10 The Role of Modified Forms of LDL and Corresponding Autoantibodies in the Development of Complications in Diabetes

Maria F. Lopes-Virella and Gabriel Virella

Oxidative stress is believed to be a critical factor in the initiation of pathogenic pathways that lead to the development of complications in diabetes mellitus [1]. Hyperglycemia plays a key role by inducing mitochondrial overproduction of reactive oxygen species (e.g., superoxide anion, hydrogen peroxide, and others), which, in turn, will lead to a variety of modifications of proteins, enzymes, and other substrates, including the formation of advanced glycation end-products (AGE) and oxidation [1, 2].

Lipoproteins are polymolecular assemblies that can be modified as a consequence of oxidation and glycation. Endothelial cells, monocytes/macrophages, lymphocytes, and smooth muscle cells (SMC) are all able to enhance the rate of oxidation of low-density lipoprotein (LDL). Reactive oxygen species and sulfur-centered radicals initiate metal ion-dependent lipid peroxidation resulting in the generation of aldehydes that interact with lysine residues in ApoB-100. Myeloperoxidase, a heme enzyme secreted by activated macrophages, is able to catalyze lipid peroxidation independent of free metal ions. Oxidation of arachidonic acid, usually secondary to oxidative stress, prostaglandin synthesis by endothelial cells (EC) and platelet activation, leads to the formation of aldehydes that interact with the lysine residues of ApoB100 causing its aggregation, and the resulting modification is generally referred to as malondialdehyde (MDA)-modified LDL [3].

M.F. Lopes-Virella, M.D., Ph.D. (✉)
Department of Medicine, Medical University of South Carolina, 114 Doughty Street, Charleston, SC 29425, USA

Ralph A. Johnson VA Medical Center, Charleston, SC, USA
e-mail: virellam@musc.edu

G. Virella
Department of Microbiology and Immunology, Medical University of South Carolina, Charleston, SC, USA

The Pathogenic Role of Modified LDL

The pathogenic role of modified LDL in the progression of atherosclerosis is well-established. It has been investigated from two different angles: the direct pro-atherogenic effect of modified forms of LDL [2, 4] and the consequences of the immune response directed against neoepitopes resulting from lipoprotein modification [5]. Both types of effects have been extensively characterized in the case of oxidized LDL (oxLDL). Oxidized LDL is taken up by macrophages via receptor-mediated pathways involving primarily CD36 [2, 6, 7] and it induces cholesteryl ester (CE) accumulation and the transformation of macrophages into foam cells [8, 9]. In addition, high concentrations of oxLDL are cytotoxic and experimental data suggests that oxLDL can injure vascular cells, both endothelial and smooth muscle cells (SMC) [10, 11]. Furthermore, oxLDL induces enhanced synthesis of growth factors

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including platelet-derived growth factor-AA (PDGF-AA) and PDGF receptor in SMC, as well as of granulocyte-monocyte colony stimulating factor, macrophage colony stimulating factor (M-CSF) and granulocyte-colony stimulating factor in aortic endothelial cells from humans and rabbits [12]. In addition, oxidized LDL may affect fibrinolysis by inhibiting the secretion of tissue plasminogen activator (tPA) by human endothelial cells [13] and stimulating the secretion of plasminogen activator inhibitor (PAI)-1 [13]. Thus, oxLDL is unable to stimulate the endothelium-dependent activation of fibrinolysis and may promote a chronic prothrombotic state. The cell-mediated immune system is also activated by the presentation of oxLDL oligopeptides by antigen presenting cells, activating T helper 1 cells (Th1) cells in the vascular wall. As a consequence of their activation, Th-1 cells release interferon-γ that activates macrophages and induces the release chemokines that attract more T cells to the area. The process becomes self-perpetuating, resulting in a chronic inflammatory reaction [14, 15].

Oxidized LDL has also been found to have pro-inflammatory effects relevant to the atherosclerotic process. It has chemotactic effects on monocytes [16], enhances monocyte adhesion to EC in culture [17, 18], enhances the expression of vascular cell adhesion molecule 1 (VCAM 1) and intercellular adhesion molecule 1 (ICAM 1) by human aortic endothelial cells induced by tumor necrosis factor-alpha (TNF alpha) [19] and of ICAM-1 in resting human endothelial vein cells [20]. These proinflammatory effects are the result of the activation of a variety of functional pathways. Oxidized LDL has been shown to activate a variety of cell types expressing CD36 and other scavenger receptors and contribute to the generation of reactive oxygen species (ROS) [21]. On macrophages, the interaction of oxLDL and CD36 (mediated by oxidized phospholipids) results in activation of the src family members Fyn/Lyn, and of several components of the MAP kinase pathway, including MKKK, MKK, FAK, and MAPK (JNK) [7]. The activation of these kinases and associated proteins such as Vav are associated with foam cell formation as well as with unregulated actin polymerization and loss of cell polarity causing a migration defect and the trapping of activated cells in the atheromatous lesions [7]. In platelets the same signaling events lead to enhanced platelet reactivity and enhanced formation of thrombi [22]. Recently it has been reported that ligation of CD36 by oxLDL leads to the formation of a toll-like receptor heterodimer (TLR-4-TLR-6) that, in turn, will activate MyD88 and nuclear factor kappa B (NFkB), a critical step in inducing the synthesis and release of proinflammatory cytokines [23].

The advanced glycation end-products (AGE) LDL (as well as other AGE-modified proteins) have also been shown to have pro-inflammatory properties [24, 25]. AGE-modified proteins will impact endothelial cells eliciting increased permeability and pro-coagulant activity [26] as well as overexpression of VCAM-1 [27]. AGE also contributes to fibroblast proliferation and T lymphocyte activation, which results in the release of increased amounts of interferon-γ that will activate monocytes and macrophages, inducing in turn the release of pro-inflammatory cytokines and chemokines [26], thus creating the conditions for a chronic inflammatory reaction in the arterial wall. The impact of AGE in the atherosclerotic process associated with diabetes was confirmed in streptomycin-induced diabetic ApoE −/− mice. Administration of soluble forms of AGE receptors (RAGE) resulted in reduction of vascular permeability and reduced the progression of atheromatous lesions [28].

The Adaptive Immune Response Elicited by Modified LDL

The pro-inflammatory properties of modified LDL appear to be considerably enhanced as a consequence of their immunogenicity. The immunogenicity of modified LDL was first reported by Steinbrecher et al. based on the immunization of laboratory animals with modified lipoproteins [29]. Of all the modified forms of LDL, oxLDL has been studied in greatest detail from the immunological point of view. Steinbrecher as well as Palinski et al. character-

ized its immunogenic epitopes [30, 31]. Furthermore, human autoantibodies to oxLDL were the first to be purified and characterized [32–34]. Immune complexes (IC) containing modified LDL have been isolated from the peripheral blood of patients with diabetes, cardiovascular disease, and healthy individuals [35, 36]. Both oxidized LDL and corresponding antibodies have been isolated from atheromatous human tissue [32, 37]. Thus, it seems reasonable to use circulating IC as a sampling of the IC that are deposited in the vessel wall. The formation of LDL-IC in circulation is likely to be inconsequential, but those IC formed in the vessel wall will result in enhanced phagocytosis and increased presentation of peptides derived from modified LDL to T helper cells, which are a critical step in the perpetuation vascular inflammation, as described above.

In several studies we have consistently found that the predominant isotype of modified LDL antibodies is IgG [33, 34, 38–40]. This is a significant finding because IgG antibodies are proinflammatory [33, 34, 38–40]. As reported by our group, predominance of circulating IgG antibodies with higher avidity over IgM antibodies in isolated oxLDL-IC is associated with parameters indicative of deteriorating renal function in the DCCT/EDIC cohort [40, 41]. Several groups have reported data suggesting that IgM antibodies to oxidized phospholipids and oxidized LDL have protective effects with relation to the development of atherosclerosis [42–47], although whether this protective effect extends to antibodies recognizing modified peptides seems questionable based on data published by Fredrickson and co-workers [48]. We have carried out two studies on the correlation between the levels of IgG and IgM antibodies to oxLDL contained in isolated IC from patients with type 1 diabetes and the development of nephropathy. In one of the studies we found that the predominance of immune complexes containing IgG antibodies to oxLDL with relatively high avidity was associated with abnormal albuminuria [40, 41]. In a more recent study we found significant positive associations of IgG oxLDL antibody concentration in isolated IC with serum creatinine and albumin excretion rate, as well as a negative correlation with estimated glomerular filtration rate were observed. IgM oxLDL antibody concentrations did not show any correlation with those parameters [40]. Both studies, however, were based on small groups of patients (33 and 34 patients, respectively). We have studied a much larger population of 932 patients with type 2 diabetes, and while the study confirms the predominance of IgG over IgM oxLDL antibodies in isolated immune complexes (Table 10.1), 28 patients had IgG/IgM ratios ≤2 and 9 had ratios <1. That subpopulation may be relatively protected against development of atherosclerosis but the data analysis of that study is still in progress, and it can become complicated by the relatively small number of patients with low IgG/IgM antibody ratio. In conclusion, at this point a solid conclusion about the protective role of IgM modified LDL antibodies in humans is not warranted. If a predominant IgM response has protective effects against the development of atherosclerosis, it is difficult to see how that information can be translated into the clinical setting.

Table 10.1 Quantitative distribution of IgG- and IgM-oxidized LDL antibodies contained in immune complexes isolated from the serum of 929 patients with type 2 diabetes

	IgG[a]	IgM[a]	IgG/IgM ratio
Mean	84.2	4.5	34.0
S.D.	82.8	7.1	43.6
Median	60.2	2.6	19.7
Range	0.2–588	0–135	0.2–482

[a]Values in μg/mL

The Composition of Circulating Modified LDL Immune Complexes and Diabetic Complications

Besides studying the pathogenic role of modified LDL antibodies [40, 49–51], we developed methodology that allows the measurement of modified forms of LDL and the corresponding antibodies involved in IC formation through the isolation and fractionation of circulating IC [36, 40, 41, 52]. This is an important methodological improvement over the direct assay of modified

LDL or their corresponding antibodies in serum or plasma samples because most modified LDL in circulation is associated with the corresponding antibodies, and the measurements of either component of the circulating complexes is inaccurate due to the mutual saturation of antigen and antibody binding sites [36, 39, 52].

In contrast with the conflicting data generated by studies of modified LDL or antibodies to modified LDL [39, 53], data generated in clinical studies carried out on the DCCT/EDIC cohort (type 1 diabetes) with our assay have shown that high levels of oxLDL and AGE-LDL in isolated and fractionated IC are associated with increased risk for developing diabetic nephropathy [54]. Using coronary artery calcification (CAC) indices and carotid intima-media thickness (IMT) as endpoints indicative of cardiovascular disease progression we also found that increased levels of oxLDL and of AGE-LDL in circulating IC are associated in the DCCT/EDIC cohort with the development of coronary calcification and with increased levels and progression of carotid IMT. The levels of MDA-LDL in isolated IC show a significant but weaker correlation with increased carotid IMT [55, 56]. In contrast, in patients with type 2 diabetes (VADT cohort), the levels of oxLDL and AGE-LDL in circulating IC are not significantly associated with the occurrence of acute events, but high concentrations of MDA-LDL in IC are strong predictors of acute events, especially myocardial infarction (MI) [57]. In agreement with our data, Holvoet et al. reported in two separate studies a link between high levels of oxLDL and established CAD and between elevated plasma MDA-LDL levels and plaque instability [58, 59].

The correlation between MDA-LDL levels and plaque instability is particularly significant because it has been well-established that atherosclerotic plaque rupture is a critical event triggering thrombus formation, arterial luminal obstruction, and subsequent acute coronary syndromes [60]. Plaques that are prone to rupture consist of a larger intimal lesion with abundant macrophages and foam cells and a thinned fibrous cap [61]. Necropsy studies have demonstrated that atherosclerosis in diabetic patients is more extensive and accelerated than that in non-diabetic patients [62]. Furthermore, studies have also shown that atherosclerotic lesions in diabetic patients were more vulnerable as they had larger intimal lesions and more macrophage infiltration as compared to those in non-diabetic patients [63]. Analysis of gene expression in atherosclerotic plaques showed that when compared to stable plaques, vulnerable plaques have higher expression of matrix metalloproteinases (MMP) with collagenase activity, which contribute to the thinning of the fibrous cap, causing plaque instability and rupture [64]. Among the metalloproteinases, MMP-9 has been the object of considerable interest in recent years and according to some studies is an independent risk factor for atherothrombotic events [65, 66]. MMP-9 synthesis and release can be induced through TLR-4 stimulation, usually involving bacterial endotoxins [67] but also by minimally modified LDL [68]. The association of circulating MDA-LDL and IC-associated MDA-LDL specifically with plaque instability/acute CV events raises interesting questions such as whether IC containing different modified forms of LDL may lead to distinct gene regulation and in the case of MDA-LDL lead to plaque instability by inducing macrophage apoptosis and/or increased synthesis of matrix metalloproteinases, such as MMP-9 [69]. OxLDL-IC, in contrast, induce the release of proinflammatory cytokines [50] and promote collagen synthesis by smooth muscle cells [70], and therefore are more likely to contribute to atheroma progression without a significant effect on plaque stability (Fig. 10.1).

Considerable interest has been raised by the accumulation of apoptotic macrophages around the necrotic core of vulnerable plaques [69]. A variety of pro-apoptotic insults has been proposed to play a significant role in the evolution of atheromas, including oxidative stress, endoplasmic reticulum (ER) stress, accumulation of non-esterified (free) cholesterol, and effects of pro-inflammatory cytokines released by activated macrophages [69]. Accumulation of free cholesterol in macrophages in combination with signals delivered through scavenger receptors or with interferon-γ, known to be released by activated T lymphocytes in atheromas [15, 71], leads to serine phosphorylation of STAT-1 which is a critical element in the induction

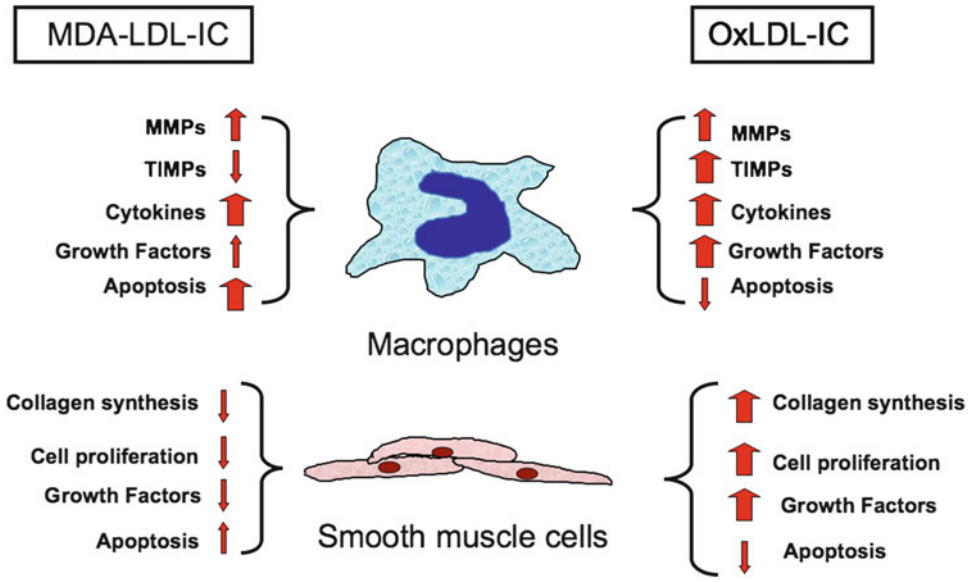

Fig. 10.1 Diagrammatic representation of the different effects of immune complexes prepared with human copper-oxidized malondialdehyde-modified LDL and the corresponding human antibodies reported by several groups (see text). While both types of immune complexes induce the release of pro-inflammatory cytokines, MDA-LDL-IC are pro-apoptotic while oxLDL-IC are anti-apoptotic and induce the release of proliferation and growth factors by macrophages and smooth muscle cells, and only oxLDL-IC induce collagen synthesis by smooth muscle cells

of apoptosis secondary to ER stress [72]. The apoptotic macrophages in atheromas are ingested by functional macrophages (efferocytosis). Efferocytosis in early lesions seems to result in suppression of inflammation, while in advanced lesions is associated with enhanced inflammation [69]. This evolution appears to be a result of defective efferocytosis, allowing the apoptotic cells to undergo necrosis, resulting in the accumulation of cell fragments that promote inflammation and plaque instability [69].

Pathogenic Mechanisms of Modified LDL IC

We have published extensive data proving that oxLDL-IC are more potent activators of human macrophages than oxLDL [50, 51, 73, 74]. The uptake of IC prepared with native or copper-oxidized LDL by human monocyte-derived macrophages is primarily mediated by Fcγ receptors, primarily FcγRI [75–77] and it has been shown that the binding of oxLDL antibody blocks the interaction of oxLDL with CD36 [78], so scavenger receptors are not involved in the process. The dependency of the vascular inflammatory process on the activation of phagocytic cells via Fcγ receptors has been demonstrated in double-knockout (DKO) mice generated by crossing apolipoprotein E-deficient mice (apoE(−/−)) with FcγR γ-chain-deficient mice (gamma(−/−)) [79]. The progression of atherosclerosis in the DKO mice is significantly reduced in comparison with apoE(−/−) mice. For MDA-LDL IC and AGE-LDL-IC FcγRI is also involved but possible involvement of scavenger receptors or receptors for AGE-modified proteins has not been excluded.

One fundamental property of LDL-IC is their ability to deliver large concentrations of free and esterified cholesterol to macrophages [51, 75, 80]. The intracellular accumulation of free cholesterol is a known inducer of ER stress, which is believed to be the prime stimulus for the chain of events that results in modification of LDL and atheroma formation. However, experimental studies have shown that ER stress usually protects against apoptosis [69]. In fact, both oxLDL at concentrations

not exceeding 75 μg/mL and oxLDL-IC prevent macrophage apoptosis [77, 81]. Whether the anti-apoptotic effect of oxLDL is a consequence of the induction of ER stress is not clear, because in addition to enhanced generation of reactive oxygen and nitrogen species [82], several other mechanisms seem to be involved, including the release of M-CSF mediated by the activation of a PI3K-dependent pathway, upregulation of the anti-apoptotic Bcl-XL gene by NFkB activation, activation of sphingosine kinase, which causes the levels of anti-apoptotic sphingosine-1-phosphate to increase, and inhibition of acid sphingomyelinase, which prevents pro-apoptotic ceramide generation [81, 83]. The anti-apoptotic effect is more pronounced with oxLDL-IC [77, 84] and is not unique to oxLDL-IC, because it has also been reproduced with KLH-anti-KLH IC [77]. However, there are significant differences between oxLDL-IC and other IgG-containing IC. Only oxLDL-IC can induce foam cell formation and the magnitude of the pro-inflammatory response induced in human macrophages is greater with oxLDL-IC than with KLH-IC, for example [50].

While oxLDL cell signaling is mediated by scavenger receptors, oxLDL-IC deliver activating signals via Fcγ receptors. The cross-linking of Fcγ receptors by IC induces phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) by kinases of the Src family, and consequent activation of the Syk pathway [85, 86]. Activation of Syk triggers the mitogen-activated protein kinase (MAPK) signaling cascade, which includes ERK1/2, p38 MAPK, and c-Jun N-terminal kinase (JNK). MAPK activation is also essential for Fc-mediated activation of NFκB [87]. Following the general rule, oxLDL-IC primarily engage FcγRI and induce the activation of the MAPK pathway [88], which is responsible for the expression of pro-inflammatory gene products. In addition, cross-linking of FcγRs by oxLDL-IC activates PI3K and c-Akt [77]. Activated c-Akt promotes cell survival by at least four different mechanisms: (1) phosphorylating the Bad component of the Bad/Bcl-X_L complex which results in its dissociation and cell survival, (2) caspase 9 inactivation, (3) regulation of the expression of transcription factors, and (4) activating IKK kinases which phosphorylate IκB and, as a consequence, release the active form of NFkB, which induces the expression of genes favoring cell survival [89] (Fig. 10.2). The repertoire of oxLDL-IC-induced pro-survival genes is much wider than that induced by oxLDL alone [74]. Also, oxLDL-IC induce HSP70B expression in macrophages. This protein binds to the internalized lipid moiety of oxLDL-IC and prevents its degradation, while at the same time inducing sphingokinase-1 [82, 90].

In contrast to oxLDL, there is no published information concerning pathways of cell activation triggered by MDA-LDL or MDA-LDL-IC. The association of MDA-LDL with acute coronary syndromes [3, 59] and the association of high levels of MDA-LDL in the circulating IC isolated from patients with type 2 diabetes who had acute CVD events, mainly MI [57], strongly suggest that MDA-LDL and MDA-LDL-IC have proapoptotic activity. The different effects of cellular uptake of oxLDL-IC and MDA-LDL-IC (Fig. 10.1) could be a result of structural differences between MDA-LDL and oxLDL. The extent of MDA-lysine modification is much greater in laboratory produced MDA-LDL than in copper-oxidized LDL [52]. This difference results in the generation of epitopes unique to MDA-LDL, and the fact that MDA-LDL antibodies obtained by immunization of rabbits with laboratory-prepared MDA-LDL react with LDL isolated from IC proves that MDA-LDL with identical epitopes and, therefore, with similar structural characteristics, is generated in vivo. Also, while copper oxidation predominantly results in ApoB fragmentation, MDA modification is associated with ApoB aggregation [91]. Obviously, these differences in ApoB could determine different biological properties of the two forms of modified LDL. For example, it has been reported that the processing of heavily oxidized and aggregated LDL by macrophages is defective [92]. Thus, the uptake of MDA-LDL IC could result in a variety of conditions that could promote apoptosis, including: (1) the release of much higher concentrations of free cholesterol in the cell, (2) intracellular accumulation of aggregated LDL, (3) cytoplasmic release of lipoprotein degradation

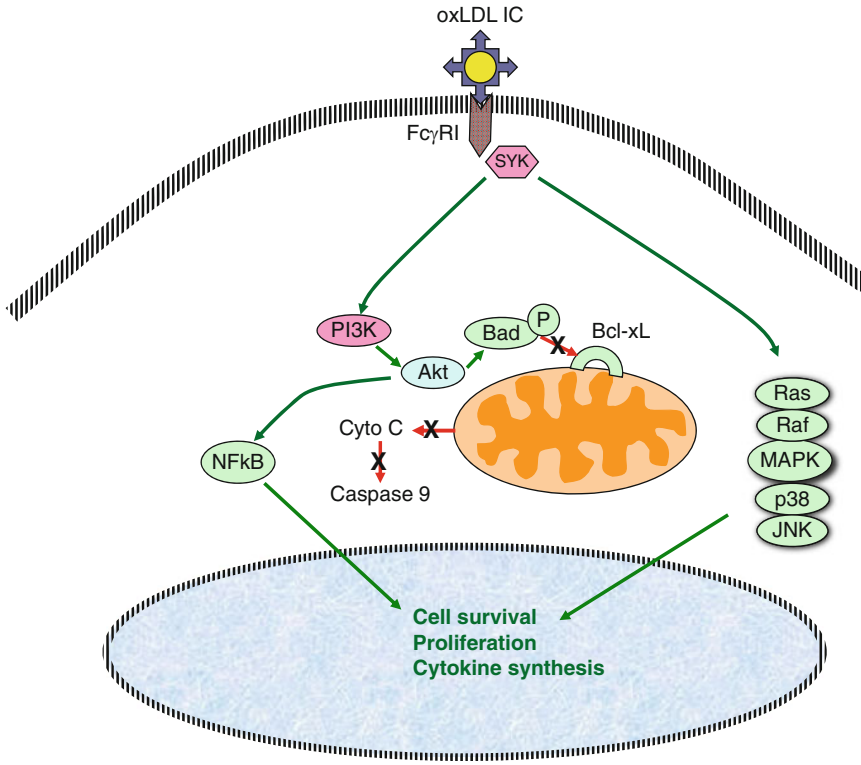

Fig. 10.2 Diagrammatic representation of the activation pathways triggered by oxLDL-IC through the engagement of FcγRI. Two main pathways are activated, the MAPK pathway which is important for the activation of cell proliferation and cytokine synthesis, and the Akt pathway, which also contributes to the induction of cell proliferation and cytokine synthesis through NFκB activation and also promotes cell survival through the dissociation of the Bad/Bcl-X_L complex, blocking the pathway that leads to the activation of caspase 9

products and oxidized phosphatidylcholine, which could be transported to the extracellular compartment and then react with scavenger receptors and/or TLRs, delivering signals that would favor the activation of pro-apoptotic pathways.

There is considerable interest in identifying biomarkers indicative of plaque instability. A variety of proteins and enzymes have been proposed as candidates, as reviewed recently by Koenig. [93] Besides MMPs, reactive proteins (CRP), cytokines (IL-6, IL-18), enzymes (glutathione peroxidase, lipoprotein-associated phospholipase A-2 (Lp-PLA2)), myeloperoxidase, chemotactic proteins (monocyte chemotactic protein-1), and modified lipoproteins have been proposed as indicators of plaque instability [3, 58, 59, 66, 94, 95]. Our data suggest that modified forms of LDL can also be useful biomarkers for cardiovascular disease [54–56] and plaque vulnerability risk [57].

In conclusion, modified LDL plays a key role as a persistent insult leading to chronic vascular inflammation. The pro-inflammatory effects of modified LDL are significantly enhanced as a consequence of the formation of immune complexes as a consequence of the reactivity of different LDL modification with specific antibodies. In general, modified LDL IC have proinflammatory properties, but both clinical and experimental data suggest that there are differences in the consequences of cellular uptake of IC depending on the predominant type of LDL modification. This novel finding opens a variety of basic and clinical research perspectives, ranging from the investigation of the molecular mechanisms that are responsible for the different cellular effects of

different LDL modifications to the definition of specific LDL modifications as risk factors able to discriminate between patients with different types or degrees of diabetes-associated complications.

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