SHOULD WE MEASURE ROUTINELY OXIDIZED AND SMALL DENSE LOW-DENSITY LIPOPROTEINS IN SUBJECTS WITH TYPE 2 DIABETES?

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SHOULD WE MEASURE ROUTINELY OXIDIZED AND SMALL DENSE LOW-DENSITY LIPOPROTEINS IN SUBJECTS WITH TYPE 2 DIABETES?

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Running title: Oxidized and small, dense LDL in diabetes

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ABSTRACT

Beyond LDL-cholesterol concentrations, in recent years several clinical studies have shown that both oxidized and small, dense low-density lipoproteins (LDL) have a strong predictive role for the presence of vascular atherosclerosis. These two lipid parameters seem to have a synergistic impact on cardiovascular risk, with a greater importance in patients at higher-risk, such as those with type-2 diabetes. Increased levels of oxidized and small, dense LDL levels are a feature of diabetic dyslipidemia and small, dense LDL have been shown to be good predictor of future cardiovascular events, at both univariate and multivariate analyses. On the other hand, although the association of oxidized LDL with surrogate markers of atherosclerosis is consistent, the correlation with hard clinical end points seems to be smaller. Yet, measurement of these two lipid parameters has not been widely used in daily practice due to the limited availability of clinical data and to methodological problems: lack of availability of easy, cheap and reproducible essays for measurement of oxidized and, particularly, small, dense LDL have reduced their assessment in large clinical end-points trials. However, on the basis of available data, the therapeutic modulation of small, dense LDL is significantly associated with reduced cardiovascular risk, even after adjustment for confounding factors. In conclusion, the routine measurement of oxidized and small, dense LDL in patients with type-2 diabetes cannot be recommended in daily clinical practice so far; yet, their measurement is strongly encouraged, in order to better understand their role on the cardiovascular risk of patients with type-2 diabetes.

Key words: diabetes; oxidized LDL; small, dense LDL; cardiovascular risk; prevention.

How did you gather, select and analyze the info you considered in your review?

We searched for and reviewed all the available evidence in a systematic way. A literature search (by Medline and Scopus) was performed using the following headings: “diabetes”, “LDL”, “lipoproteins”, “oxidized” “small, dense”, “size”, “subfractions”, “subclasses”, and “therapy” up to 30 December 2009. The authors also manually reviewed the references of selected articles for any pertinent material.

What is the take-home message for the clinician?

Beyond LDL-cholesterol concentrations, oxidized and small, dense LDL seems to have a synergistic impact on the cardiovascular risk of subjects with type-2 diabetes. Although the routine measurement of these two lipid parameters in these patients cannot be recommended so far, their measurement is strongly encouraged, in order to better understand their role on cardiovascular risk of patients with type-2 diabetes.

1. Introduction

Diabetes mellitus (DM) and its vascular complications represent a significant and growing problem in the Western World. Patients with type-2 diabetes develop more frequently accelerated atherosclerosis compared to the general population and mortality for ischemic heart disease is common [**1**]. Oxidative stress, as a consequence of the combined effect of various cardiovascular risk factors such as dyslipidemia, hypertension and DM, plays a crucial role in the atherogenic process [**2**]. The increase in reactive oxygen species leads to the formation of oxidized (ox) low-density lipoproteins (LDL). Nowadays, there is enough evidence supporting the hypothesis that oxLDL are highly immunogenic and atherogenic. Reactive oxygen species (ROS) are generated through several metabolic pathways. These cascades act as modifiers in fatty acids, lipoproteins and amino acids, resulting in the formation of atherogenic particles such as oxLDL [**3**].

Native LDL particles are characterized by heterogeneity in their physical properties like size and density. Based on various analytical methods such as gradient gel electrophoresis, high-performance gel-filtration chromatography and nuclear magnetic resonance spectroscopy, subclasses of larger, more buoyant or smaller and more dense LDL have been identified. These small, dense LDL particles are more likely to form oxLDL, are less readily cleared and highly atherogenic [**4**]. Insulin resistance, which is central in type-2 DM, leads to high serum levels of very low density lipoproteins (VLDL) enriched in triglycerides, which are metabolized predominantly to small dense LDL. Thus, small dense LDL particles are an integral part of diabetic dyslipidemia that is also characterized by increased triglyceride and reduced high density lipoproteins (HDL)-cholesterol concentrations [**5**].

Clinical studies have shown that both small, dense LDL and oxLDL may have a distinct predictive role for the presence of vascular atherosclerosis in patients with type-2 DM. However, measurement of these two lipid parameters has not been widely used in daily practice probably due to the limited availability of clinical data and to methodological problems. The aim of the present

article is to discuss the most recent clinical evidence about the role of small dense LDL and oxLDL in the development and progression of atherosclerosis and cardiovascular diseases, mainly in patients with type-2 DM. In order to do so, a systematic literature search (by Medline and Scopus) was performed using the following headings: “diabetes”, “LDL”, “lipoproteins”, “oxidized” “small, dense”, “size”, “subfractions”, “subclasses”, and “therapy” up to 30 December 2009. The authors also manually reviewed the references of selected articles for any pertinent material.

2. Oxidation of LDL

LDL can be oxidized by metal ions (e.g. Cu^{+2}), lipoxygenases, myeloperoxidase and reactive nitrogen species. Under oxidative stress, lipid molecules containing polyunsaturated fatty acids in LDL are easily oxidized. *In vitro*, this process includes three phases and a number of changes in lipid composition occur, including the substantial loss of free and esterified cholesterol and the generation of oxidation products, such as oxysterols [**6**]. Oxysterols seems to have many potent and diverse effects *in vitro*, several of which may be important in the atherosclerosis process. The initial phase (lag phase) includes the consumption of the endogenous LDL antioxidants (e.g. vitamin E). The second phase (propagation phase) includes the rapid oxidation of esterified fatty acids to lipid hydroperoxides and up to 90% of the steryl ester acyl groups are modified. In details, in mildly oxidized LDL, cholesteryl hydroxyperoxy-octade-cadienoate (Chol-HPODE) and cholesteryl hydroxyl-octadecadienoate (Chol-HODE) were detected as the main oxidation products. Chol-HPODE was reported to inactivate platelet–derived growth factor. The third phase is the decomposition phase where the hydroperoxides are converted to reactive aldehydes. These aldehydes react with lysine residues in apolipoprotein (apo) B-100, resulting in oxLDL, which has decreased affinity for the LDL receptor and increased affinity for scavenger receptors of subendothelial macrophages [**7**].

Oxidized LDL represents a variety of modifications of both the lipid and protein components of LDL that occur when ROS induce lipid peroxidation. This oxidation process is thought to occur mainly in the arterial wall, rather than in plasma. Although fully oxLDL are cleared from the circulation within minutes by the reticuloendothelial system, the presence of small amounts of minimally modified LDL denotes the presence of circulating oxLDL. Therefore, the measurable circulating plasma levels of oxLDL represent only a small fraction of total LDL [**8**]. However, oxLDL antibodies are present in the circulation and can be readily measured. In the normal arterial wall without evidence of atherosclerosis, oxLDL levels are very low but in the early stages of atherosclerosis, oxLDL are clearly present. Although concentrations of oxLDL in the arterial wall are not measurable, circulating levels of oxLDL or oxLDL antibodies are theoretically the biomarker of total oxLDL, and consequently of total atherosclerosis burden [**9**].

Currently, a directly image oxLDL in human arteries is unavailable [**10**]. Yet, several specific oxLDL assays based on monoclonal antibodies have been developed since the 1990s, when clinical studies established the strong relationship of circulating oxLDL with cardiovascular diseases. These antibodies can bind unique oxidation-specific epitopes but the methods are not easily comparable, as they have different binding epitopes. More recently enzyme-linked immunoabsorbent assay (ELISA) methods to measure plasma levels of oxLDL are commercially available [**11-13**]. Oxidized phospatidylcholines (oxPC) are produced after lipid oxidation and promote the expression of monocyte chemoattractant protein-1 (MCP-1) in endothelial cells and oxLDL recognition by macrophage scavenger receptors, mainly cluster of differentiation (CD) 36. In the first of the above mentioned ELISA methods, the murine immunoglobulin (Ig)M monoclonal antibody DLH3 recognizes oxPC epitopes and uses isolated LDL rather than plasma to measure oxLDL [**14**]. Another IgM monoclonal antibody, the E06, recognizes phosphorylcholine, the hydrophilic moiety in phospatidylcholines, and by design this second method is independent of LDL cholesterol or apoB levels [**15,16**]. Finally, the third method uses the antibody 4E6 which binds to aldehydes-modified lysine groups on LDL. OxLDL levels measured with this method are

closely correlated with total LDL-cholesterol levels, and this may limit its clinical utility [**17**]. It is clear that these three assays use antibodies detecting different epitopes and they are set up in different formats and units of measurement. Thus, their results are not easily comparable, limiting the interpretation of the results in clinical studies that assessed plasma levels of oxLDL with different essays. For this reason Tsimikas et al. have suggested that investigators should present their data by linking their antibody to the type of oxLDL they have measured (i.e. "oxLDL-E06" for the E06 antibody) [**8**].

3. Oxidized LDL and cardiovascular risk

The majority of cardiovascular diseases are linked to the atherosclerosis process. One of the most important steps in this cascade is the transfer of oxLDL across the endothelium into the arterial wall [**18**]. This probably takes place at sites of endothelial rupture, induced by oxLDL itself as well as by physical forces and inflammation. OxLDL can damage the continuity of endothelial wall and further induce the expression of adhesion molecules such as MCP-1 and the macrophage colony stimulating factor [**19**]. Therefore, monocytes are activated and attached together with T lymphocytes on the surface of the endothelial cells. Subsequently, leucocytes as well as endothelial and smooth muscle cells secrete growth factors, which cause chemoattraction and, thus, the enhanced migration of monocytes and leucocytes into subendothelium. Modified monocytes form macrophages, which generate ROS that contribute to the conversion of oxLDL into highly oxidized particles.

This form of LDL is considered the most atherogenic and is taken up by macrophages to form the foam cells, which can secrete growth factors and, therefore, inducing smooth muscle cells migration and proliferation into the intima. Thus, the initial lesion, i.e. the fatty streak, is converted to a more advanced lesion with a fibrous cap that covers the lipid rich core. In acute coronary

syndromes, some of these plaques (vulnerable plaques) are thinner than the stable plaques, and more prone to rupture. A ruptured plaque is the nidus for thrombus formation and, therefore, for arterial lumen occlusion [**20,21**].

Beyond their crucial role in the formation of the atherosclerotic plaque, oxLDL seems to affect other aspect of the atherogenic process. For instance, endothelial dysfunction is clearly promoted by oxLDL. Apoptosis, a process in endothelial and smooth muscle cells that contributes to plaque vulnerability, seems to be affected by oxLDL and their products, such as phospatidylocholine and oxysterols [**22**]. Also, oxLDL promotes thrombus formation by inducing the release of tissue factor by both endothelial cells and smooth muscle cells, and further stimulates the coagulation cascade by reducing thrombomodulin trancription, suppressing protein C activation and modulating tissue factor pathway inhibitor [**7**]. In addition, oxLDL increases expression of matrix metalloproteinase-9, which causes vascular remodeling and rupture of the fibrous cap in the atherosclerotic lesions [**23**].

Elevated plasma oxLDL levels have been reported in patients with coronary artery disease (CAD), carotid atheromatosis, and chronic hemodialysis. However, since oxidation of LDL is mediated by several risk factors and present in both early and late lesions, there is no clear evidence that oxLDL can be used as a marker of preclinical atherosclerosis. Nevertheless, in asymptomatic individuals, elevated oxLDL levels have been associated with increased carotid intima-media thickness and familial hypercholesterolemia [**24**]. Moreover, some clinical studies have shown an inverse correlation between levels of oxLDL and HDL cholesterol [**7**].

The association of plasma oxLDL with the presence and the severity of CAD has been established from many clinical studies with the use of the ELISA procedures mentioned above. Several reports have shown an increase in oxLDL levels in the acute phase immediately after acute myocardial infarction, cerebral infarction, or percutaneous transluminal coronary angiography (PTCA) procedure [**25**]. One possible explanation for this temporal increase is that oxLDL can be

released from the ruptured fibrous cap of the atherosclerotic lesions at the time of occurrence of infarction. Moreover, this observation suggests that plasma oxLDL could reflect the conditions of atherosclerotic lesions and vulnerability of the plaque.

This association was previously documented in cross-sectional studies showing a correlation among severity of acute coronary syndrome and levels of plasma oxLDL. Hayashida et al. demonstrated elevated levels of serum soluble lectin-like oxidized LDL receptor-1 (the endothelial receptor for oxLDL) in patients with acute coronary syndromes, suggesting their potential prognostic role [**26**]. Ehara et al. also reported that circulating oxLDL-DLH3 levels were elevated in acute coronary syndromes and that this was correlated with the presence of oxLDL in atherectomy material removed after PTCA [**27**]. In addition, in a follow-up study, Tsimikas et al. suggested that iatrogenic rupture of the plaque during PTCA increases plasma levels of oxLDL-E06 **[16]**. In this latter study, a strong correlation was documented between lipoprotein(a) [Lp(a)] and oxLDL-E06 levels, supporting the novel observation from the same group that Lp(a) ultimately binds most of the oxidized phospholipids measured by monoclonal antibody oxLDL-E06. These authors suggested that Lp(a) may be a part of the innate immune system, having a role in clearing proinflammatory oxidized phospholipids; yet, when Lp(a) levels are elevated, as under oxidative stress, this lipoprotein may become an atherogenic molecule in the arterial wall [**28**].

The same research group has also recently confirmed the association of circulating levels of oxLDL with stable CAD, a relationship which had been already reported in previous studies [**29-31**]. In a population of 504 patients undergoing coronary angiography for stable CAD, they reported a close correlation of oxLDL/apoB-100 ratio with the presence and the extent of coronary atherosclerosis [**28**]. Interestingly, a close correlation between Lp(a) and the above mentioned ratio was found in this study; nevertheless, in patients under 60 years old, oxLDL/apoB levels were independent of Lp(a), suggesting that oxLDL may mediate atherogenicity beyond its association with Lp(a).

In the stent implantation era, restenosis is considered a serious complication, although it has been significantly reduced using drug-eluting stents. There are conflicted results about the usefulness of oxLDL in predicting in stent restenosis and secondary cardiovascular events after a successful PTCA. Naruko et al. enrolled 102 patients with acute myocardial infarction and showed that high levels of oxLDL-DLH3 prior to discharge were correlated with highly percentages of restenosis [**32**]. On the other hand, Braun and colleagues in 687 patients who suffered from unstable and stable angina or myocardial infarction, did not show a significant correlation between oxLDL-4E6 levels and cardiovascular outcomes, including cardiovascular death, myocardial infarction or angiographically-determined restenosis [**33**]. In consistent to these findings, Segev et al. have shown that oxLDL-E06 levels, despite an increase after uncomplicated PTCA for stable angina, were not associated with restenosis, in a 6-month angiographic follow-up study [**34**].

4. Oxidized LDL in patients with type 2 diabetes mellitus

Recent evidences suggest that diabetic atherosclerosis cannot simply be attributed to the adverse lipid profile of these patients, but is mainly an oxidation and inflammation mediated process. It is well known that individuals with type-2 DM exhibit enhanced LDL oxidizability, leading to accelerated atherosclerosis. Glycemia itself induces oxidative stress and advanced glycation end products, formed by glucose-induced modification of proteins, and further acts as ligands for multiple receptors, such as the lectin-like oxidized LDL receptor-1 (LOX-1), inducing oxidative stress, inflammation and vascular dysfunction in diabetes. LOX-1 has been identified as the major oxLDL receptor that is present primarily in the endothelial cells; it has been found up-regulated in the vascular endothelium of diabetic animals and seems to play an important role in the pathogenesis of hypertension, diabetes and atherosclerosis (**Figure 1**).

Also, oxLDL interacts with β2-glycoprotein I (β2GPI), and oxLDL/β2GPI complexes have been considered as putative autoantigens in the autoimmune-mediated atherosclerotic vascular

disease. Tabuchi et al. [**35**] reported a potent interaction of C-reactive protein with oxLDL/β2GPI complexes and its association with arterial inflammation, hyperglycemia and hypercholesterolemia in patients with diabetes mellitus. Further, an increase in the *in vivo* LDL oxidation has been clearly shown in patients with type-2 diabetes, as reflected by the higher prevalence of highly oxidized LDL [**36**]. The association between glycemia and LDL oxidation is in agreement with the observation that even in healthy individuals transiently increased plasma glucose levels correlate with a higher susceptibility of *in vitro* oxidation of LDL [**37**].

Yet, oxLDL themselves may also promote glycemia and the development of diabetes. Recent data from the CARDIA study showed that in healthy young individuals high concentrations of oxLDL were associated with abdominal obesity, hypertriglyceridemia and hyperglycemia [**38**], which are essential components of the metabolic syndrome. Notably, subjects at the highest quartile of oxLDL concentrations had a threefold higher risk for the development of metabolic syndrome compared to those who were at lowest quartile during the 5-year follow up. In addition, in a recent clinical study that enrolled women having normal glucose levels, impaired glucose tolerance, impaired fasting glucose or diabetes type-2, levels of oxLDL were independent predictors for the development of type- 2 diabetes after the 6-year follow-up [**39**]. This may be explained by the fact that oxLDL can reduce the insulin signaling [**40**] as well as the glucose uptake [**41**].

On clinical grounds, the role of oxLDL as predictor of increased cardiovascular morbidity and mortality in patients with type-2 diabetes has not been clearly delineated. Data from the Health, Aging and Body Composition study, including 3,033 elderly individuals, failed to demonstrate a significant association between oxLDL levels and total cardiovascular risk. Nevertheless, subjects with high oxLDL levels showed a trend to suffer more frequently of acute myocardial infarction [**42**]. Luoma et al. further reported that oxLDL autoantibodies were not predictive of severe chest pain attacks in patients with type-2 diabetes and CAD who were part of this large cohort. By contrast, a predictive role of oxLDL for cardiac events in patients with diabetes type-2 was demonstrated by Shimada et al. [**43**]. They found in patients with type-2 diabetes and

angiographically documented CAD that high levels of oxLDL were an independent and significant predictor of cardiovascular events. In line with these findings, Stephens et al. [**44**] reported a significant association between plasma oxLDL:LDL ratio and CAD status in a cross sectional study of 3,012 middle-aged men with type-2 diabetes

Although the correlation with hard clinical end points seems to be weak, the association of with softer end points, such as surrogate markers of atherosclerosis, is more stronger. A clinical study, which included patients with type-2 DM and control subjects, suggested that in patients with long duration of the disease the levels of oxLDL were significantly higher, and that the ankle-brachial index was lower than in control or newly diagnosed patients [**45**]. Similarly, greater intima-media thickness (IMT) of common carotid artery was correlated with higher levels of oxLDL [**45**]. Data from the Chennai Urban Rural Epidemiology Study showed a strong association between oxLDL and IMT, even after adjusting for age, sex, and glucose intolerance. Ujihara et al. [**46**] further investigated the association between diabetic nephropathy and high levels of oxLDL in patients with type-2 DM. They found that patients with macroalbuminuria had higher values of oxLDL in comparison to the other patients of the cohort with either microalbuminuria or normal renal function. Similar findings were reported by Hsu et al [**47**]: in comparison to controls, increased titers of oxLDL antibodies were found in patients with type-2 DM and macrovascular disease, but not in those without macrovascular disease.

OxLDL has also been implicated in the hypertrophy of adipocytes from lipid accumulation and proliferation of adipose tissue [**48**]. It has been also reported that oxLDL may modulate the production of adiponectin, which is able to reduce the excess ROS production in hyperglicemic states, an effect that has implications for vascular protection in diabetes [**49**]. In addition, oxLDL may impair triglyceride storage and secretion [**50**]. To complete this circle of associations between LDL peroxidation and obesity, it has been proposed that the increase in adipose tissue may promote the synthesis of oxLDL, by either increasing production of arachidonate-5-lipoxygenase which catalyzes LDL oxidation, or by decreasing production of superoxide dismutase, which prevents

LDL oxidation [**48**]. However, a more relevant pathophysiologic explanation may be the increased production by the adipose tissue of small dense LDL, which is reputedly more prone to oxidation than buoyant LDL particles [**51**]. Both diabetes and the metabolic syndrome may promote LDL oxidation through glycemia, development of end-glycation products and increased adiposity. On the other hand, oxLDL may further deteriorate insulin resistance and promote the proliferation of adipocytes. Notably, Scheffer et al. [**52**] have shown that smaller LDL size was associated with increased levels of oxidized LDL, providing additional evidence for the role of small, dense LDL in the etiology of atherosclerosis in diabetes.

5. Formation of small, dense LDL

LDL comprises multiple subclasses with discrete size and density, different physicochemical composition and metabolic behaviour. Based on their characteristic appearance in analytical ultracentrifugation and gradient gel electrophoresis, up to seven distinct subclasses have been defined. It has been suggested that there are parallel metabolic channels within the delipidation cascade from VLDL to LDL [**53**] and a metabolic relationship between large VLDL particles and small LDL particles has been demonstrated using stable isotopes in subjects with a predominance of small, dense LDL [**54**]. These studies have not yet identified the specific precursors of individual LDL subclasses; however there are data from animal models suggesting that separate pathways may be responsible for the generation of distinct LDL particles [**55**]. Dietary intervention studies also demonstrated inverse correlations between changes in large and small LDL, as well as between changes in medium sized and very small LDL, which raise the possibility of precursor-product relationships between distinct LDL subclasses [**56**].

Activity of lipolytic enzymes is related to the size of LDL particles. A significant inverse relationship between post-heparin lipoprotein lipase activity and small, dense LDL has been demonstrated and induction of lipoprotein lipase by high fat diet was associated with an increase of

large LDL and decrease of small, dense LDL. Reduced activity of lipoprotein lipase and increased activity of hepatic lipase has been shown in subjects with a predominance of small, dense LDL particles [**57**]. Hepatic lipase has a higher affinity for LDL than lipoprotein lipase and is positively correlated with plasma triglycerides, apoB, mass of large VLDL and small, dense LDL, but not with the mass of large LDL [**58**], suggesting a central role for hepatic lipase in the lipolytic conversion of these particles.

Triglyceridaemia seems to be the main determinant of LDL subclass distribution [**59**]. In fact, the formation of small, dense LDL particles is mostly observed in hypertriglyceridemic state, with an increased transfer of triglycerides from triglyceride-rich lipoproteins to LDL and HDL particles, in exchange of cholesteryl esters through the action of cholesteryl esters transfer protein [**60**]. This phenomenon leads to the generation of VLDL particles enriched in cholesteryl esters and smaller, triglyceride-rich LDL particles that are good substrates for hepatic lipase. As a consequence, predictive value of small, dense LDL is usually reduced when triglycerides levels are taken into account [**61**]. For example, in a published analysis from the EPIC-Norfolk prospective population study, predictive power for cardiovascular events of LDL particle number and size was lost after adjustment for HDL-cholesterol and triglycerides levels [**62**].

6. Small, dense LDL and cardiovascular risk

Several reasons have been suggested to explain the enhanced atherogenicity of small, dense LDL. These particles are taken up more easily by arterial tissue than larger LDL [**63**], suggesting greater transendothelial transport. In addition, small, dense LDL have decreased receptor-mediated uptake and increased proteoglycan binding [**64**]. Sialic acid, due to its exposure at the LDL surface, plays a determinant role in the *in vitro* association of LDL with the polyanionic proteoglycans [**65**] and it has been shown that sialic acid content of LDL particles of subjects with the predominance of

small, dense LDL is reduced. Further, oxidative susceptibility increases and antioxidant concentrations decreases with decreasing LDL size [**66**]. Altered properties of the surface lipid layer associated with reduced content of free cholesterol [**67**] and increased content of polyunsaturated fatty acids [**68**] might also contribute to enhanced oxidative susceptibility of small dense LDL.

To date, the association of LDL size with cardiovascular diseases has been tested in over fifty studies, including cross-sectional and prospective epidemiologic, as well as clinical intervention trials; these studies overall suggest that the quality of LDL exerts a direct influence on cardiovascular risk. Due to previously described atherogenic properties, the predominance of small, dense LDL has been accepted as an emerging cardiovascular risk factor by the National Cholesterol Education Program Adult Treatment Panel III. It has also been shown that even small increases in the concentration of these particles may substantially contribute to the determination of total cardiovascular risk [**69**]. Other studies have investigated if the therapeutic modification of LDL size may be significantly associated with reduced cardiovascular risk. Such investigations used arteriographic changes as outcome variables and have reported that benefit was concentrated in patients with a predominance of small, dense LDL who received treatment that tend to reduce the amount of such particles [**70-72**].

These findings overall suggest that the therapeutic modification of LDL size may be significantly associated with reduced cardiovascular risk, even after multivariate adjustment for confounding factors. In addition, it has been reported [**73**], although not directly demonstrated, that the modulation of LDL size with fibrates probably contributed to the reduction of cardiovascular risk in large clinical trials [**74-76**]. Yet, it cannot be fully excluded that the increased cardiovascular risk associated with smaller LDL size may also be a consequence of the broader pathophysiology of which small, dense LDL are a part (e.g. high triglycerides, low HDL-cholesterol, increased LDL particle number, obesity, insulin resistance, diabetes, metabolic syndrome) [**77-80**].

7. Small, dense LDL and type-2 diabetes

Small, dense LDL represent one the main dislipidemic features in type-2 diabetes [**81**]. Subjects with a predominance of small dense LDL have a greater than two fold increased risk for developing type-2 diabetes independently of age, sex, glucose tolerance and body mass index, and it has been calculated that an increase in LDL size may be associated with about a 16% decrease in the risk of developing diabetes [**82**]. Subjects with the insulin resistance syndrome have an elevated prevalence of small, dense LDL [**83**] and this has been confirmed in both men and women with type-2 diabetes [**84,85**]. In addition, using a euglycemic clamp technique to categorise individuals as insulin-sensitive, insulin-resistant, or with diabetes, more severe states of insulin resistance were associated with smaller LDL size [**86**].

We have investigated the clinical significance of LDL size and LDL subclasses in patients with type-2 diabetes: those with manifest CAD had decreased LDL size and increased small, dense LDL particles as compared to subjects without established CAD. Multivariate analysis revealed that LDL size was the strongest marker of CAD as compared to other established cardiovascular risk factors, including plasma lipids and lipoproteins [**87**]. Increased IMT is considered a reliable surrogate marker of early atherosclerosis and it has been shown to significantly correlate with the presence of CAD and to predict coronary events [**88-90**]. In the previously mentioned study [**87**] LDL size was significantly associated with carotid IMT and LDL size was the second strongest predictor of IMT when compared to nine other cardiovascular risk factors, and the strongest of all lipid parameters. Thus, small, dense LDL is a key feature of subjects with type-2 diabetes and seems further to represent a strong marker of their clinical and subclinical atherosclerosis.

Hypolipidemic treatments are able to favourably modulate LDL size and subclasses in patients with type-2 diabetes but the effects are varying among the different agents (**Table 1**). Notably, in the "Diabetes Atherosclerosis Intervention Study" [**91**] the therapeutical modulation of LDL size by fenofibrate was significantly associated with reduced cardiovascular risk at univariate analysis; in addition, using multivariate analyses with adjustments for confounding factors, changes

in LDL size were strong predictors for cardiovascular events. Yet, although fibrates are more powerful than statins in improving LDL size and subclasses, existing evidence suggest that statins are more powerful agents in reducing hard clinical end points (i.e. cardiovascular morbidity and mortality). In the same context, while fenofibrate seems to be the best fibrate in lowering small dense LDL, the FIELD study [**92**] showed no significant reduction in the primary end point in patients with type-2 diabetes who were randomised to receive fenofibrate or placebo.

8. Conclusions

The most important link between lipid metabolism and inflammation is based on the formation of foam cells (first step of plaque generation) from altered, oxidized LDL [**93**]. Yet, LDL are very heterogeneous particles, with distinct subclasses that differ in size, density, physicochemical composition, metabolic and oxidative behaviour, as well as atherogenicity [**94**]. Increasing evidence suggests that the ‘‘quality’’ of LDL has a direct influence on cardiovascular risk, with smaller, more dense LDL particles being more susceptible to oxidation and greatly atherogenic [**95**].

The inflammation process is induced from oxidized lipoproteins, mainly oxLDL, that are independent predictors of the progression of the atherosclerosis cascade, possibly by binding monocytes to the endothelium [**96**]. Additionally, oxLDL seems to be responsible for the expression of pro-inflammatory genes in the subendothelium of vascular bed, including leukocyte adhesion molecules (e.g. intercellular adhesion molecule-1, vascular cell adhesion molecule-1 and P-selectin), chemotactic molecules, (e.g. MCP-1) and mitogenic growth factors, such as the macrophage-colony-stimulating factor, which has a pivotal role in the recruitment and activatation of inflammatory cells in the vessel wall [**97,98**]. Furthermore, oxLDL has a potential role in the formation of reactive aldehyde and phospholipid species with proinflammatory properties, such as the lysophosphatidylcholine [**99**]. In patients with type-2 diabetes hyperglycemia enhances the

oxidative stress, further modifying LDL into oxLDL. In such patients the decreased antioxidant activity is also linked to the lower plasma levels of anticoagulant proteins, as well as to the increased levels of hydroperoxides [**100,101**].

Yet, the relationship between autoantibodies to oxLDL and atherosclerosis remains controversial. There are studies showing high levels of autoantibodies to oxLDL in subjects with coronary artery disease, carotid atherosclerosis or peripheral arterial disease [**102,103**]. Current evidences support the concept that IgG autoantibodies are associated with atherogenesis enhancement while IgM have a natural protective role [**104**]. Circulating IgM autoantibodies have been found in plasma of apparent healthy subjects without any atherosclerosis manifestation [**105**]. It has been also demonstrated a positive correlation of IgG autoantibodies to angiographically documented coronary artery disease and an inverse association of IgM with it [**106**]. Further, in patients with type-2 diabetes high levels of IgG autoantibodies were not associated with subclinical atherosclerosis, but with manifest atherosclerotic lesions [**45**].

Therefore, oxidized and small, dense LDL seems to have a synergistic impact on cardiovascular risk (**Figure 2**), with a greater importance in patients at higher-risk, such as those with type-2 diabetes. Increased levels of oxidized and small, dense LDL levels are a feature of patients with type-2 diabetes and small, dense LDL have been shown to be good predictor of future cardiovascular events, even after multivariate adjustment for confounding factors. On the other hand, although the association of oxidized LDL with surrogate markers of atherosclerosis is consistent, the correlation with hard clinical end points seems to be smaller.

In addition, lack of availability of easy, cheap and reproducible essays for measurement of oxidized and, particularly, small, dense LDL have reduced their assessment in large clinical end-points trials. However, on the basis of available data, the therapeutic modulation of small, dense LDL is significantly associated with reduced cardiovascular risk, even after multivariate adjustment for confounding factors. Since small, dense LDL are difficult to be measured in non-specialized laboratories, in the last years alternative approaches have included the use of lipid indices as their

surrogate markers; yet, recent studies have shown that these lipid indices are not good predictors of small, dense LDL [**107**] and, therefore, their use should be discouraged.

In conclusion, on the basis of available data, the routine measurement of oxidized and small, dense LDL in patients with type-2 diabetes cannot be recommended in daily clinical practice so far. Yet, their measurement is strongly encouraged, in order to better understand their role on cardiovascular risk of patients with type-2 diabetes.

AUTHORS' CONTRIBUTIONS

Manfredi Rizzo: Concept/design, Data analysis/interpretation, Drafting article, Critical revision of article, Approval of article.

Kaspar Berneis: Concept/design, Data analysis/interpretation, Drafting article, Critical revision of article, Approval of article.

Spyridon Koulouris: Drafting article, Critical revision of article, Approval of article.

Socrates Pastromas: Concept/design, Data analysis/interpretation, Drafting article, Critical revision of article, Approval of article.

Giovam Battista Rini: Drafting article, Critical revision of article, Approval of article.

Dimitrios Sakellariou: Drafting article, Critical revision of article, Approval of article.

Antonis S. Manolis: Drafting article, Critical revision of article, Approval of article.

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Table 1. Therapeutic modulation of LDL size and subclasses by different agents in subjects with type-2 diabetes (as modified from **108,109**).

Authors	Year	Drug	Benefit
Winkler et al.	2002	Fluvastatin	Yes
Kazama et al.	2003	Pravastatin	No
Geiss et al.	2002	Simvastatin	No
Freed et al.	2002	Atorvastatin	No
Pontrelli et al.	2002	Atorvastatin	Yes
Wagner et al.	2003	Atorvastatin	Yes
Ikejiri et al.	2004	Atorvastatin	Yes
Frost et al., Geiss et al.	2001	Atorvastatin	No
Soedamah et al.	2003	Atorvastatin	No
Lahdenpera et al.	1993	Gemfibrozil	Yes
O'Neal et al.	1998	Gemfibrozil	Yes
Wagner et al.	2003	Gemfibrozil	Yes
Kazama et al.	2003	Bezafibrate	Yes
Kondo et al.	2004	Bezafibrate	No
Hayashi et al.	1998	Bezafibrate	Yes
Feher et al.	1999	Fenofibrate	Yes
Frost et al.	2001	Fenofibrate	Yes
Tan et al.	2001	Fenofibrate	Yes
Vakkilainen et al.	2003	Fenofibrate	Yes
Pan et al.	2002	Nicotinic acid	Yes
Pan et al.	2002	Nicotinic acid	Yes
Patti et al.	1999	Fish oil	No
Petersen et al.	2002	Fish oil	No
Mostad et al.	2007	Fish oil	No
Woodman et al.	2003	Fish oil (EPA)	No
Woodman et al.	2003	Fish oil (DHA)	Yes

Farnier et al.	2005	Ezetimibe	Yes
Winkler et al.	2002	Pioglitazone	Yes
Perez et al.	2004	Pioglitazone	Yes
Parhofer et al.	2005	Pioglitazone	Yes
Deeg et al.	2007	Pioglitazone	Yes
Berneis et al.	2008	Pioglitazone	Yes
Lautamaki et al.	2006	Rosiglitazone	No
Yu et al.	2006	Rosiglitazone	No
Albaladejo Oton et al.	2009	Rosiglitazone	No
Deeg et al.	2007	Rosiglitazone	No
Berneis et al.	2008	Rosiglitazone	No

EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid.

Figure 1. The vicious cycle between glycemia, LDL oxidation, advanced end glycation products (AEGs), lectin-like oxidized LDL receptor-1 (LOX-1) and atherosclerosis process in type-2 diabetes,

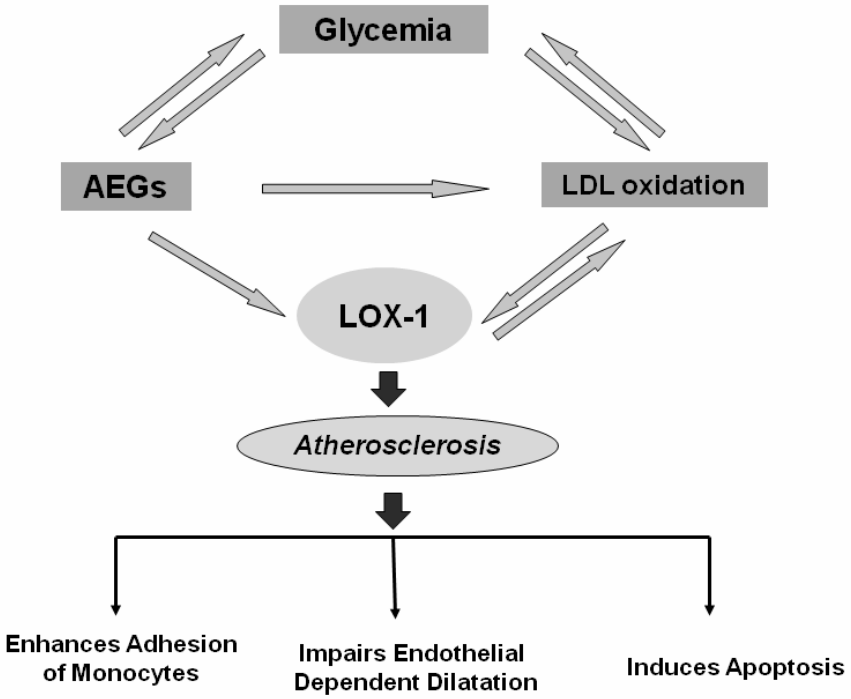

Figure 2. The synergistic role of oxidized and small, dense LDL in coronary artery disease.