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C-Reactive Protein and Other Emerging Blood Biomarkers to Optimize Risk Stratification of Vulnerable Patients

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Several emerging plasma biomarkers may ultimately prove useful in risk stratification and prognosis of cardiovascular disease. The clinical utility of these biomarkers will depend on their ability to provide a reflection of the underlying atherosclerotic burden or activity; the ability to provide reliable, accurate, and cost-effective information; and the ability to predict future events. High-sensitivity C-reactive protein (hs-CRP) fulfills many, if not all, of these criteria, and blood levels of hs-CRP are now commonly used in clinical practice to improve vascular risk prediction in primary and secondary prevention across all levels of low-density lipoprotein-cholesterol (LDL-C), all levels of the Framingham Risk Score, and all levels of metabolic syndrome. High-sensitivity C-reactive protein may also have clinical relevance as an adjunct to LDL-C for both the targeting and monitoring of statin therapy. Accumulating evidence suggests that several other selected emerging biomarkers may also potentially prove useful in the diagnosis and prognosis of cardiovascular disease. Specifically, data are accumulating on the potential clinical utility of lipoprotein-associated lipoprotein-associated phospholipase A_2, myeloperoxidase, oxidized LDL, lipoprotein (a), isoprostanes, and small, dense LDL. This review focuses on hs-CRP and these emerging plasma biomarkers, and their potential diagnostic and prognostic utility in cardiovascular disease. Plasma biomarkers that reflect the clinical potential of atherothrombotic disease may allow more precise risk stratification and prognostication in high-risk populations, and perhaps earlier diagnosis and intervention in patients at risk for or with occult cardiovascular disease. (J Am Coll Cardiol 2006;47:C19–31) © 2006 by the American College of Cardiology Foundation

Biomarkers are generally considered to be plasma measurements of molecules, proteins, or enzymes that provide independent diagnostic or prognostic value by reflecting an underlying disease state or condition. In the case of coronary heart disease, they must reflect the underlying biology of the vessel wall and, in particular, the atherosclerotic process and/or its sequelae. The clinical utility of a biomarker depends on its ability to account for a significant proportion of the disease being evaluated; be accurate and reliable; provide good sensitivity, specificity, and predictive value; and be available for widespread application (1). Clinical application further requires the demonstration, in multiple prospective cohorts, that evaluation of the biomarker is not only predictive of disease, but substantively adds to traditional risk factors such as those used in the Framingham risk score. This review evaluates several selected blood-based biomarkers that have the potential for being clinically useful in assessing cardiovascular risk.

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LOW-DENSITY LIPOPROTEIN-CHOLESTEROL (LDL-C) AS A BIOMARKER OF CARDIOVASCULAR RISK

Historically, the cholesterol content of lipoproteins, and in particular LDL-C, is the prototypical biomarker of coronary artery disease (CAD). Cholesterol content of lipoproteins serves as a convenient, yet inadequate, surrogate marker for quantitating concentrations of specific lipoproteins. It was appreciated in 1903 by Anitschkow that feeding rabbits purified cholesterol resulted in typical atherosclerotic lesions, which were postulated to occur by entrance of plasma cholesterol into the vessel wall (reviewed in reference [2]). The most convincing clinical evidence that elevated LDL-C is causal in the pathogenesis of CAD comes from patients with homozygous familial hypercholesterolemia, who usually have LDL-C levels of >500 mg/dl, where myocardial infarction and ischemic death have been reported in the first decade of life (3). Cholesterol analysis was first reported in 1885 (4), but it was not until 1974 that a fully enzymatic procedure using cholesterol esterase and oxidase coupled to a colorimetric agent became widely available clinically (5). Ironically, until just recently, most values of LDL-C in clinical laboratories were not directly measured, because of the complexities of separating various lipoprotein fractions, but were actually estimated from the Friedewald formula. Even measures of direct LDL-C contain the cholesterol content of lipoprotein (a) (Lp[a]), which may be significant in patients with high Lp(a) levels. Despite these limitations, LDL-C has been consistently confirmed as a major risk factor for CAD and is the basis for treatment with statins.

Abbreviations and Acronyms

ACS	= acute coronary syndrome
apo	= apolipoprotein
CAD	= coronary artery disease
CETP	= cholesterol ester transfer protein
ELISA	= enzyme-linked immunoabsorbent assay
HDL-C	= high-density lipoprotein cholesterol
HOCl	= hypochlorous acid
hs-CRP	= high-sensitivity C-reactive protein
LDL-C	= low-density lipoprotein cholesterol
Lp(a)	= lipoprotein (a)
Lp-PLA_2	= lipoprotein-associated phospholipase A_2
MPO	= myeloperoxidase
OR	= odds ratio
OxLDL	= oxidized LDL
OxPLs	= oxidized phospholipids
PAF-AH	= platelet-activating factor acetylhydrolase
PCI	= percutaneous coronary intervention

Nonetheless, lipoproteins alone do not explain all of the risk inherent in CAD; one-half of all heart attacks and strokes occur among individuals without hypercholesterolemia as currently defined, and one-fifth of all cardiovascular events occur in the absence of any of the major risk factors (6). Thus, additional biomarkers are needed to more fully and non-invasively diagnose and prognosticate CAD.

HIGH-SENSITIVITY C-REACTIVE PROTEIN (hs-CRP)

C-reactive protein is a circulating pentraxin that plays a major role in the human innate immune response (7) and provides a stable plasma biomarker for low-grade systemic inflammation. C-reactive protein is produced predominantly in the liver as part of the acute phase response. However, CRP is also expressed in smooth muscle cells within diseased atherosclerotic arteries (8) and has been implicated in multiple aspects of atherogenesis and plaque vulnerability, including expression of adhesion molecules, induction of nitric oxide, altered complement function, and inhibition of intrinsic fibrinolysis (9,10).

Among patients with stable angina and established CAD, plasma levels of hs-CRP have consistently been shown associated with recurrent risk of cardiovascular events (11–13). Similarly, during acute coronary ischemia, levels of hs-CRP are predictive of high vascular risk even if troponin levels are non-detectable, suggesting that inflammation is associated with plaque vulnerability even in the absence of detectable myocardial necrosis (14–16). The ability of hs-CRP to predict vascular risk has also been consistently demonstrated among patients undergoing elective angioplasty as well as surgical coronary revascularization (17,18), and the clinical combination of hs-CRP, troponin, and brain natriuretic peptide has been advocated as a generalized means to risk stratification (19).

Despite these data, the most relevant use of hs-CRP remains in the setting of primary prevention. To date, over two dozen large-scale prospective studies have shown baseline levels of hs-CRP to independently predict future myocardial infarction, stroke, cardiovascular death, and incident peripheral arterial disease (20,21). Moreover, eight major prospective studies have had adequate power to evaluate hs-CRP after adjustment for all Framingham covariates, and all have confirmed the independence of hs-CRP (22–28). For example, in the Women's Health Study (23), baseline levels of hs-CRP classified as <1, 1 to 3, and >3 mg/l have been shown to provide important prognostic information on risk across all levels of LDL-C (Fig. 1, right) and all levels of the Framingham Risk Score (Fig. 1, left). As shown in Figure 2, the poorest event-free survival in this cohort was, as expected, among those with elevated levels of both LDL-C and hs-CRP. However, as also shown in Figure 2, cardiac event-free survival was worse for those with elevated levels of hs-CRP and low levels of LDL-C as compared to those with elevated levels of LDL-C and low levels of hs-CRP. More recent analyses from this cohort further demonstrate the additive value of hs-CRP across all levels of the total cholesterol to high-density lipoprotein cholesterol (HDL-C) and apolipopro-

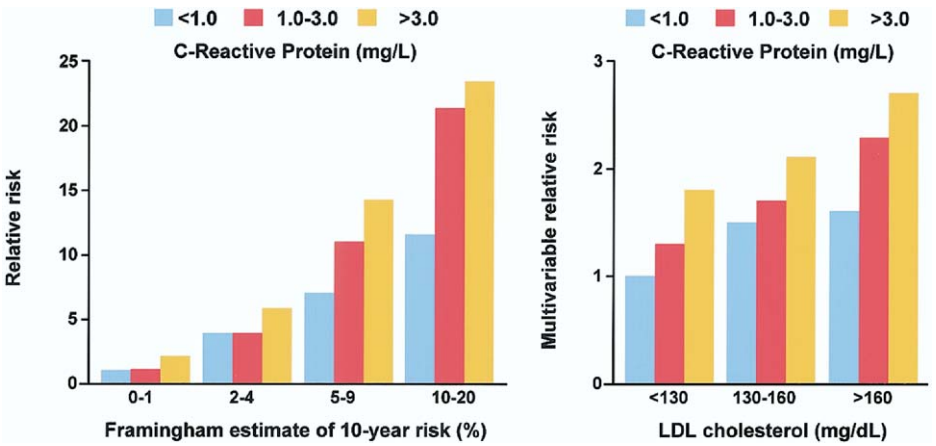

Figure 1. Additive value of high-sensitivity C-reactive protein after adjustment for traditional risk factors. Data are shown across all levels of low-density lipoprotein (LDL) cholesterol **(right)** and across all levels of calculated Framingham Risk **(left)**. Adapted, with permission, from Ridker et al. (23).

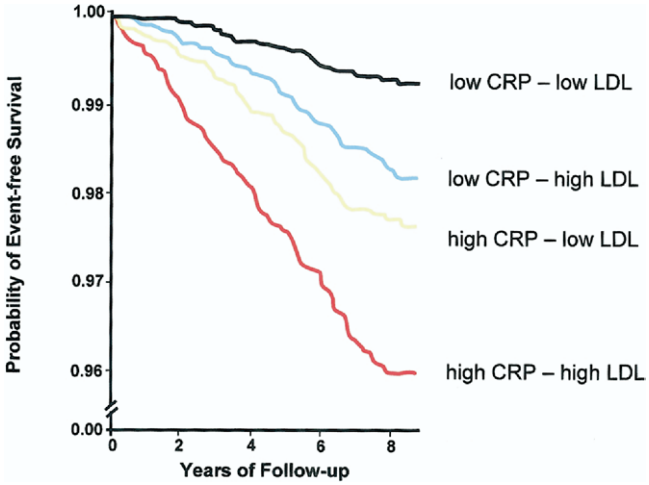

Figure 2. Cardiovascular event-free survival in apparently healthy American women according to plasma levels of low density lipoprotein (LDL)-cholesterol and high sensitivity C-reactive protein (CRP). Adapted, with permission, from Ridker et al. (23).

tein (apo)B to apoA ratios, even after adjustment for age, smoking, blood pressure, obesity, and diabetes (29). Similar data are now available from the Physicians Health Study (22), the Atherosclerosis Risk in Communities (ARIC) Study (25), the Nurses Health Study and Health Professionals Follow-Up Studies (24), the MONItoring of Trends and Determinants in CArdiovascular Disease (MONICA)-Augsberg cohort (26), the Reykjavik Heart Study (27), the Coronary Heart Study (28), and even the Framingham cohort, at least with regard to stroke (30). Multiple cohort studies further indicate that hs-CRP provides additive risk information across all levels of the metabolic syndrome (31–33) and is predictive of incident type 2 diabetes (34,35).

Beyond CRP's ability to predict risk among both primary and secondary prevention patients, interest in it has increased with the recognition that statins lower CRP in a manner largely independent of LDL-C reduction (36–38) and that the efficacy of statin therapy may be related to the underlying level of vascular inflammation as detected by hs-CRP. This phenomenon, first observed within the Cholesterol and Recurrent Events trial of secondary prevention (11), has since been observed within the Air Force/Texas Coronary Atherosclerosis Prevention Study of primary prevention (39) and, most recently, among acute coronary syndrome (ACS) patients enrolled in the Pravastatin or Atorvastatin Evaluation and Infection Therapy–Thrombolysis In Myocardial Infarction 22 (PROVE IT–TIMI 22) trial (40). In this latter study, the level of hs-CRP achieved after initiation of statin therapy proved to be of equal importance for subsequent vascular events as was the achieved level of LDL-C. However, the best overall survival was observed among those who not only lowered LDL-C below 70 mg/dl, but who also lowered hs-CRP below 2 mg/l (Fig. 3). This effect was present regardless of the statin regimen used. Further, because the level of hs-CRP achieved after initiation of statin therapy could not be predicted by the level of LDL-C, these data have lead to the hypothesis that measuring and monitoring of hs-CRP following initiation of statin therapy may be required to maximize benefit in a manner analogous to the way clinicians currently measure and monitor LDL-C (40). Support for this concept is provided by data from the Reversal of Atherosclerosis With Lipitor (REVERSAL) trial, in which atherosclerotic progression defined by intravascular ultrasound was reduced more when levels of both hs-CRP and LDL-C were lowered (41). Indeed, in the REVERSAL data for atherosclerotic regression, as in the PROVE IT–TIMI 22 data for clinical events, the greatest benefits were observed among those who not only achieved the lowest levels of LDL-C, but who also achieved the lowest levels of hs-CRP.

Despite the evidence described above, it is important to recognize that there remain no firm data to date that lowering CRP levels *per se* will lower vascular risk. Further, as with other biomarkers of inflammation, it remains controversial whether CRP plays a direct causal role in atherogenesis (42), and ongoing work with targeted CRP-lowering agents will be required to fully test this hypothesis. However, the clinical utility of hs-CRP has been well established, and on the basis of data available through 2002, the Centers for Disease Control and Prevention and the American Heart Association endorsed the use of hs-CRP as an adjunct to global risk prediction, particularly among those at "intermediate risk"(43). Those guidelines also suggested use of hs-CRP for risk stratification in the setting of acute ischemia and for secondary prevention. Data available since 2002 strongly reinforce these recommendations and suggest expansion to lower-risk groups, as well as those taking statin therapy. Perhaps most importantly, data for hs-CRP provides evidence that biomarkers beyond those traditionally used for vascular risk detection and monitoring can play important clinical roles in prevention and treatment.

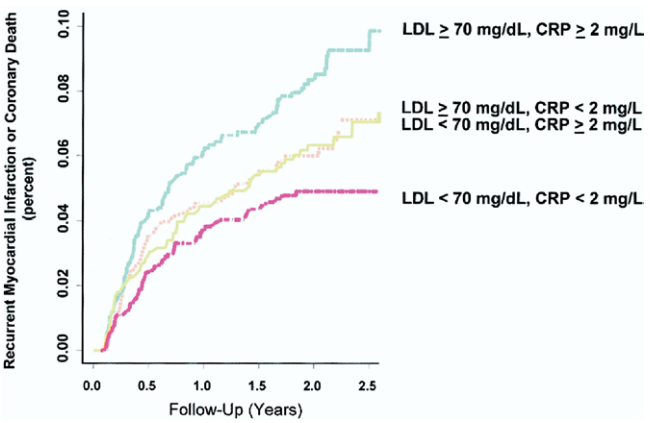

Figure 3. Rates of recurrent myocardial infarction and cardiovascular death among acute coronary syndrome patients treated with statin therapy according to achieved levels of low density lipoprotein (LDL)-cholesterol and high sensitivity C-reactive protein (CRP) in the Pravastatin or Atorvastatin Evaluation and Infection Therapy–Thrombolysis In Myocardial Infarction 22 trial. Adapted, with permission, from Ridker et al. (40).

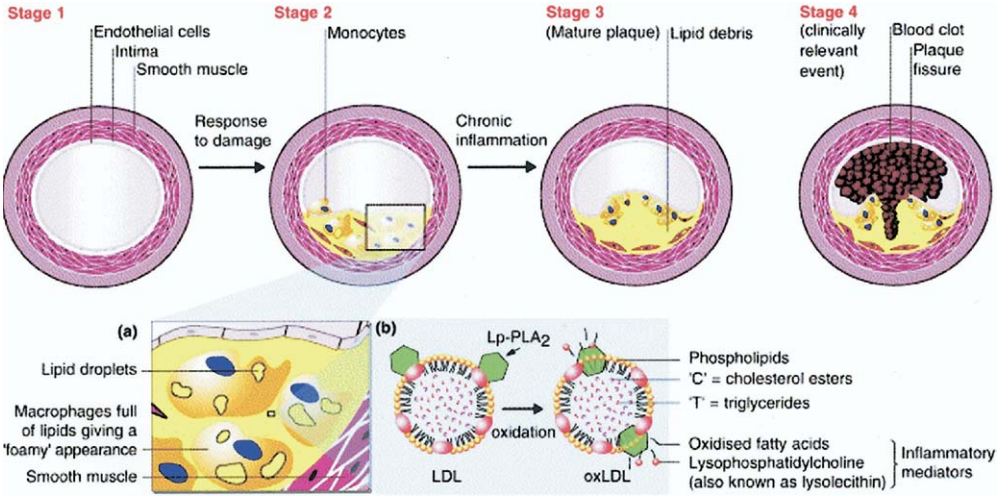

Figure 4. The role of lipoprotein-associated phospholipase A_2 (Lp-PLA_2) in the breakdown of oxidized phospholipids. Reprinted, with permission, from Macphee (44). LDL = low-density lipoprotein; oxLDL = oxidized low-density lipoprotein.

LIPOPROTEIN-ASSOCIATED PHOSPHOLIPASE A_2

Lipoprotein-associated phospholipase A_2 (Lp-PLA_2), also known as platelet-activating factor acetylhydrolase (PAF-AH), hydrolyzes sn-2 side chains of oxidized phospholipids (OxPLs) to yield a free oxidized fatty acid and lysophosphatidylcholine (Fig. 4). This activity creates two potentially pro-inflammatory and pro-atherogenic particles from one, although it has not been determined if this is a proatherogenic or anti-atherogenic property (44). Lipoprotein-associated phospholipase A_2 is secreted by monocyte-macrophages, T-cells, and mast cells, and has high affinity, resides in, and is transported by lipoproteins. It is primarily present on LDL, and small amounts are found on HDL, but it also seems to have a particular predilection for binding to Lp(a), where it is found in up to seven times higher content than equimolar amounts of LDL (45). The phospholipase activity of HDL was recently shown to be entirely due to Lp-PLA_2 rather than paraoxonase, suggesting that Lp-PLA_2 accounts for the anti-atherogenic and anti-inflammatory properties of HDL (46). Several studies have shown protective effects of overexpression of PAF-AH, including resistance against oxidative stress (47), reduced levels of oxidized lipoproteins (48), and a reduction in atherosclerosis (49). Interestingly, in Japanese populations, an inherited deficiency of Lp-PLA_2, present as a heterozygous trait in 27% of the population, appears to confer increased risk of myocardial infarction, stroke, and peripheral arterial disease (50). However, other data suggest that it may be pro-atherogenic, as it is present within atherosclerotic plaques and co-localizes with macrophages (51). Animal studies suggest that inhibitors of Lp-PLA_2, interestingly, may confer protection against atherosclerosis (52), and phase II studies are currently under way to test such compounds in humans.

Recently, an antibody-based sandwich enzyme-linked immunoabsorbent assay (ELISA) has been developed to measure Lp-PLA_2 levels in plasma. This plasma mass assay shows a high correlation with Lp-PLA_2 activity. Several prospective epidemiological studies have reported that Lp-PLA_2 is a predictor of CAD, although controversy persists as to its independence from LDL-C. The relationship of Lp-PLA_2 to LDL-C is also supported by several studies showing equivalent decreases in Lp-PLA_2 and LDL-C levels in response to several different classes of lipid-lowering agents (53). Further, in contrast to CRP, which is reduced by statin therapy in a manner independent of effects on LDL-C, there is little evidence that statins lower Lp-PLA_2 once LDL-C reduction is accounted for (54). Nonetheless, in a nested case-control study of hyperlipidemic patients from the West of Scotland Coronary Prevention Study (WOSCOPS), elevated baseline Lp-PLA_2 levels were found to be independent predictors of death, myocardial infarction, and revascularization in males, although the odds ratio (OR) was only ~1.2 (55). In the ARIC study, Lp-PLA_2 was an independent predictor of incident CAD, but only in patients with LDL-C levels below 130 mg/dl (25), an observation that conflicts somewhat with that from the WOSCOPS data. In the MONICA study, Lp-PLA_2 levels predicted hard coronary events in hypercholesterolemic middle-aged males at 14-year follow-up (56). In this study, LDL-C was not measured and thus, the independence of Lp-PLA_2 to LDL-C could not be evaluated. Brilakis et al. (57) recently showed that elevated Lp-PLA_2 at baseline in patients undergoing clinically indicated coronary angiography predicted cardiovascular events at four-year follow-up, independent of traditional risk factors and CRP. In that study, Lp-PLA_2 did not correlate with angiographic CAD, suggesting it might be more involved in CAD progression or plaque destabilization. However, in a case-control analysis from the Women's Health Study (58), Lp-PLA_2 was not an independent predictor of cardiac events after adjusting for LDL-C levels. In all of the above studies, the predictive value of Lp-PLA_2 was attenuated

substantially after adjusting for LDL-C and traditional risk factors.

The current data support continued research evaluating Lp-PLA_2 as a potential predictor of cardiovascular disease. In particular, further evidence is needed to quantitate the extent to which Lp-PLA_2 measures are independent of traditional risk factors, particularly LDL-C. In addition, additional data are needed in more diverse populations, as most of the data are derived from middle-aged males.

MYELOPEROXIDASE (MPO)

Myeloperoxidase is a heme peroxidase that is present in and secreted by activated phagocytes at sites of inflammation. Myeloperoxidase can generate several reactive, oxidatively derived intermediates, all mediated through a reaction with hydrogen peroxide, to induce oxidative damage to cells and tissues. For example, MPO reacts with hydrogen peroxide and chloride anion at physiological concentrations to produce hypochlorous acid (HOCl), a potent metal ion-independent chlorinating oxidant that can lead to formation of chlorotyrosine and other chlorinated products, resulting in adverse biological effects (59). Myeloperoxidase can also induce formation of tyrosyl radicals, resulting in dityrosine and generation of nitrotyrosine and nitrated lipids through reactions of hydrogen peroxide and nitric oxide, leading to consumption of nitric oxide and nitric-oxide derived oxidants in vitro and in vivo. Oxidation products from MPO are found at significantly increased rates (up to 100-fold higher compared to circulating LDL) on LDL isolated from atherosclerotic lesions (60) and lead to accelerated foam-cell formation through nitrated apoB-100 on LDL and uptake by scavenger receptors (61). Such distinct products of MPO are also clearly documented within lipid-rich, foam-cell-rich areas of human atherosclerotic lesions (62). More recently, several studies have shown that HDL is susceptible to oxidative modification with MPO-mediated nitration, chlorination, and tyrosilation. Such modified HDL levels were higher in patients than in control subjects, were present within atherosclerotic lesions, and resulted in decreased cholesterol efflux out of cells via an ABCA1-mediated mechanism, implicating oxidized HDL as an additional mechanism of lesion formation (63–65).

Accumulating evidence suggests that MPO may play a causal role in plaque vulnerability (66) (Fig. 5). Sugiyama et al. (67) showed that advanced ruptured human atherosclerotic plaques, derived from patients with sudden cardiac death, strongly expressed MPO at sites of plaque rupture, in superficial erosions and in the lipid core, whereas fatty streaks exhibited little MPO expression. In addition, MPO macrophage expression and HOCl were highly colocalized immunochemically in culprit lesions of these patients. Several inflammatory triggers, such as lysophosphatidylcholine, cholesterol crystals, and CD40 ligand, induced release of MPO and HOCl production from MPO-positive macrophages in vitro. In an in vitro study, HOCl induced

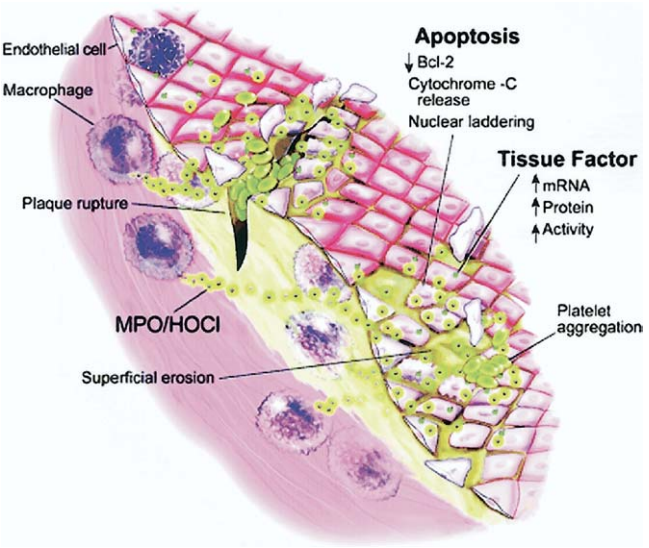

Figure 5. The role of myeloperoxidase in plaque vulnerability. Reprinted, with permission, from Hazen (66). HOCl = hypochlorous acid; MPO = myeloperoxidase; mRNA = messenger ribonucleic acid.

endothelial cell death and tissue factor expression, suggesting a link between endothelial erosion and thrombosis (68). There is also evidence that neutrophils, which secrete MPO, infiltrate fissured and thrombosed plaques in patients with ACSs (69). Consistent with MPO's potential role in the atherosclerotic process, genetic polymorphisms resulting in MPO deficiency or diminished activity are associated with lower cardiovascular risk, although the generalizability of these findings is uncertain (70,71).

In parallel with MPO's effects on nitric oxide, LDL oxidation, and presence within ruptured plaques, several recent clinical studies have suggested that MPO levels may provide diagnostic and prognostic data in endothelial function, angiographically determined CAD, and ACSs. Serum levels of MPO were recently noted to independently predict brachial artery flow-mediated dilation in 298 subjects with CAD or multiple risk factors for CAD (72). In a case control study of 175 patients with angiographically determined CAD, Zhang et al. (73) showed that the highest quartiles of both blood and leukocyte MPO levels were associated with ORs of 11.9 and 20.4, respectively, for the presence of CAD compared to the lowest quartiles. In the c7E3 Anti-Platelet Therapy in Unstable Refractory Angina study, Baldus et al. (74) showed that MPO levels were strong independent predictors of increased risk for subsequent cardiovascular events at six months in patients with ACSs and seemed to particularly identify patients at higher risk initially presenting with low troponin T levels. In this ACS study, the combination of CRP and MPO appeared to provide better prognostic information then either used alone. Brennan et al. (75) obtained MPO levels in the emergency department in 604 patients presenting with chest pain but no initial evidence of myocardial infarction, and showed that MPO levels predicted the in-hospital development of myocardial infarction, independent of other mark-

ers of inflammation, such as CRP. In addition, they showed that MPO levels were strong predictors of death, myocardial infarction, and revascularization at six months after the initial event. Interestingly, atorvastatin has been shown to reduce levels of MPO-derived oxidants, independent of the lipid-lowering effects, suggesting specific antioxidant properties (76,77).

The current data suggest that MPO may serve as both a marker of disease, providing independent information on diagnosis and prognosis of patients with chest pain, and also as a potentially causal entity in the progression and destabilization of atherosclerotic lesions at the time of acute ischemia. It is not yet clear how much additional predictive power MPO levels provide above and beyond standard ischemia markers such as troponin, and further work is required to develop a commercial standardized assay. Moreover, as outlined above, almost all data for MPO is in the setting of ACS or established CAD, so the ability of MPO to provide information beyond traditional atherothrombotic risk factors used in the Framingham Risk Score is unknown. Additional studies are also needed to confirm its diagnostic and prognostic ability.

OXIDIZED LDL (OxLDL)

Oxidized LDL is generated during lipid peroxidation when oxygen free radicals abstract a hydrogen atom (H^+) from a methylene (C=C) carbon present on polyunsaturated fatty acids, resulting in generation of reactive species that modify both the lipid and protein components of LDL (as well as bystander proteins). Oxidation of LDL occurs primarily in the vessel wall, activating many inflammatory and atherogenic pathways (78,79).

Oxidized LDL is not present in normal arteries and is present only in markedly diminished amounts in fibrotic or regressing lesions (80). However, it is present in almost all other lesion types, including very early lesions of fetal aortas whose mothers were hypercholesterolemic during gestation even before monocyte entry into the vessel wall, suggesting that LDL oxidation is involved a priori in the development of atherosclerotic lesions (81). Human carotid and coronary arteries are significantly enriched in OxLDL and, importantly, unstable plaques appear to be preferentially enriched in OxLDL (82,83). In animals, OxLDL within plaques is preferentially depleted in response to regression/antioxidant diets (84–86), and in humans OxLDL becomes depleted after treatment with statins (87). In addition, OxLDL may be imaged in the vessel wall of animal models using radiolabeled oxidation-specific antibodies (84,88,89).

Oxidized LDL is not one homogeneous entity, but represents multiple chemical and immunogenic modifications of the lipid and apoB-100 on LDL. The term "OxLDL" has been used in a generic sense to describe many different types of OxLDL. To improve clarity in this rapidly evolving field of OxLDL biomarkers, we have suggested that investigators designate the particular type of OxLDL they describe with the antibody used to detect it (i.e., OxLDL-E06 measures OxPL epitopes on apoB using the murine antibody E06) (90–92).

Currently, three major OxLDL plasma ELISAs have been developed based on murine monoclonal antibodies DLH3, 4E6, and E06 that recognize various epitopes of OxLDL. Antibody DLH3 detects oxidized phosphatidylcholine epitopes on LDL and is generally performed on isolated LDL, which is not amenable to high-throughput sample measurement (OxLDL-DLH3) (93). Antibody 4E6 binds to a derivatized apoB moiety when at least 60 lysine groups have been modified, but it binds to both copper OxLDL and to malondialdehyde-LDL (OxLDL-4E6). Oxidized LDL-4E6 is available commercially for research purposes as a sandwich ELISA. A high correlation has been noted between OxLDL-4E6 and LDL-C ($r = 0.65$ to 0.70) in several studies (94–97). The natural murine antibody E06 specifically binds to the phosphorylcholine head group of oxidized but not native phospholipids (91,92,98,99) to measure OxLDL-E06. This assay is performed by capturing a constant, saturating amount of each patient's plasma apoB-100 on a microtiter plate with murine antibody MB-47, which specifically binds human apoB-100. Because each plate binds an equal fraction of each patient's apoB-100, it is by definition independent of LDL-C. Biotinylated E06 is then added to detect the presence of OxPL, e.g., to yield OxPL/apoB (Fig. 6). This assay is currently not standardized to absolute units of OxPL and is read out in relative light units per 100 ms with chemiluminescent techniques.

In the last five years, an increasing number of studies have evaluated the role of OxLDL in pre-clinical atherosclerosis, endothelial dysfunction, stable CAD, ACS, percutaneous coronary intervention (PCI), and response to statins. Elevated plasma OxLDL levels have been associated with increased carotid intima-media thickness in asymptomatic

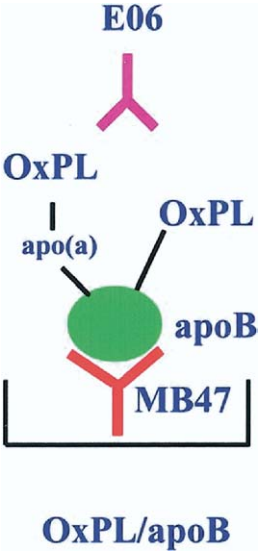

Figure 6. Schematic representation of the chemiluminescent enzyme-linked immunoabsorbent assay to measure oxidized LDL-E06.

subjects with relatives with familial hypercholesterolemia, asymptomatic middle-aged males, and even in children (97,100–102). Increased OxLDL levels have been recently associated with the metabolic syndrome and small, dense LDL (94,95,103). Coronary and brachial endothelial function is strongly correlated with plasma OxLDL levels (104–106), and improvement of brachial endothelial function was strongly correlated with reduced plasma OxLDL-DLH3 levels after LDL apheresis (107).

Oxidized LDL plasma levels correlate with the presence of CAD (92,95,108). Toshima et al. (108) have shown that plasma OxLDL-DLH3 levels were elevated in patients with CAD compared to normal control subjects, and that the receiver operating curves showed that the area under the curve was higher for OxLDL-DLH3 than for total cholesterol, apoB, HDL-C, and triglyceride levels (108). Similarly, Holvoet et al. (95) also showed that OxLDL-4E6 levels were higher in older (mean age 74 years) patients with CAD, CAD-risk equivalent, and the metabolic syndrome. Oxidized LDL-4E6 correlated with CRP and hypercholesterolemia, which likely explains why subjects with CAD had lower levels of OxLDL-4E6 than those with CAD-risk equivalents, since they also had lower LDL-C levels after treatment. Our group recently demonstrated that OxLDL-E06 strongly correlated with the presence and extent of CAD and was an independent predictor of CAD in a model with all traditional risk factors, including LDL-C and CRP (92).

Similarly, elevated OxLDL levels are significantly higher in patients with ACS (82,98,109). Oxidized LDL-4E6 predicted the presence of ACS in conjunction with troponin levels; OxLDL-DLH3 appeared to differentiate the severity of the underlying clinical presentation, and plasma levels were correlated with the presence of immunochemically detected OxLDL in coronary atherectomy specimens; and OxLDL-E06 levels showed significant increases in patients after acute myocardial infarction and subsequently declined near basal levels by seven months, confirming a temporal rise and fall of OxLDL due to plaque rupture and/or infarction. These studies emphasize that in a setting of plaque disruption and myocardial infarction, these values will be elevated, reflecting an acute phase response, and cannot be used to ascertain baseline values.

Our group has also shown that OxLDL-E06 levels significantly increased immediately after PCI and returned to baseline by 6 h (99), suggesting iatrogenically induced efflux of OxPL from the treated plaque. Oxidized LDL autoantibody titers decreased and apoB-immune complex levels increased acutely in a classical anamnestic immunization pattern, consistent with release of OxLDL and binding by pre-formed autoantibodies and generation of immune complexes. We have also shown recently that statins affect OxLDL-E06 levels. In the Myocardial Ischemia Reduction with Aggressive Cholesterol Lowering Study, levels of total apoB-associated OxPL were significantly reduced in the atorvastatin group (91). However, despite a smaller pool of apoB, the content of OxPL in the remaining individual apoB particles (OxPL/apoB) was increased and associated with increased Lp(a) levels, which we have recently shown bind such OxPL (98,99,110). This suggests the hypothesis that mobilization of OxPL, binding to Lp(a), and ultimate clearance represents a novel mechanism contributing to rapid plaque stabilization induced by atorvastatin.

Three recent studies assessed the prognostic utility of OxLDL measures. In a cross-sectional study, Holvoet et al. (111) showed that OxLDL-4E6 levels did not predict overall coronary heart disease, but did predict incident myocardial infarction in an older cohort. In a prospective study, Shimada et al. (112) followed 238 patients with CAD for a mean of 52 months and showed that baseline levels of OxLDL-DLH3 were significantly higher in patients with subsequent development of cardiac death, non-fatal myocardial infarction, and unstable angina. In a multivariate analysis accounting for typical risk factors, including LDL-C, an independent association of OxLDL-DLH3 and future events was noted with an OR of 3.15 in the highest quartile versus those in the lowest quartile. In a second prospective study, Wallenfeldt et al. (113) showed that baseline OxLDL-4E6 levels predicted the progression of carotid intima-media thickness in asymptomatic and presumably healthy 58-year-old Swedish males, independent of other cardiovascular risk factors.

In summary, OxLDL may serve as an attractive biomarker as it may provide a link between lipoprotein disorders and inflammation. Clinical research on OxLDL biomarkers is rapidly accelerating, and our understanding of its potential clinical utility is expanding. However, before such measures can be incorporated into routine clinical practice, additional work is needed to compare the different assays in their clinical utility, and more insights are needed into their ability to provide independent prognostic information, particularly in relation to other risk factors such as LDL-C and CRP. Additionally, more prospective studies are needed with serial measurements to assess changes in these markers with various treatments.

LIPOPROTEIN (a)

Lipoprotein (a) is a lipoprotein of unknown physiological function composed of apolipoprotein (a) covalently attached by a single disulfide bond on apolipoprotein B-100 (Fig. 7). Apolipoprotein (a) is composed of kringles IV and V and an inactive protease domain and has high homology (75% to 98%) to plasminogen, which has been postulated to confer pro-thrombotic effects, although these have been demonstrated conclusively only in vitro (114,115). Kringle IV is composed of 10 subtypes, of which kringle IV-type 2 repeats are very polymorphous and may exist in 2 to 43 copies, whereas the other 9 kringle IV-2 subtypes are present in 1 copy. Lipoprotein (a) is transmitted in an autosomal dominant fashion, and values range from 0 to more than 250 mg/dl. Plasma levels are largely determined

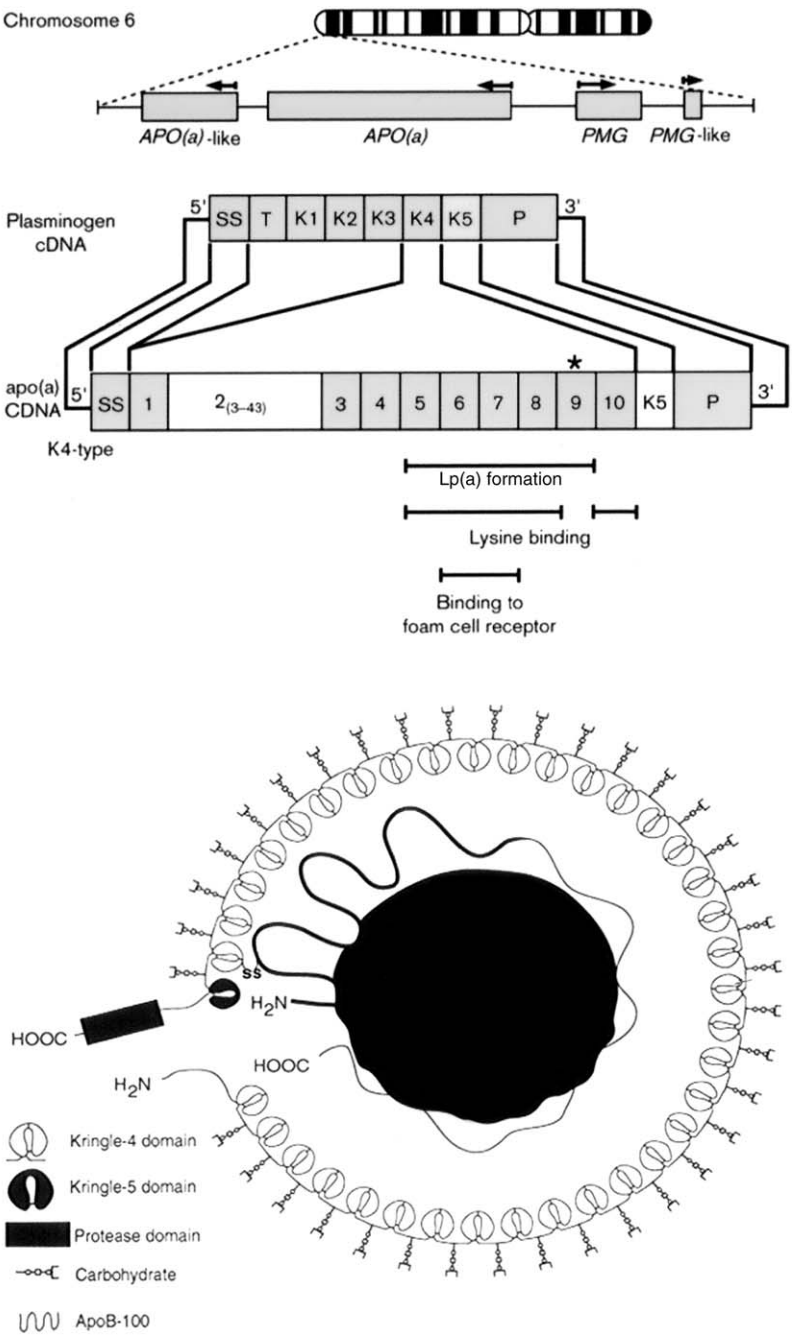

Figure 7. Genetic structure of apolipoprotein (a) **(top)** and structure of lipoprotein (a) **(bottom)**. Reprinted, with permission, from Hobbs and White (115).

by the rate of hepatic apoA synthesis, with smaller isoforms (less kringle IV-2 repeats) being secreted much faster than larger isoforms and therefore resulting in higher plasma concentrations. Thus, the number of kringle IV-2 repeats is moderately inversely related to plasma levels of Lp(a)(114). Lipoprotein (a) concentrations within atherosclerotic lesions of saphenous vein grafts have been noted to be significantly higher than plasma levels, suggesting an accumulation in the vessel wall (116).

The association of Lp(a) with CAD and its ability to act as biomarker of risk appears to be strongest in patients with hypercholesterolemia and, in particular, in young patients with premature atherosclerosis. Part of the reason for this reflects the observation that there appear to be important threshold effects such that only very high Lp(a) levels are associated with elevated vascular risk; in this regard, increased plasma levels of Lp(a) (>30 mg/dl) independently predict the presence of symptomatic and angiographic CAD, particularly in patients with elevated LDL-C levels (117,118). Interestingly, recent data from the prospective Cardiovascular Health Study, following 3,972 randomly chosen and apparently healthy subjects >65 years old for an average of 7.4 years, showed a relative risk of approximately three-fold for death from vascular events and stroke in the highest quintile compared to the lowest quintile

of Lp(a), but for males only, whereas no such relation existed for women (119). The increased risk appears to be accentuated in high-risk (120) as opposed to low-risk populations (121), and several studies have shown that young patients are particularly affected, compared to older patients (92,122,123).

The underlying mechanisms of Lp(a) contributing to the pathogenesis of atherosclerosis are not well understood. In a series of basic (110) and clinical studies (91,92,98,99), we have shown that Lp(a) is highly associated with OxPL (r = ~0.85 to 90), suggesting that some of its atherogenic properties may be mediated by its ability to bind and transport OxPL. For example, Lp(a) rises acutely in a similar fashion to OxLDL-E06 after ACS (98) and immediately after PCI (99), and correlates with the presence and extent of CAD to a similar extent as OxLDL-E06 (92). In addition, we have shown that 80 mg atorvastatin increases Lp(a) levels ~10% from baseline, which has been noted in several other studies (reviewed in [91]). In association with this increase in Lp(a) was an ~10% increase in OxLDL-E06 (OxPL/apoB), which we have interpreted as a marker of efflux of OxPL from the vessel wall and binding of OxPL by upregulated Lp(a) in the circulation.

Because the excess cardiovascular risk of Lp(a) seems to abate with lowering LDL-C levels (124) or with aging (92,122,123), it seems most appropriate to focus Lp(a) evaluation to younger patients at high risk, particularly males, such as those with multiple risk factors, and particularly those with a family history of premature CAD. When Lp(a) levels are elevated (>30 mg/dl), the primary objective is to treat elevated LDL-C or low HDL-C aggressively with a statin, perhaps in conjunction with niacin, which is the only drug that reliably lowers Lp(a) by ~30% at the higher doses. In addition, these types of patients would presumably derive the greatest benefit from early detection and treatment, as Lp(a) levels, except in acute-phase response states, remain relatively constant throughout life, and therefore these patients are exposed to the cardiovascular risk of Lp(a) starting at birth, similarly to patients with familial hypercholesterolemia. Screening for Lp(a) in such high-risk individuals would theoretically be cost-effective, as it needs to be measured only once, but this has not been evaluated as no optimal and specific treatment exists to lower Lp(a) levels. Use of Lp(a) measurements in low-risk populations is not currently recommended.

ISOPROSTANES

Isoprostanes are non-enzymatic, free-radical catalyzed isomers of cyclooxygenase-derived enzymatic products of arachidonic acid (125,126). In contrast to enzymatically generated prostaglandins, which are generated from free arachidonic acid, isoprostanes can be generated on intact cholesteryl esters and phospholipids, which are major components of lipoprotein particles and cell membranes. Following generation, isoprostanes are released by a phospholipase activity, circulate in plasma, and are ultimately excreted in urine. F2 isoprostanes are stable, specific, and unique end-products (up to 64 species can be generated) of lipoprotein metabolism and can be measured with high sensitivity and specificity with gas chromatography/mass spectrometry. However, measurement of F2 isoprostanes does not necessarily reflect LDL oxidation as they are also generated basally at low levels under normal physiological functions and are also elevated in most inflammatory disorders.

Elevated F2 isoprostane levels have been documented in patients with hypercholesterolemia, diabetes mellitus, smoking, renovascular hypertension, and hyperhomocysteinemia (125,126). Evidence of enhanced presence of F2 isoprostanes has been detected within carotid atherosclerotic plaques. In addition, they colocalize with foam cells immunocytochemically (127), and their levels correlate with unstable carotid plaques (128). Urinary F2 isoprostane levels correlate with plasma LDL-C levels and LDL-associated isoprostanes. Interestingly, unlike in mouse models, vitamin E does not seem to affect urinary isoprostane levels in humans (129). In addition, isoprostanes are elevated in unstable angina and in the coronary sinus and urine of patients undergoing PCI or thrombolysis and reperfusion during acute myocardial infarction (125,126).

Measurement of F2 isoprostanes has become the gold standard in measuring oxidative stress in vivo. However, despite the promising clinical data, no prognostic information is currently available. In addition, the relative sophistication and expense required to perform isoprostane assays in specialized laboratories in an efficient and cost-effective manner inhibits widespread use. Although ELISA kits are now available, little information is available to ascertain their precision and accuracy, and they are limited by reduced specificity in the presence of biological fluids, such as plasma.

LDL PARTICLE SIZE HETEROGENEITY—SMALL, DENSE LDL

Small, dense LDL is thought be a particularly proatherogenic compared to more buoyant LDL because it is more amenable to oxidative modification and is easily transported to the subendothelial space and trapped by proteoglycans (130). Small, dense LDL particles are generated when excess triglycerides on very low-density lipoprotein are exchanged for cholesterol esters on LDL by cholesterol ester transfer protein (CETP), producing triglyceride-rich LDL, which then undergoes lipolysis by hepatic lipase to produce smaller and denser LDL particles. At least seven subclasses of LDL particles have been described by several techniques, including density gradient gel electrophoresis, ultracentrifugation, and chromatographic and nuclear magnetic resonance spectroscopy techniques based on particle buoyant density (1.019 to 1.060 mg/dl), size, charge, and lipid and apoprotein content (130,131). Pattern A represents larger, more buoyant LDL,

and pattern B small, dense LDL, which is present in 30% to 35% of adult males, primarily with metabolic syndrome. Variations in genes involved in lipoprotein metabolism, such as apoA-I, C-III, A-IV, CETP, and manganese-superoxide dismutase, may explain 35% to 45% of the variability in LDL particle size (132). In addition, dietary fat, and particularly increased carbohydrate intake, can induce small, dense LDL phenotype (133).

Several prospective and case-control studies have shown that small, dense LDL is associated with approximately three-fold increased risk of CAD (130,134). In addition, angiographic studies have also shown increased CAD progression in patients with excess small, dense LDL particles. In the Familial Atherosclerosis Treatment Study, treatment with colestipol/lovastatin and colestipol/niacin significantly decreased hepatic lipase activity with a concomitant conversion of small, dense LDL to buoyant LDL, which was the strongest predictor of angiographic regression (135). Similarly, cholestyramine and niacin reduced progression of angiographic CAD, but only in those patients with predominantly small, dense LDL (present in 40% of the cohort) (136). However, in most of these and other studies, an independent relationship could not be demonstrated because of the strong correlation to triglyceride levels. Therefore, it is debatable whether measuring small, dense LDL at the current state of the art provides enough additional clinical utility above and beyond measuring triglyceride levels and/or diagnosing the metabolic syndrome. However, further studies are needed to evaluate the predictive value of other lipoprotein particles in prospective studies.

CONCLUSIONS

Plasma biomarkers are likely to have an important role in the future in risk stratification and prognosis of cardiovascular disease, particularly when complementing established risk factors. Compared to invasive or noninvasive imaging modalities, they offer the advantage of being relatively risk-free, less expensive, and applicable to a wide range of populations at risk. Further, like cholesterol evaluation, plasma biomarkers can be performed and interpreted in outpatient primary care settings that are the most appropriate for patient follow-up and discussions of preventive interventions. However, as they are necessarily measured in the blood, their tissue or organ etiology often cannot be determined and, when abnormal, it may be difficult to localize the site of abnormality. As atherosclerosis is a diffuse process, plasma biomarkers have the ability to provide a general measure of risk that may allow more targeted assessment with a variety of focused techniques in those with abnormal levels. In addition, they may offer a more global assessment of the response to therapy to various interventions. Coupled with novel imaging modalities, plasma biomarkers may allow more precise risk stratification and prognostication in high-risk populations that may allow physicians the ability to intervene sooner in the disease process and reduce mortality and morbidity from cardiovascular disease (137). High-sensitivity C-reactive protein has already moved into clinical practice and proven to have utility in primary prevention, acute coronary ischemia, and in the monitoring of statin therapy. As described in this review, several other biomarkers of inflammation, thrombosis, and lipid oxidation are also emerging and may ultimately prove to have utility in a variety of clinical settings.

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