and a high content of oxidized cholesterol, lipoperoxides, conjugated dienes, and thiobarbituric acid reactive substance (TBARS) [6,16,22,37]. LDL(−) from subjects with either familial hypercholesterolemia or normolipidemia is relatively rich in free cholesterol and triglycerides, and its ApoB content is lower than that in the non-electronegative LDL fraction [14]. LDL(−) also contains increased amounts of ApoE, ApoC-III, sialic acid, and non-esterified fatty acids (NEFA) [14]. LDL(−) and α-tocopherol levels showed an inverse and significant correlation [30]. Regarding lipid profile, the percentage of LDL(−) was positively correlated with LDL cholesterol [39].

Differently from *in vitro* oxidized LDL, LDL(−) particles show altered structure of the surface lipids and a denatured ApoB-100 backbone that appears to be buried into the lipophilic environment [11,37,40,41]. However, LDL(−) did not show ApoB fragmentation or other changes arising from excessive oxidation. This observation was confirmed by our group, who found structural modifications in ApoB of LDL(−) by circular dichroism spectroscopy [37]. According to our previous results, LDL(−) recognized by anti-LDL(−) monoclonal antibodies (clone 3D1036), shows a slight alterations of secondary structure. In addition, it is well established that differ-

ences in the lipid composition of LDL promote ApoB conformational changes that are relevant for LDL interaction with B/E receptor [42]. Binding of native LDL to its receptor is mediated by positively charged Lys residues in ApoB that have affinity for negatively charged Cys residues in the receptor binding domain. In contrast, LDL(−) has low binding affinity for the LDL receptor [43]. The increased electronegativity and low number of active surface-accessible Lys residues in this particle certainly contributed to its decreased affinity for the LDL receptor [35]. The unfolding of ApoB-100 and its sinking into the particle surface lipids can also contribute for this effect [40]. In addition, interaction with PGs is an essential step in the retention of LDL by subendothelial extracellular matrix, and it appears that this binding involves basic residues of amino acids (ApoB) and negatively charged proteoglycans (PGs). Previously, Borén et al. [44] developed a model based on mutagenesis of the large ApoB protein to assess its functional domains within native LDL. In short, recombinant LDL thus obtained showed chances in lysine to glutamic acid (K3363E), basic to neutral amino acids (RK3359-3369A) in site B, where arginine residues were changed to serine ones, and lysine to alanine or arginine was substituted with to glutamine (R3500Q). The same authors described that arginine at residue 3500 stabilizes the carboxyl terminus, permitting normal interaction between LDL and its receptor [45]. Nevertheless, only recombinant lysine (K3363E) LDL and lysine and arginine (RK3359-3369A) LDL were associated with decreased PGs binding [45]. Therefore, these amino acids residues appear to be important for the atherogenic potential of LDL. Previously, it was shown that native LDL has two lysine populations, i.e., "normal" Lys residues have a pK_a of 10.4 whereas "active" Lys residues, which have been suggested to be involved in receptor binding, have a pK_a of 8.8 [43]. Interestingly, LDL(−) sub-fraction showed a third type of Lys residues, named "intermediate" Lys, with a different microenvironment and higher basicity (pK_a 10.7) [43]. These differences between native LDL and LDL(−) indicate a distinct conformation of ApoB-100 with a possible loss of affinity of LDL(−) for the B/E receptor [43]. In fact, LDL(−) from subjects with normolipidemia and familial hypercholesterolemia shows impaired binding to the LDL receptor, a characteristic that could lead to decreased *in vivo* clearance of this particle [35]. Lower clearance of LDL(−) results in increased residence time in blood circulation, which in turn could favor further modifications of LDL(−) resulting in increased inflammatory and atherogenic potential.

As compared to native LDL, LDL(−) presents increased binding affinity to arterial PGs, the main component of the subendothelial extracellular matrix. This property would favor retention of LDL(−) on the PG-rich surface layer of the arterial intima thus contributing to progression of atherosclerosis [36]. The interaction occurs between positively charged residues of ApoB-100 and the negatively charged sulfate and carboxyl groups in the glycosaminoglycan (GAG) chains of PGs [46,47]. Regarding that the negative net charge of LDL(−) is a common characteristic in all LDL(−) subfractions, their interaction with PGs could be decreased. However, it has been previously reported that the LDL(−) subpopulation shows different size, density, and composition that contributes to distinct levels of binding to PGs [13,36].

In contrast to the oxidative origin of LDL(−), Sánchez-Quesada et al. verified that both native and electronegative LDL present low levels of lipid peroxidation products, an indication that LDL(−) is not only produced by oxidative modification [48]. Physical and biological characteristics of LDL(−) consist of nonenzymatic glycosylation, increased content and activity of platelet-activating factor acetylhydrolase (PAF-AH) and phospholipase A_2 (PLA_2), enrichment of NEFA, hemoglobin and ApoB-100 cross-linking, and increase in ApoC-III and ApoE in LDL [48].

Glycation and oxidation of LDL reduces its affinity for the LDL receptor, leading to reduced hepatic catabolism, increased content of cholesteryl esters in macrophages, and altered endothelial function [49]. These events seem to be closely interrelated. In addition, as a result of hyperglycemia, tissue and plasma proteins are modified and their physiological function is disturbed [50]. Protein glycation that occurs in diabetic patients is regarded as one of the key factors in the pathogenesis of diabetic complications, including accelerated atherosclerosis [51]. Although nonenzymatic glycosylation and oxidation are increased in type 2 diabetes (DM2), these features would not be directly involved in the generation of LDL(−) [22]. LDL(−) properties suggest that high content of this particle in plasma could promote accelerated atherosclerosis in DM2 patients through both an increase in its residence time in plasma and induction of an inflammatory response in the arterial wall cells [22]. Optimization of glycemic control in DM2 subjects increased native LDL resistance to oxidation (longer lag-phase time) but no effect was observed in oxidizability of LDL(−) [22]. In a recent study conducted in patients with DM2, LDL(−) decreased significantly as compared to baseline levels after treatment with oral antidiabetic drugs (both pioglitazone and metformin for 12 weeks), suggesting that hypoglycemic drugs may have an antiatherosclerotic effect [52]. Previously, Sánchez-Quesada et al. [21] described that high level of glycation is necessary for LDL to achieve its electronegativity. These findings clearly show that further physicochemical changes in LDL contribute to the generation of LDL(−). Therefore, events such as aggregation [36] and high PAF-AH activity [21] contribute to the generation of LDL(−) by an oxidative-independent mechanism. Nevertheless, LDL(−) from diabetic patients shows inflammatory potential associated with chemokine release in endothelial cells. This proatherogenic effect could be related to the high PAF-AH activity observed in LDL(−) [21].

The lipoprotein-associated phospholipase A_2 (Lp-PLA_2), also known as PAF-AH, is considered a member of the group of phospholipases A_2, which are specific for hydrolysis of phospholipids [53]. PAF-AH is produced by inflammatory cells and is mostly transported by LDL (85%), where it hydrolyzes oxidized phospholipids. Several studies propose a proinflammatory role for PAF-AH that acts by forming noxious bioactive lipid mediators (lysophosphatidylcholine and oxidized NEFA) in the lesion-prone vasculature [54]. Asatryan et al. observed that LDL incubated with low-molecular weight phospholipases A_2 (PLA_2) induced formation of LDL(−) without evidence of significant increase in lipid peroxidation [55]. The action of PAF-AH produces lysophospholipids and mainly lysophosphatidylcholine (LPC) that links this enzyme to early coronary atherosclerosis [56]. Müller et al. showed that presence of lysophosphatidylcholine implies that an additional way is available for the organism to modulate the intensity of production of reactive oxygen species at the inflammatory site [57]. Although increased PAF-AH activity appears to be strongly related to high cardiovascular risk [58], this particle displays an important role in the preventing additional oxidation of LDL(−) [21], thus reinforcing the non-oxidative mechanism for the generation of LDL(−). Recently, we observed that obese adolescents have increased PAF-AH activity, although it did not show correlation with LDL(−) levels (unpublished data). These results are consistent with the presence of other mechanisms contributing for the generation of LDL(−).

In addition, Benítez et al. [35] observed that enrichment of LDL with NEFA promotes a concentration-dependent loss of affinity for its receptor, although PLA_2 treatment had been more effective in generating LDL(−) than NEFA-induced modification. *In vitro* modification of LDL by PLA_2 or NEFA enhanced its electronegativity and resulted in an increase in the lysophosphatidylcholine content [34,35]. NEFA enrichment of LDL and apolipoprotein released by lipolysis could also increase LDL electronegativity [48]. In humans, increased NEFA content in LDL(−) from subjects with familial

hypercholesterolemia and diabetes is likely to play a major role in the loss of affinity of LDL(−) for the LDL receptor [22].

In addition, Ziouzenkova et al. observed that high degree of ApoB-100 modification resulted from the formation of covalent bond between hemoglobin (Hb) and LDL, which promoted formation of dityrosine but not malondialdehyde epitope [25]. Hb-mediated reactions can be implicated in the oxidative stress that arises during hemodialysis (HD). These modifications were probably induced by inflammatory processes occurring after contact between the blood and the HD membranes [25]. Hb-mediated oxidation induces the formation of cross-linking between ApoB and Hb backbones and increase in LDL electrophoretic mobility. These reactions also yielded a marked dose-dependent increase in the levels of LDL(−) and LDL(2−) with a preferential conversion to LDL(−), whereas the proportion of LDL(2−) was approximately 10 times lower than that of LDL(−) [25]. Interaction between LDL(−) and free Hb (total and metHb) promotes modifications in ApoB and an increase in the negative net charge of LDL, although with not strong lipid oxidation of LDL (TBARS and hydroperoxides). Therefore, modification of LDL by Hb occurs through a lipid peroxidation-independent mechanism [59]. These observations were recently reinforced after analysis of LDL(−) from patients undergoing hemodialysis. These subjects showed high levels of LDL(−) when compared with both patients undergoing peritoneal dialysis and normal healthy individuals. On the other hand, most pronounced lipid abnormalities were shown by patients undergoing peritoneal dialysis [26,32].

Furthermore, recent evidence suggests that enrichment of LDL with ApoC-III contributes to the generation of electronegative, proinflammatory and atherogenic particles, which are compatible with the properties of LDL(−) [60]. This possibility was reinforced by Mauger et al. [61] who verified a strong correlation of ApoC-III_1 and ApoC-III_2 with the small dense LDL phenotype. Recently, Mello et al. [62] observed that LDL(−) is mainly associated with smaller and denser LDL particles. Furthermore, Flood et al. [63], Hiukka et al. [60] and Camejo et al. [46] proposed that interaction of modified LDL and small dense LDL with PGs is influenced by ApoC-III. Regarding the presence of sialic acid in LDL(−) it is likely that binding of this particle to the arterial PGs depends on the degree of sialylation of ApoC-III. However, recently Bancells et al. [64] showed that presence of ApoC-III and ApoE do not influence LDL(−) binding affinity for PGs.

In addition to the biochemical mechanisms described above, Védie et al. previously proposed that variations observed in the genes coding for apolipoproteins (ApoB and ApoC-III) could change the electrophoretic behavior of LDL [65]. As ApoB is the main structural apolipoprotein of LDL, variations in the ApoB gene could result in differences in the electric charge of this particle in individuals with similar LDL lipid profile.

According to the response-to-retention hypothesis proposed by Williams and Tabas [47,66], lipoprotein retention is a key event in provoking initial damage to the normal artery and thus promoting atherosclerotic lesions. Regarding this hypothesis, retention of LDL(−) could contribute to atherosclerotic process by different mechanisms related to both the oxidative properties of the particle and modification-induced conformational changes (size and density) affecting lipids and proteins [67]. Recently, Bancells et al. [36] proposed that aggregation appears to cause increased LDL(−) binding affinity to PGs favoring the retention of LDL(−). In addition, previous results support the idea that retained LDL(−) contributes to the development of atherosclerosis by different ways, such as, apoptosis, inflammation, cytokines release and cytotoxicity [39,48].

Therefore, independently of the multiple and complex origin of the LDL(−), its interaction with PGs is essential for its retention and its participation in the atherosclerotic process.

3. Inflammatory and immune response

Both LDL oxidized *in vitro* by different agents and LDL(−) show pro-inflammatory characteristics associated with immune response activation. Presence of these modified lipoproteins in the bloodstream stimulates components of the immune system that are related to the acute and chronic phases of many diseases, especially atherosclerosis. These biomarkers include macrophages, T cells, monocyte chemotactic protein-1 (MCP-1), autoantibodies and autoantigens related to modified lipoproteins, interleukins (IL-1, IL-2, IL-6, IL-8, IL-12, and IL-10), tumor-necrosis factor (TNF), gamma-interferon (γ-IFN), and platelet-derived growth factor [41,68–70]. This profile is consistent with previous studies conducted by Sanchéz-Quesada et al. [14] and De Castellarnau et al. [68], who observed that LDL(−) isolated from plasma of both normolipidemic subjects and patients with FH has pro-inflammatory actions.

Regarding the immune nature of atherosclerosis, adhesion of monocytes to the arterial wall endothelium appears be one of the key events in the early development of atherosclerotic plaques. Frostegård et al. found a strong increase in the amount of adhesion molecules adhered to endothelial cells after their exposure to oxidized LDL (when compared to native LDL), suggesting that oxLDL induces adhesion as well as monocyte differentiation [71]. In this context, Fukumoto et al. [72] and Shoji et al. [73] observed that the immune system generates antibodies to oxLDL in presence of oxLDL. According to Inoue et al. [74], and Monaco et al. [75], anti-oxLDL antibodies show a deleterious effect. Afterwards, Faviou et al. [76] found that the concentration of anti-oxLDL antibodies in patients with unstable or stable angina was higher than in healthy subjects, reinforcing the idea of potential negative effect of these antibodies. In contrary, Karvonen et al. [77] showed that autoantibodies (IgM isotype) have an inverse association with carotid atherosclerosis, suggesting that activation of the humoral immune response to oxidized LDL may be beneficial. Recently, Chou et al. [78] described that oxidation-specific epitopes are the major target of natural antibodies.

Similarly to the profile of oxLDL described above, LDL(−) shows proinflammatory and immunogenic properties. According to Siqueira et al. [79], there is growing experimental evidence for the participation of acquired immunity in atherosclerosis. However, few studies link the immune response to oxLDL and the cardiovascular risk conferred by the metabolic syndrome.

Using an animal model, we previously observed that diet supplementation with soy isoflavones decreased the amount of IgG autoantibodies reactive to LDL(−) as compared to the group without supplementation. This event could be related to a lower generation of LDL(−) and, consequently, lower stimulation of the humoral immune response [80].

Further, after treating LDLr−/− mice with anti-LDL(−) monoclonal antibody (clone 31036), Grosso et al. [81] observed that their levels of circulating free LDL(−) were lower than those in either non-immunized mice or those immunized with irrelevant monoclonal antibody. This indicates that passive immunization with anti-LDL(−) monoclonal antibody had a protective effect on atherosclerotic plaque development. It is possible that the decreased levels of free LDL(−) in blood plasma were due to the formation of immune complexes between LDL(−) and the monoclonal antibody injected into mice. If formation of immune complexes had actually occurred, it could be concluded that the monoclonal antibody neutralized the circulating LDL(−) particles and their atherogenic and inflammatory effects were avoided. In addition, the authors demonstrated that mice treated with the anti-LDL(−) monoclonal antibody had less foam cells in the subintimal layer of atherosclerotic lesions than the control mice.

In subjects with CHD, Oliveira et al. [24] and Siqueira et al. [82] found that the titers of antibodies anti-LDL(−) were higher than in the control group. However, Barros et al. [83] found an opposite profile in children and adolescents. The concentration of anti-LDL(−) autoantibodies in normocholesterolemic individuals was higher than that in hypercholesterolemic subjects with or without family history of acute cardiovascular event.

In summary, LDL(−), as oxLDL, is able to activate inflammatory and immune responses, but the real impact of anti-LDL(−) autoantibodies in the atherosclerotic process and other chronic diseases is not yet clear. Fig. 1 shows a possible mechanism that links LDL(−) and inflammatory and immune responses to atherosclerosis. According to this hypothesis, native LDL (nLDL) may either be modified in blood plasma under inflammatory conditions or migrate into the sub-endothelial space where it undergoes oxidative and possibly other structural modifications that result in LDL(−). LDL(−) is internalized by macrophages through scavenger receptors generating foam cells. Further, epitopes from LDL(−) are presented to B cells by macrophages and anti-LDL(−) antibodies are produced. After this step, immune complexes to LDL(−) [IC-LDL(−)] could precipitate and stimulate the maintenance of the inflammatory and immune responses. In this condition, free LDL(−), antibodies to LDL(−), and IC-LDL(−) remain in the intima and can be effused to the lumen space. This view is compatible with the presence and detection of these biomarkers in the lumen and atherosclerotic lesions.

4. Pathophysiological properties of LDL(−)

LDL(−) is considered an important factor in the initiation and progression of atherosclerotic plaques. *In vitro* studies showed that LDL(−) in cultured endothelial cells has cytotoxic effect and stimulates apoptosis and production of leukocyte recruitment mediators, such as interleukin 8 (IL-8), monocyte chemotactic protein 1 (MCP-1), and vascular cell adhesion molecule 1 (VCAM-1) [6,14,84].

The interaction of LDL(−) with endothelial cells and the consequent release of granulocyte/macrophage colony-stimulating factor (GM-CSF) could contribute to increase the formation of foam cells, both changing oxidative stress in the intima and the characteristics and composition of extracellular matrix in atheromatous plaques [34]. The *in vitro* susceptibility of LDL to oxidative modification has been positively associated with the amount of LDL(−), which shows lipid peroxides, necessary to initiate copper-catalyzed LDL oxidation [85]. However, mechanisms independent of oxidative modifications are able to generate LDL(−) with atherogenic potential.

Besides the *in vitro* studies, considerable *in vivo* evidence has shown that LDL(−) is present in plasma and atherosclerotic lesions of humans [7], rabbits [8] and mouse [81].

De Castellarnau et al. [68] and Sánchez-Quesada et al. [14] showed that LDL(−) isolated from either normocholesterolemic or hypercholesterolemic subjects induced release of IL-8 and MCP-1 in endothelial cells, supporting the hypothesis that this particle is proinflammatory and atherogenic in humans.

In type 1 and 2 diabetes mellitus (DM1 and DM2), LDL could be differently modified. Whereas subjects with DM1 show a favorable lipid profile and presence of microangiopathy, those with DM2 exhibit a profile related to dyslipidemia and macroangiopathy. This profile is reinforced by LDL analysis, in which LDL from subjects with DM1 shows the highest electrophoretic mobility, compatible with LDL(−) content; whereas differently, subjects with DM2 show LDL(−) is in a state of higher susceptibility to oxidation and with a higher content of diene conjugates [86]. Moro et al. [18] studied patients with DM2 and found that LDL was more glycated, more susceptible to *in vitro* oxidation, and contained a higher percentage of LDL(−) when compared with native LDL. Glycation of ApoB is proposed to be associated with a significant increase in the production of *in vivo* and *in vitro* oxidized LDL.

Sánchez-Quesada et al. [13] found that LDL(−) from normocholesterolemic individuals was predominant in the dense sub-fraction (Phenotype B), whereas most of LDL(−) from patients with familial hypercholesterolemia (FH) was present in the light LDL subclasses (Phenotype A). It is likely that the differences between contents of LDL sub-fractions found in this study reflect a change in triglyceride content in these sub-fractions. A similar profile was previously described by Sevanian et al. [85]. In contrast, Chappey et al. [87] found a bimodal distribution, in which LDL(−) was present in both denser and lighter LDL particles. The increase in the production of LDL(−) is closely related to the increase in the levels of oxLDL and small and dense LDL [31]. This observation is reinforced by the association observed between negative charge in LDL and inflammatory markers of atherosclerosis [14,88].

Regarding renal disease, the levels of LDL(−) in renal patients undergoing dialysis are higher than in normal subjects. LDL(−) may be a useful marker of oxidative stress, and Lobo et al. suggested that patients undergoing hemodialysis are more susceptible to cardiovascular disease due to this condition [26].

Therefore, LDL(−) is a potential marker present in pathophysiological processes related to cardiovascular disease, diabetes mellitus, renal disease, and possibly other diseases.

5. Methods for detection of LDL(−)

Firstly, Avogaro et al. [1] isolated LDL(−) using anion-exchange chromatography in a HPLC system, and this technique was later optimized in a FPLC system [3,5,7,14]. Although these methods generate semi-quantitative results, they remain the principal tool used in studies on LDL(−).

In the late years, capillary isotachophoresis (cITP) has also been used as a technique to characterize plasma lipoprotein subfractions according to their net electric charges [89]. The cITP technique allowed separation of two major LDL sub-fractions, fast- (fLDL) and slow-migrating LDL (sLDL), according to their electrophoretic mobilities. The fLDL fractions correspond to LDL(−), β-VLDL, and small dense LDL. After the light LDL fraction was precipitated from whole serum with heparin-Mg^{2+}, electronegative LDL could be measured using cITP in the small dense LDL fraction [90,91]. Therefore, the analytical cITP technique may be useful in the routine analysis of lipoprotein profiles. It was previously shown that the absolute levels of lipoprotein sub-fractions can be determined as the peak area relative to that of an internal marker, and the levels of fLDL and sLDL were proportional to the LDL protein content [91]. These authors reported that the fLDL and sLDL levels are associated with the carotid-artery intima-media thickness and that fLDL is significantly related to the level of serum triglycerides (TG). This observation was previously demonstrated by Sánchez-Quesada et al. [13], who reported that patients with hypertriglyceridemia have an increased proportion of LDL(−). Therefore, high levels of TG could contribute to increased LDL electronegativity. Difficulties due to lack of standardized assays to measure circulating LDL(−) have been overcome by the development of monoclonal antibodies (MAb 3D1036) [37]. Our laboratory has developed an assay to measure LDL(−) in plasma, total LDL and LDL sub-fractions and tissues using a monoclonal antibody MAb (3D1036) that recognizes epitopes in LDL(−) but not in native LDL (cross-linking <1.0%).

Recently, Faulin et al. [92] developed and validated a sandwich enzyme-linked immunosorbent assay (ELISA) to measure LDL(−) in human plasma using two different monoclonal antibodies (free and biotinylated, MAb-1A3 and MAb-2C7, respectively).

Regarding use of antibody (in comparison with other techniques), its main advantages are (I) specificity and sensitivity, (II)

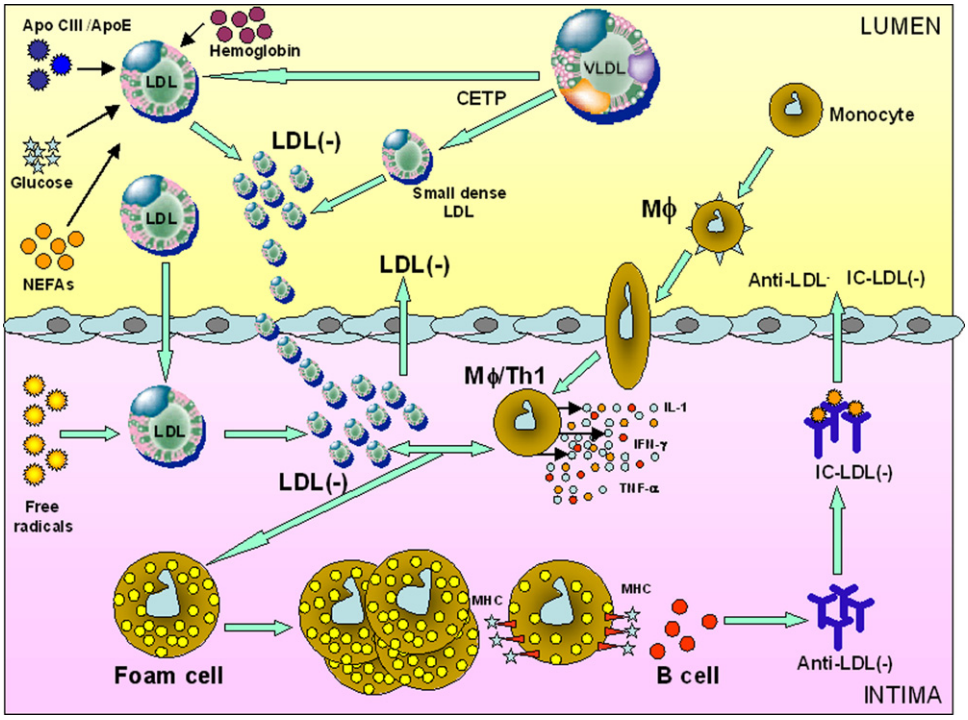

Fig. 1. Immune and cellular mechanisms involving electronegative low-density lipoprotein [LDL(−)]. Anti-LDL(−): antibodies to LDL(−), IC-LDL(−): immune complexes to LDL(−), IL-1: interleukin-1, LDL(−): electronegative LDL, MHC: major histocompatibility complex, MΦ: macrophages, MΦ/Th1: activated macrophages, nLDL: native LDL, TNF: tumor-necrosis factor, and Φ-IFN: gamma-interferon. For more information, please see text.

simultaneous analysis of large number of samples, (III) reduced time of analysis per sample, and (IV) direct detection in different biological fluids.

Therefore, the ELISA technique is a very practical tool to measure LDL(−) in human blood for both widespread research and clinical diagnosis.

The current methods used to monitor LDL(−) are specific for electronegative LDL independently of its origin. These methods are not able to discriminate LDL(−) generated by non-enzymatic glycosylation, increased expression and activity of platelet-activating factor acetylhydrolase (PAF-AH) and phospholipase A_2 (PLA_2), enriched NEFA, hemoglobin and ApoB-100 cross-linking, increase in ApoC-III and ApoE or oxidation reactions in LDL. In addition, while chromatographic and electrophoretic methods evaluate total LDL(−) particle regarding its electronegativity, ELISA (based on monoclonal antibodies – MAb) utilizes FPLC-purified human LDL(−) which is used as antigen to generate the MAbs [37]. These MAbs recognize epitopes presents in LDL(−) that was also isolated as a function of its net electric charge.

Although most studies show increased levels of LDL(−) in subjects with high cardiovascular risk, previously Barros et al. [83] and Córdoba-Porras et al. [93] did not show significant differences relative to controls. These differences are probably associated with the distinct design of the studies, in which clinical and demographic characteristics of subjects are a crucial point. Current studies in the literature are limited to a reduced number of subjects ($n < 100$), and distinct methods of detection. In addition, there are few validation and reproducible studies.

6. Effect of drugs and life style

From the classical studies conducted by Anitschkow (1913) on diet and cardiovascular disease [94], it is accepted that components of the diet are important in the development, prevention, and treatment of cardiovascular diseases. Whereas cholesterol [95], the saturated and trans fatty acids stimulate the atherogenic process [96], consumption of fiber and monounsaturated (w-9) and polyunsaturated fatty acids (w-3 and w-6) [97–100] modulate lipid metabolism and reduce the cardiovascular risk. Besides the well-established role of the nutrients describe above, involvement of isolated soy protein [101,102], isoflavones [8], phenolic components [103], phytosterols [104], and antioxidants [105–107] has shown that other diet components display important effects on the development of atherosclerosis and other diseases in which modification of LDL is present.

Soy isoflavones can both prevent lipid peroxidation by scavenging lipid-derived peroxyl radicals and inhibit copper-dependent LDL oxidation [108,109]. Damasceno et al. [80] observed that isoflavones decreased the amount of LDL(−) in plasma and aorta of dyslipidemic rabbits. This effect occurred for both high and low intake of isoflavones (7.3 and 0.73 mg/kg of body per day, respectively), but with different intensity. Previously, Wiseman et al. [110] reported that isoflavone-containing soy protein is more effective in inhibiting LDL oxidation than isoflavone-depleted soy protein. Similarly, Damasceno et al. [102] worked with rabbits and verified that consumption of a diet rich in cholesterol and casein caused an increase in the atherosclerotic lesion size in the aorta when compared to animals that received a hypercholesterolemic diet containing soy protein instead of casein. This increase observed in the casein group may be associated with an increase in the generation of LDL(−).

In addition, Natella et al. [111] reported that supplementation with selenium for a 10-day period was able to prevent both the postprandial increase in LDL(−) and susceptibility to oxidative modification in LDL. In another study, experimental data showed a postprandial increase in LDL(−) concentration after ingestion of a meal containing oxidizable lipids [112].

In vitro studies have shown that phenolic components present in coffee are able to modify lipoprotein oxidative susceptibility. In 2007, it was observed that resistance of LDL to oxidative modification significantly increased and LDL(−) concentration did not

change after coffee drinking. It is likely that these results indicate incorporation of coffee's phenolic acids into LDL particles [113].

Regarding the antioxidant potential of nutrients and the bioactive components present in diet, α-tocopherol is the most investigated of them. It is considered a chief antioxidant for the prevention of experimental atherosclerosis. It acts as a scavenger of lipid peroxyl radicals in lipoproteins protecting them against oxidation and avoiding generation of oxLDL [114]. The effect of α-tocopherol supplementation on LDL(−) content in hemodialysis (HD) patients was previously investigated by Mafra et al. [32] who observed decreased LDL(−) levels after supplementation. On the other hand, Pereira et al. [29] observed that simvastatin decreased LDL(−) levels independent of its association with α-tocopherol, suggesting that α-tocopherol does not affect the antioxidant action of simvastatin in terms of protein nitration or generation of LDL(−) in hypercholesterolemic subjects.

In fact, simvastatin therapy induced a progressive decrease in the proportion of LDL(−). Simvastatin not only decreases plasma cholesterol but also modifies the qualitative characteristics of LDL, e.g., improvement of LDL(−) affinity for LDL receptor and increase in light LDL in comparison with dense LDL [115]. A similar profile was described by Zhang et al. [90], who evaluated low-dose rosuvastatin showing that this drug reduced LDL(−) content and the small and dense LDL sub-fractions in hypercholesterolemic patients with CHD. Although reduction in the content of modified LDL may represent a novel pleiotropic effect of rosuvastatin, the mechanism of these effects is not yet clear. These authors propose that up-regulation in the number of LDL receptors is due to inhibition of cellular cholesterol synthesis in patients under statin therapy. Similarly, simvastatin therapy has been shown to increase the affinity of LDL(−) for LDL receptors in patients with familial hypercholesterolemia [16].

Recently, Tang et al. [116] evaluated the effect of LDL(−) from smokers on differentiation of endothelial progenitor cells (EPC) and observed that the most electronegative fraction (L5) was associated with upregulation of lectin-like oxLDL receptor 1 (LOX-1 receptor) and inhibition of EPC.

Besides the effect of diet and drugs, the protective role of regular exercise against atherosclerosis is well established. However, information on the effect of exercise on LDL(−) is insufficient. According to Sánchez-Quesada et al. [117], high levels of HDL in trained subjects could explain the increased resistance of LDL to oxidation and decreased generation of LDL(−) observed in these subjects. This possibility was reinforced by a study conducted by Benítez et al. [28], in which high levels of HDL were related to reduced LDL oxidation (approximately 20%). In athletes, however, LDL(−) content after aerobic exercise was higher than before [27,28]. According to Gutteridge, intense exercise promotes an increase in O_2 consumption in skeletal muscle and this event favors oxidative modification of LDL [118].

Therefore, drugs and life style components (diet, smoke, and exercise) are able to modify LDL(−) generation, possibly reducing the cardiovascular risk.

7. Conclusion

Classical risk factors for CHD include levels of total- (TC) and LDL cholesterol (LDL(−)C), low levels of HDL cholesterol (HDL-C), as well as elevated blood pressure, smoking habit, age, and recently, obesity, familial history of premature CHD, and physical inactivity.

Currently, news risk factors were added to these parameters. Qualitative characteristics of lipoproteins, such as physicochemical properties (size, electrophoretic mobility) and oxidative profile, have been the goal of many studies. In this context, LDL(−) is a potential marker. In this review, the major points focusing this particle showed that:

(I) Origin of LDL(−) is multiple and complex, and includes the oxidative process;
(II) LDL(−) is able to activate the inflammatory and immune responses;
(III) Currently, no accessible commercial "gold standard" method is available to evaluate LDL(−), and there is not any study showing correlation between methods;
(IV) Although LDL(−) is present in health and disease, its content during pathological processes is higher than 10% of total LDL;
(V) Drugs, diet, cigarette smoking, and exercise modify the content of LDL(−) in humans;

In conclusion, LDL(−) is a potential metabolic stress biomarker, which is present in health and disease. Regarding the open problems relative to this particle, we propose that evaluation of LDL(−) be included in prospective, randomized, and crossover trials, since only with large-scale information its clinical relevance will be safely analyzed.

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