Established and Emerging Plasma Biomarkers in the Prediction of First Atherothrombotic Events

Paul M Ridker, MD, MPH; Nancy J. Brown, MD; Douglas E. Vaughan, MD; David G. Harrison, MD; Jawahar L. Mehta, MD, PhD

In the current Adult Treatment Panel guidelines for cardiovascular risk detection, the plasma-based markers recommended for use in global risk assessment or in the definition of the metabolic syndrome are low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol, and triglycerides. It is widely recognized, however, that more than half of all future vascular events occur in individuals without overt hyperlipidemia. For example, in a recent large-scale analysis of >27,000 healthy American women, 77% of all future events occurred in those with LDL-C levels <4.14 mmol/L (<160 mg/dL) and 45% of all events occurred in those with LDL-C values <3.36 mmol/L (<130 mg/dL). Although risk-scoring systems that additionally evaluate traditional risk factors such as smoking, hypertension, and diabetes greatly improve risk prediction, multiple studies demonstrate that 20% to 25% of all future events occur in individuals with only 1 of these factors. Moreover, the prevalence of traditional risk factors is almost as high in those without disease as in affected individuals.

As our understanding of the pathobiology of atherothrombosis has improved, researchers have attempted to evaluate the activities of these biological processes by measuring markers in plasma or urine (ie, biomarkers). Indeed, a series of candidate biomarkers reflecting inflammation, hemostasis, thrombosis, and oxidative stress have been evaluated as potential clinical tools in an effort to improve risk prediction. To be useful in a clinical setting, the biomarker of interest must be shown in multiple prospective studies to predict future cardiovascular events. Retrospective studies are of limited value because they are prone to bias and cannot exclude the possibility that the particular biomarker is elevated as a result of, rather than a cause of, disease. To be used widely, the proposed biomarker should provide independent information on risk or prognosis beyond that available from global assessment algorithms such as the Framingham Risk Score. The biomarker additionally should be easy to measure in a cost-effective manner in outpatient settings. This typically requires an inexpensive and standardized commercial assay with low variability that does not require specialized plasma collection or assay techniques. Although not a critical issue for risk prediction, the biomarker will have broader acceptance if reduction of the biomarker leads to reduced vascular risk.

Several established and emerging novel biomarkers for vascular risk meet these criteria (Table 1), although few are ready for clinical practice. With the exception of high-sensitivity C-reactive protein (hsCRP), none has demonstrated additive value over and above Framingham risk scoring, and few are supported by commercial assays that achieve appropriate levels of standardization and accuracy for clinical use. Additionally, no clear evidence exists that lowering plasma levels of any of these biomarkers, including hsCRP, lowers vascular risk. However, many of these novel biomarkers provide important insights into the pathophysiology of atherothrombosis and serve as important research tools.

This overview focuses on established and emerging biomarkers in the prediction of atherothrombotic events in apparently healthy individuals and thus includes discussion of markers of inflammation, fibrinolysis, oxidative stress, and altered lipids (Table 1). It is important to recognize that other emerging vascular biomarkers, including brain natriuretic peptide and myeloperoxidase, have shown initial promise in the setting of acute myocardial ischemia but have yet to be evaluated in outpatient screening of healthy individuals. Other novel markers emerging in primary prevention include those related to adipocyte function, including adiponectin.

High-Sensitivity C-Reactive Protein

Inflammation characterizes all phases of atherothrombosis and provides a critical pathophysiologic link between plaque formation and acute rupture leading to occlusion and infarction. C-reactive protein (CRP) is the best characterized of the currently available inflammatory biomarkers and has emerged as a potential marker for cardiovascular risk. Composed of 5 23-kDa subunits, CRP is a circulating pentraxin that plays a major role in the human innate immune response. Although generally considered to be an acute-phase reactant, CRP is also produced in smooth muscle cells within human coronary arteries and is expressed preferentially in diseased vessels. CRP may directly affect expression of adhesion molecules, impact fibrinolysis, and alter...
endothelial dysfunction. Very recently, transgenic mice expressing human CRP have been shown to have an increased thrombotic risk and perhaps increased atherogenesis.

Clinically, CRP can be measured with several standardized, validated, and inexpensive high-sensitivity assays. More than 20 prospective, epidemiologic studies demonstrate that hsCRP is an independent predictor of risk of myocardial infarction (MI), stroke, peripheral arterial disease, and sudden cardiac death, even in apparently healthy individuals. hsCRP is surprisingly specific for the prediction of vascular events, and elevated levels do not predict noncardiovascular mortality or the development of classical inflammatory disorders. In several studies, hsCRP has been shown to add prognostic information at all levels of LDL-C and at all levels of risk as determined by the Framingham Risk Score (Figure 1). However, hsCRP levels do not correlate with lipids; combined assessment of both hsCRP and lipids appears to improve risk prediction. Individuals with elevated levels of hsCRP but low levels of LDL-C are at higher absolute risk for future cardiovascular events than those with elevated levels of LDL but low levels of hsCRP (Figure 2).

Recently, the American Heart Association and the Centers for Disease Control and Prevention issued clinical guidelines for the use of hsCRP and suggested that evaluation be considered for those deemed by global risk prediction to be at "intermediate risk." Levels of hsCRP of <1 mg/L, 1 to 3 mg/L, and >3 mg/L should be interpreted as lower, moderate, and higher vascular risk, respectively. Screening for hsCRP should be performed at the discretion of the physician as a part of global risk evaluation, not as a replacement for LDL-C and high-density lipoprotein cholesterol testing. The relationship between hsCRP and risk appears linear across a full range of values, so individuals with hsCRP levels >10 mg/L are at higher absolute risk for future cardiovascular events than those with elevated levels of LDL but low levels of hsCRP (Figure 2). Additionally, hsCRP levels provide incremental risk information at all levels of the metabolic syndrome (Figure 3).

**TABLE 1. Clinical Epidemiology of Proposed Novel Biomarkers in the Prediction of Future Cardiovascular Events**

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Prospective Studies Convincing?</th>
<th>Standardized Commercial Assay Available?</th>
<th>Additive to Lipid Screening?</th>
<th>Additive to Framingham Risk Score?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inflammation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hsCRP</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>sICAM-1</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SAA</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Interleukin-6</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Interleukin-18</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Myeloperoxidase</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>sCD40 ligand</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Altered Thrombosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tPA/PAI-1</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>+</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Homocysteine</td>
<td>+</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D-dimer</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Oxidative Stress</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxidized LDL</td>
<td>+/-</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Altered lipids</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipoprotein(a)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL particle size</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

hsCRP indicates high-sensitivity C-reactive protein; LDL, low-density lipoprotein; PAI-1, plasminogen activator inhibitor; SAA, serum amyloid A; t-PA, tissue-type plasminogen activator.
may be at even higher levels of risk than those with levels between 3 and 5 mg/L. Any clinical use of hsCRP, however, is best limited to those at “intermediate risk,” that is, individuals with anticipated 10-year event rates between 6% and 20%. Several studies additionally show that hsCRP levels >3 mg/L also predict recurrent coronary events, thrombotic complications after angioplasty, poor outcome in acute ischemia, and complications after coronary bypass surgery. In acute myocardial ischemia, hsCRP levels predict poor outcome when troponin levels are normal, suggesting that an enhanced inflammatory response is a factor in determining subsequent plaque rupture. Clinical data suggest that individuals with elevated hsCRP levels may be more likely to benefit from aggressive interventions.

It is critical to recognize that there is no evidence yet that lowering CRP will necessarily lower vascular risk. However, weight loss, exercise, and smoking cessation all lower hsCRP levels. Further, statins, fibrates, and other lipid-lowering therapies lower hsCRP. Thus, any clinical use of hsCRP at this time should be for improved targeting of primary prevention efforts directed at these modifiable risk factors. The efficacy of statins appears greater in those with elevated hsCRP levels, an observation that has lead to the hypothesis that hsCRP might be useful for targeting statin therapy in primary prevention. Aspirin has also been reported to have greater clinical benefit in terms of risk reduction in those with elevated levels of hsCRP. However, hsCRP levels do not correlate well with imaging measures of underlying atherosclerosis. Rather, elevated levels appear to provide insight into the likelihood of developing plaques more prone to rupture. Evidence for this is the finding that elevated hsCRP levels are more often observed in the presence of frankly ruptured plaques than in those with erosive disease or in those who died of nonvascular causes. Thus, hsCRP may be a better-related to clinical events than actual disease burden.

Other Markers of Inflammation

Plasma fibrinogen is an important acute-phase reactant and multiple epidemiologic studies demonstrate that baseline fibrinogen levels predict future risk of MI and stroke. Fibrinogen also plays a major role in hemostasis and traditionally has been classified among novel hemostatic and thrombotic risk factors. In direct comparisons, however, fibrinogen appears to be a weaker predictor of coronary events than hsCRP, and the measurement of fibrinogen is less well-standardized. Fibrinogen-lowering trials, such as the Bezafibrate Infarction Prevention study, failed to demonstrate clinical efficacy in terms of event reduction, although subgroup analyses suggested benefit in those with diabetes or hypertriglyceridemia. Ongoing clinical trials with fenofibrate will help reconcile these issues.

Several other markers of inflammation show clinical promise. These include alternative acute-phase reactants such as serum amyloid A, the inflammatory cytokines interleukin-6 (IL-6), IL-18, and tumor necrosis factor α, the leukocyte adhesion molecules ICAM-1, VCAM-1, P-selectin, and soluble CD40 ligand, lipoprotein-associated phospholipase A2, and biomarkers of leukocyte activation, including myeloperoxidase. 

With the exception of fibrinogen, evidence that these alternative inflammatory biomarkers are effective in population-based screening is currently modest. Nonetheless, there is considerable evidence that many have usefulness in the setting of acute myocardial ischemia. In particular, several recent studies demonstrate that myeloperoxidase levels can differentiate high risk from low risk in patients presenting with acute chest pain. Further, myeloperoxidase levels predict poor vascular outcome in the setting of acute coronary syndrome even when troponin levels are low. Thus, although clinical chemistry issues regarding variability and standardization remain, it is probable that some form of inflammatory evaluation may be adopted in the setting of acute coronary syndrome.

Two emerging biomarkers for vascular risk are IL-18, and matrix metalloproteinase-9 (MMP-9). IL-18 is an inflammatory marker produced by macrophages that stimulates release of interferon gamma by T cells. This propagates an inflammatory response, including immune cell recruitment, which promotes formation and progression of the atherosclerotic plaque. Administration of IL-18 in animal models of atherosclerosis resulted in increased atherosclerotic lesion size and T-cell number while inhibiting binding of IL-18 to its receptor-diminished plaque formation. In human studies, IL-18 expression was greater in atherosclerotic lesions, particularly in unstable plaque, compared with normal arteries. The IL-18 serum concentration was an independent predictor of cardiovascular death in patients with cardiovascular disease. In addition to this evidence, recent data from the Prospective Epidemiological Study of Myocardial Infarction (PRIME) showed an independent association between plasma IL-18 concentration and future coronary events. These initial results suggest that measurement of IL-18 may add prognostic information to lipid and inflammatory markers; its effect was not attenuated by adjustment for classic risk factors. Matrix metalloproteinase-9, a member of the metalloproteinase family, breaks-down collagen fragments and is local-
ized in the shoulder and foam cell-rich regions of atherosclerotic plaque. Variations in the MMP-9 expression have been related to the presence and severity of atherosclerosis. In cell studies, degradation of the matrix by MMP-9 at the endothelial layer promotes recruitment of monocyte-derived cells into the subendothelial space. In experimental models of atherosclerosis, degradation of the matrix surrounding smooth muscle cells also promotes smooth muscle cell migration, whereas further expression of MMP-9 results in destabilization of the plaque. These data support a role for MMP-9 in several stages of atherosclerosis. Results of clinical studies now show an association of MMP-9 serum concentrations with premature coronary atherosclerosis as well as MI and unstable angina.

Homocysteine

Homocysteine is a sulfhydryl-containing amino acid derived from dietary methionine. In individuals with rare genetic disorders of methionine metabolism, severe elevations of homocysteine can develop and lead to premature atherothrombosis in several vascular systems. Multiple mechanisms relate hyperhomocystinemia to vascular risk, including endothelial dysfunction, platelet activation, a proinflammatory response, and accelerated oxidation of LDL-C. In the general population, modest elevations of homocysteine are common and reflect poor dietary intake of folate as well as a common polymorphism in the methylene tetrahydrofolate reductase (MTHFR) gene. From a pharmacogenetic perspective, however, the clinical impact of the MTHFR polymorphism appears modest. Those homozygous for the MTHFR 677 TT variant have, at most, an increased risk of only 15% to 20%, and this effect has not been seen in countries where folate fortification has been implemented as a means to reduce the incidence of neural tube defects. In heterozygous individuals, there is little evidence of elevated homocysteine levels, even in those with low folate intake.

Plasma homocysteine can be measured by high-performance liquid chromatography or by immunoassay. Despite relative ease of measurement and a series of prospective studies showing association, screening for homocysteine has not been recommended in recent guidelines. In part, this conservative approach reflects the observation that after fortification in the United States, the magnitude of predictive value of homocysteine has decreased. Although early studies reported strong positive associations between plasma homocysteine levels and risk, in recent meta-analyses, a 25% lower homocysteine level has been associated with an 11% lower risk of coronary heart disease (CHD), an estimate smaller than anticipated. This reduced effect is not caused solely by multivitamin intake, because prospective studies have shown risk relationships in those using and not using multivitamin supplements. It is uncertain whether homocysteine evaluation adds to the Framingham Risk Score and there is little evidence that homocysteine evaluation can identify those at high risk (Figure 4). However, it is not clear that any specific intervention is required in such individuals. At this time, several trials of folate supplementation in primary and secondary prevention are ongoing.

Lipoprotein(a)

Lipoprotein(a) [Lp(a)] consists of an LDL particle with its apo B-100 component linked by a disulfide bridge to apo(a), a complex variable-length protein that has sequence homology to plasminogen. Plasma Lp(a) concentrations vary depending on apo(a) isoform size; many isoform variants have been described in different genetic kindreds. More than 25 heritable forms of Lp(a) exist, demonstrating the importance of the genome in determining plasma levels, an important issue for risk prediction across different population groups. Homology between Lp(a) and plasminogen initially increased.

the possibility that Lp(a) may inhibit endogenous fibrinolysis through plasminogen competition.78 Lp(a) also appears to act on tissue factor and plasminogen expression, to colocalize within atherosclerotic lesions, and to induce chemotactic activity at the vascular endothelium.79,80 However, the biologic function of Lp(a) remains uncertain.

Many retrospective and cross-sectional studies suggest a positive association between Lp(a) and vascular risk. However, Lp(a) levels increase after acute myocardial ischemia and with the acute-phase response; thus, these studies cannot determine a causal relation. Most but not all prospective studies that avoided this bias have shown baseline levels of Lp(a) to predict future vascular risk.81 In an overview of 27 studies with a mean follow-up of 10 years, those with Lp(a) levels in the top one-third of the distribution had a risk 1.6-times higher than those with Lp(a) levels in the bottom one-third.81 These effects were only modestly attenuated when adjusted for classical cardiovascular risk factors, partly because there is little correlation between Lp(a) and other risk markers. No studies have been performed to assess whether Lp(a) adds to risk as determined by Framingham scoring. It is also controversial as to whether Lp(a) evaluation adds to LDL screening.

Whether the assessment of Lp(a) adds prognostic information to overall risk in primary prevention remains uncertain, because Lp(a) appears to be an important marker primarily in individuals with markedly elevated risk caused either by diabetes or by hyperlipidemia.82–84 Other comparative studies have found Lp(a) to predict risk but to have modest magnitude compared with several other novel markers. Some of this effect is caused by the nonlinearity of Lp(a) in that risk primarily increases at very high levels.85 As with homocysteine, assessment of Lp(a) is likely to have greater benefit in certain high-risk groups such as those with premature atherosclerosis and in the setting of renal failure. For example, in one study, the risks of stroke, cardiovascular death, and death from all causes associated with the highest quintile level of Lp(a) were 2- to 3-times higher than the lowest quintile in a large cohort of men aged 65 years and older.86 In this study, Lp(a) level did not predict outcomes in older women.

Despite promising data, an impediment to wider use of Lp(a) in clinical practice is relatively poor standardization of commercial assays. Much of the inaccuracy of commercial Lp(a) determinations results from the use of techniques sensitive to apo(a) size.87 The introduction of assays unaffected by apo(a) heterogeneity should improve this limitation. Another concern is that few interventions lower Lp(a), with the exception of niacin, a difficult-to-tolerate drug that also lowers LDL concentrations and thus reduces any adverse hazard associated with Lp(a).88 No randomized evidence exists that lowering Lp(a) lowers vascular risk.

PAI-1 and Other Biomarkers of Fibrinolysis

Thrombosis plays a pivotal role in the pathogenesis of acute vascular occlusion, and impaired fibrinolysis can result from an imbalance between clot-dissolving enzymes such as tissue-type plasminogen activator (t-PA) or urokinase-type plasminogen activator and their endogenous inhibitors, primarily plasminogen activator inhibitor (PAI-1).89 PAI-1, a member of the superfamily of serine protease inhibitors, circulates with a half-life of ≈6 minutes and is derived from several sites, including the liver, the vascular endothelium, and adipose.90 Experimentally, there is strong evidence that PAI-1 directly contributes to arterial thrombosis in specific vascular beds. Transgenic mice that overexpress a stable form of human PAI-1 have age-dependent coronary arterial thrombosis and subendothelial infarction.91

Several environmental and genetic factors further modulate PAI-1 and fibrinolytic balance. In particular, a common single 4/5 guanine (4G/5G) polymorphism located upstream from the transcription site of the PAI-1 gene has been described that influences plasma PAI-1 levels.92 PAI-1 antigen and activity are highest in individuals homozgyous for the 4G allele and lowest in those homozygous for the 5G allele. Although the 4G allele is less frequent in blacks, the relationship between PAI-1 4G/5G genotype and plasma levels is similar across ethnic groups.93 This genotype further influences the relationship of insulin resistance and circulating PAI-1 concentrations,94 as well as the effects of activation and interruption of the renin-angiotensin system (RAS) on PAI-1 antigen.95,96 Beyond genetic predisposition, a number of factors are known to directly influence PAI-1 production, including glucose, insulin, neurohormonal factors, type II diabetes, and the acute-phase response.97–102 As such, PAI-1 can be considered as a composite indicator of inflammation, metabolic control, and neurohormonal activation, all of which may contribute independently or synergistically to cardiovascular risk.

In terms of clinical risk prediction, several studies have linked abnormalities of fibrinolysis to increased risk of future arterial thrombosis.103–106 For example, elevated levels of PAI-1 predict cardiovascular risk in middle-aged men,103 and circulating PAI-1 concentrations are elevated in young men at increased risk for recurrent infarction.103 Similar prospective data show baseline levels of t-PA antigen and clot lysis time to predict first-ever MI and stroke,107,108 whereas D-dimer, a marker of fibrin turnover, is consistently elevated in those at risk for future events.109–111 However, whether the predictive value of measures of fibrinolysis is truly independent of traditional risk factors remains uncertain. With specific regard to PAI-1, there are no population-based data demonstrating that knowledge of baseline levels substantially adds prognostic information to lipid screening or to Framingham risk scoring. Moreover, the use of PAI-1 antigen or activity levels as a clinical predictor is limited by practical considerations, including circadian variation, sensitivity to neurohormonal and metabolic factors, and the challenges of specimen collection to minimize release of PAI-1 from platelets.

Despite these limitations, there are intriguing data relating changes in PAI-1 that follow the use of agents known to reduce vascular risk. Most importantly, numerous studies indicate that angiotensin-converting enzyme inhibitors decrease PAI-1 antigen concentrations in men and women, in different ethnic groups, in patients with coronary risk factors, and in patients after MI.100,112–115 By contrast, AT1 receptor antagonists that did not reduce the risk of cardiovascular death or MI in either the Irbesartan Diabetic Nephropathy Trial116 or the Reduction of Endpoints in NIDDM with the
Angiotensin II Antagonist Losartan (RENAAL) trial\textsuperscript{117} do not consistently reduce PAI-1 antigen.\textsuperscript{98} Activation of the RAS by thiazide diuretics or salt-depletion increases PAI-1 antigen in patients with normal to high renin concentrations.\textsuperscript{99,101} This effect of diuretics on PAI-1 antigen concentrations may seem paradoxical when one considers that diuretics reduce mortality in the setting of essential hypertension.\textsuperscript{99,101} However, the effect of activation of the RAS on PAI-1 antigen is attenuated in individuals in whom diuretics are most likely to reduce blood pressure, compared with individuals with normal to high renin hypertension.\textsuperscript{118} Thus, although PAI-1 measurement may have limited value for clinical event prediction in broad populations, the use of PAI-1 as an informative biomarker of thrombosis and fibrinolysis in studies of the RAS should not be overlooked.

**Tissue-Type Plasminogen Activator**

Because of its association with impaired glucose tolerance and the biological plausibility discussed previously, a great deal of attention has been given to PAI-1 as a contributor to and as a marker of risk of ischemic cardiovascular events. However, its counterpart in the fibrinolytic system, t-PA, has also been examined as a potential marker of cardiovascular risk. Although t-PA and PAI-1 belong to the fibrinolytic system of proteins, there are major physiological differences between the two; t-PA is primarily synthesized and secreted by the vascular endothelium. It has a short half-life (~6 minutes) in the blood and circulates in trace concentrations in plasma. Whereas PAI-1 production is driven by the molecular clock and exhibits a relatively robust circadian variation, t-PA production is not driven by circadian factors and plasma levels fluctuate little during a 24-hour period. The t-PA is stored in small dense granules in the vascular endothelium, and it is secreted in relatively large quantities in response to specific stimuli, including bradykinin and substance P. Release of t-PA is not induced by increases in flow alone or increases in local nitric oxide (NO) or prostacyclin production.

Although it is likely counterintuitive to the casual reader that elevated plasma levels of t-PA might be associated with risk, this relationship has been demonstrated in multiple epidemiological studies over the past decade. The first study to identify this relationship used plasma samples from Physician’s Health Study participants.\textsuperscript{107} In an accrued matched-pair analysis of prospective data, t-PA antigen was associated with increased risk of MI in apparently healthy men. Specifically, men in the highest quintile of t-PA antigen level at baseline had an almost 3-fold increase in risk of a first MI, compared with men in the lowest quintile (relative risk 2.81, \(P=0.002\)). In multivariate analyses that controlled for all available atherosclerotic risk factors, the association between t-PA antigen and risk of MI was no longer statistically significant. The t-PA was also shown in the Physician’s Health Study to be a strong marker of risk of stroke.\textsuperscript{108} This analysis examined 88 samples from healthy participants in the Physician’s Health Study who subsequently had a first stroke and 471 samples from participants who remained free of cardiovascular disease during the 5-year follow-up. The age-adjusted relative risk for stroke in men with baseline t-PA concentrations above the 95th percentile was 3.51 (\(P=0.0006\)) for total stroke and 3.89 for thromboembolic stroke. These findings did not change substantially in analyses that also controlled for risk factors.

These results linking elevated plasma levels of t-PA antigen with risk of MI and stroke have been confirmed and extended in other epidemiological studies. In an observational study of 141 patients with angiographically proven coronary artery disease followed-up for 13 years, t-PA antigen was the only marker predicting coronary events, with subjects in the 5th quintile of t-PA levels having a relative risk of 7.3 to compare to that of the first quintile.\textsuperscript{105} Similar results have been reported in the prospective Atherosclerosis Risk In Communities (ARIC) study of middle-aged adults in which stored baseline plasma samples from 326 subjects with CHD were compared with a stratified random sample of the entire cohort of 720 subjects. Again, plasma levels of t-PA and PAI-1 were associated positively with CHD incidence in analyses adjusted for age, race, and sex, but were not associated with CHD after adjustment for other risk factors.\textsuperscript{119}

Clearly, abundant clinical epidemiological data suggest that elevated plasma t-PA levels are a marker of subsequent cardiovascular events, both MI and stroke. It has been suggested that elevated plasma levels of t-PA serve as a marker of preclinical atherosclerosis. However, the more widely accepted explanation for the relationship between t-PA and risk is derived from the fact that t-PA in plasma rapidly interacts with and complexes with active PAI-1 in plasma. Importantly, these covalent complexes have a longer circulating half-life than unbound t-PA. Therefore, increased t-PA antigen levels are generally viewed as a correlate of increased plasma PAI-1 activity. Studies are now underway to examine more dynamic and potentially informative aspects of t-PA and its potential association with risk of cardiovascular disease. As noted, t-PA is stored in endothelial cells and is secreted in large quantities in response to specific stimuli such as bradykinin. In healthy human subjects, arterial t-PA release can increase >60-fold acutely, whereas arterial t-PA release is markedly diminished in cigarette smokers and in the presence of atherosclerosis. This potentially serves as a relatively sensitive marker of endothelial dysfunction that complements information gathered by measuring flow-dependent or endothelial-dependent vasodilation. Prospective studies are now underway to define the relationship between endothelial dysfunction and acute t-PA release, and the impact of common risk factors on arterial t-PA release.

**Biomarkers of Oxidative Stress**

Reactive oxygen species (ROS) are a family of molecules including oxygen and its derivatives that are produced in aerobic cells. Excessive ROS production, outstripping endogenous antioxidant defense mechanisms, is commonly referred to as oxidative stress.\textsuperscript{120} A large body of literature has linked oxidative stress with hypertension and atherosclerosis.\textsuperscript{121} An initial focus of oxidative stress is related to its role in ischemia/reperfusion, in which phagocytic cells are stimulated to produce “bursts” of ROS that exacerbate tissue damage.\textsuperscript{122} It is now clear, however, that many cells contain
enzymes that constitutively generate low levels of ROS. An important family of enzymes is the NADPH oxidases, multicompartment enzyme systems that are the predominant source of ROS in vascular cells.\(^{123}\) The NADPH oxidases continuously produce low levels of superoxide. However, their activity can be increased by common stimuli such as angiotensin II, inflammatory cytokines, and mechanical stimuli. Experimental data indicate that these low levels of ROS contribute to many aspects of the atherosclerotic process. Importantly, the superoxide anion reacts in a diffusion-limited manner with NO. This not only leads to loss of the beneficial effects of NO (eg, vasodilatation, inhibition of adhesion molecule expression, inhibition of platelet adhesion, prevention of lipid oxidation) but also results in formation of the strong oxidant peroxynitrite (ONOO\(^{-}\)), particularly when NO production is excessive. Peroxynitrite potently oxidizes many compounds, damages cell membranes, and transfers NO\(_2\) to the 3-OH group on tyrosines in a variety of proteins.\(^{124,125}\) This last action alters the catalytic function of enzymes, forms antigenic substrates, inhibits tyrosine phosphorylation, and alters metabolic pathways.\(^{126–129}\) In addition to nitration by peroxynitrite, nitrotyrosine formation can occur as a result of formation of NO\(_2\) from nitrite via reactions of hydrogen peroxide with peroxidases.\(^{130}\)

A particularly important aspect of the biochemistry of ROS is lipid oxidation.\(^{131}\) A number of studies suggest that the oxidatively modified LDL (ox-LDL) is a more potent proatherosclerotic mediator than the native unmodified LDL. Endothelium exposed to ox-LDL develops early signs of injury, such as apoptosis.\(^{132}\) Ox-LDL decreases the gene expression of the endothelial cell NO synthase (eNOS) and enhances ROS generation in endothelial cells.\(^{133}\) Ox-LDL also activates inflammatory cells and facilitates release of a number of growth factors from monocytes/macrophages.\(^{134,135}\) Vascular smooth muscle cells exhibit intense proliferation when exposed to ox-LDL.\(^{136}\) Platelet eNOS activity is diminished in the presence of ox-LDL, and these cells demonstrate intense activation when exposed to small amounts of thrombin.\(^{137}\) LDL, especially when oxidized, enhances the formation of MMPs in vascular endothelial cells and fibroblasts,\(^{138}\) thus setting the stage wherein oxidative stress may lead to rupture of a soft plaque. Recent studies have indicated that ox-LDL stimulates expression of CD40/CD40L in endothelial cells, leading to cytokine release.\(^{139}\) In other studies, ox-LDL upregulates expression of various components of the RAS, such as angiotensin-converting enzyme and Ang II AT1 receptors in endothelial and vascular smooth muscle cells.\(^{140,141}\) RAS activation is a critical component of atherosclerosis and myocardial ischemia.\(^{142,143}\)

A number of ox-LDL receptors have been identified in the monocytes/macrophages, smooth muscle cells, and endothelial cells. These include SR-A1, SR-AII, CD36, macroisialin, and the lectin-like receptor for ox-LDL (LOX-1). Blockade of these receptors inhibits many of the processes leading to atherosclerosis. Of all these receptors, LOX-1 is predominantly expressed in endothelial cells, and to a small extent in monocytes/macrophages, smooth muscle cells, and platelets.\(^{144}\) Use of a LOX-1–blocking antibody reduces almost all of the effects of ox-LDL on endothelial cell biology described.\(^{144}\)

Altered cellular function resulting from protein, lipid, and DNA oxidation has been implicated in vascular inflammation. Of note, many signaling pathways, including the MAP kinases, PI3 kinase, Akt, protein kinase C, and protein phosphatases are activated by ROS.\(^{123}\) In addition, ROS such as peroxynitrite, hydrogen peroxide, and lipid peroxides activate DNA binding factors (eg, AP-1, NF\(_{κB}\)) that, in turn, lead to transcription of several proinflammatory genes, including VCAM-1, MCP-1, ICAM, and E-selectin.\(^{145}\) ROS also have been shown to activate MMPs and angiogenesis hallmarks of unstable atherosclerotic lesions.\(^{58}\) Thus, ROS have been implicated in virtually every step in the atherosclerotic process.

Collectively, these observations suggest that oxidative stress, either in the form of ROS per se or via modification of LDL, plays an integral role in the initiation of atherosclerosis (characterized by accumulation of inflammatory cells), to its progression (smooth muscle cell growth and proliferation), and the end event (rupture of atherosclerotic plaque and activation of platelets resulting in formation of a thrombus). Given such strong evidence in favor of proatherogenic role of ROS, it has been puzzling that trials of antioxidant vitamins have generally proven ineffective in reducing coronary events.\(^{146}\) One explanation for these negative trials may be that there is no well-accepted marker of oxidant stress that allows therapy to be targeted to the appropriate individuals and the efficacy of treatment to be followed. Further, the correct antioxidants that penetrate the cell membrane and have long-lasting effect are not yet available. In this context, identification of an easily measured and accurate marker of oxidative stress might prove useful in future trials of antioxidant therapy for atherosclerosis.

Despite these considerations, no clear “best” marker of oxidation has yet emerged. Based on the site of ROS release and their activity, a large number of biomarkers of oxidative stress have been proposed (Figure 5, Table 2).\(^{147–150}\) Given its obvious pathophysiologic significance, there has been a great deal of interest in detection of ox-LDL or antibodies to ox-LDL. In a recent nested case-control study in individuals with and without elevated LDL-C levels, ox-LDL levels were increased in patients in whom acute MI subsequently developed at a mean of 2.8 years.\(^{161}\) In another study, 178 patients with angiographic coronary artery disease had a higher level of ox-LDL and higher global risk assessment scores than 126 age-matched controls.\(^{162}\) This finding has been corroborated in a larger study comprising 385 individuals with CHD and 1183 patients at high risk for CHD. In this latter analysis, the odds ratio for high CHD risk in the highest quartile of ox-LDL was 2.79, compared with the lower quintile and after adjusting for age, sex, race, LDL-C, smoking status, and CRP.\(^{163}\) Although of interest, the cross-sectional design of these studies makes it impossible to discern whether ox-LDL elevations are a cause or a result of prevalent atherosclerosis. No study to date has prospectively examined the usefulness of oxidized LDL as a risk factor for the subsequent development of atherosclerosis in otherwise healthy subjects.
been found to be elevated in some but not all studies of subjects with cardiovascular disease. Oxidation of intracellular glutathione (GSH) leads to accumulation of oxidized glutathione (GSSG). The enzyme uses hydrogen peroxide and other peroxides as cosubstrates for this reaction. In the plasma and interstitial tissues, oxidation of cysteine leads to formation of cystine. The ratios GSSG/glutathione and cysteine/cystine may reflect oxidative processes in vivo. On the right of this Figure are reaction products of NO. NO released from the endothelium and is oxidized to vasoinactive nitrite \( \text{NO}_2^- \). The reaction of either nitrite or NO with the heme group of hemoglobin leads to formation of nitrate \( \text{NO}_3^- \), which can be detected in plasma. Nitrogen dioxide \( \text{NO}_2 \) and other nitrosating species can react with thiols to form nitrosothiols \( \text{RS-NO} \), such as S-nitrosoglutathione, S-nitrosohemoglobin, and S-nitrosoalbumin, which may serve as circulating NO donors. Myeloperoxidase in inflammatory lesions and in atherosclerotic vessels, on reaction with hydrogen peroxide, can form a highly reactive \( \pi \) radical that can oxidize NO to nitrite and nitrogen dioxide, which can react with tyrosines to form nitrotyrosine.

Of other markers of oxidative stress, F-2 isoprostane detection has been one of the best characterized, and levels are elevated in smokers, hypercholesterolemic patients, and in those with diabetes. F-2 isoprostanes can be measured in urine and plasma using gas chromatography/mass spectrometry. However, this method is impractical for large-scale use and plasma measurements are of questionable value. A recently developed enzyme-linked immunosorbent assay shows promise for large-scale use but needs validation.

Another potentially useful oxidative stress marker is the plasma level of oxidatively modified tyrosines, including nitrotyrosines, cysteine/cystine, and bromotyrosines. As noted, these are formed as a result of hydrogen peroxide interactions with peroxides, and recent data indicate that plasma levels of nitrotyrosine correlate with the severity of coronary artery disease. Interestingly, statin treatment lowers plasma levels of these modified tyrosines, and a drawback to these assays is that they are performed using sophisticated and expensive technology that is not routinely available.

Two other assays of oxidative stress that are technologically simpler are potentially useful. Plasma levels of thiols such as glutathione and the ratio of reduced to oxidized glutathione can be readily determined using high-performance liquid chromatography. Recent work by Jones et al has shown that this “glutathione redox ratio” varies with age in a manner compatible with the onset of vascular disease. Recently, a commercially available assay of “organic peroxides” known as the Free Oxygen Radical Test (FORT) or d-ROMs test has become available. This assay is relatively inexpensive and can be performed in minutes. In a recent report, the d-ROMs assay was used to assess the effectiveness of various antioxidant treatment strategies.

Another potential marker is erythrocyte levels of the antioxidant enzyme glutathione peroxidase-1. In mammalian cells, glutathione peroxidase is the predominant enzyme responsible for catalysis of hydrogen peroxide and lipid peroxides; therefore it is an antioxidant defense marker rather than a marker of oxidant stress. In a recent study of 636 patients referred for angiography and evaluation of suspected coronary artery disease, the baseline levels of erythrocyte glutathione peroxidase-1 were inversely associated with increased risk of cardiovascular events during a 5-year follow-up. Although related to gender and smoking status, levels of glutathione peroxidase remained significantly associated with risk after adjustment for major vascular risk factors (Figure 6).

The large number of proposed biomarkers of oxidant stress present clinicians with a substantial diagnostic challenge. A technological concern is that biomarkers of oxidation often are at risk for auto-oxidation and their measurement cannot easily be made on stored samples. In other cases (eg, lipid hydroperoxides, glutathione), the analyte being measured is short-lived and unstable and therefore requires immediate
### TABLE 2. Proposed Biomarkers of Oxidative Stress

<table>
<thead>
<tr>
<th>Oxidant Stress Marker</th>
<th>Previous Use</th>
<th>Method of Measurement</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>F2-isoprostanes(^{147})</td>
<td>(\uparrow) in smokers, diabetes, COPD, hypercholesterolemia, scleroderma</td>
<td>GS/mass spectroscopy; ELISA (new kits need widespread validation); Urine and plasma</td>
<td>Best characterized</td>
<td>GC/mass spectroscopy method impractical for large studies; ELISA kit measurements promising but need validation; Currently not well-accepted; Plasma measurements of questionable value</td>
</tr>
<tr>
<td>Thiobarbituric acid reactive substances (TBARS)(^{146})</td>
<td>(\uparrow) in a variety of systemic illnesses (multiple sclerosis, hemodialysis, malaria, diabetes), after hyperbaric oxygen exposure</td>
<td>Spectrophotometric reaction between malondialdehyde (end product of lipid oxidation) with TBA</td>
<td>Simple assay; Extensively used in basic studies.</td>
<td>Spectrophotometric method is nonspecific and may detect other aldehydes; HPLC modification is more specific but impractical for large studies</td>
</tr>
<tr>
<td>Oxidized LDL (ox-LDL)(^{146})</td>
<td>Ox-LDL (\uparrow) in acute coronary syndromes, heart failure, after MI; ox-LDL antibodies correlate inversely with endothelial function in transplantation subjects and coronary artery disease</td>
<td>ELISA and related antibody-based assays; ox-LDL can be measured with murine antibody EO6</td>
<td>Relatively straightforward assays may be applied to large number of subjects</td>
<td>Specificity of ox-LDL measurements questionable and may reflect oxidized fatty acids that have been exchanged with the LDL particle; ox-LDL rapidly cleared from plasma, low levels may not reflect level of the underlying disease; Exercise (\uparrow) LDL oxidation acutely</td>
</tr>
<tr>
<td>Free oxygen radical monitor (FORM assay or D-ROMs test)(^{153})</td>
<td>(\uparrow) in peripheral vascular disease, can be (\downarrow) by antioxidants; (\uparrow) in hypertension and can be (\downarrow) by antihypertensive treatment; (\uparrow) by heavy alcohol use</td>
<td>Measure of lipid peroxides and lipid alcohols; Depends on Fenton-like reaction leading to formation of lipid peroxo and alkoxo-radicals that in turn react with a chromogenic substrate</td>
<td>Simple assay can be completed in minutes</td>
<td>Specificity not established; Probably not suitable for samples stored for prolonged periods; EDTA and EGTA interfere with assay</td>
</tr>
<tr>
<td>8-Hydroxy 2’-deoxyguanosine (8OHdG)(^{132–154})</td>
<td>(\uparrow) in smokers, implicated in carcinogenesis; (\uparrow) in blood and mononuclear cells in hypertensive subjects</td>
<td>Oxidation of guanine at the C8 position leads to a G to T substitution; Can be measured using HPLC or ELISA; Urine samples most commonly used</td>
<td>Potentially very important mechanism underlying oxidative modification of gene expression</td>
<td>Can be altered by gene excision rather than oxidation; Increased by enhanced metabolic rate. ELISA may not be specific for 8OHdG</td>
</tr>
<tr>
<td>Protein carbonyl(^{155–157})</td>
<td>(\uparrow) in tissues and plasma in aging, Alzheimer disease, cystic fibrosis, cataracts, Parkinson disease, and muscle after exercise; Plasma levels (\downarrow) by antioxidant treatment</td>
<td>Formed by oxidation of side chains of lysine, praline, arginine, and threonine; also by reactions with hydroxynonalenal (product of lipid oxidation); Colorimetric reaction with 2,4 dinitrophenylhydrazine; Antibody tests also available</td>
<td>Simple assays; May reflect oxidation of both proteins and lipid oxidation; Pathophysiologically relevant targets</td>
<td>Tissue levels may not be reflected in plasma samples; Not specific for cardiovascular disease</td>
</tr>
<tr>
<td>Modified tyrosines (nitrotyrosine, chlorotyrosine, bromotyrosine)(^{158,159})</td>
<td>Nitrotyrosine levels (\uparrow) in coronary artery disease; Statins (\downarrow) nitrotyrosine levels</td>
<td>Most accurate measurement requires quadrupole GC/mass spectroscopy; ELISA assays also available for nitrotyrosine</td>
<td>May reflect generation of peroxynitrite or reactions of peroxides with hydrogen peroxide</td>
<td>Not widely available; ELISA assays need validation</td>
</tr>
<tr>
<td>Plasma glutathione levels; Ratio of oxidized to reduced glutathione(^ {160})</td>
<td>(\uparrow) in hypertension, experimental models of atherosclerosis</td>
<td>Glutathione is most prevalent intracellular thiol; Oxidation may occur on direct reaction with oxidants or may reflect reaction of glutathione peroxidase and (H_2O_2); Requires HPLC;</td>
<td>Physiologically very relevant; May reflect oxidative status in nonlipid compartments (eg, cytoplasm, intracellular space)</td>
<td>Measurement difficult; Samples must be collected in specific buffer</td>
</tr>
</tbody>
</table>

COPD indicates chronic obstructive pulmonary disease; ELISA, enzyme-linked immunosorbent assay; GC, gas chromatography; HPLC, high-pressure liquid chromatography; LDL, low-density lipoprotein; MI, myocardial infarction; TBA, thiobarbituric acid.
assay or dilution in a preservation buffer. This represents a challenge when assays cannot be performed on site within hours or days. Another major issue is that oxidation, like inflammation, almost certainly occurs in numerous conditions, including cancer, immune disorders, asthma, smoking, diabetes, and aging.\(^{169}\) Thus, elevated markers of oxidation will almost certainly not be specific for atherosclerosis. Despite these limitations, optimism remains that biomarkers of oxidant stress will play a role in clinical detection of risk.

### Measurements of NO

There is general agreement that the small-molecule gas NO has an antiatherogenic effect in the vasculature.\(^{170,171}\) Further, many common risk factors for atherosclerosis lead to impairment in the ability of the endothelium to produce “bioactive” NO.\(^{172}\) In many cases, this is because NO is destroyed by a reaction with O\(_2^-\) after it is produced.\(^{173}\)

Given the predominant role of NO in modulation of vascular disease, and the fact that numerous diseases result in NO loss, the ability of the endothelium to modulate vasodilatation by production of NO may reflect a summation of processes focusing on the endothelium, and one might be able to use endothelium-dependent vasodilatation as a readout of the summation of many diseases that ultimately cause vascular disease. Indeed, endothelium-dependent vasodilatation, either in the coronary arteries or in the forearm, seems to reflect prognosis, at least in retrospective studies.\(^{174–176}\)

These considerations have resulted in substantial interest in being able to measure NO metabolites in the plasma. Most efforts have focused on the oxidative degradation products of NO, nitrite (NO\(_2^-\)) and nitrate (NO\(_3^-\)), nitrosothiols (including nitroso-hemoglobin and nitrosalbumin), and nitrosyl hemoglobin (Figure 5). Nitrite and nitrate can be measured using either the well-known Greiss reaction, one of its modifications, or high-performance liquid chromatography.\(^{177}\) Recent studies have suggested that the acute NO release in response to acetylcholine leads to increases in plasma NO\(_2^-\).\(^{178}\) Both NO and NO\(_3^-\) are oxidized to NO\(_2^-\) by hemoglobin in red blood cells.\(^{179,180}\) Plasma levels of NO\(_2^-\) and NO\(_3^-\) are increased by exercise training and are even higher in elite athletes.\(^{181}\) Conversely, these levels are low in hypertensive subjects.\(^{182}\)

Despite these examples, such measurements are fraught with difficulty. The diet is a potential source of NO\(_2^-\) and NO\(_3^-\), and to obtain reliable results, experimental subjects should ingest a diet low in green leafy vegetables for several days before measurements.\(^{183}\) Intestinal bacteria can also be a source of plasma nitrate. Nitrite and nitrate are common contaminants on laboratory glass and plastic ware and can be tremendously higher than the low levels present in biological samples, making it necessary to use substantial caution in handling such samples. Another issue regarding measurement of NO in the blood is that 3 different NO synthases exist, and plasma levels of NO metabolites might be the product of any one. Indeed, in inflammatory disorders, levels of plasma NO metabolites are increased, likely because of high activity of the inducible NOS.\(^{184}\) Additionally, if loss of NO is caused by oxidative inactivation (which ultimately leads to NO\(_2^-\) and NO\(_3^-\) formation), levels of NO\(_2^-\) and NO\(_3^-\) will not reflect this process. In experimental animals, measurement of nitrosyl-hemoglobin levels correlate with endothelial function; however, nitrosyl-hemoglobin levels have proven difficult to detect in human blood.\(^{185}\) Thus, although promising from a pathophysiologic perspective, multiple barriers to broad clinical evaluation of NO exist that must be overcome before testing in general populations is possible.

### Summary

There is considerable pathophysiologic and clinical interest in the development of novel biomarkers for inflammation, hemostasis, thrombosis, and oxidative stress that may help in the detection of individuals at high risk for future vascular events. However, as outlined in Table 1, few of these markers have demonstrated an ability to predict risk over and above information available from global assessment tools such as the Framingham Risk Score, and no evidence is available demonstrating that specific reductions in any of these novel markers will lower vascular risk. Although this overview has focused on the role of biomarkers for prognosis in primary prevention, it remains possible that several biomarkers will prove useful for demonstrating efficacy of therapy or in predicting specific patient groups more or less likely to benefit from targeted interventions. It also remains probable that no single biomarker will emerge that provides appropriate information for all clinical settings; thus, multimarker approaches also need evaluation. Ongoing efforts in plasma-based biomarker research will simultaneously need to address novel pathways of disease and carefully evaluate clinical applications and clinical efficacy.

### References


Established and Emerging Plasma Biomarkers in the Prediction of First Atherothrombotic Events
Paul M Ridker, Nancy J. Brown, Douglas E. Vaughan, David G. Harrison and Jawahar L. Mehta

Circulation. 2004;109:IV-6-IV-19
doi: 10.1161/01.CIR.0000133444.17867.56

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/109/25_suppl_1/IV-6

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org/subscriptions/