[Frontiers In Bioscience, Landmark, 23, 1422-1444, March 1, 2018]

# Modified lipoproteins as biomarkers of atherosclerosis

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## 1. ABSTRACT

Pathogenesis of atherosclerosis and the search for novel therapies and diagnostic markers remain major problems of modern medicine. Currently available therapeutic approaches are often not sufficiently effective, probably due to the complexity of the disease mechanisms. This review focuses on the evaluation of low density lipoprotein (LDL) and high density lipoprotein (HDL) as risk factors of atherosclerosis. We summarize the current paradigm of LDL involvement in atherogenesis and HDL presumably protective properties. We next discuss the available evidence for the protective effect of HDL and the consequences of HDL dysfunction. Finally, we question the currently widely accepted hypothesis of the central role of oxidized LDL in atherogenesis and present an alternative concept of multiple modification of LDL that confers its pro-atherogenic properties.

## 2. INTRODUCTION

### 2.1. The paradigm of lipid theory of atherogenesis and the value of low density lipoprotein and high density lipoprotein as risk factors

Atherosclerosis is the morphological basis of the most dangerous cardiovascular diseases (1). Atherosclerotic lesion is formed in the artery wall and consists of a lipid core and a fibrous cap separating the lipid core from the lumen. Lipid deposition in the innermost arterial intimal layer is one of the earliest manifestations of atherosclerosis, which is also readily visible (2, 3). In the intima, lipids accumulate both inside the vascular wall cells and in the extracellular space, where they associate with the extracellular matrix components (4). Formation of “foam” cells with cytoplasm filled with lipid droplets (hence the name) is recognized as a trigger of atherogenesis (5-7). According to current understanding, low density lipoprotein (LDL)

circulating in human blood is the major source of lipids accumulated in the arterial wall cells. On the other hand, high-density lipoprotein (HDL) appears to be protective, since it facilitates the outflow of lipids from cells, thereby preventing the formation of foam cells (8). Lipoproteins can be both effectors (mechanistic factors) and biomarkers of atherosclerosis, which makes them attractive therapeutic targets or candidate molecules for developing novel diagnostic tools, or both.

### 2.2. The role of modified LDL in atherosclerosis

The ability of HDL to participate in reverse transport of lipids has been demonstrated in a large number of studies and is reviewed elsewhere (9). LDL has been regarded as the likely principal source of lipids accumulating in the arterial wall, and was used for modeling atherogenesis. However, the attempts to induce intracellular lipid accumulation in cultured arterial cells by incubating them with native LDL were not successful (10). On the other hand, LDL chemically modified *in vitro* by acetylation, malondialdehyde treatment, oxidation, and other methods, was capable to induce lipid deposition in cultured cells, indicating that modified LDL is atherogenic (11). Based on these data, it was hypothesized that not native, but modified LDL is responsible for foam cell formation and plays a trigger role in atherogenesis. Various forms of atherogenic modification of LDL have been discovered in the blood of atherosclerotic patients. In particular, small dense LDL, electronegative, oxidized, and desialylated particles were found (12, 13). It was shown that in the bloodstream, the same LDL particle can undergo multiple modifications acquiring the properties of small dense, electro-negative, oxidized and desialylated lipoproteins (14). It can be suggested that the risk of onset and development of atherosclerosis is dependent not as much on the total content of LDL in the blood as on the level of multiply modified. Accordingly, it is reasonable to assume that the level of multiply modified LDL is a better biomarker of atherosclerosis in comparison with the total LDL level. Similar conclusions can be drawn for HDL. It was found that different subfractions of HDL differ in their ability to cause lipid outflow from cells. Moreover, impaired cholesterol efflux capacity of HDL correlated with atherosclerosis, including early stages of disease development (15, 16). Therefore, abnormal lipid transport and, as a result, foam cell formation can be attributed to the dysfunctional HDL rather than alterations of blood levels of normal lipoprotein particles. Perhaps, the level of dysfunctional HDL can be considered a better biomarker of atherosclerosis than the total level of HDL.

### 2.3. Evaluation of LDL and HDL as risk factors: AHA recommendations

The expected roles of LDL and HDL in atherogenesis could be confirmed by clinical studies. However, the obtained results did not always correspond to theoretical expectations. The concept of evidence-based medicine postulates that mechanistic role of any biomarker should be supported by findings from clinical studies and trials (17). Therefore, the question to be answered is “whether any kind of marker modulation will affect the established endpoints?” In studying atherosclerotic diseases, study endpoints usually include all fatal and nonfatal cardiovascular events, angina, revascularization, fatal and nonfatal myocardial infarction, stroke, *etc*. To assess direct effects on atherosclerosis, surrogate instrumental measures, namely, coronary artery calcification and carotid intima media thickness are often used as the endpoints. It is possible that the results of such studies would be more encouraging if atherogenic multiply modified LDL and dysfunctional HDL were considered as pharmacological targets and biomarkers. However, the knowledge accumulated to date does not allow justifying the use of modified LDL and HDL as mechanistic markers of atherosclerotic disease due to insufficient evidence base. Therefore, these lipid parameters are not mentioned in the latest and most widely used American and European recommendations and guidelines for management of dyslipidemias to reduce atherosclerotic cardiovascular risk (18-20).

During the recent years, a wide spectrum of conventional and experimental biomarkers has been considered for evaluating cardiovascular risk. Modified LDL and dysfunctional HDL should obviously be regarded as lipid-related markers, along with apolipoproteins. Other important biomarkers include inflammatory molecules, such as fibrinogen and high-sensitivity C-reactive protein, thrombotic markers, such as lipoprotein-associated phospholipase A2 and homocysteine, markers of glucose metabolism, and organ-specific markers. It is also important to evaluate whether a given biomarker can have a causative relationship with atherosclerosis or is just a marker of preclinical disease (21). The problem of developing novel biomarkers is related not only to the complexity of the network of different markers and risk factors, but also to the fact that the majority of novel suggested biomarkers usually has unknown or little proven ability to contribute to the existing methods of cardiovascular risk assessment on top of the conventional risk factors, having therefore practically negligible diagnostic or prognostic value (22-25).

The aim of this review is to evaluate whether modified LDL and dysfunctional HDL can be considered as markers and/or effectors of atherosclerosis. We will discuss possible reasons for the discrepancies between theoretical concepts and study results and provide suggestions on how the studies should be organized to obtain clearly interpretable results.

## 3. HIGH DENSITY LIPOPROTEIN IN ATHEROGENESIS

### 3.1. Epidemiological data demonstrating negative association of HDL with atherosclerotic disease

Numerous epidemiological studies have clearly demonstrated an inverse relationship between HDL plasma levels and the risk of atherosclerotic cardiovascular disease. These studies share a rich history, dating back to over six decades. The famous Framingham study was the first to introduce the concept that HDL does protect against coronary heart disease, at least, in healthy subjects (26). Another evidence of atheroprotective role of HDL came from studies demonstrating lower HDL cholesterol levels in patients with clinical atherosclerosis (27). However, further studies showed that the association between atherosclerotic disease and HDL cholesterol was not linear. High and very high HDL cholesterol levels (above 1.5. mmol/l) did not improve the prognosis, but were even associated with increased risk in a retrospective assessment of the results of IDEAL and EPIC Norfolk studies; at the same time, hazard ratio <1 for relationship between HDL and atherosclerotic cardiovascular disease was also revealed (28, 29). The most recent study reported the lowest overall (total) mortality in persons with HDL cholesterol level of approximately 50 mg/dl. Therefore, the association between the parameters was U-shaped: the mortality risk was attributed to both low and very high HDL cholesterol. However, this conclusion could not be specifically linked to CVD-associated mortality (30).

Moreover, the relationship between HDL cholesterol and cardiovascular risk appears to be complex, since many other pro-atherogenic factors, including triglyceride-rich lipoproteins, lipoprotein remnants, small dense LDL, and inflammation may act as confounders (31). It is important to note, however, that none of the studies attempted to analyze the impact of different HDL subclasses on atherosclerotic and cardiovascular risk.

The balance between HDL and LDL concentrations seems to be important. Therefore, calculating the ratio of LDL cholesterol to HDL cholesterol is still performed in different studies, as a better estimate of lipoprotein misbalance. However, März with co-authors questioned the usefulness of such parameter, because of the non-linear association between HDL cholesterol and cardiovascular risk. Indeed, such calculations may lead to erroneous conclusions on non-elevated risk, because the predictive value of HDL cholesterol is modified by both triglycerides and LDL cholesterol (32); one should also consider possible technical errors in the measurements of HDL cholesterol in hypertriglyceridemia. European guidelines point out that values obtained with direct measurements may over- or underestimate LDL cholesterol and HDL cholesterol levels. Calculation of non-HDL cholesterol level may partially overcome this problem, but a correct analysis of HDL cholesterol is still required, especially in hypertriglyceridemic subjects. In any case, the ratio of non-HDL cholesterol to HDL cholesterol may be considered as an alternative to solely HDL cholesterol measurement, but this lipid-related parameter has only IIb class recommendation (that is, usefulness is less well established by evidence), and the lowest level of evidence (that is, consensus of opinion of the experts and/or small studies, retrospective studies, registry studies) (19).

disorders due to pathogenic mutations in *ABCA1*, *APOA1* and *LCAT* genes and phenotypically characterized by very low HDL levels did not show dramatic elevation of cardiovascular risk, sometimes even independent of HDL levels; however, the risk of premature atherosclerotic cardiovascular disease in specific cohorts of patients with Tangier disease or familial *LCAT* deficiency, and further in *APOA1* deficiency is generally elevated (33-36). Genome-wide association studies that have identified 46 loci independently associated with HDL cholesterol levels, together with Mendelian randomization studies, demonstrated that genetic risk scores do not impact cardiovascular risk, thus providing a strong argument against anti-atherogenic causal role of HDL (37-44).

Taken together, these findings indicate that some genetic variants possessing the impact on HDL cholesterol also influence cardiovascular risk, while others do not. Therefore, it can be speculated that HDL level is much less important than the intrinsic mechanisms by which the observed level is achieved (45).

### 3.2. Possible mechanisms of anti-atherogenic effects of HDL

The mechanistic role of HDL in inhibition of atherogenesis seemed obvious for many years, since it laid on the top of the Glomset's concept of reverse cholesterol transport, i.e. of the ability of HDL particles to induce cholesterol efflux from cells back to circulation (46). Indeed, the logical extension of this concept was the idea that the increased level of HDL cholesterol inequitably means the increased release of cholesterol from cells (including foam cells, with the respect to atherosclerosis). From this point of view, to provide for the anti-atherogenic effect, the quantity of released cholesterol should be equal or higher than that taken up by cells. The inverse associations between HDL cholesterol and cardiovascular risk observed in the epidemiological studies should be strictly related to the mass of transported cholesterol. However, current understanding of HDL metabolism and function is far from complete. Apart from the ability to stimulate efflux of excess cholesterol from cells, HDL can have several

anti-atherogenic functions (47). The antioxidant and anti-thrombotic effects of HDL, as well as its role in the maintenance of vascular endothelium, should be considered along with the induction of reverse cholesterol transport. All these properties are related to the composition of HDL particles, most importantly, to the content of apolipoprotein apoA-I (48, 49). Therefore, the putative anti-atherogenic effect of HDL is likely to depend on the functional properties of the particles, rather than on the total HDL content.

The efflux of cholesterol from cells to HDL particles occurs via different pathways, predominately mediated by two transmembrane proteins, ATP-binding cassette transporters. The first one, ABCA1, facilitates cholesterol efflux to lipid-free apoA-I-containing pre-β-HDL particles. The second one, ABCG1, releases cholesterol to mature α-HDL particles (50). The efflux of cholesterol specifically depends on the cell type and cellular microenvironment. Resident macrophages possess both ABCA1- and ABCG1-mediated pathways. The difference between them is that ABCA1-mediated pathway is predominant in foam cells, whereas ABCG1-mediated pathway facilitates redistribution of intracellular sterols from the endoplasmic reticulum. Therefore, ABCG1-mediated pathway promotes cholesterol efflux to various lipoproteins and other acceptors. It was shown that lipid-free apoA-I and HDL, while promoting cholesterol efflux, also induce intracellular signaling for triggering anti-inflammatory responses, thus opening an intriguing link between inflammation and intracellular cholesterol retention (51-54).

Recent but very important findings indicate that microRNAs are present in the HDL, and thus can be involved in the mechanisms by which HDL particles participate in the regulation of cardio-metabolic disease, especially due to alterations of HDL-microRNA composition observed in atherosclerosis (55). One of the proposed mechanisms is the transfer of microRNAs between the body compartments by HDL particles (56).

### 3.3. Unsuccessful attempts to use HDL increase against atherosclerosis

Taking into account the complexity of the mechanisms by which HDL may provide anti-atherogenic action, using therapy-induced increase of HDL levels to reduce the risk of atherosclerosis seems to be ungrounded (57). One of the first therapeutic options to increase HDL cholesterol was nicotinic acid. A recent meta-analysis was performed on 11 randomized controlled clinical trials that enrolled, in total, 2682 patients in the active group and 3934 in the control group. It demonstrated that niacin significantly reduced major coronary events and any cardiovascular events, induced the regression of coronary atherosclerosis and carotid intima thickness (58). However, in the HPS2-THRIVE study, which enrolled 25,673 patients, the combination of niacin with laropiprant did not provide significant reduction in cardiovascular events (59). Fibrates were considered as appropriate agents to increase HDL cholesterol in an indirect way, by lipoprotein lipase activation. A meta-analysis of 18 prospective randomized clinical studies has demonstrated a reduction of severe cardiovascular events incidence, but the effect could be attributed neither to the increase of HDL cholesterol, nor to the decrease of triglycerides or LDL cholesterol reduction (60). The Federal Drug Administration had concluded that the use of fibrates and niacin for the reduction of cardiovascular events has no sufficient evidence (61, 62).

HDL catabolism via cholesteryl ester transfer protein (CETP) inhibition failed to demonstrate the effect on major cardiovascular endpoints, thus allowing further skepticism on the HDL hypothesis. Drug-based inhibition of CETP resulted in a significant increase in HDL cholesterol along with a drop in LDL cholesterol (63). Unfortunately, 3 out of 4 prospective clinical studies with CETP inhibitors were prematurely terminated because of increased mortality in the treatment group or futility of study continuation. ILLUMINATE Study with torcetrapib in approximately 15,000 study participants was started in 2004 and terminated in 2006 due to increased mortality in the treatment group. Dal-OUTCOMES study with dalcetrapib in approximately 15,000 study participants was started in 2008 and terminated in 2012 due to the lack of effect. ACCELERATE Study with evacetrapib in approximately 11,000 study participants was started in 2012 and terminated in 2015 due to the lack of effect (32). The latest study, REVEAL Study with anacetrapib in approximately 30,000 study participants, was started in 2011. The first published paper reported that 30,449 individuals in Europe, North America, and China were randomized to receive anacetrapib (100 mg) daily or placebo, and the results were anticipated in 2017 (64). It is expected that REVEAL trial will provide a proper evaluation of the clinical efficacy and safety of the addition of anacetrapib to the effective statin regimen. However, it is currently not possible to make any conclusions on the ability of the latest CETP inhibitor to reduce atherosclerosis progression and cardiovascular risk.

### 3.4. Evaluation of the emerging potential biomarkers.

A study from the Emerging Risk Factors Collaboration has shown that the addition of combination apolipoprotein B and AI or lipoprotein-associated phospholipase A2 to risk evaluation, which already takes into account total and HDL cholesterol, provided slight improvement in cardiovascular disease prediction (65). Replacing the information on total and HDL cholesterol with apolipoprtein B or

AI worsened cardiovascular disease risk prediction in persons without known disease. Therefore, the clinical relevance of these novel biomarkers remains unclear (65, 66). Several explanations of the disappointing performance of CETP inhibitors were proposed. One is focused on the differences in pharmacokinetics and pharmacodynamics of drug molecules, thus inspiring the development of new inhibitors that either reduce CETP production or modify its function (67). Another explanation also proposes the recruitment of inadequate cohorts of study participants, thus focusing on proper identification of patients who get benefit from CETP inhibition in previous clinical trials (68). In our point of view, the most convincing explanation proposed so far is that, due to inhibition of transfer of cholesteryl esters from HDL to LDL, the lifespan of so-called "dysfunctional HDL" is extended. In this case, HDL may even gain adverse and non-beneficial properties (69, 70). However, none of the clinical trials has evaluated the HDL functionality, apolipoprotein and lipid composition, or HDL subfractions. Therefore, the idea of dysfunctional HDL remains only an attractive hypothesis. On the other hand, from the point of view of cellular mechanisms of atherosclerosis, it is possible to speculate that HDL, in spite of its functionality, is able to perform reverse cholesterol transport only of normally metabolized cholesterol. The excess of cholesterol that was internalized from modified LDL via scavenger receptor pathway or phagocytosis of LDL aggregates, and further deposited in cells in the form of lipid droplets, may not be accessible for the mechanisms of normal regulation of intracellular cholesterol homeostasis.

Finally, it can be concluded that HDL cholesterol can not currently be considered as an appropriate therapeutic target for novel drugs. Therefore, anti-atherogenic role of HDL remains challenged by skepticism, and the question on whether HDL is a biomarker, a mechanistic factor, or just an innocent bystander, remains still unanswered. However, the anticipated positive results of the REVEAL study may strongly affect this conclusion and open more room for HDL-rising therapy.

## 4. MODIFIED LDL IN ATHEROGENESIS

### 4.1. Oxidized LDL: myth or reality?

Currently, PubMed lists more than 9000 articles indexed under "oxidized LDL", and around 4295 under "oxidized LDL and atherosclerosis." More than 1000 reviews have been written on oxidized LDL. Thousands of researchers have studied and are currently studying oxidized LDL and its role in atherogenesis. For a long time, D. Steinberg and his co-workers remained the undisputed leaders in this field. This group was the first to demonstrate that LDL incubated with cultured cells undergoes oxidative modification (71). In parallel, Prescott with co-authors showed that incubation of cultured macrophages with oxidized LDL leads to accumulation of cholesterol esters in cells, while native LDL does not cause a similar effect (72). Until now, it is widely accepted that it is oxidized LDL that causes the accumulation of cholesterol in the intimal subendothelial cells, thereby triggering atherogenesis (73-75). Oxidized LDL was detected in atherosclerotic lesions (76), but could not detected in the bloodstream for a long time. As a consequence, it is believed that LDL is oxidized not in the blood, but in the vascular wall, where it accumulates in tissue macrophages. On the other hand, traces of oxidation were also found in circulating LDL (77), although this fact does not prove that the lipoprotein particle was oxidized in the blood and not in the solid tissue. The following arguments support the ideas on the key role of oxidized LDL in atherogenesis: 1) autoantibodies to malondialdehyde-LDL (MDA-LDL) were found in the blood (78); 2) antibodies to *in vitro* oxidized LDL recognize oxidation products co-localized with LDL in the atherosclerotic lesions (76); 3) lipoprotein isolated from atherosclerotic lesion is similar to oxidized LDL (79). We provide below counter-arguments to all of these suggestions.

1) MDA-LDL is a poor model of oxidized LDL. Commercially available monoclonal and polyclonal anti-LDL antibodies are produced against LDL purified from human plasma and further modified *in vitro* by MDA or copper oxidation. Both methods of modification lead to a random and non-specific exposition of immunogenic sites, and MDA-modified LDL appears to be different from LDL oxidized by other methods.

2) While oxidized LDL is not detected in the blood, a circulating, multiply modified atherogenic LDL is detected and can be isolated from the blood of atherosclerotic patients (13).

3) Anti-LDL antibodies isolated from the blood of atherosclerotic patients show cross-reactivity at least with MDA-LDL and desialylated LDL. Moreover, these antibodies have an order of magnitude greater affinity for desialylated LDL as compared to oxidized LDL (80).

4) Incubation of native LDL with serum from atherosclerotic patients leads first to desialylation of the lipoprotein particle, then to changes in the lipid composition. As a result, the particle becomes smaller and denser. Much later the particle becomes more electronegative, and, finally, signs of LDL oxidation appear (81).

Comparison of the above *pro* and *contra* arguments draws us to the conclusion that circulating oxidized LDL does exist, but its role in atherogenesis

might be greatly exaggerated. Atherogenic modification of LDL is not limited to oxidation, but is a multiple modification affecting the carbohydrate, lipid and protein moieties of the lipoprotein particle. Multiple modification of LDL leads to change in many of the physical and chemical characteristics of the lipoprotein particle. It is likely that oxidation is not a determining factor in LDL atherogenicity, since other events occur sufficiently earlier for the lipoprotein particle to become atherogenic, i.e., capable of inducing accumulation of intracellular lipids. Because anti-LDL autoantibodies primarily recognize desialylated LDL, it is plausible that autoantibodies are produced in response to the appearance of desialylated LDL, and not oxidized LDL. This and other facts lead us to the understanding that non-oxidative modifications of LDL can be more important for the onset and progression of atherosclerotic lesions.

### 4.2. Known modified LDL types present *in vivo*

LDL is generally known lipoprotein fraction with density range from 1.0.19 to 1.0.63 g/l. Using ultracentrifugation or gradient gel electrophoresis, LDL particles can be separated into large, intermediate, small, and very small LDL (82, 83). Small and very small LDL is often considered as small dense LDL (sdLDL). There is a correlation between LDL particle size and density; however, these parameters are not identical. The lifetime of sdLDL is longer than that of large LDL (42, 43). It was reported that sdLDL contains less antioxidants and is therefore more susceptible to oxidation than larger forms of LDL (84). sdLDL possesses lower sialic acid content as compared to large LDL (85). As a result of desialylation, binding of sdLDL to arterial proteoglycans is increased. This binding leads to prolonged retention time of desialylated sdLDL in the subendothelial intima (86). The ability of sdLDL to accumulate in the cells and extracellular matrix of the intima leads us to a conclusion that this fraction of LDL corresponds to naturally occurring atherogenic lipoprotein circulating in the bloodstream.

Using methods of separation that are sensitive to particle's electric charge, such as agarose gel electrophoresis, isotachophoresis or ion exchange chromatography, electronegative LDL (LDL(-)) can be revealed in circulation (12, 87). LDL(-) was first discovered by Avogaro and co-authors (12). Currently, at least five subfractions of LDL(-) with different degrees of electronegativity can be determined (88, 89). LDL(-) is associated with cardiovascular risks and myocardial infarction (90-92). LDL(-) particles are also prone to aggregation (12, 93), which promotes accumulation of intracellular cholesterol (94-97). It was demonstrated that atherogenic modification of lipoprotein is responsible for its aggregation (97). Moreover, LDL(-) is also characterized by altered structure of its protein content (98). In addition, changes in the lipid moiety of LDL(-) may also contribute to its tendency to aggregate (99-101). Modifications of apoprotein structure in LDL(-) impairs its binding to the LDL receptor, and thus leads to prolonged circulation times (102, 103). On the other hand, LDL(-) binds to the lectin-like oxidized LDL receptor 1 (LOX-1) (90, 104). LDL(-) retention takes place in the subendothelial intimal space due to its binding to the proteoglycans. Subendothelial intimal cells take up LDL(-) aggregates via a nonspecific way, which contributes to foam cell formation. Complexes of LDL(-) with anti-LDL autoantibodies also participate in this process (105).

The optimal method to study naturally occurring modified LDL is isolation of LDL from the blood of atherosclerotic patients. Indeed, LDL isolated from patients with coronary atherosclerosis in most cases caused accumulation of the intracellular lipids in cultured cells, while LDL obtained from healthy individuals possessed no such effect (106, 107). In 1989 it was discovered that total content of sialic acid (N-acetylneuraminic acid) in atherogenic LDL from atherosclerotic patients was significantly lower than that in non-atherogenic LDL (108, 109). Later, the existence of LDL in the plasma was confirmed by other groups (110, 111). Different values of the degree of LDL desialylation were reported in different groups, but this is most likely the result of methodological shortcomings (112, 113). Therefore, circulating atherogenic LDL is most likely desialylated.

Desialylated LDL subfraction could be purified by lectin affinity chromatography (114). This fraction induced intracellular lipid accumulation in cultured cells, while sialylated LDL possessed no atherogenicity. The atherogenicity of desialylated LDL, that is, its ability to induce accumulation of intracellular cholesterol, has been shown by several groups (108, 115, 116). Desialylated LDL differs considerably from native LDL with respect to carbohydrate and lipid composition, including N-acetylgalactosamine, N-acetylglucosamine, galactose and glucose contents (117). The levels of free cholesterol, cholesteryl esters and triglycerides were found to be lower in desialylated subfraction of LDL, while the levels of monoglycerides and free fatty acids were higher than in native LDL (118, 119). The levels of free fatty acids, mono- and diglycerides were lower in desialylated LDL. Desialylated LDL is also denser than native LDL (120). Analysis of LDL subfractions showed that the increase of LDL density is accompanied with decreased content of phospholipids, free and esterified cholesterol and triglycerides and smaller particle size, decreased content of sialic acid and higher atherogenicity. It was shown that desialylated LDL has higher electrophoretic mobility than native LDL (120).

In desialylated LDL a part of amino groups of apoB is chemically modified, while another part is

**Table 1.** Affinity constants of lipoprotein-anti-LDL Interaction

| Lipoprotein | Affinity constant (x $10^{-7}$ $M^{-1}$) | LDL-induced Cholesterol increment (% over control) |
|---|---|---|
| LDL from healthy subjects | 2.4 | 2±10 |
| Glycosylated LDL | 2.6 | 105 ±14[1] |
| Acetylated LDL | 2.8 | 163±15[1] |
| Cu2+-oxidized LDL | 3.5 | 250±18[1] |
| Lipoprotein(a) | 3.6 | 102±7[1] |
| MDA-LDL | 10.9 | 305±21[1] |
| LDL from atherosclerotic patients | 11.3 | 246±31[1] |
| Desialylated LDL | 89.4 | 290±32[1] |

[1]Significant difference from LDL from healthy subjects (p<0.0.5). Data from one of three representative experiments are presented. The affinity constant of anti-LDL was determined using native and modified ($^{125}$I)LDL of known specific activity (200-1,000 cpm/ng apolipoprotein B). Known amounts of added radioactive and nonradioactive LDL and the amount of ($^{125}$I)LDL bound to anti-LDL were used to calculate the affinity constant. Adapted with permission from (80).

masked due to the changes in tertiary structure of apoB (120).

The degree of LDL oxidation is determined by measuring apoB-lipid adducts: the content of apoB-bound cholesterol in LDL reflects the degree of LDL oxidation. This parameter was found to be considerably higher in desialylated than in native LDL (121). Thus, desialylated LDL is oxidized lipoprotein. Desialylated LDL possesses higher oxidizability and susceptibility to *in vitro* oxidation compared to native LDL (120). Increased degree of oxidation and oxidizability of desialylated LDL may be explained by reduced contents of the major fat-soluble antioxidants in lipoprotein particles, such as coenzymeQ10, α-and y-tocopherols, β-carotene and lycopene.

Anti-LDL autoantibodies were isolated from the sera of atherosclerotic patients (80). Table 1 shows the affinity constants of isolated anti-LDL to different lipoproteins. As compared to LDL obtained from healthy individuals, anti-LDL autoantibodies show higher affinity for LDL isolated from atherosclerotic patients, as well as for MDA-LDL. LDL desialylated *in vitro* by neuraminidase possessed the highest affinity constant. It can be concluded that autoantibodies against LDL are produced in response to desialylated LDL in the bloodstream. Cross-reaction with the MDA-LDL may be explained by the similar conformation of certain epitopes of desialylated LDL and MDA-LDL.

### 4.3. Mechanisms of modified LDL-induced atherogenesis

Intracellular lipid accumulation, enhanced proliferative activity and increased synthesis of fibrous components by subendothelial intimal cells are considered as manifestations of atherosclerosis at the arterial cellular level. In addition to intracellular lipid accumulation, circulating desialylated LDL increases proliferative activity and total protein synthesis, including that of collagen and glycosaminoglycans by cells cultured from human aortic subendothelial intima (122). By contrast, native LDL does not affect cellular proliferation and extracellular matrix synthesis.

It was shown that pre-incubation of cultured cells with desialylated LDL that induced accumulation of intracellular lipids was sufficient to stimulate both proliferation and extracellular matrix synthesis. All forms of modified LDL have a tendency to self-association, and the large associates of LDL, but not the dispersed particles, cause the accumulation of intracellular lipids. Even native LDL in large associates with naturally occurring (collagen, elastin, fibronectin) or artificial (latex particles, dextran sulfate) compounds induced intracellular lipid accumulation and stimulated both proliferative and synthetic activities when added to cultured cells. The increase of proliferative and synthetic activities in cultured cells correlated with the amount of accumulated intracellular cholesterol (122, 123).

Therefore, intracellular lipid accumulation induced by modified LDL causes an increase of proliferative activity and synthesis of the connective tissue matrix components. Accumulation of intracellular lipids does not occur without complex formation of LDL particles, which is dependent on LDL modification. It can be concluded that modified LDL participates enter the cells bypassing the specific LDL receptor and thereby induce abnormal accumulation of intracellular lipids, which in turn can lead to all known atherosclerotic manifestations at the cellular level.

### 4.4. Probing for the role of modified LDL in atherosclerosis: *in vitro* and clinical studies

To date, a considerable amount of experimental data supporting the mechanistic role of modified LDL in atherogenesis is accumulated, at least, regarding the intracellular lipid deposition. Most of these data were obtained in *in vitro* experiments in different

cell culture-based models, and have demonstrated that modified low density lipoprotein, in contrast to LDL from healthy donors, is able to induce intracellular lipid accumulation as the first manifestation of atherogenesis at the cellular level. Therefore, a consensus has been reached that LDL must undergo some kind of chemical modification rendering it atherogenic (124). However, all the available studies had several limitations. First, there were too many possible types of LDL *in vitro* modification, including acetylation, maleylation, metal-dependent oxidation, reactive oxygen species-dependent oxidation, non-enzymatic glycation, enzymatic deglycosylation, methylation, malonic dialdehyde treatment, etc. Most of these reactions have not been observed in circulation, except for non-enzymatic glycation and enzymatic modification (e.g. desialylation) (125-132). Moreover, LDL containing increased amounts of oxidized lipids and phospholipids, and, LDL presumably modified by phospholipase A2, have been described (133, 134).

We should also mention sdLDL and LDL(-) with demonstrated atherogenicity that are definitely present in circulation (135-139). Noteworthy, desialylated, electronegative and sdLDL are likely to represent the same LDL subfraction. The difference between these subfractions can be explained by using different methods of isolation (140-143).

Furthermore, in most studies that utilized *in vitro* oxidized LDL as a source of accumulated lipids, no special focus was made on how and in what manner modified LDL were obtained; however, it is difficult to believe that different methods of LDL modification produce the same type of modified LDL.

Next, not all cell types are suitable for studying atherosclerosis-related processes. The most reliable cells are monocytes-macrophages and subendothelial arterial cells, i.e. cell types that immediately participate in the formation of atherosclerotic lesions in humans (144, 145). To some extent, linear cells of monocytic origin may also represent adequate models.

In general, the available *in vitro* studies conducted on different cell culture-based models have provided a sufficient background to support the hypothesis that modified LDL may be the actor in the processes of atherogenesis, but provided no information for characterization of modified LDL as the biomarkers of atherosclerosis.

Studies in animal models provided additional data to favor the hypothesis on atherogenic modified LDL. However, it should be mentioned that no animal model that fully replicates the human atherosclerosis could be created. At present, widely used mouse models of atherosclerosis can be used to mimic a single pathogenic aspect of human atherosclerosis, namely, the dyslipidemia. The most widely used mouse models are the apoE-knockout mice that develop atherosclerotic lesions on a chow diet, and the LDL-receptor-knockout mice on a high-cholesterol diet, with LDL receptor deficiency causing a strong increase of LDL level and the development of atherosclerotic lesions (146). Therefore, animal models reproduce the effects of hypercholesterolemia, and may be applicable for the studies of hereditary disorders, but not of human atherosclerosis in general.

Numerous clinical studies that were focused mainly on the role of modified LDL as the biomarker of atherosclerosis-related diseases had also certain limitations. One of them was the wide use of the term “oxidized LDL”, which caused some confusion. In the clinical studies assessing clinical relevance of modified LDL, confusing methodology of measurement was used (e.g., the use of anti-MDA-LDL antibodies to measure ostensibly “oxidized” LDL). Failing to directly demonstrate the presence of oxidized LDL subfraction in the bloodstream, the term “minimally oxidized LDL” was introduced, which should mean some hypothetical particle that cannot be distinguished or characterized by existing methods, but which actually exists, penetrates the arterial wall, undergoes further modification, and causes atherosclerosis (133). Unfortunately, such an understanding of the processes of atherogenesis has become widely accepted and left no room for further revision. The experimental and clinical data on oxidative LDL modification are based either on indirect evidence, such as the results of measurements of oxidized LDL levels with anti-MDA-LDL or anti-copper-oxidized LDL antibodies, or on direct markers of LDL oxidative damage, such as oxidized lipids and phospholipids. The first aspect has already been discussed above. The second one may be due to the retarded LDL catabolism, thus reflecting the presence of senescent LDL particles in the bloodstream, disregarding their pathogenic impact. Therefore, in contrast to the *in vitro* and animal experiments, clinical studies omitted the mechanistic significance of modified LDL in atherosclerosis, but tried to position it as a biomarker. It has to be admitted that the attempts made were unsuccessful, leading to no final conclusions on the sensitivity and specificity of modified/oxidized LDL.

More decisive information on the role of modified (oxidized) LDL in atherosclerosis was expected to come from the results of several clinical randomized trials. It was an attempt to solve the abovementioned problem, based on the hypothesis of oxidative proatherogenic LDL damage. Indeed, if LDL oxidation is the main reason for launching atherogenesis, the antioxidant treatment should be a radical and effective preventive measure. To provide a background for these trials, data from early observational studies were taken into account, demonstrating an inverse relationship between the intake of dietary antioxidants,

**Table 2.** Characteristics of atherogenic LDL found in human blood plasma

| Parameter | Desialylated LDL | LDL(-) | Small dense LDL |
|---|---|---|---|
| Atherogenicity | ↑ | ↑ | ↑ |
| Size | ↓ | ↓ | ↓ |
| Density | ↑ | ? | ↑ |
| (-) Charge | ↑ | ↑ | ↑ |
| Sialic acid | ↓ | ↓ | ↓ |
| Cholesteryl esters | ↓ | ↓ | ↓ |
| Phospholipids | ↓ | ↓ | ↓ |
| Protein/lipids | ↑ | ↑ | ↑ |
| Oxidizability | ↑ | ↑ | ↑ |
| Antioxidants | ↓ | ↓ | ↓ |
| Amino group modification | ↑ | ↑ | ? |
| Self-association | ↑ | ↑ | ↑ |

like α-tocopherol, β-carotene, and ascorbic acid, and major cardiovascular events. However, these randomized trials yilded disappointing results, with a lack of risk reduction, or even risk elevation, in antioxidant-treated patients (147). Despite the several plausible explanations for negative results that have been proposed (antioxidant choice, dosage, combinations of antioxidant drugs, patient selection, etc.), the topic of oxidized LDL in atherosclerosis was closed. Finally, American Heart Association did not recommend antioxidant vitamin supplements to prevent cardiovascular diseases (148). However, some serious omissions were made in the conducted clinical studies. None of the large studies was focused on the monitoring of modified LDL level or aimed to eliminate the modified LDL from circulation. None of the studies used drugs capable of preventing lipid deposition in the arterial wall. Therefore, the question on the role of modified LDL as a mechanistic factor or the comprehensive biomarker of atherosclerosis remains open.

## 5. STUDIES OF DESIALYLATED LDL

As mentioned above, atherogenic LDL circulating in the blood is small, dense, desialylated and more electronegative. It was shown that LDL(-) isolated by ion-exchange chromatography corresponded to desialylated LDL (75). On the other hand, desialylated LDL, which is a small dense lipoprotein, was also more electronegative (118, 121). On the other hand, it was demonstrated that sdLDL has a low content of sialic acid, i.e., it is desialylated (85). These findings point to a similarity between the three types of modified LDL. Table 2 shows changes in chemical and physical parameters of atherogenic LDL discovered in the blood of atherosclerotic patients. These data demonstrate a remarkable similarity between characteristics of all three kinds of LDL, suggesting that sdLDL, desialylated LDL and LDL(-) are similar if not identical. All kinds of naturally occurring atherogenic LDL determined by different methods are represented by the same lipoprotein particle that had undergone multiple modification.

Naturally occurring atherogenic LDL particles have modified lipid, protein and carbohydrate moieties. Therefore, such the lipoprotein particles may be regarded as multiplemodified LDL. The correlations between alteration of chemical and physical parameters of modified LDL and its ability to induce intracellular lipid accumulation have analyzed to reveal what kind of modification determines atherogenicity of LDL. The only significant negative correlation was found between LDL atherogenic potential and the sialic acid level. Such parameters as LDL size and charge, amount of free lysine amino groups, the contents of phospholipids and neutral lipids, fat-soluble antioxidants and lipid peroxidation products, the degree of oxidation and oxidizability of LDL, did not correlate significantly with atherogenicity (117, 120). This suggests that desialylation is the most important modification resulting in LDL atherogenicity.

The special importance of desialylation in the atherogenic modification of circulating LDL was demonstrated when LDL was incubated with the blood plasma of atherosclerotic patients (149). A decrease of the sialic acid content in initially non-atherogenic LDL was observed after 1 h of incubation (Table 3).

Further incubation led to a continued decrease of the sialic acid content, and, after 3 hours, LDL became atherogenic *i. e.* was able to cause accumulation of lipids in cultured cells. After 6 hours, a decrease in the content of free cholesterol was observed, and after 12 hours the content of cholesteryl esters and phospholipid dropped. By this time, the particle size of the LDL has

**Table 3.** Scheme of LDL modification

| Hour | | | | | | |
|---|---|---|---|---|---|---|
| **1** | **3** | **6** | **12** | **24** | **36** | **48** |
| ↓ Sialic acid | ↑ Atherogenicity | ↓ Free cholesterol | ↓ Size | ↓ Triglycerides | ↑ Electronegativity | ↑ apo B-bound cholesterol |
| ↑ % of desialylated LDL | | | ↓ Cholesteryl esters | | | ↑ Susceptibility to oxidalation |
| | | | ↓ Phospholipids | | | ↑ Fluorescence |
| | | | | | | ↓ Vitamin E |

Adapted with permission from (149).

decreased. After 24 hours, the content of triglycerides decreased. After 36 hours, LDL particles became more electronegative. The content of triglycerides decreased after 24 hours. After 36 hours of incubation, LDL particles became more electronegative. A longer incubation of LDL with plasma for 48-72 hours resulted in a loss of α-tocopherol, increase of LDL susceptibility to oxidation and to accumulation of cholesterol covalently bound to apoB and increase of auto-fluorescence: markers of lipoperoxidation of LDL particle. In parallel, degradation of apoB was observed.

Therefore, desialylation of LDL particles is one of the first, or even the primary event in the cascade of multiple modifications. Presumably, desialylation is a necessary and sufficient condition for the appearance of atherogenic properties in LDL. The cascade of multiple modifications of LDL in blood plasma leads to significant physico-chemical changes in LDL: desialylation, loss of lipids, reduction in the particle size, increase of its electronegativity and to peroxidation of lipids. This concept can explain the presence of desialylated LDL, sdLDL, LDL(-) and oxidized LDL in the bloodstream. It should be specially emphasized that, contrary to the widespread belief, the oxidation of LDL is neither the only, nor the most important atherogenic modification, because oxidation occurs at the final stages of multiple modification cascade and does not significantly increase the atherogenic potential of already multiply modified LDL.

Since desialylation appears to be the most important atherogenic modification in the cascade of the multiple modification of LDL, it is essential to establish its mechanism. In the blood of atherosclerotic patients, sialidase (141) and trans-sialydase activity was found (130). Isolated trans-sialidase caused desialylation of native LDL. LDL treated with trans-sialydase induced cholesteryl ester accumulation cultured cells (130). Therefore, in can be concluded that trans-sialidase is responsible for LDL desialylation and for foam cell formation. Trans-sialidase activity is present both in lipoproteins and in free form. Isolated trans-sialidase is a protein of about 65 kDa. The content of enzyme in blood serum varies from 20 to 200 μg/ml. There are three pH optima: 3.0., 5.0. and 7.0. Calcium and magnesium ions stimulate trans-sialidase activity. Presence of SH-groups is essential for enzyme activity. LDL, IDL, VLDL, and HDL particles can serve as donors for trans-sialidase. Sialylated LDL is a preferable substrate in comparison to sialylated VLDL, IDL and, especially, HDL.

Trans-sialidase may play a very important role in atherogenesis. LDL desialylated by trans-sialidase causes intracellular lipid accumulation and of proliferation and synthesis of extracellular matrix. Thus, LDL desialylation induced by trans-sialidase leads to all known cellular manifestations of atherosclerosis.

Theoretical calculation has established that the time needed to transformation of arterial cells into foam cells under the influence of native LDL is around 130 years. Under the influence of atherogenic modified LDL, this time is shortened up to 15 years, which is still slower than the known rate of atherosclerosis development. The real rate of the foam cell formation should be much higher than the calculated value. It is suggested that the mechanisms potentiating the atherogenicity of modified LDL may come into play. At least three such the mechanisms have been identified: self-association of LDL particles, formation of LDL-containing immune complexes and formation of LDL associates with the connective tissue matrix components.

Modified LDL particles spontaneously associate *in vitro* under the conditions of cell culture while native LDL remains dispersed (94-96), with a positive correlation between atherogenicity of modified LDL and the degree of LDL association (95, 96). Removal of LDL associates from the incubation medium by filtration completely prevented intracellular lipid accumulation. Therefore, self-association increases atherogenic potential of LDL. The uptake of associated LDL is considerably higher than that of non-associated LDL particles (94). Cytochalasin B, an inhibitor of cellular phagocytosis, and latex beads, phagocytosis cargo, inhibit the uptake of LDL associates, which indicates that LDL

associates are taken up by phagocytosis (94-96). The rate of intracellular degradation of associated modified LDL was significantly lower than that of non-associated particles. Thus, the high atherogenicity of lipoprotein associates is a result of enhanced uptake by phagocytosis and a low rate of intracellular degradation.

Multiple modification of LDL particles may provoke production of autoantibodies. Indeed, circulating immune complexes consisting LDL and anti-LDL autoantibodies were found in blood of most coronary atherosclerosis patients (150-154). A positive correlation between blood serum levels of LDL containing immune complexes and the severity of coronary and extra-coronary atherosclerosis has been demonstrated (153, 154). LDL isolated from circulating immune complexes was desialylated, small dense, more electronegative, with low contents of neutral lipids and phospholipids, as well as neutral saccharides; conformational changes in tertiary structure of apoB were also observed (152). Thus, LDL from circulating immune complexes was identical to multiple modified LDL.

The complexes of autoantibodies with native LDL stimulate lipid accumulation in cultured cells and potentiate atherogenicity of desialylated LDL (80, 153). Binding of C1q complement component and fibronectin to the LDL-autoantibody complex resulted in a more pronounced intracellular lipid accumulation. It was also demonstrated that LDL can form associates with cellular debris, collagen, elastin and proteoglycans of human aortic intima that cause lipid accumulation in cultured cells (155, 156).

Therefore, formation of large associates containing modified LDL (self-associates, immune complexes and associates with the connective tissue matrix) markedly increases atherogenic potential of modified lipoproteins.

## 6. CONCLUSION

The current knowledge on modified LDL and dysfunctional LDL invites us to focus on several aspects. First of them is the role of modified LDL as comprehensive biomarkers of atherosclerosis. It is noteworthy that each year, tens of biomarkers are claimed to be added to the laboratory repertoire, including modified LDL and dysfunctional HDL. However, the number of novel biomarkers that reach common clinical acceptance and implementation into the laboratory practice remains very small. There is no established set of criteria or requirements that should be used to define any measurable parameter as a biomarker. However, we can point to such characteristics of biomarkers as diagnostic effectiveness (sensitivity and specificity, proven significant diagnostic efficiency or beneficial change in treatment, or, ideally, both), analytical stability (evidence-based analytical performance of the assay, pre-analytical sample handling factors and storage stability, the ability to be measurable in routine clinical laboratory practice, easiness of laboratory handling), plausibility (well-understood pathobiology of a biomarker, its origins and the relationship to the medical condition), and cost-effectiveness (cost minimization, life years gained, health state preference value, monetary gains). Nothing of the above is known for modified LDL and dysfunctional HDL. Moreover, the following questions are still unanswered. Had these markers been measured with an appropriate method and been shown to be additive to or replace conventional tests? Have there been independent studies? Has there been a multicenter study? Has meta-analysis of evidence been performed? Have there been randomized clinical trials? Is it possible to measure these biomarkers in a conventional laboratory without additional equipment and/or staff? (157). Unfortunately, the answer to all these questions is negative. Thus, current knowledge does not allow for positioning modified LDL and dysfunctional HDL as biomarkers of atherosclerosis, even formally.

However, just the same current knowledge may allow formulating the hypothesis that modified LDL may have a mechanistic effect at a certain or even initial stage of atherogenesis, that is, to act as a trigger of the pathologic process. Further course of atherosclerotic pathology may be less evident due to the presence and impact of modified LDL, and over the time of atherosclerosis progression the significance of modified LDL as a biomarker may even continue to decline. In any case, modified LDL should not be considered as “innocent witnesses”, since there is a sufficient evidence base indicating its mechanistic role. By contrast, the role of dysfunctional HDL remains completely unclear, thus providing a wide field and perspective for research.

## 7. ACKNOWLEDGEMENT

The authors declare no conflict of interest. This work was supported by Russian Science Foundation (Grant # 14-15-00112).

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**Key Words:** Atherosclerosis; cardiovascular disease; LDL; HDL; modified LDL; lipidosis

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