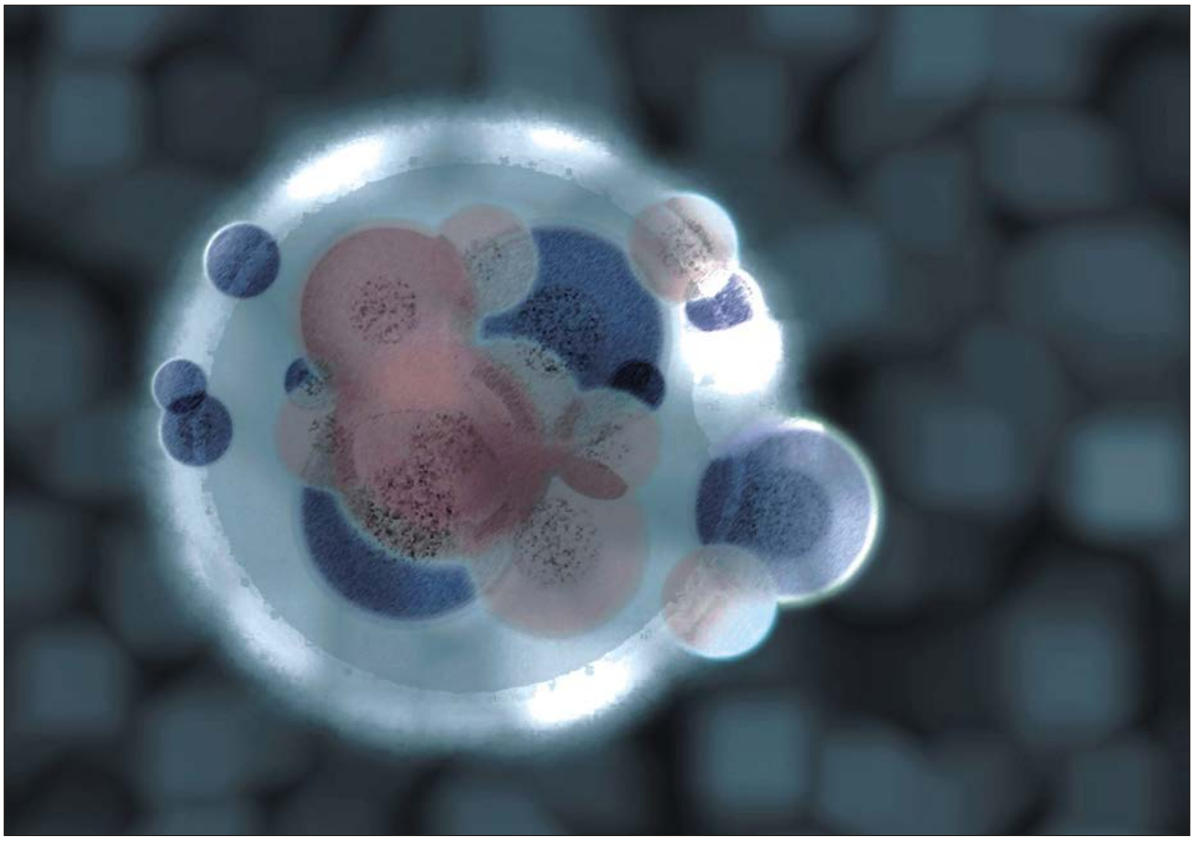

Journal of Biological Sciences

ISSN 1727-3048

science alert

ANSInet
an open access publisher
http://ansinet.com

Journal of Biological Sciences 10 (8): 785-789, 2010
ISSN 1727-3048

A Modification Method for Isolation and Acetylation of Low Density Lipoprotein of Human Plasma by Density Discontinuous Gradient Ultracentrifugatio

[1]J.Z. Reza, [2]A. Nikzamir, [3]M. Doosti and [4]M.S. Pour
[1]Department of Biochemistry, Faculty of Medicine,
Shahid Sadoughi University of Medical Sciences, Yazd, Iran
[2]Department of Biochemistry, Faculty of Medicine,
Ahwaz Jondi Shapour University of Medical Sciences, Ahwaz, Iran
[3]Department of Clinical Biochemistry, Faculty of Medicine,
Tehran University of Medical Sciences, Tehran, Iran
[4]Islamic Azad University, Parand Branch, Iran

Abstract: There is now a consensus that atherosclerosis represents a state of heightened oxidative stress characterized by lipid and protein oxidation in the vascular wall. The purpose of this study was to evaluate a simple and precise manner in which to assess the LDL and modified LDL for clinical and experimental application. A step density gradient was constructed from NaCl and KBr solutions varying in density(d) from 1.035 to 1.1 g mL^{-1} and from 5.5 mL of pool plasma of 32 healthy normolipedemic men (23-25 years old) was adjusted to d 1.09 g mL^{-1}. The sample was Separated was after a single ultracentrifugation 40,000 g for 10 h at 4°C in a swinging bucket rotor. Centrifugation showed four density layers d = 1.1 g mL^{-1}, d = 1.09 g mL^{-1}, d = 1.065 g mL^{-1}, 1.035 g mL^{-1}. Isolated LDL was acetylated by saturated sodium acetate and acetate anhydride. Acetylated LDL (ac-LDL) has different physico-chemical properties than native LDL. The acetate cellulose electrophoresis was applied for analysis of electrophoretic properties native and ac- LDL. Ac-LDL moved 1.5 times faster than native LDL. This study support a simple and precise manner in which to assess the LDL and modified LDL (oxidized -LDL and acetylated-LDL) for clinical and research purposes.

Key words: Atherosclerosis, lipoproteins, native-low density lipoprotein, modified low density lipoprotein

INTRODUCTION

Atherosclerosis is the major source of morbidity and mortality in the developed world. Atherosclerosis is characterized by the accumulation of cholesterol deposits in macrophages in large- and medium-sized arteries. This deposition leads to a proliferation of certain cell types within the arterial wall that gradually impinge on the vessel lumen and impede blood flow. The association between LDL cholesterol and atherosclerosis has been established based, in part, on an experiment of nature (Stocker *et al*., 2004; Mackness and Mackness, 2004).

Triacylglycerols (TG) and Cholesterol Esters (CE) not enable to move within the water based solution of the Blood stream, so they carry out in blood by Lipoproteins (Colpo, 2005). Lipoproteins are five major groups (Chylomicrons (CM), VLDL, LDL, HDL). Low-density lipoprotein (LDL) is a lipoprotein that transports CE and TG from the liver to peripheral tissues (Brousseau *et al*., 1993).

Most studies have indicated that modified LDL particles contribute in cardiovascular Diseases (Virella *et al*., 2000). The most modified LDL is oxidized LDL. Oxidized LDL modifications are a prerequisite for macrophage uptake and their transformtion into foam cells (Yla-Herttuala *et al*., 1990). The LDL that has extracted from human and rabbit atherosclerotic lesions, indicates nearly all of the physicochemical and immunologic properties of LDL oxidized (Yla-Herttuala *et al*., 1989). Furthermore, modified LDL can trigger an autoimmune response that leading to the formation of autoantibodies (Salonen *et al*., 1992). *In vivo* condition commonly use for acetylated LDL because its construction is very fast and simple. Also, the effects of ox-LDL and ac-LDL in many aspects are similar. Several procedures are present for acetylating LDL. The oxidative modification of low density lipoprotein (LDL) may play an important role in the pathogenesis of atherosclerosis.

Therefore nowadays modified LDL-C has very application in molecular biology of Coronary Heart

Corresponding Author: Abdolrahim Nikzamir, Department of Biochemistry, Faculty of Medicine,
Ahwaz Jondi Shapour University of Medical Sciences, Ahwaz, Iran Tel: +986113332036

Diseases (CHD) and for *in vivo* cellular model (Berliner and Suzuki, 1996; Kamps *et al*., 1992; Higaki *et al*., 1993; Wang *et al*., 2007; McPherson *et al*., 2007). Ac-LDL internalized by monocyte-derived Macrophages that associate with atherosclerotic lesions. In contrast, ac-LDL was not located in nonactivated monocytes adherent to the lesion surface (Landers and Lewis, 1993). Preconditioning cells with glutathione-ethyl ester (GSH-Et) indicate that Protein Kinase-C (PKC) pathway regulates ac-LDL binding and decrease reactive oxygen intermediate generation and apoptotic conversion in foam cells. These effects can be prevented by a PKC inhibitor (calphostin C) (Rosenson *et al*., 2005). Ac-LDL used for identifying macrophage like cells in variety of tissues (Victor *et al*., 2002). Therefore preparation these molecules is important in clinical and research goals.

MATERIALS AND METHODS

Blood sample: Human LDL was obtained from the blood of healthy volunteers of healthy, normolipidemic males, in the department of Clinical Biochemistry, Faculty of Medicine, Tehran University of Medical Sciences, Tehran, Iran from May 2006 through April 2008. Pool plasma was isolated from healthy, normolipidemic male volunteers 23 to 35 years old and all donors had fasted overnight for 12 to 14 h.

The venous blood (10-15 mL) was collected from every volunteers in bottles containing Na_2EDTA (final concentration 1 mg mL^{-1}) and plasma was separated by centrifugation (1500 g, 30 min) after 10 min at 4°C. Plasma sample for lipoprotein isolation was stored in -70°C.

Parameter of plasma lipids: Total plasma lipid and TG concentrations were estimated by using an enzymatic method (Pars Azmon, Tehran, Iran), apolipoproteins (apo) and CRP by ELISA methods (Pars Azmon, Tehran, Iran) and Lp(a) by turbidmetric Kit (Bohringer Minhayme, Germany).

Isolation of plasma low density lipoprotein

Gradient construction: For LDL isolation the gradient making construction is necessary. The salt stock solution contains NaCl (153 g L^{-1}) and KBr (354 g L^{-1}) and its density was 1.346 g mL^{-1}. Solutions with lower density were made by dilution of the stock with 0.15 M NaCl solution (d = 1.005) according to this formula:

$$M_{KBr} = \frac{V\,(d_2 - d_1)}{(1 - \bar{v}_5)\,d_2}$$

Where:

M_{KBr} = Mass of Kbr
V = Volume to be adjusted
d_1 = Density of the first solution at 5°C
d_2 = Density of the second solution at 4°C
$\bar{v}_5$ = Partial specific volume of KBr at 4°C

First, the density of plasma was raised by addition KBr and NaCl to 1.09 g mL^{-1} (Mori *et al*., 2001; Geiss *et al*., 2004; Chapman *et al*., 1988).

Isolation of LDL: Discontinues density gradients were constructed in a polyallomer tube (9/61 diam × 31/2), 2 mL of a NaCl-KBr solution of density 1.1 g mL^{-1} then, were pumped into button tube. The following solutions were layered onto the letter with the aid of the density-flow: 5.5 mL plasma (d = 1.09 g mL^{-1}), 1.5 mL of NaCl-KBr solution (d = 1.065 g mL^{-1}) and 1mL of NaCl-KBr solution (d = 1.035 g mL^{-1}). Immediately upon complication, the gradients were centrifuged at 40000 rpm for 10 h at 4°C in a SW41-ti swing bucket rotor (Beckman Coulter, Inc., Palo Alto, CA) (Havel *et al*., 1955).

Acetylating of LDL: Isolated LDL was acetylated by Fraenkel-Conrat (1957). Then 0.5 mL of 0.15 M NaCl solution containing 14 mg of LDL protein was added to 0.5 mL saturated sodium acetate and starring in ice-water bath, next over a period of 1 h with continuous stirring aliquots (2 mL) of acetate anhydride. After addition acetic anhydrate equal to 1.5 times the mass of LDL used, mixture was stirred for 30 min. Finally, the mixture was dialyzed for 24 h at 4°C against 12 L of 0.15 M NaCl, 0.3 EDTA and pH 7.4.

Separation of LDL by electrophoresis on acetate cellulose: Aliquots (1-2 μL) of whole plasma, native LDL or isolated LDL and acetylated LDL were elcterophoresed for 40 min with 200 volt on acetate cellulose films (Magnani and Howard, 1971) barbiturate buffer (pH=8.65) using Bio-Rad system. The cellulose acetate plate was placed in buffer for about 15 min before the electrophoretic separation. On completion of electrophoresis, slides were stained for lipoprotein with Pansue S (0.5 g/100 H_2O) for 10 min and were destained with Acetic acid (5%) (Bradford, 1976).

Assay: The content of protein was determined by Bradford Method that can be found elsewhere (Basu *et al*., 1976).

RESULTS

Ultra centrifugation plasma was done at the following densities: 1.035 to 1.1 g mL^{-1}, total cholesterol,

Table 1: The parameters of plasma lipids, apolipoproteins and TG concentrations in male blood donors

Variables	Mean (mg dL^{-1})±SD
Total cholesterol	161.56±24.45
Triglyceride	129.15±73.68
HDL-C	43.12±9.20
LDL-C	92.15±15.91
ApoA	165.25±16.51
ApoB	79.59±19.65
Lp(a)	17.40±12.56
CRP	1.47±1.74

Data are Mean±SD

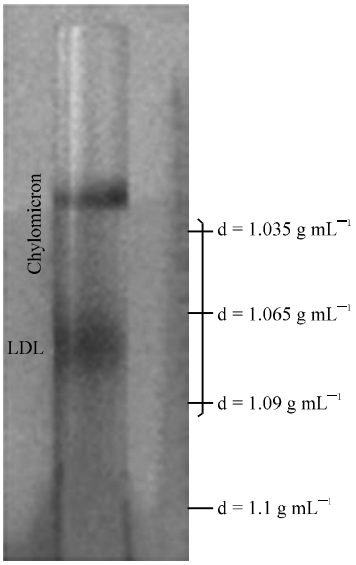

Fig. 1: Schematic representation of the distribution of Lipoproteins bands in the density discontinuous gradient at 10 h ultracentrifugation (40000 rpm). LDL located between 1.035 and 1.09 g mL^{-1}

Triglycerides, HDL-C, LDL-C, Apo A, Apo B, LP(a) and CRP contents in fractions were 161.56±24.45, 129.15±73.68, 43.12±9.20, 92.15±15.91, 165.25±16.51, 79.59±19.65, 17.40±12.56 and 1.47±1.74, respectively (Table 1).

Gradient ultracentrifugation: After density discontinuous gradient ultracentrifugation, four bonds observed: d = 1.1 g mL^{-1}, d = 1.09 g mL^{-1}, d = 1.065 g mL^{-1}, 1.035 g mL^{-1} (Fig. 1).

Electrophoresis of AC-LDL on cellulose acetate slides: Plasma and the obtained lipoprotein fractions were submitted to electrophoresis in acetate cellulose slide. Two lipoprotein fractions were seen in the plasma sample (Fig. 2), corresponding to AC-LDL and Native-LDL.

Electrophoresis of the acetylated-LDL prepared in acetate cellulose at barbiturate buffer with pH 8.65, indicated that the acetylated-LDL have a enhanced charge as compared with native LDL (Fig. 2).

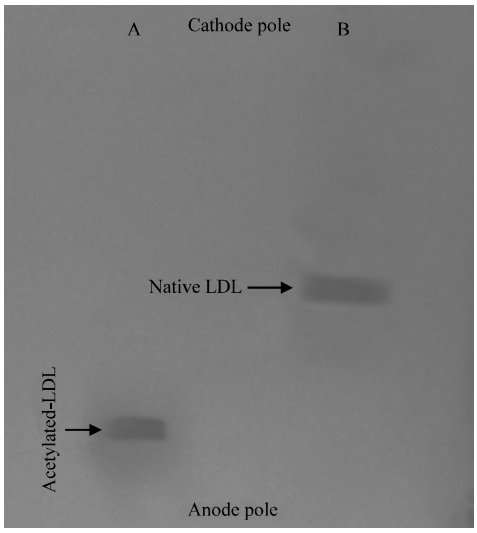

Fig. 2: Electerophoretic pattern of AC-LDL. Five micrograms of each LDL preparations and plasma were subjected to electrophoresis in acetate cellulose slide at barbiturate buffer, pH 8.65. Slides stained with Pansue S. (A) Acetylated-LDL; (B) native LDL

DISCUSSION

Most studies have indicated that the modified lipoproteins trigger an immune response that leading to the production of autoantibodies and subsequently to the formation of atherogenic immune complexes (Virella *et al*., 2000; Yla-Herttuala *et al*., 1989, 1990; Salonen *et al*., 1992; Mironova *et al*., 2000; Bergmeier *et al*., 2004). Therefore modified LDL has many applications in Coronary Artery Disease (CAD) studies. Varity of modified LDL, included ox-LDL and ac-LDL, are present. These modified partical have very similar effects, so *in vitro* studies used ac-LDL because simple and fast preparations. Sequential flotation ultracentrifugation is commonly used in the preparation of plasma lipoproteins. However, protocols often require prolonged centrifugation time (48-72 h) and large plasma volumes (2-20 mL), which makes them unsuitable for studies on small laboratory animals (Victor *et al*., 2002).

Several preparative and analytical methods have been proposed to isolate plasma lipoproteins by ultracentrifugation. Most of them are very time consuming and laborious and damage the particle integrity. But our procedure describes a fast and safe method for preparation and modifying of Low Density Lipoprotein (LDL) of human plasma in large scale and lower time.

Albumin apparently contaminates the VLDL, IDL and HDL fractions, but cannot made problem in our method because the presence of albumin does not interfere with

lipid and apolipoprotein determination, acetylation or electrophoresis processes. Therefore we did not performed further purification for our subsequent analytical studies.

Ultracentrifugation time in this study was 10 h but in other experiments, this time was 18 h (Chapman *et al*., 1988). Therefore we showed that LDL isolation in shorter times can mad without effect on the electerophoretic and sedimentation properties.

Chapman *et al*. (1988) isolated the major lipoproteins classes, VLDL (d<1.016 g mL^{-1}), LDL (d = 1.028-1.050 g mL^{-1}), HDL_2(d = 1.066-1.1 g mL^{-1}) and HDL_3 (d = 1.1-1.53 g mL^{-1}) from human plasma and analyzed the chemical, physical and immunological features of this classes. In this study we used chapman method with minor modifications and isolated LDL band (Fig. 1).

Brousseau *et al*. (1993) described a micromethod for separation of plasma Lipoproteins. They showed that centrifugation in short time has less deleterious effects on the lipoprotein structure.

Basue *et al*. (1976) studied electerophoretic properties of ac-LDL used the agarose gel at pH 8.6 and indicated that the acetyl-LDL has enhanced negative charge as compared with native LDL. Present study showed that acetate cellulose electrophoresis has excellent efficacy. This method is very fast and simple; therefore acetate cellulose is better than agarose electrophoresis. Basu *et al*. (1976) showed that size and configuration of the acetyl-LDL are similar to that of native LDL, as were revealed by electron microscopy after negative staining of the lipoproteins with uranyl acetate (Basu *et al*., 1976).

Pietzsch *et al*. (1995) applied a very fast sequential separation method for LDL isolation (100 min), but their method required to very high speed centrifugation (625,000 g). This speed can harmful for equipment. Also with increasing rotor speed, VLDL and LDL lipid constituents principally tended to decrease. The procedure was suitable for the assessment of lipid and protein constituents in lipoproteins from very small plasma samples (500 μL) but no for huge amounts.

In conclusion, analysis of electerophoretic and ultracentrifugation data indicate that our method can used for extraction and modifying LDL and these preparations are suitable for use in molecular and cellular studies in cardiovascular diseases.

ACKNOWLEDGMENT

This research is supported by a grant from the Tehran University of Medical Sciences, Tehran, Iran.

REFERENCES

Basu, S., J. Goldstein, R. Anderson and M. Brown, 1976. Degradation of cationized low density lipoprotein and regulation of cholesterol metabolism in homozygous familial hypercholesterolemia fibroblasts. Proc. Nati. Acad. Sci., 73: 3178-3182.

Bergmeier, C., R. Siekmeier and W. Gross, 2004. Distribution spectrum of paraoxonase activity in HDL fractions. Clin. Chem., 50: 2309-2315.

Berliner, J.A. and Y. Suzuki, 1996. The role of oxidized lipoproteins in atherogenesis. Free Radic. Biol. Med., 20: 707-727.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem., 72: 248-254.

Brousseau, T., V. Clavey, J. Bard and J.C. Fruchart, 1993. Sequential ultracentrifugation micromethod for separation of plasma lipoproteins and assays of lipids, polipoproteins and lipoprotein particles. Clin. Chem., 39: 960-964.

Chapman, M., M. Laplaud, G. Luc, P. Forgez, E. Bruckert, S. Goulinet and D. Lagrange, 1988. Further resolution of the low-density lipoprotein spectrum in normal human lasma: Physicochemical characteristics of discrete subspecies separated by density gradient ultracentrifugation. J. Lipid Res., 29: 442-458.

Colpo, A., 2005. LDL cholesterol: Bad cholesterol or bad science. J. Am. Phys. Surgeons, 10: 83-89.

Fraenkel-Conrat, H., 1957. Methods for investigating the essential groups for enzyme activity. Methods Enzymol., 4: 247-269.

Geiss, H.C., S. Bremer, P.H.R. Barrett, C. Otto and K.G. Parhofer, 2004. *In vivo* metabolism of LDL subfractions in patients with heterozygous FH on statin therapy: Rebound analysis of LDL subfractions after LDL apheresis. J. Lipid Res., 45: 1459-1467.

Havel, R., H. Eder and J. Bragdon, 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human plasma. J. Clin. Invest., 34: 1345-1353.

Higaki, M., K. Sato, N. Miyasaka and K. Nishioka, 1993. Uptake of acetylatedlow density lipoprotein (ac-LDL) by synovial cells. Scandinavian J. Rheumatol., 22: 102-106.

Kamps, J., J.K. Kruijt, J. Kuiper and J.C. van Berkel, 1992. Characterization of the interaction of acetylated LDL and oxidatively modified LDL with human liver parenchymal and kupffer cells in culture. Arteriosclerosis Thrombosis, 12: 1079-1087.

Landers, S.C. and J.C. Lewis, 1993. AcLDL binding and endocytosis by macrophages and macrophage foam cells *in situ*. Exp. Mol. Pathol., 59: 38-50.

Mackness, B. and M. Mackness, 2004. Paraoxonase-1: Biochemistry and contribution to atherosclerosis. Int. Congr. Ser., 1262: 91-94.

Magnani, N. and N. Howard, 1971. A quantitative method for blood lipoproteins using cellulose acetate electrophoresis. J. Clin. Pathol., 24: 837-845.

McPherson, P., I. Young, B. McKibben and J. McEneny, 2007. High density lipoprotein subfractions: Isolation, composition and their duplicitous role in oxidation. J. Lipid Res., 48: 86-95.

Mironova, M., R. Klein, G. Virella and M. Lopes-Virella, 2000. Anti-Modified LDL antibodies, LDL-containing immune complexes and susceptibility of LDL to *in vitro* oxidation in patients with type 2 diabetes. Diabetes, 49: 1033-1041.

Mori, M., H. Itabe, Y. Higashi, Y. Fujimoto and M. Shiomi *et al*., 2001. Foam cell formation containing lipid droplets enriched with free cholesterol by hyperlipidemic plasma. J. Lipid Res., 42: 1771-1781.

Pietzsch, J., S. Subat, S. Nitzsche, W. Leonhardt, K.U. Schentke and M. Hanefeld, 1995. Very fast ultracentrifugation of plasma lipoproteins: Influence on lipoprotein separation and composition. Biochim. Biophys. Acta, 1254: 77-88.

Rosenson, S.R.S., E. Chnari, T. Brieva, A. Dang and P. Moghe, 2005. Glutathione preconditioning attenuates Ac-LDL-induced macrophage apoptosis via protein kinase C-Dependent Ac-LDL trafficking. Exp. Biol. Med., 230: 40-48.

Salonen, J.T., S. Yta-Herttuala, R. Yamamoto, S. Butler and H. Korpel *et al*., 1992. Autoantibody against oxidized LDL and progresión of carotid atherosclerosis. Lancet, 339: 883-887.

Stocker, R. and J.F. Jr. Keaney, 2004. Role of oxidative modifications in atherosclerosis. Physiol. Rev., 84: 1381-1478

Victor, R.S., J. Josep, L. Miquel and P.O. Julia, 2002. Ultracentrifugation micromethod for preparation of small experimental animal lipoproteins. Anal. Biochem., 303: 73-77.

Virella, G., S. Koskinen, G. Krings, J.M. Onorato, S.R. Thorpe and M. Lopes-Virella, 2000. Immunochemical characterization of purified human oxidized low-density lipoprotein antibodies. Clin. Immunol., 95: 135-144.

Wang, M.D., R. Kiss, V. Franklin, H.M. McBride, S.C. Whitman and Marcel, 2007. Different cellular traffic of LDL-cholesterol and acetylated LDL-cholesterol leads to distinct reverse cholesterol transport pathways. J. Lipid Res., 48: 633-645.

Yla-Herttuala, S., W. Palinski, M.E. Rosenfeld, D. Steinberg and J.L. Witztum, 1990. Lipoproteins in normal and atherosclerotic aorta. Eur. Heart J., 11: 88-99.

Yla-Herttuala, S., W. Palinski, M.E. Rosenfeld, S. Parthasarathy and T.E. Carew *et al*., 1989. Evidence for the presence of oxidatively modified low density lipoprotein in atherosclerotic lesions of rabbit and man. J. Clin. Invest., 84: 1086-1095.