REVIEW

LDL electronegativity index: a potential novel index for predicting cardiovascular disease

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Abstract: High cardiovascular risk conditions are frequently associated with altered plasma lipoprotein profile, such as elevated low-density lipoprotein (LDL) and LDL cholesterol and decreased high-density lipoprotein. There is, however, accumulating evidence that specific subclasses of LDL may play an important role in cardiovascular disease development, and their relative concentration can be regarded as a more relevant risk factor. LDL particles undergo multiple modifications in plasma that can lead to the increase of their negative charge. The resulting electronegative LDL [LDL(−)] subfraction has been demonstrated to be especially atherogenic, and became a subject of numerous recent studies. In this review, we discuss the physicochemical properties of LDL(−), methods of its detection, atherogenic activity, and relevance of the LDL electronegativity index as a potential independent predictor of cardiovascular risk.

Keywords: low-density lipoprotein, LDL, LDL electronegativity index, cardiovascular disease, atherosclerosis

Introduction

The risk of cardiovascular disease (CVD) development is closely associated with altered lipoprotein profile of blood plasma. Lipoprotein particles circulating in human blood vary in their chemical and physical properties. It is well documented that individuals with high CVD risk have elevated amounts of circulating low-density lipoprotein (LDL) and decreased proportion of high-density lipoprotein (HDL). LDL is the major source of cholesterol and the lipids that contribute to the development of the atherosclerotic plaque, whereas HDL is not atherogenic, and its concentrations inversely correlate with the CVD risk.[1] Total cholesterol and LDL cholesterol lowering with statins is therefore standard therapeutic approach for treating patients with atherosclerosis and increased CVD risk.[2,3] This strategy, however, did not reduce the CVD risk beyond ~70% in most cases, and in some clinical studies lowering LDL cholesterol was not efficient.[4–7] This pointed to the existence of other important factors promoting atherosclerosis development that have to be taken into account.

The LDL pool is heterogenic and consists of several subclasses that can be separated based on the differences in their density, size, chemical composition, and electrical charge.[8,9] Among them, small dense LDL (sdLDL) was demonstrated to be highly atherogenic, and its level strongly correlated with CVD risk.[10–12] Study of the significance of LDL subclasses for atherosclerosis progression has been hindered by the lack of standardization of the analytical methods. LDL subfraction analysis has been performed by different groups, using gradient density ultracentrifugation,[13–15]

gradient gel electrophoresis,[16] nuclear magnetic resonance (NMR) analysis,[17] homogeneous assays,[18–20] and other methods. All these methods deliver slightly different results, and variations are possible even within one method due to the modifications of experimental conditions.[21,22] It is, however, generally accepted that sdLDL fraction has a density of 1.044–1.063 g/mL and a particle size around 15–20 nm.[23] LDL subclasses analysis is currently being considered as an important diagnostic tool for improvement of CVD risk and treatment efficacy assessment.[24]

Apart from the size and density, LDL subfractions differ by their chemical composition. LDL particles can undergo multiple modifications in the blood plasma that increase their atherogenicity.[25–27] One of the first types of atherogenic modified LDL that has been discovered is oxidation.[25,28] Oxidized LDL particles are recognized by a number of receptors, including CD36 and TLR-4 and can induce the immune response and inflammation that contribute to the atherosclerosis progression.[29,30] Oxidized LDL was demonstrated to induce the lipid storage in cultured endothelial cells (ECs), but could only be prepared in vitro.[31,32]

Other forms of modified LDL have been discovered during the last two decades. Desialylated LDL could be detected in the blood plasma of atherosclerosis patients using a lectinsorbent assay.[33,34] Desialylation of LDL particles is performed in plasma by *trans*-sialidase that participates in the metabolism of glycoconjugates.[35] Glycation of apoB lipoprotein in LDL particles has also been described.[36,37]

Another form of atherogenic modified LDL is electronegative LDL [LDL(−)] that can be distinguished using methods sensitive to the particle charge, such as agarose gel electrophoresis, isotachophoresis, or ion-exchange chromatography.[38,39] LDL(−) is characterized by an enhanced ability to aggregate and is associated with the increased CVD risk.[40]

It has been demonstrated that sdLDL is especially susceptible to atherogenic modifications that occur in blood plasma. sdLDL particles have a much longer circulation time than larger LDL subfractions that are cleared from the bloodstream through interaction with the LDL receptor.[11,41] For instance, glycation of LDL particles was observed preferentially in the sdLDL fraction, and small LDL had a decreased sialic acid content.[42,43] sdLDL particles contained less antioxidative vitamins that make them less protected against oxidation.[44] Moreover, the elevated LDL(−) was associated with higher oxidized LDL and sdLDL levels.[45] Isolated LDL(−) particles had a decreased sialic acid content in comparison with native LDL, and this difference was more pronounced in LDL(−)

fractions from patients with atherosclerosis.[27,46] It is likely that desialylated and electronegative LDL fractions are closely linked or even identical. Converging evidence points to the existence of a cascade of multiple modifications of sdLDL particles, starting with desialylation and minimal oxidation, followed by further oxidation and formation of highly atherogenic and proinflammatory complexes.

LDL electronegativity index

LDL(−) is a heterogeneous population of particles that vary by their density and size, but share the increased negative charge.[47] The origins of LDL(−) are not yet clearly understood. Incubation of LDL with blood plasma at 37°C resulted in the formation of LDL(−) that could be blocked by 4-bromophenacyl bromide, an inhibitor of secretory phospholipase A_2 (sPLA$_2$), suggesting that sPLA$_2$ plays a primary role in LDL(−) formation.[48] Desialylation of LDL during incubation with blood plasma has also been described.[35] It is likely that the acquisition of the negative charge occurs as a result of successive changes in the LDL particle, including desialylation, loss of lipids, reduction of particle size, and peroxidation in the blood plasma. A summary of such modifications leading to the formation of LDL(−) is presented in Figure 1.

LDL(−) detection

The separation of LDL into electropositive [LDL(+)] and electronegative [LDL(−)] fractions was first performed by Avogaro et al[39] using ion-exchange chromatography. More recently, Chen et al[49] described five subfractions of plasma LDL with various degrees of electronegativity, from L1 (least electronegative) to L5 (most electronegative).[50] The authors found that the plasma levels of the most electronegative (L5) fraction were moderately elevated in individuals with high CVD risk, such as smokers, patients with hypercholesterolemia, type 2 diabetes, and myocardial infarction.[51–53] Anion exchange chromatography remains the standard method for LDL(−) extraction for further analysis that allows a more detailed study of chemical and physical properties of these particles. Another approach to analyze the LDL subfractions based on the particle charge is the capillary isotachophoresis.[54] In this method, LDL(−) is detected as a fraction of fast migrating LDL, which is separated from slowly migrating LDL. Some authors used capillary isotachophoresis to analyze the sdLDL fraction obtained by heparin precipitation.[55] Finally, recently created monoclonal antibodies to LDL(−) allows distinguishing the LDL(−) fraction from native LDL particles by the specific epitopes.[9,56,57]
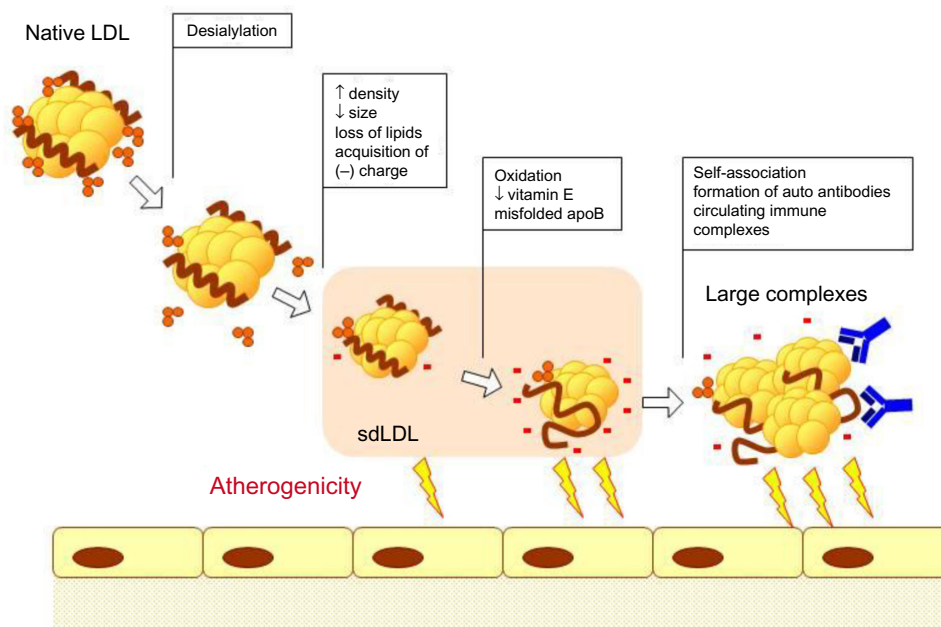
Figure 1 Atherogenic modifications of LDL.
Notes: Multiple atherogenic modifications of LDL particles have been detected in human blood plasma: desialylation was the first event, followed by loss of free cholesterol and cholesterol esters, phospholipids and triglycerides, increase of particle density, and decrease of its size; next, negative charge of particles was increased, leading to the formation of electronegative LDL fraction, in which misfolded apoB was reported; at later stages, increased oxidation and decreased antioxidant content were observed; finally, large, highly atherogenic complexes were formed due to self-association of modified LDL particles and the formation of autoantibodies.
Abbreviations: LDL, low-density lipoprotein; sdLDL, small dense LDL.

Enzyme-linked immunosorbent assay (ELISA) assay with monoclonal antibodies is especially useful for fast and direct detection of LDL(−) in clinical practice.[58] It has to be taken into account that the existing LDL(−) detection methods are only sensitive to the electrical charge and do not distinguish the different origins of the LDL(−) particles. For instance, they cannot discriminate particles that vary by the relative amount of lipoproteins and/or nonesterified fatty acids, particles that underwent oxidation etc. Most of the available monoclonal antibodies were generated against LDL(−) particles isolated based on their charge by ion-exchange chromatography, and they do not help to overcome this problem.

Tendency to aggregate

LDL(−) particles are characterized by a number of specific chemical and physical features in comparison to native LDL. It is well known that LDL(−) is prone to aggregation.[39] Spontaneous aggregation of LDL(−) particles has been investigated in more detail in a recent study.[59] The authors report that LDL(−) formed amyloid-like structures and even possessed amyloidogenic properties, promoting native LDL particles to aggregate. Moreover, amyloid-β peptide enhanced the aggregation of LDL(−) particles. LDL particle aggregation is closely related to their atherogenic activity, and aggregated LDL(−) is characterized by an increased proteoglycan binding.[60,61]

The nature of this increased affinity is currently being investigated. Some authors proposed that the N-terminus of apoB in the LDL(−) particles play the key role as monoclonal antibodies to this region interfered with the binding of particles to proteoglycans.[62] Thus, this region could be an additional proteoglycan-binding site present in LDL(−), next to the constitutive binding site in apoB from native LDL.[63,64]

Misfolded apoB

The electronegative L5 fraction had a decreased content of apolipoprotein B (apoB) and increased content of other lipoproteins.[65] A misfolded conformation of apoB in LDL(−) particles has also been reported.[66,67] As demonstrated by circular dichroism studies, secondary structure of apoB is disturbed in LDL(−) particles, with decreased content of α-helices and increased content of β-sheets.[68] Studies of tryptophan fluorescence spectroscopy also pointed to the misfolded conformation of apoB, as the fluorescence emission was decreased in LDL(−) in comparison to native LDL, indicative of the abnormal exposure of tryptophan residues to the aqueous environment.[59,68] Another evidence of lipoprotein misfolding in LDL(−) particles comes from 2D-NMR analysis that demonstrated a number of lysine residues in LDL(−) with altered ionization status probably due to their exposure to the solvent.[69] Interestingly, estradiol prevented the misfolding of

apoB in LDL(−) particles and decreased their aggregation ability without affecting the negative charge.[70] Therefore, the misfolded lipoprotein appears to be the decisive factor responsible for LDL(−) aggregation. LDL(−) fractions also demonstrate changes in their lipid composition, such as increased content of nonesterified fatty acid.[71,72] This may explain the altered properties of LDL(−) particles' surface and contribute to their enhanced aggregation ability.[73]

Lipolytic activity

LDL(−) particles are characterized by the enzymatic activity that modulates their lipid composition and inflammatory properties. It has been demonstrated that the particles are enriched with platelet-activating factor acetylhydrolase (PAF-AH), a phospholipase that targets the oxidized phospholipids.[74] PLC-like and SMase activities have also been detected in LDL particles, although their origins remain to be elucidated.[75,76] Proteomic studies detected a higher content of non-apoB proteins in LDL(−) in comparison to native LDL, including apoA-I, apoE, apoC-III, apoA-II, apoD, apoF, and apoJ.[77] Although the absolute amounts of these proteins remain very low, they could still play a role in LDL(−) properties and lipolytic activities. For instance, ApoJ acts as a chaperone that binds the misfolded proteins in blood.[78] Its binding to the misfolded apoB in LDL(−) particles could have a protective role against particle aggregation. The combination of PAF-AH and PLC-like activities could regulate the concentrations of proinflammatory oxidized LDL to attenuate their deleterious effects in human plasma.[64] More studies are needed however to determine the origin and significance of these lipolytic activities.

Inflammatory and atherogenic properties

Several features of LDL(−) account for the increased atherogenicity of this LDL subtype (Figure 2). Altered protein structure of the LDL(−) particles leads to a decreased affinity to the LDL receptor as compared to native LDL and prolonged circulation times of the particles.[79,80] On the other hand, the most electronegative (L5) fraction of LDL(−) was shown to interact with the lectin-like oxidized LDL receptor 1 (LOX-1), causing endothelial dysfunction, apoptosis, and atherogenic response in cultured ECs.[51,81] A recent study demonstrated that L5 LDL(−) fraction induced the production of reactive oxygen species and increased the C-reactive protein levels in cultured cells via LOX-1 signaling in cultured aortic ECs, which can further contribute to the atherogenesis.[82] Circulating LDL(−) can activate inflammatory and immune responses that contribute to the atherosclerosis progression. Because of its increased ability to bind proteoglycans, LDL(−) particles and their aggregates have a prolonged residence time in the subendothelial space. Internalized by macrophages through scavenger receptors, LDL(−) serves as a source for the lipid storage and foam cell formation in the arterial wall. The production of anti-LDL(−) autoantibodies may also play an important role in the disease progression, which currently remains to be studied.[9] Along with the autoantibodies to LDL, LDL-containing circulating immune complexes have been found in blood plasma of atherosclerosis patients. These complexes are characterized by smaller sizes and more negative electrical charge than native LDL and have pronounced atherogenic properties, as demonstrated by the accumulation of cholesterol esters in cultured smooth muscle cells and monocyte-derived macrophages.[83] In vitro studies have demonstrated that LDL(−) had a cytotoxic effect on the EC, stimulating apoptosis and cytokine production, including IL-8, MCP-1, and VCAM-1.[49,84,85] Incubation of cultured human monocytes with LDL(−), but not with LDL(+), resulted in cytokine release mediated by TLR4 and CD14, which was the main receptor recognizing LDL(−).[86] Similar cytokine-inducing effect has also been described
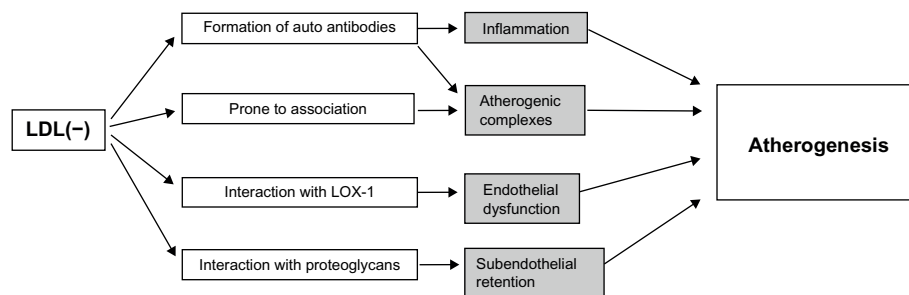

Figure 2 Atherogenic properties of electronegative LDL.
Notes: Several features account for the increased atherogenicity of electronegative LDL [LDL(−)]: formation of autoantibodies and susceptibility to self-association lead to formation of complexes with high atherogenicity. LDL(−) can cause endothelial dysfunction through interaction with LOX-1 receptors. Interaction of LDL(−) with proteoglycans in the subendothelial intima causes increased retention of the particles that serve as a source of lipid storage.
Abbreviations: LDL(−), electronegative low-density lipoprotein; LOX-1, LDL receptor 1.

for the ceramide-enriched LDL, which shares most of the properties of LDL(−).[87,88]

Clinical importance of the LDL electronegativity index in prediction of CVD

Recent studies strongly suggest that LDL electronegativity can be considered as an important factor in evaluating cardiovascular risk in many pathological conditions. As mentioned earlier, the elevated levels of electronegative LDL fractions have been reported in many conditions associated with high cardiovascular risk, including hyperlipidemia, diabetes, renal disease, and coronary syndromes. In patients with hypercholesterolemia, the most electronegative, mildly oxidized L5 LDL fraction was increased in comparison with healthy individuals.[49] Elevated L5 LDL fraction has also been reported in familial hypercholesterolemia patients[50] and in smokers,[51] both cases being associated with high cardiovascular risk. Recent studies demonstrated that L5 LDL fraction was significantly increased in patients with ST-segment elevation myocardial infarction, where it was demonstrated to play a role in platelet activation and thrombosis.[52,53] Elevated levels of L5 were observed in the plasma of patients with metabolic syndrome, a group of metabolic abnormalities that are associated with CVDs, which is used to identify individuals with high cardiovascular risk.[89] Together these results suggest that LDL electronegativity might be considered as a novel predictor of cardiovascular risk.

The clinical importance of LDL electronegativity index has recently been explored in a study analyzing the correlation between the plasma level of L5 fraction and cardiovascular risk estimated using the Framingham risk score.[90] This score has been developed during a large epidemiological Framingham Heart Study and is calculated using such predictors as sex, age, blood pressure, treatment of hypertension and diabetes mellitus, smoking, body mass index, and levels of total cholesterol and HDL.[91,92] According to this classification, "general" CVD risk included coronary death, myocardial infarction, coronary insufficiency, angina, ischemic and hemorrhagic stroke, transient ischemic attack, peripheral artery disease, and heart failure. The "hard" CVD category included coronary death, myocardial infarction, and stroke. The study was performed on patients with metabolic syndrome and healthy individuals. Comparison of these two groups revealed no statistically significant difference in total cholesterol and LDL levels, whereas L5 fraction was significantly higher in patients with metabolic syndrome. No association was registered between L5 level and total

cholesterol or LDL. Regression analysis demonstrated association between L5 content and fasting plasma glucose level and body mass index. L5 level and waist circumference were associated with CVD risks, and the independent contribution of L5 content (with controlled variance of waist circumference) was 11% of 30-year "general" CVD risk and 8% of 30-year "hard" CVD risk. Therefore, L5 content was shown to strongly correlate with different CVD risk factors and with CVD risk. Moreover, the authors argued that plasma L5 levels in asymptomatic individuals with metabolic syndrome also correlated with the number of fulfilled metabolic syndrome criteria and therefore with CVD progression. However, it has not been studied, whether or not the elevated L5 fraction was accompanied by an increase of other known atherogenic LDL modifications. Future studies should compare the diagnostic values of electronegative LDL and other atherogenic LDL types, including modified LDL fractions, such as oxidized LDL and desialylated LDL. Quantitative analysis of various types of modified LDL currently remains challenging because of low amounts of circulating particles. For instance, the association of atherogenic oxidized LDL with CVD risk was difficult to establish, making its use as a biomarker inconvenient.[93] Therefore, LDL electronegativity was suggested as a novel index for CVD prediction because it is relatively easy to assess, although more studies are needed to strengthen this concept.

It remains to be determined whether the elevated level of L5 LDL actually plays a causative role in CVD. Nevertheless, properties of electronegative LDL attracted attention as potential direction of therapy improvement. As elevated levels of L5 are associated with such conditions as smoking, they may partly be ameliorated by lifestyle corrections that are generally recommended for individuals with high CVD risk. More directed therapeutic strategies emerge as a result of better understanding of electronegative LDL pathogenicity. In vitro studies on cultured human aortic epithelial cells explored the possibility of blocking L5 internalization through LOX-1 receptor using the naturally occurring compound sesamol. Study of the sesamol effect on Syrian hamsters fed a high-fat diet demonstrated that the compound addition could reduce plasma L5 levels and atherosclerotic lesion size.[94] These results encourage the search for compounds specifically targeting the electronegative LDL fraction and related signaling. In another study, the authors demonstrated a decrease of LDL(−) uptake by macrophages and foam cell formation caused by anti-LDL(−) single-chain variable antibody fragments (scFv). The exposure of macrophages to LDL(−) resulted in enhanced

expression of CD36, which promoted the lipid uptake and foam cell formation.[95] The addition of the fragments led to a dose-dependent inhibition of the LDL(–) uptake as well as to the decrease of *Cd36* expression at mRNA level. The protective effect of the fragments has also been demonstrated on animal model (*Ldlr* knockout mice), where it caused reduction of atherosclerotic lesion at the aortic sinus in comparison with untreated animals.[96] However, it is important to evaluate the effects of agents specifically targeting electronegative LDL on the production, metabolism, and uptake of other atherogenic LDL subtypes.

The increase of LDL electronegativity can therefore be considered as an emerging independent risk factor of CVD, as confirmed by the analysis of various groups of patients with elevated CVD risk. The possibility of targeted therapy aiming to reduce the LDL(–) fraction has been explored in experiments on animal models. More studies are needed however to confirm the clinical utility of LDL electronegativity index on a larger scale and to design appropriate therapeutic approaches for reducing LDL(–) fraction in humans.

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Disclosure

The authors report no conflicts of interests in this work.

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