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GENETICS (AJ MARIAN, SECTION EDITOR)

# The Underlying Chemistry of Electronegative LDL's Atherogenicity

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**Abstract** Electronegative low-density lipoprotein (LDL) found in human plasma is highly atherogenic, and its level is elevated in individuals with increased cardiovascular risk. In this review, we summarize the available data regarding the elevation of the levels of electronegative LDL in the plasma of patients with various diseases. In addition, we discuss the harmful effects and underlying mechanisms of electronegative LDL in various cell types. We also highlight the known biochemical properties of electronegative LDL that may contribute to its atherogenic functions, including its lipid and protein composition, enzymatic activities, and structural features. Given the increasing recognition of electronegative LDL as a potential biomarker and therapeutic target for the prevention of cardiovascular disease, key future goals include the development of a standard method for the detection of electronegative LDL that can be used in a large-scale population survey and the identification and testing of strategies for eliminating electronegative LDL from the blood.

**Keywords** Atherogenesis · Cardiovascular disease · Endothelial cell apoptosis · Electronegative LDL · Platelet activation · Thrombogenesis

## Introduction

During the last few decades, the lowering of plasma levels of low-density lipoprotein (LDL) cholesterol (LDL-C) has been the primary therapeutic approach in the prevention of cardiovascular events [1–3]. However, the lowering of LDL-C levels in some clinical trials has been shown to have no effect on hazard ratios or cardiovascular mortality [4, 5]. Furthermore,

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in patients without traditional cardiovascular risk factors, other factors have been shown to affect the development of atherosclerosis and cardiovascular diseases, such as lifestyle [6, 7], inflammation [8], rheumatic diseases [9, 10], and genetic polymorphisms [11, 12]. Alternative hypotheses have suggested that LDL variants, such as oxidized LDL (oxLDL), small dense LDL, and lipoprotein (a), may be important constituents of LDL that determine its atherogenicity and may be associated with atherosclerosis and cardiovascular diseases [13–16]. Although oxLDL has been shown to produce atherogenic responses in cultured endothelial cells (ECs), it can only be artificially prepared in vitro [17, 18]. Another form of minimally oxidized LDL that has gained increasing recognition for its role in the development of atherosclerosis is electronegative LDL [19, 20]. Since the initial characterization of human-atheroma-derived LDL by Hoff et al. [21, 22] more than two decades ago, the term "electronegative LDL" has been used to describe LDL with relatively high electrophoretic mobility on agarose gel electrophoresis. In 1988, Avogaro et al. [23] separated human LDL into electropositive and electronegative LDL fractions called LDL(+) and LDL(–), respectively, by using fast protein liquid chromatography with ion-exchange columns. They confirmed that LDL(–) particles are heterogeneous in morphology and size and have a tendency to aggregate [23]. In 2003, Chen et al. [24, 25] sequentially divided plasma LDL into five subfractions with increasingly negative charge, designated as L1–L5, by using fast protein liquid chromatography with anion-exchange columns. With this technique, which has become a standard method for the isolation of electronegative LDL, the least electronegative subfraction of LDL (i.e., L1) and the most electronegative subfraction of LDL (i.e., L5) are distinctly separate, and the intermediary subfractions (i.e., L2–L4) are excellent materials with which to study the transitional electronegativity-based changes in LDL. In a study that compared the subfraction composition of LDL from normocholesterolemic subjects and patients with hypercholesterolemia, L1 composed 85.9 % and 69.4 % of total LDL-C, respectively, L2 composed 5.2 % and 9.2 %, L3 composed 5.9 % and 12.8 %, L4 composed 2.0 % and 4.9 %, and L5 composed 1.1 % and 3.9 % [25]. L5 has been shown to be particularly atherogenic in ECs, and its level is elevated in patients with increased cardiovascular risk [26, 27•, 28•]. Therefore, understanding the underlying chemistry of L5 is essential for uncovering its pathogenesis in cardiovascular disease. In this review, we will summarize the available data regarding the elevation of plasma L5 levels in patients with increased cardiovascular risk and the known atherogenic effects of L5 in various cell types. In addition, we will highlight the known biochemical properties of L5 that may contribute to its atherogenic functions.

## Patient Groups with Elevated Plasma Levels of Electronegative LDL

In Table 1, the patient groups in which LDL(–) or L5 levels have been shown to be elevated are shown. Plasma levels of L5 were first found to be elevated in hypercholesterolemic patients [24], and, in a later study, L5 was reported to compose 8.1 % of total LDL [28•], which was 3.5 times higher than the level of L5 in normolipidemic individuals. Similarly, in a study of patients with familial hypercholesterolemia, L5 composed 3.7 % of total LDL, which was 3.4 times higher than the level of L5 in normolipidemic individuals [25]. In a study of patients with diabetes mellitus, L5 composed 1.7 % of total LDL, which was 2.2 times higher than the level of L5 in healthy control subjects [29]. In smokers and patients with metabolic syndrome, L5 levels were reported to be increased compared with those in control individuals and correlated with atherogenicity and risks of cardiovascular disease [27•, 30]. In a study in which LDL was subdivided into three subfractions according to charge, the level of the most electronegative subfraction (i.e., LDL-3) was increased in patients with hypertension [31]. In patients with ST-segment-elevation myocardial infarction (STEMI), L5 composed 15.4 % of total LDL and was present at a plasma concentration ten times higher than that in subjects without metabolic syndrome, regardless of LDL-C level [32••]. Using antibodies to quantify LDL(–), other researchers have shown that the level of LDL(–) is increased in patients with acute coronary syndrome, such as unstable angina and stable angina [33], and in patients undergoing hemodialysis and peritoneal dialysis [34]; however, these approaches have not been verified by using previously established techniques.

The consistent pattern of elevated L5 levels that has emerged in patients with cardiovascular risks has suggested that the index of LDL electronegativity represents a novel predictor of atherosclerosis. To further demonstrate the importance of electronegative LDL and its relationship with cardiovascular disease, a rapid, quantitative protocol is needed that can be used for a large-scale epidemiologic survey.

## Atherogenic Effects of Electronegative LDL on Different Cell Types

L5 is not recognized by LDL receptor (LDLR) [31], but rather, it signals through lectin-like oxLDL receptor 1 (LOX-1) and platelet-activating factor receptor (PAFR) (Fig. 1) [24, 26, 32••]. LOX-1, initially identified as the major receptor for oxLDL in ECs, is expressed at a high level in proatherogenic settings and has been shown to have a critical role in atherogenesis [35•].

The known effects of L5 on the cell types studied to date are summarized in Table 1. Nearly all in vitro studies with L5 have been performed by incubating cell cultures with 50 μg/mL L5 for 24 h to maintain consistency. L5 has been shown to

**Table 1** Patient groups with elevated plasma levels of L5 or LDL(–) and the harmful effects of L5 on various cell types

| Patient group | Plasma levels of L5 or LDL(–) | Method of quantification | EC apoptosis (%)[a] | Effect of L5 | Effect on cell signaling | References |
|---|---|---|---|---|---|---|
| HLP | L5 increased by 3.5 times; HLP, 8.1±2 % ($n$=7) vs control, 2.3±1 % ($n$=7) | FPLC | 36±8 | Direct effect on vascular ECs | Decreases EC migration, tube formation, MMP-2 and MMP-9 expression, and increases VEGF and CRP[a] expression | [24, 26, 28•] |
| FH | L5 increased by 3.4 times; FH, 3.7±1.7 % ($n$=3) vs control, 1.1±0.2 % ($n$=5) | FPLC | 40 | Direct effect on vascular ECs | Inhibits PI3K/Akt signaling, decreases FGF-2 and Bcl2 expression, and increases Bad, Bax, and TNFα expression | [25, 29, 37–39, 42] |
| | | | | Indirect effect on monocytes | Increases adhesion to HUVECs by increasing expression of VCAM-1 and CXC (including GRO-*x*, IL-8, ENA-78, and GCP-2) from ECs | [38, 42, 43] |
| | | | | Direct effect on monocytes | Increases release of IL-6, IL-10, and MCP-1 | [25, 41] |
| DM | L5 increased by 2.2 times; DM, 1.7±1.0 % ($n$=5) vs control, 0.8±0.6 % ($n$=5) | FPLC | 65 | Direct effect on aortic ECs | Disrupts *FGF-2* transcription | [29, 36, 45] |
| Smokers | L5 increased[b] | FPLC | 40–45 | Direct effect on EPCs | Impairs differentiation by inhibiting Akt phosphorylation and downregulating KDR and CD31 | [30] |
| MetS | L5 increased[b] | FPLC | 17[c] | Indirect effect on CMs | Increases release of IL-8, CINC-2α/β, ENA-78, MIP-3α, and TIMP-1 from ECs | [27•] |
| HTN | Most electronegative subfraction (LDL-3) increased[b] | HPLC | ND | ND | ND | [31] |
| STEMI | L5 increased by10.3 times; STEMI, 15.4±14.5 % ($n$=30) vs control, 1.5±1.1 % ($n$=30) | FPLC | 40 | Direct effect on platelets | Increases platelet activation and aggregation through PKCα signaling | [32••] |
| | | | | Direct effect on aortic ECs | Increases tissue factor and P-selectin expression from dysfunctional ECs | [32••, 93] |
| UA and SA | LDL(–) increased by 1.2–1.9 times; UA, 40.7 μg/mL ($n$=29) and SA, 35.0 μg/mL ($n$=19) vs control, 21.6 μg/mL ($n$=15) | ELISA | ND | ND | ND | [33] |
| HD and PD | LDL(–) increased by 4–10.5 times; HD, 575±233 μg/mL ($n$=25) and PD, 223±117 μg/mL ($n$=11) vs control, 54±3 μg/mL ($n$=30) | ELISA | ND | ND | ND | [34] |

*CINC-2α/β* cytokine-induced neutrophil chemoattractant 2α/β, *CM* cardiac myocyte, *CRP* C-reactive protein, *DM* diabetes mellitus, *EC* endothelial cell, *ELISA* enzyme-linked immunosorbent assay, *EPC* endothelial progenitor cell, *FGF-2* fibroblast growth factor 2, *FH* familial hypercholesterolemia, *FPLC* fast protein liquid chromatography, *GCP-2* granulocyte chemotactic protein 2, *GRO* growth-related oncogene, *HD* hemodialysis, *HLP* hypercholesterolemia, *HPLC* high performance liquid chromatography, *HTN* hypertension, *HUVEC* human umbilical endothelial cell, *IL* interleukin, *KDR* kinase insert domain receptor, *LDL* low-density lipoprotein, *MCP-1* monocyte chemotactic protein 1, *MetS* metabolic syndrome, *MIP-3α* macrophage inflammatory protein 3α, *MMP* matrix metalloproteinase, *ND* not determined, *PD* peritoneal dialysis, *PI3K* phosphoinositide 3-kinase, *PKCα* protein kinase Cα, *SA* stable angina, *STEMI* ST-segment-elevation myocardial infarction, *TIMP-1* tissue inhibitor of matrix metalloproteinase 1, *TNFα* tumor necrosis factor α, *UA* unstable angina, *VCAM-1* vascular cell adhesion molecule 1, *VEGF* vascular endothelial growth factor

[a] Data obtained from cells treated with 50 μg/mL L5

[b] Quantitative comparison not reported

[c] Cardiac myocyte apoptosis (%) indirectly induced through EC signaling

induce EC apoptosis via LOX-1 and PAFR [24, 26]. In cultured vascular ECs, L5 from patients with hypercholesterolemia induced apoptosis in a dose- and time-dependent manner by reducing fibroblast growth factor 2 transcription via the suppression of the phosphoinositide 3-kinase/Akt signaling pathway [24, 36]. Furthermore, L5 induced the expression of Bad, Bax, and tumor necrosis factor α and the release of cytochrome *c* from mitochondria (Fig. 1). Similarly, L5 from patients with diabetes mellitus was shown to induce as much as 65 % apoptosis in ECs [36]. In a study of the physicochemical characteristics of L5 from patients with familial hypercholesterolemia, L5 was found to be heterogeneous in density and composition among individuals, and the induction of EC apoptosis was more strongly associated with LDL electronegativity than with LDL size or density [37]. In addition to apoptosis, L5 [and in some cases LDL(–)] has been shown to induce the release of the chemokines monocyte chemotactic protein 1 and interleukin (IL)-8 [38] from ECs, inhibit vascular EC migration and tube formation via increased vascular endothelial growth factor signaling, and suppress the expression of matrix metalloproteinases 2 and 9 in ECs (Fig. 1) [39]. Because damage to ECs contributes to increased permeability, inflammation, and atherogenesis [40] in the vascular endothelium, L5 is believed to have an important role in the initiation of atherosclerosis.

L5 has also been shown to impair endothelial progenitor cell differentiation by inhibiting Akt phosphorylation and downregulating kinase insert domain receptor and CD31 [30]. Although cardiac myocytes and smooth muscle cells are not directly exposed to circulating L5, L5 can, nevertheless, induce cardiomyocyte apoptosis indirectly through endothelial cytokines, including IL-8, cytokine-induced neutrophil chemoattractant 2α/β, lipopolysaccharide-induced CXC chemokine (CXCL5/ENA-78), macrophage inflammatory protein 3α, and tissue inhibitor of matrix metalloproteinase 1 [27•]. In monocytes, L5 directly increases the release of IL-6, IL-10 [41], and monocyte chemotactic protein 1 and indirectly enhances mononuclear leukocyte adhesion to ECs by increasing the expression of vascular cell adhesion molecule 1 and CXC chemokines, including growth-related oncogene (GRO) α, GRO-β, IL-8, ENA-78, GRO-γ, and granulocyte chemotactic protein 2 from ECs (Fig. 1) [38, 42, 43].

Recently, L5 has been shown to activate platelets through LOX-1 and PAFR (Fig. 1). L5 enhanced adenosine diphosphate (ADP)-induced signaling, which leads to platelet aggregation and the attachment of platelets to damaged endothelium [32••]. L5 has been shown to create a thrombophilic state by triggering platelet activation and aggregation with L5-activated ECs [44] and, thus, may also have a substantial role in promoting thrombogenesis that leads to STEMI [32••].

L5 is the only charge-defined subfraction of LDL that can induce endothelial dysfunction, monocyte inflammation, and platelet activation [30, 32••, 36, 42]. L5 is believed to accelerate atherosclerosis through increased residence time in the

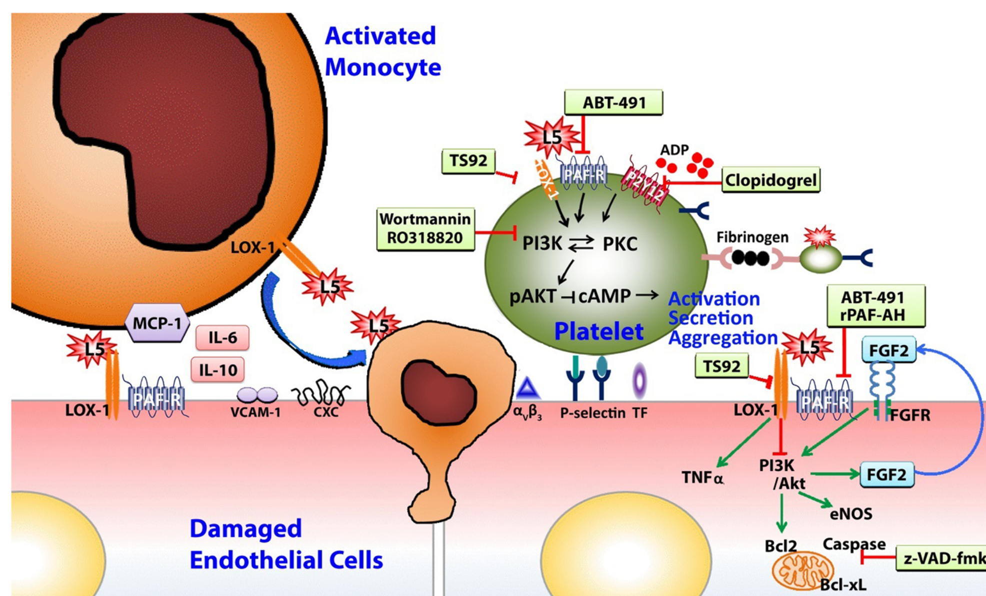


**Fig. 1** Mechanisms of L5-induced atherosclerosis via multiple signaling pathways in endothelial cells, platelets, and monocytes. L5 activates endothelial cells through lectin-like oxidized LDL receptor 1 (*LOX-1*) and platelet-activating receptor (*PAF-R*), suppressing phosphoinositide 3-kinase (*PI3K*)/Akt signaling and increasing the release of tumor necrosis factor α (*TNFα*). In platelets, L5 augments adenosine diphosphate (*ADP*)-induced platelet activation and aggregation through LOX-1 and PAF-R. In monocytes, L5 promotes adhesion with endothelial cells and induces the release of interleukin (*IL*)-6, IL-10, monocyte chemotactic protein 1 (*MCP-1*), and other mediators to induce systemic inflammation. *cAMP* cyclic adenosine monophosphate, *eNOS* endothelial nitric oxide synthase, *FGF2* fibroblast growth factor 2, *FGFR* fibroblast growth factor receptor, *PAF-AH* platelet-activating factor acetylhydrolase, *pAKT* phosphorylated Akt, *PKC* protein kinase C, *TF* tissue factor, *VCAM-1* vascular cell adhesion molecule 1, *z-VAD-fmk* a caspase inhibitor

plasma and by the induction of inflammatory responses in artery wall cells [45]. Notably, the mean plasma levels of L5 that have been identified in patients with cardiovascular risks (e.g., 150 μg/mL in patients with STEMI) far exceed the level of L5 used in in vitro studies (50 μg/mL). The atherogenic effects of L5 observed in vitro support the notion that the index of LDL electronegativity may potentially be used to predict and monitor the development of atherosclerosis.

## Biochemical Properties of Electronegative LDL

### Lipid Moieties of Electronegative LDL

To understand the different receptor affinities for L5, as well as the proatherogenic activity of L5, it is important to consider the biochemical properties of L5, including its chemical composition [46••] (Table 2). Because the in vitro oxidation of LDL primarily results in the modification of surface phospholipids, lipid moieties may be an important factor in the antigenic epitopes of electronegative LDL. Avogaro et al. [23] compared the chemical composition of LDL(+) and LDL(–) and showed that the level of phospholipids was dramatically decreased (23.2±1.7 % vs 5.5±2.8 %) and that the level of free cholesterol was increased (11.0±1.6 % vs 17.4±5.4 %) in LDL(–) [23]. No significant variation was observed in cholesteryl ester or triglyceride content. Avogaro et al. suggested that decreased phospholipid levels may be the result of oxidation, given that oxidized phospholipids are more susceptible to hydrolysis by phospholipases than are non-oxidized phospholipids. However, these findings regarding the phospholipid content of LDL(–) have not been replicated. In 2003, Yang et al. [25] reported that L5 from patients with familial hypercholesterolemia compared with L5 from normolipidemic individuals had reduced cholesteryl ester content and increased triglyceride content, whereas free cholesterol and phospholipid levels were not significantly different between the groups [25]. In 2007, results similar to those of Yang et al. were shown in patients with diabetes mellitus [29]. The discrepancy between these findings (Table 2) may be attributed to the use of different commercial kits from Menarini (Florence, Italy) and Merck (Darmstadt, Germany). Sanchez-Quesada et al. [38] used a third commercial method (Wako Chemicals, Richmond, VA, USA) for quantifying lipid components and compared LDL(–) with LDL(+) in patients with familial hypercholesterolemia and in normolipidemic individuals. They showed that in patients with familial hypercholesterolemia, triglyceride and nonesterified fatty acid (NEFA) levels were increased in LDL(–) compared with LDL(+) from these patients. The same was observed in normolipidemic individuals, but esterified cholesterol and phospholipid levels were comparable between LDL(–) and LDL(+). When lipid oxidation parameters were examined, no differences were observed in the levels of antioxidants (α-tocopherol, α-carotene, β-carotene, and lycopene),

**Table 2** Changes in lipid components in L5 versus L1 or LDL(–) versus LDL(+) reported in various study groups

| | PL (%) | TG (%) | Chol (%) | CE (%) | NEFA (mol/mol apoliprotein B) | References |
|---|---|---|---|---|---|---|
| Native LDL from healthy NL subjects (*n*=18) | 23.2±1.7 | 5.4±1.7 | 11.0±1.6 | 43.2±3.8 | ND | [23] |
| Modified LDL from healthy NL subjects (*n*=18) | 5.5±2.8* | 8.2±3.7 | 17.4±5.4* | 46.7±8.0 | ND | |
| L1 from NC subjects (*n*=4) | 25.0±0.7 | 7.1±0.9 | 9.8±1.0 | 33.6±2.4 | ND | [25] |
| L5 from NC subjects (*n*=4) | 26.2±3.0 | 13.6±1.2* | 9.7±0.5 | 24.2±3.9* | ND | |
| L1 from FH patients (*n*=3) | 25.9±0.7 | 3.7±0.8 | 11.7±0.2 | 34.0±0.3 | ND | |
| L5 from FH patients (*n*=3) | 24.9±1.9 | 16.9±0.6* | 11.4±0.6 | 22.5±9.2* | ND | |
| L1 from NC subjects (*n*=5) | 26.3±4.2 | 3.9±0.6 | 8.9±1.3 | 35.0±7.2 | ND | [29] |
| L5 from NC subjects (*n*=5) | 30.0±3.2 | 7.1±3.1* | 8.3±1.0 | 20.0±2.6* | ND | |
| L1 from DM patients (*n*=5) | 24.5±0.8 | 4.1±0.9 | 8.5±1.5 | 37.9±4.3 | ND | |
| L5 from DM patients (*n*=5) | 26.9±6.5 | 6.7±2.4* | 8.2±1.0 | 18.3±1.0* | ND | |
| LDL(+) from NC subjects (*n*=31) | 25.6±0.9 | 6.8±1.3 | 11.6±0.7 | 28.9±1.7 | 12.3±7.7 | [38, 41, 51] |
| LDL(–) from NC subjects (*n*=31) | 25.8±1.7 | 8.0±1.5* | 12.2±0.8 | 28.6±1.8 | 24.7±12.0* | |
| LDL(+) from FH patients (*n*=31) | 25.9±0.8 | 5.5±1.0 | 11.8±0.5 | 30.1±1.4 | 12.8±4.3 | |
| LDL(–) from FH patients (*n*=31) | 24.9±1.1 | 7.5±1.4* | 12.2±0.7 | 30.6±1.6 | 22.5±9.3* | |
| LDL(+) from NC subjects (*n*=48) | 27.2±2.0 | 7.2±1.8 | 11.4±0.8 | 29.1±1.9 | 14.6±7.2 | [45] |
| LDL(–) from NC subjects (*n*=48) | 27.3±1.3 | 9.5±1.9* | 11.3±1.0 | 28.7±1.8 | 36.2±11.5* | |
| LDL(+) from DM patients (*n*=68) | 27.0±2.7 | 7.7±2.6 | 11.1±1.2 | 28.7±1.8 | 14.9±7.2 | |
| LDL(–) from DM patients (*n*=68) | 26.4±2.0 | 10.2±2.8* | 11.3±0.8 | 28.6±2.1 | 23.2±6.7* | |

Data are expressed as the mean value ± the standard deviation

*CE* cholesteryl ester, *Chol* free cholesterol, *DM* diabetes mellitus, *FH* familial hypercholesterolemia, *NC* normal control, *ND* not determined, *NEFA* nonesterified fatty acid, *NL* normolipidemic, *PL* phospholipid, *TG* triglyceride

*$P<0.05$ versus L1, LDL(+), or native LDL within the same group

**Table 3** Changes in apolipoprotein (*apo*) and protein content in L5 versus L1 or LDL(–) versus LDL(+) reported in various study groups

| | LC–MS$^E$ [46••] | | Coomassie blue staining [56] | | Immunoturbidimetry [51] | | LC–ESI/MS/MS [51] | |
|---|---|---|---|---|---|---|---|---|
| | L1 | L5 | L1 | L5 | LDL(+) | LDL(−) | LDL(+) | LDL(−) |
| Apo(a) | U | 0.805±1.016 | ND | ND | ND | ND | U | 0.006±0.002 |
| Albumin | 0.004±0.006 | 0.095±0.096 | ND | ND | ND | ND | 0.005±0.004 | 0.011±0.010 |
| Apo E | 0.020±0.023 | 0.525±0.317* | Low | High | 0.023±0.021 | 0.093±0.030* | 0.042±0.016 | 0.217±0.051* |
| Apo AI | 0.052±0.091 | 0.402±0.167* | Low | High | 0.068±0.015 | 0.233±0.017* | 0.039±0.027 | 0.146±0.032* |
| Apo AII | ND | ND | ND | ND | 0.010±0.008 | 0.056±0.012* | 0.029±0.009 | 0.141±0.056* |
| Apo CII | ND | ND | ND | ND | 0.033±0.005 | 0.043±0.005 | 0.028±0.034 | 0.049±0.049 |
| Apo CIII | U | 0.348±0.353 | Low | High*[a] | 0.049±0.027 | 0.210±0.145* | 0.090±0.055 | 0.374±0.278* |
| Apo J | U | 0.041±0.047 | ND | ND | 0.002±0.002[b] | 0.001±0.001[b] | 0.001±0.001 | 0.011±0.005* |
| PAF-AH | U | 0.004±0.009 | ND | ND | ND | ND | ND | ND |
| PON1 | U | 0.036±0.054 | ND | ND | ND | ND | ND | ND |

Data are expressed as the mean value (number of moles of indicated lipoprotein or protein per mole of apo B) ± the standard deviation.

*LC–ESI/MS/MS* liquid chromatography–electrospray ionization multistage mass spectrometry, *LC–MS$^E$* liquid chromatography–mass spectrometry, *ND* not determined, *PAF-AH* platelet-activating factor acetylhydrolase, *PON1* paraoxonase 1, *U* undetectable

**P*<0.05 versus L1 or LDL(+)

[a] *P* determined following densitometric analysis

[b] Measured by using an enzyme-linked immunosorbent assay

fatty acid hydroxides, or malondialdehyde between LDL fractions or between normolipidemic individuals and familial hypercholesterolemia patients. These data confirmed those of a previous study in which thiobarbituric acid reactive substances were measured [47]. Similar results were also reported in patients with diabetes mellitus [45]. However, modifications in LDL(–) from diabetes mellitus patients were reported to be increased, including nonenzymatic glycosylation and oxidation [48].

## Lipoprotein and Protein Components of Electronegative LDL

A summary of the changes in lipoprotein and protein content documented in L5 versus L1 and in LDL(–) versus LDL(+) is shown in Table 3. The protein framework of L1 is composed almost entirely of apolipoprotein B-100 (apo B-100), which has an isoelectric point (pI) of 6.620. L5 is composed largely of apo B-100, but it contains other proteins, including apolipoprotein E (apo E; pI 5.5), apolipoprotein AI (apo AI; pI 5.4), apolipoprotein CIII (pI 5.1), and lipoprotein (a) (pI 5.5) [46••]. The proportional increase in the levels of these low-pI proteins in L5 compared with L1, observed by using sodium dodecyl sulfate–polyacrylamide gel electrophoresis and 2D electrophoresis, may contribute to the overall negative charge of L5. Data derived from liquid chromatography–mass spectrometry (LC–MS$^E$)—a more detailed technique for the quantification of proteins—have shown that L1 contains 99.71±0.37 % apo B-100 on the basis of weight percentage, as well as minute amounts of albumin, apo E, and apoAI [49, 50]. In contrast, L5 contains only 61.26±21.44 % apo B-100 and approximately 30 % apolipoprotein (a) [apo(a)]. Apo E and apo AI compose about 3 % and 2 % of the total weight of L5, respectively. Sizable amounts of albumin, apolipoprotein CIII, apolipoprotein J, platelet-activating factor acetylhydrolase (PAF-AH), and paraoxonase 1 have also been detected in L5.

The differences observed between L5/L1 and LDL(–)/LDL(+) (Table 3) may have resulted from variations in the different chromatographic separation techniques used. For example, L5 is extracted from other LDL subfractions by the progressive elevation of the salt gradient during anion-exchange chromatography, and it represents a small, but highly negatively charged LDL. In contrast, LDL(–) is a product of the dichromatic division of total LDL [23–25]. The major differences between the L5/L1 and LDL(−)/LDL(+) pairs are in their differential levels of apo E and apo AI content. LDL(–), which was analyzed by using either immunoturbidimetry or liquid chromatography–electrospray ionization multistage mass spectrometry (LC–ESI/MS/MS), contains approximately fivefold more apo E and fourfold more apo AI than does LDL(+) [51]. In contrast, L5, which was analyzed by using LC–MS$^E$, contains 24-fold more apo E and eightfold more apo AI than does L1 [46••]. The significance of the distinctly higher levels of apo E and apo AI content in L5 is currently under investigation in our laboratory. Another major disparity between the L5/L1 and LDL(−)/LDL(+) pairs is that whereas apo(a) was detected in high amounts in L5, it was not detectable in LDL(–) by using immunoturbidimetry, and it is detected only in a minute amount by using LC–ESI/MS/MS [51]. A possible explanation for this difference is that the density range used for the

separation of LDL differs between the techniques used. The LDL density range used by Bancells et al. [51] to separate LDL(−) from LDL(+) is from 1.019 to 1.050 g/mL, whereas in our studies, we used a range from 1.019 to 1.063 g/mL for LDL subfractions. The density of apo(a)-containing particles ranges between 1.020 and 1.120 g/mL, with values mostly falling between 1.050 and 1.120 g/mL [52].

## Enzymatic Activities of Electronegative LDL

Lipoprotein-associated phospholipase $A_2$ (Lp-PLA2) has a pathogenic role in endothelial inflammation, atherosclerosis, and other cardiovascular diseases [53, 54]. LDL(–) isolated from patients with familial hypercholesterolemia or diabetes has been shown in proteomic studies to contain Lp-PLA2 [29, 55, 56]. Lp-PLA2 can cleave phospholipids to generate lysophosphatidylcholine (lysoPC) and oxidized NEFA [54]. In 2007, Gaubatz et al. [29] showed that LDL(–) subfractions (D6 and D7) were enriched with Lp-PLA2, as well as with lysoPC and NEFA. Similarly, Yang et al. [56] quantified Lp-PLA2 in LDL subfractions from patients with diabetes mellitus and showed that L5 contained about one Lp-PLA2 molecule for every 237 L5 particles, whereas L1 contained one Lp-PLA2 molecule for every 152,000 L1 particles [56].

LysoPC and NEFA are bioactive, proinflammatory lipid metabolites [54]. Because Lp-PLA2 is associated with LDL(–), increased lipolysis by Lp-PLA2 results in a high lysoPC and NEFA content that impairs the ability of LDL to bind to LDLR [57]. Furthermore, NEFA promotes the pathogenesis of diabetes mellitus [53, 58, 59•] and inflammatory responses [60]. The reduction in the level of the secreted form of phospholipase $A_2$ has been studied as a therapeutic target in the prevention of cardiovascular disorders, but the phase III clinical trial was stopped prematurely for lack of efficacy [61].

PAF-AH is another powerful immediate-response molecule that elicits physiologic and pathophysiologic responses both independently and through the activation of specific G-protein-coupled receptors [62]. In 2003, Benítez et al. [63] reported that LDL(–) from both hypercholesterolemic patients and normolipidemic individuals had PAF-AH activity that was five times higher than that in LDL(+). Furthermore, our proteomic studies showed that PAF-AH was detectable in L5 but not in L1 [46••] (Table 3). The presence of PAF-AH is believed to be related to the inflammatory capacity of LDL(–) [63, 64]; however, the genetic deficiency of PAF-AH in humans increases the severity of atherosclerosis and other syndromes [65, 66], and recombinant PAF-AH has been shown to lessen the proapoptotic effect of L5 and its ability to inhibit fibroblast growth factor 2 transcription [24]. There is not presently an explanation for the discrepancy in these findings, but the possibility remains that PAF-AH content is increased in LDL(–) as the result of a compensatory mechanism against the development of atherosclerosis.

Sphingomyelinase (SMase) activity is detectable in LDL(–) [67, 68•, 69] and is elevated in patients with diabetes mellitus, sepsis, and chronic heart failure [70, 71]. SMase hydrolyzes sphingomyelin to yield ceramide and phosphocholine. Increased intracellular levels of ceramide lead to aging, inflammation, insulin resistance, mitochondrial dysfunction, and monocyte activation [41, 72–74]. A strong association has been demonstrated between SMase activity at pH 7.4 and extracellular LDL [75, 76]. LDL(–) has SMase activity [69] that leads to proinflammatory effects and aggregation [67]. In addition, in vitro studies have shown that SMase-modified LDL induces the aggregation and subendothelial retention of atherogenic lipoproteins [77] and contributes to the increased gene expression of inflammatory molecules from monocytes [78]. In SMase-deficient mice in an apo E-deficient background, the early foam cell aortic root lesion area was found to be reduced in size [79]. Because soluble forms of SMase have not been detected in LDL(–) lipoproteins after extensive proteomic analysis [51, 80, 81], we hypothesize that the SMase activity observed is inherent to one of the apolipoproteins of LDL(–). Although SMase has potential as a therapeutic target for the prevention of atherosclerosis, known inhibitors for SMase have shown a weak ability to attenuate the enzymatic activity of SMase. In a study in which isolated human LDL was shown to catalyze the formation of ceramide from either fluorescently labeled sphingomyelin or from $[^{14}C]$sphingomyelin, the putative catalytic sites for SMase activity were found to be the His2230–Ser2306–Asp2359 triads located in the $\alpha_2$ region of apo B-100 [76]. However, further structural and functional studies are needed to confirm the activity associated with these triads and to delineate the mechanism for the activation of SMase activity in LDL(–).

## Structure of Electronegative LDL

The majority of LDL is composed of apo B-100, which is a large protein consisting of 4,536 amino acid residues [82, 83]. Apo B-100 has a pentapartite structure with alternating $\alpha$ helixes and $\beta$ pleated sheets ($\alpha_1$–$\beta_1$–$\alpha_2$–$\beta_2$–$\alpha_3$) [46••]. The $\alpha_2$ and $\alpha_3$ domains maintain the structural integrity of LDL by stabilizing electrostatic interactions with the phospholipid belt of the LDL particle [84]. Various types and degrees of modifications can occur in the amino acid residues of apo B-100 [85–88]. Hamilton et al. [80] have shown that treatment of LDL with peroxynitrite alters amino acid residues of apo B-100 and leads to changes in the secondary structure with loss of $\alpha$ helices and gain in $\beta$ sheets, resulting in increased random coil content. A misfolded conformation of apo B-100 in LDL(–) was first characterized by using tryptophan fluorescence spectroscopy, which provides information on tertiary structure [89]. Bancells et al. [67, 69] further confirmed this by circular dichroism studies and also found that LDL(–) has a high affinity for human aortic proteoglycans and exhibits

phospholipolytic activities. Furthermore, results obtained by using 2D nuclear magnetic resonance have revealed different populations of exposed lysine residues in apo B-100 of LDL(–) and LDL(+) fractions of LDL [67]. Lysine residues are involved in the recognition between LDL and LDLR, indicating that alterations in basicity may be responsible for the weak binding affinity between L5 and LDLR [23]. The chemical alterations that result from modifications such as oxidation [75, 76, 90] and nitration [91] may cause apo B-100 to lose regular secondary elements and gain random coil conformations that generate a more flexible structure, which may also account for the increased affinity of L5 for scavenger receptors [80].

## Targeting Electronegative LDL and Its Atherogenic Effects

The known pharmacologic inhibitors of L5-induced signaling pathways are not sufficient for blocking the multitude of interactions triggered by L5. Neutralizing antibodies against LOX-1 can attenuate but not eliminate many of the harmful effects of L5 [26, 32••]. Furthermore, LOX-1 also binds to C-reactive protein, bacteria, heparin, and electronegative materials in a nonspecific manner [92], suggesting that L5 may have its own more specific receptor. Although PAFR is also a receptor for L5, antagonists of PAFR cannot completely block the effects of L5 [24, 32••]. Therefore, directly targeting L5 or eliminating it from the plasma may be the most effective strategy for preventing atherosclerosis. In a recent study [28•], we showed that atorvastatin significantly decreased plasma L5 levels in hypercholesterolemic patients treated with atorvastatin for 6 months (10 mg/day). Furthermore, plasma L5 levels returned to the baseline in two noncompliant patients 3 months after discontinuation of treatment. This study suggests that the beneficial effects of atorvastatin may be attributed to its ability to reduce the vascular toxicity of L5. Given that there are several known side effects of lipid-lowering drugs, identifying a therapeutic agent that exclusively eliminates plasma L5 may be of great clinical value for patients with increased risk of atherosclerosis.

## Conclusions

Studies have collectively indicated that L5 may be a putative biomarker for monitoring the development of atherosclerosis. To more clearly understand the importance of electronegative LDL, a large-scale epidemiological survey of L5 levels is needed, which would require the development of a standardized clinical method for measuring plasma L5 levels. In addition, more studies are needed to identify and test strategies for eliminating L5 from the blood.

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**Compliance with Ethics Guidelines**

**Conflict of Interest** Liang-Yin Ke, Nicole Stancel, Henry Bair, and Chu-Huang Chen declare that they have no conflict of interest.

**Human and Animal Rights and Informed Consent** This article does not contain any studies with human or animal subjects performed by any of the authors.

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- • Of importance
- •• Of major importance

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