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Review

Future Biomarkers for Detection of Ischemia and Risk Stratification in Acute Coronary Syndrome

Fred S. Apple,[1*] Alan H.B. Wu,[2] Johannes Mair,[3] Jan Ravkilde,[4] Mauro Panteghini,[5] Jillian Tate,[6] Franca Pagani,[7] Robert H. Christenson,[8] Martin Mockel,[9] Oliver Danne,[10] and Allan S. Jaffe,[11] on behalf of the Committee on Standardization of Markers of Cardiac Damage of the IFCC

Background: **Evaluation of patients who present to the hospital with a complaint of chest pain or other signs or symptoms suggestive of acute coronary syndrome (ACS) is time-consuming, expensive, and problematic. Recent investigations have indicated that increases in biomarkers upstream from biomarkers of necrosis (cardiac troponins I and T), such as inflammatory cytokines, cellular adhesion molecules, acute-phase reactants, plaque destabilization and rupture biomarkers, biomarkers of ischemia, and biomarkers of myocardial stretch may provide earlier assessment of overall patient risk and aid in identifying patients with higher risk of an adverse event.**

Approach and Content: **The purpose of this review is to provide an overview of the pathophysiology and clinical and analytical characteristics of several biomarkers that may have potential clinical utility to identify ACS patients. These biomarkers (myeloperoxidase, metalloproteinase-9, soluble CD40 ligand, pregnancy-associated plasma protein A, choline, ischemia-modified albumin, unbound free fatty acids, glycogen phosphorylase isoenzyme BB, and placental growth factor) have demonstrated promise and need to be more thoroughly evaluated for commercial development for implementation into routine clinical and laboratory practice.**

Summary: **Specifications that have been addressed for cardiac troponins and natriuretic peptides will need to be addressed with the same scrutiny for the biomarkers discussed in this review. They include validating analytical imprecision and detection limits, calibrator characterization, assay specificity and standardization, preanalytical issues, and appropriate reference interval studies. Crossing boundaries from research to clinical application will require replication in multiple settings and experimental evidence supporting a pathophysiologic role and, ideally, interventional trials demonstrating that monitoring single or multiple biomarkers improves outcomes.**

[1] Department of Laboratory Medicine and Pathology, Hennepin County Medical Center, Minneapolis, MN.
[2] Department of Laboratory Medicine, University of California, San Francisco, San Francisco General Hospital, San Francisco, CA.
[3] Clinical Division of Cardiology, Medical University Innsbruck, Innsbruck Austria.
[4] Department of Cardiology, Aarhus University Hospital, Aarhus, Denmark.
[5] Dipartmento di Scienze Cliniche "Luigi Sacco", Univesita degli Studi di Milano, Milan, Italy.
[6] Department of Chemical Pathology, Queensland Health Pathology Service, Princess Alexandra Hospital, Brisbane, Australia.
[7] Laboratorio Analisi Chimico Cliniche 1, Azienda Ospedaliera Spedali Civili, Brescia, Italy.
[8] Department of Pathology, University of Maryland, Baltimore, MD.
Departments of [9] Medicine and [10] Cardiology, University Hospital Charite, Berlin, Germany.
[11] Department of Laboratory Medicine and Cardiology, Mayo Clinic, Rochester, MN.
*Address correspondence to this author at: Hennepin County Medical Center, Clinical Laboratories P4, 701 Park Ave., Minneapolis, MN 55415. Fax 612-904-4229; e-mail fred.apple@co.hennepin.mn.us.
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Clinical Importance

Millions of patients with chest pain present annually to hospitals, and many more present with other symptoms potentially indicative of ischemia *(1–4)*. A considerable proportion have suspected acute coronary syndromes

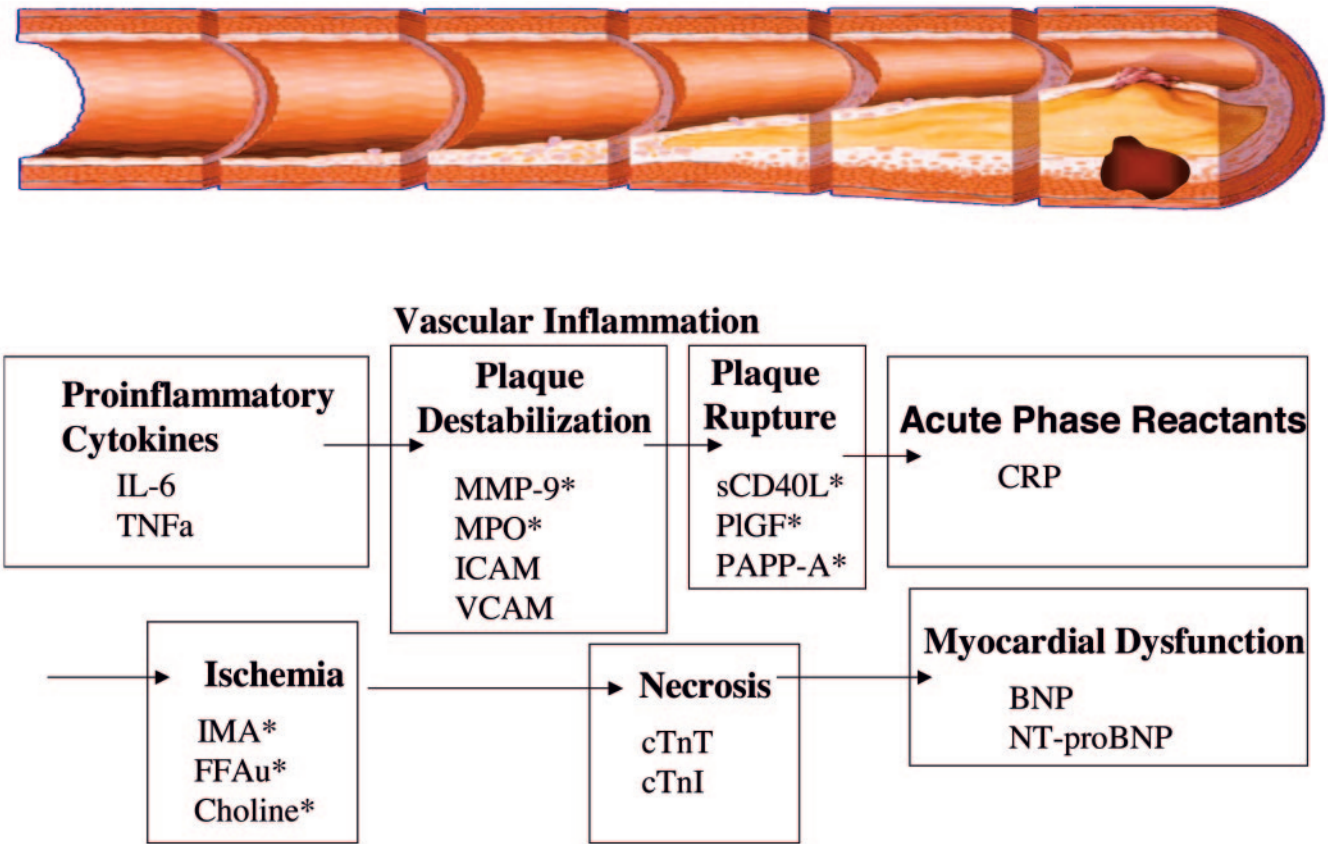

Fig. 1. Biochemical profile in ACS patients: vascular inflammation to plaque rupture to ischemia to cell death to myocardial dysfunction [adapted from Refs. *(17)* and *(18)*].

Biomarkers indicated by an ∗ are covered in this review. *TNFa*, tumor necrosis factor-α; *MPO*, myeloperoxidase; *ICAM*, intercellular adhesion molecule-1; *VCAM*, vascular adhesion molecule; *BNP*, B-type natriuretic peptide; *NT-proBNP*, N-terminal proBNP.

(ACS).[12] Although new biomarkers, e.g., cardiac troponins, have increased our abilities to detect and or exclude cardiac injury *(5–7)*, a normal troponin measurement is not synonymous with a lack of risk. Accordingly, there are still unnecessary admissions to expensive coronary care units, step-down units, and non-intensive care beds when discharge might be equally appropriate. A low threshold for consideration of a myocardial etiology of chest pain (or related symptoms) is necessary, because the presentation of an ACS is often atypical and because a missed diagnosis of ACS carries considerable risk to patients. In addition, 1–2% of patients who are sent home have a myocardial infarction (MI) and an adverse outcome. If we had markers that would further define risk, it would not only reduce the number of patients kept in the emergency department (ED), in a chest pain center, or admitted to the hospital, but would also allow for prevention of substantial numbers of new events.

Recent investigations have indicated that increases in biomarkers upstream from markers of necrosis, such as inflammatory cytokines, cellular adhesion molecules, acute-phase reactants, plaque destabilization and rupture biomarkers, biomarkers of ischemia, and biomarkers of myocardial stretch may provide an earlier assessment of overall patient risk and aid in identifying patients with higher risk of having an adverse event.

This review was developed to address a need that the committee perceived to clarify the state of the art in this important area. Its purpose is to provide an overview of the pathophysiology and clinical and analytical characteristics of several biomarkers (Fig. 1) that may have potential clinical utility to identify patients at risk. These biomarkers have demonstrated promise and need to be more thoroughly evaluated before implementation into routine clinical and laboratory practice. However, it

[12] Nonstandard abbreviations: ACS, acute coronary syndrome; MI, myocardial infarction; ED, emergency department; CAD, coronary artery disease; UA, unstable angina; MMP, matrix metalloproteinase; CI, confidence interval; CRP, C-reactive protein; sCD40L, soluble CD40 ligand; cTnT and cTnI, cardiac troponin T and I, respectively; TIMP, tissue inhibitor of metalloproteinase; IL, interleukin; PCI, percutaneous coronary intervention; FDA, US Food and Drug Administration; PAPP-A, pregnancy-associated plasma protein A; IGF, insulin-like growth factor; proMBP, proform of eosinophil major basic protein; CK-MB, creatine kinase MB; WBCHO and PLCHO, whole-blood and plasma choline, respectively; PLD, phospholipase D; ACB, albumin cobalt binding; IMA, ischemia-modified albumin; ECG, electrocardiogram; PTCA, percutaneous transluminal coronary angioplasty; DCCV, direct-current cardioversion; FFA, free fatty acid; FFA_u, unbound free fatty acids; GPBB, GPLL, and GPMM, glycogen phosphorylase isoenzyme BB, LL, and MM, respectively; and PlGF, placental growth factor.

should be appreciated that initial studies, as impressive as they may appear, are preliminary at best. We point out the following issues to keep in mind when evaluating these preliminary studies reviewed here:

- Often the analytical characteristics of the assays are not adequately described. For immunoassays, the specificities of the antibodies, the types of fragments captured, the degree of nonspecific binding, matrix issues, and/or the propensity of the assay for false-positive and/or false-negative results need to be defined.
- We need to know how the samples need to be collected and/or preserved for accurate measurements.
- We need to know the stability of the samples over time; this is key to the analysis of so many sample sets, which are often many years old.
- These same preanalytical and analytical issues likely apply to the measurement of other analytes often evaluated so that the "independent contribution" of the new analyte can be calculated.
- We need established reference intervals in general and in key subsets of patients.
- Cutoff values for the analyte in question and/or other analytes that are used in multivariable models may or may not be used optimally. A recent editorial has called attention to the critical nature of this issue *(8)*.
- Populations studied are often convenience populations for initial studies. As such, they may overrepresent those with disease and thus make evaluation of true sensitivity and specificity problematic. In addition, if the samples are older, they may provide data that are not relevant given contemporary changes in the treatment of patients.
- There is a publishing bias in favor of positive rather than negative reports. A negative evaluation is unlikely to be the first one published, and once a highly positive report is published, negative reports often have difficulty finding their way into print because they are invariably compared with the original positive report.

Evaluations have to start somewhere, but often these critical caveats are not reflected on when the results are discussed by authors who are invested in the development of the new analyte.

Myeloperoxidase

Myeloperoxidase is a hemoprotein (molecular mass of 140 kDa) consisting of a pair of heavy and light chains. It is stored in azurophilic granules of polymorphonuclear neutrophils and macrophages and functions to catalyze the conversion of chloride and hydrogen peroxide to hypochlorite. Myeloperoxidase is released into the extracellular fluid and general circulation during inflammatory conditions. This enzyme has been implicated in the oxidation of lipids contained within LDL *(9)*. Myeloperoxidase activity can be measured in blood and tissues by assays using hydrogen peroxide and *o*-dianisidine dihydrochloride as substrates *(10)*. Recently, mass assays based on an enzyme-linked immunoassay have been developed for research use only (Oxis Research and Assay Design). In addition, myeloperoxidase content can be measured in neutrophils as an index of degranulation with the Coulter counter and flow cytometry.

Oxidative stress and inflammation play important roles in the pathogenesis of the destabilization of coronary artery disease (CAD) leading to ACS. Infiltrating macrophages and neutrophils participate in the transformation of stable coronary artery plaques to unstable lesions with a thin fibrous cap. These cells are found more frequently and in higher concentrations in the culprit lesions of patients with acute MI and unstable angina (UA) than in patients with stable coronary disease *(11)*. Macrophages secrete matrix metalloproteinases (MMPs) and metal-independent myeloperoxidase, which degrade the collagen layer that protects atheromas from erosion or abrupt rupture *(12)*. As a result, plaques that have been highly infiltrated with macrophages have a thin fibrous cap and are vulnerable to erosion or rupture, precipitating events to ACS. This is particularly true in the shoulder regions of coronary artery lesions, where the shear stresses of arterial blood are highest. Neutrophilic infiltration has also been implicated as the cause of myocardial reperfusion injury after successful recanalization of previously occluded coronary arteries *(13)*. Therapeutic trials of drugs designed to minimize reperfusion injury rely in part on demonstrating a reduction in neutrophil infiltration, as documented by tissue myeloperoxidase concentrations *(14)*.

There have been a few clinical studies examining the role of myeloperoxidase as a marker of risk for ACS. Using an enzyme assay, Zhang et al. *(15)* showed that blood and leukocyte myeloperoxidase activities were higher in patients with CAD than angiographically verified normal controls, and that these increased activities were significantly associated with presence of CAD [odds ratio, 11.9; 95% confidence interval (CI), 5.5–25.5]. Results were independent of the patient's age; sex; hypertension, smoking, or diabetes status; LDL concentration; leukocyte count; and Framingham Global Risk Score. There was no tabulation of the subsequent rate of adverse events in that report.

A key study by Buffon et al. *(16)* involved 65 patients who underwent cardiac catheterization with coronary sinus sampling. The myeloperoxidase content of the leukocytes collected from the arterial circulation and the coronary sinus effluent were compared. Myeloperoxidase content was determined on the Coulter counter, which measures the neutrophil count by flow cytometry and subsequently calculates the mean myeloperoxidase content in that population. Not only was there a gradient for myeloperoxidase across the coronary sinus in patients with ACS, but that gradient was present even when the culprit lesion involved with the ACS was in the distribution of the right coronary artery, a situation in which the venous effluent from the right coronary artery does not drain into the coronary sinus. Increases in myeloperoxi-

dase correlated with values of high-sensitivity C-reactive protein (CRP). This finding in the 33 patients with ACS but not those with variant or stable angina strongly suggests that neutrophil adhesion and inflammation are not unique to the presumed culprit coronary lesion identified by angiography, but more likely identify a systemic predilection. Such data are in keeping with the recent concept that, in addition to vulnerable plaques, the vascular milieu, i.e., the blood, contributes to the propensity to unstable coronary disease *(17, 18)*.

The potential usefulness for risk stratification of blood concentrations of myeloperoxidase was examined in 2 recent studies. In the CAPTURE trial *(19)*, myeloperoxidase mass concentration was measured in 1090 patients with ACS. The death and MI rates were determined at 6 months of follow-up. With a cutoff of 350 μg/L, the adjusted hazard ratio was 2.25 (95% CI, 1.32–3.82). The effects were particularly impressive in patients with undetectable cardiac troponin T (cTnT; <0.01 μg/L), where the hazard ratio was 7.48 (95% CI, 1.98–28.29). However, only the admission cTnT was used to define this group. Myeloperoxidase and the other markers studied—cTnT, soluble CD40 ligand (sCD40L), CRP, and vascular endothelial growth factor—were independent predictors of adverse cardiac events.

In a study of 604 sequential patients presenting to the ED with chest pain, Brennan et al. *(20)* demonstrated a progressive increase in odds ratios for major adverse events at 30 days and 6 months with each quartile increase in myeloperoxidase concentration. The 6-month outcome data were similar to the results in the CAPTURE trial: corresponding odds ratios were 1.6 (95% CI, 1.0–2.7), 3.6 (2.2–5.8), and 4.7 (2.9–7.7) for the second, third, and fourth quartiles, respectively (cutoffs of 119, 198, and 394 pmol/L, respectively). The maximum odds ratio for patients who were persistently negative for cTnT was not as high as in the CAPTURE trial, at 4.4 (2.3–8.4). Differences in the designs of the two studies may account for this. The study by Brennan et al. *(20)* included the "need for revascularization" within the definition of major adverse cardiac events and used an inappropriately higher cTnT cutoff of 0.10 μg/L. All 4 studies described above used different assays and reporting units that cannot be converted to each other. A standardization effort will be needed if this marker is to be used beyond research purposes.

In summary, although myeloperoxidase participates in the inflammatory process of ACS, neutrophil activation is apparently not induced by ischemia *(21)*. Thus, myeloperoxidase is more of a marker of plaque instability and unlike a marker of oxidative stress and damage. Increased myeloperoxidase is not likely to be specific to cardiac diseases, as activation of neutrophils and macrophages can occur in any infectious, inflammatory, or infiltrative disease process.

MMP-9

MMPs are a class of 24 endopeptidases that are physiologic regulators of the extracellular matrix *(22)*. They are found in most tissues. These compounds are regulated by transcription, by certain precursors, and by interaction with ground substance and have specific endogenous inhibitors known as tissue inhibitors of metalloproteinases (TIMPs). In the heart, these substances participate in vascular remodeling, plaque instability, and ventricular remodeling after cardiac injury. MMP-9 is zinc-dependent and is known as gelatinase B. Gelatinases have 3 repeats of the fibronectin-binding domain that allow them to bind to gelatin, collagen, and laminin. Activation of proMMP-9 to the active moiety can occur as a result of exposure to NO or via proteolytic activation. MMP-9 can generate angiostatin via an interaction with plasminogen, increase its affinity for collagen, interact with intercellular adhesion molecule-1, and be antiinflammatory by processing interleukin (IL)-1β from its precursor and by reducing IL-2 response. TIMPs regulate the effects of MMP-9. Thus, one cannot understand the biological effects of MMPs without taking into account the effects of TIMPs. TIMPs also can have a variety of independent biological effects on inflammation and angiogenesis, which may have important consequences for cardiac structure and function *(22)*.

In vascular tissue, MMP-9 and several other MMPs are localized at the shoulder of a plaque. That area, which is thinner, is thought to be the area prone to rupture. Experimentally, monocytes are a rich source of MMPs and can be stimulated by specific T-cell populations via CD40 ligand signaling *(23)*. In heart tissue, MMP-9 is partially responsible for the degradation of ground substance after cardiac injury. Transgenic animals susceptible to ventricular rupture are protected by deletion of MMP-9 *(24)*. In other models, that inhibition of MMPs, including MMP-9, inhibits ventricular remodeling after acute MI, and there is therapeutic interest in testing such a strategy clinically. It is clear that some degree of MMP production is likely essential as part of the reparative process after acute cardiac injury *(25)*.

For all of these reasons, there has been interest in developing an assay for measurement of MMP-9 in peripheral blood in patients with ACS. The first report was published in 1998 by Kai et al. *(26)*. They collected blood and prepared both serum and EDTA-plasma samples for analysis by a sandwich enzyme immunoassay (marketed by Fuji Chemical Industries Ltd). Thirty-three ACS patients were studied. The criterion for MI was based on total CK activity. The mean (SD) MMP-9 concentrations in presumably healthy controls was 27 (8) μg/L, which was similar to what was found in patients with stable angina. Patients with acute MI had either very increased or normal MMP-9 concentrations (6 with high and 7 with normal concentrations) on day 1, whereas patients with UA all had high concentrations [87 (26) μg/L] initially. MMP-9 concentrations then gradually decreased in those patients. In the patients with acute MI, those with high concentrations had decreasing concentrations between day 1 and day 7. Those without increased concentrations

initially developed increased concentrations that peaked at day 3 and then began to return to baseline. Treadmill-induced ischemia did not lead to further increases. The authors hypothesized that the MMP-9 measured was from the myocardium and not the vessel wall.

Inokubo et al. *(27)*, using a similar assay, evaluated 29 ACS patients (20 with acute MI) and measured values of MMP-9 and TIMP-1 across the coronary sinus. Although aortic concentrations initially were similar to those in patients with stable angina and healthy people, there was a substantial gradient across the heart with secretion of both MMP-9 and TIMP-1. Subsequently, MMP-9 concentrations decreased and TIMP-1 concentrations increased. Using a different ELISA approach, which detected both proMMP-9 and the active moiety, a Finnish group *(28)* correlated blood concentrations with the extent of CAD in a cohort of 61 patients. They found a distribution of reference values similar to that reported by the Japanese group with a mean (SD) value of 32.2 (16.1) μg/L. Those with single- or double-vessel disease had a mean value of 40.4 (25.1) μg/L, and those with triple-vessel disease, a mean of 57.3 (39.1) μg/L. There were no differences between those with and those without previous MI. The authors hypothesized that the increased concentrations were related to inflammation in the plaques, which in aggregate might be related to the extent of the CAD.

Recently, a study from the AetheroGene investigators on 1127 patients with either stable (n = 795) or unstable (n = 332) CAD reported that values of MMP-9 were related to future cardiovascular death *(29)*. They used the Fuji assay originally reported by the Japanese to stratify the patients into quartiles. Those in the highest quartile had a significantly increased risk of cardiac death whether in the stable or unstable group. Although there was some association with other inflammatory markers, MMP-9, after correction for CRP, fibrinogen, IL-6, and IL-18, maintained its prognostic significance. This group also examined the influence of two known polymorphisms (C−1562T and R279Q) on both MMP-9 concentrations and events and found that the C−1562T polymorphism seemed to influence concentrations but that neither polymorphism was associated with increased events. These data again suggest the potential importance of MMP-9 in predicting eventual coronary events. For this reason, there have been several studies evaluating the effects of therapies on MMP-9. Some have evaluated blood concentrations and some the response that can be induced from peripheral blood mononuclear cells, and each has used different ELISAs. It now has been demonstrated that the renin–angiotensin system *(30)*, aspirin *(31)*, atorvastatin *(32)*, and doxycycline *(33)* lower MMP-9 concentrations. Outcome studies attempting to evaluate these strategies are ongoing.

Of additional interest has been the possibility that MMP-9 concentrations might be helpful in evaluating the remodeling that occurs after MI. The experimental literature for this type of test application is robust. However, only recently, Squire et al. *(34)* reported their initial experience relating blood concentrations of MMP-9 and TIMP-1 to changes in ventricular function over a 6-week time period in patients with ST-elevation MI. MMP-9 manifested a biphasic pattern with peaks at 1 and 4 days and a nadir at day 2. In contrast, TIMP-1 decreased from day 1 to day 5. More increased concentrations of MMP-9 were associated with larger ventricles and higher amino-terminal brain natriuretic peptide concentrations. These findings were not what was anticipated because in much of the animal work, it was MMP-2 that was associated with adverse remodeling, and in this study, exactly the opposite was observed.

In summary, preliminary studies suggest that MMP-9 may be of value in evaluating patients with CAD and after acute coronary events. However, more rigorous corrections with other biomarkers and clinical variables are necessary before any suggestion is made that MMP-9 may have unique clinical application.

sCD40L

Circulating sCD40L is derived largely from activated platelets and has biological activity that can trigger an inflammatory reaction in vascular endothelial cells by the secretion of cytokines and chemokines *(35)*. Membrane-bound CD40L and sCD40L forms interact with the CD40 receptor molecule, which is present not only on B cells but also on monocytes, macrophages, and endothelial and smooth muscle cells in atheromas, leading to release of matrix MMPs and subsequent destabilization of the plaque *(36)*. Release of tissue factor then activates platelets, which produce more sCD40L and perpetuate the inflammatory and prothrombotic environment in the vasculature *(37)*.

Increased sCD40L concentrations have been demonstrated in inflammatory disorders, e.g., autoimmune diseases, multiple sclerosis, and inflammatory bowel disease, as well as in stroke, hypercholesterolemia, and diabetes *(36, 37)*. Up-regulation of the CD40L–CD40 system may play a pathogenic role also in triggering ACS. Increased sCD40L has been demonstrated in patients with MI and UA *(38, 39)* as a consequence of procedures such as percutaneous coronary intervention (PCI) *(38, 40)*, and in apparently healthy women who went on to develop MI, stroke, or cardiovascular death *(41)*. In a cohort of 195 ACS cases and matched controls from the OPUS-TIMI16 trial, the median sCD40L concentration was significantly higher in patients (0.78 μg/L) than in controls (0.52 μg/L). In this study, increased sCD40L was associated with a higher risk for future recurrent cardiac events, and in combination with cardiac troponin I (cTnI; Beckman AccuTnI assay; cutoff >0.06 μg/L), it improved risk stratification for death and MI *(42)*. Unfortunately, rather than using conventional sampling for cTnI to evaluate the data, the authors used the only available sample, which was the one taken at the time of enrollment into the study.

In the CAPTURE study of unstable refractory angina,

increased sCD40L concentrations were associated with a higher incidence of cardiac events in patients on placebo *(43)*. Those treated with abciximab did not manifest this outcome, likely because abciximab treats the inflammatory–coagulation complexes that occur in such patients. Among treated patients with increased sCD40L concentrations (>5 μg/L), there was a significant reduction in cardiovascular risk independent of the presence or absence of detectable cTnT (5.5% vs 13.6% for patients on placebo) *(43)*. However, also in this trial, a cutoff value for cTnT of 0.1 μg/L was used for risk stratification, which was based only on an initial sample. In a separate population of patients presenting to the ED with acute chest pain, sCD40L concentration at a decision limit of >5 μg/L also reliably identified patients at increased cardiovascular risk by the same analytical approach *(42)*. Multivariable correction in this study used a more appropriate cardiac troponin cutoff, but a somewhat high value for CRP. Therefore, sCD40L concentration may identify patients with an increased risk of thrombosis and may be a useful indicator of plaque instability in ACS in addition to the markers of cardiac ischemia.

Plasma sCD40L is measured by commercial ELISAs [none are cleared by the US Food and Drug Administration (FDA) for clinical use]. Imprecision from clinical studies is variable with CVs up to 15% being reported. Heparin-, citrate-, and EDTA-plasmas are the recommended sample types, as prolonged clotting of serum may cause activation of platelets and overestimation of circulating sCD40L. Removal of platelets by sample centrifugation at 2000*g* is crucial for the accurate measurement of sCD40L. In 40 healthy blood donors, sCD40L concentrations ranged from 0.03 to 3.98 μg/L (mean, 2.13 μg/L), and in 130 healthy middle-aged women, concentrations at the 95th and 99th percentiles were 3.71 and 5.54 μg/L, respectively *(41)*. In a real-time patient population with acute chest pain, sCD40L concentrations were 3.19–5.87 μg/L in ACS, 1.99–3.52 μg/L in stable angina, and 0.88–1.76 μg/L in persons with no evidence of heart disease *(43, 44)*.

In summary, preliminary data demonstrate promise for commercialization of sCD40L as an independent biomarker for risk and prognosis.

Pregnancy-Associated Plasma Protein A

Pregnancy-associated plasma protein A (PAPP-A) is a high–molecular-mass (~200 kDa) glycoprotein synthesized by the syncytiotrophoblast and is typically measured during pregnancy for screening of Down syndrome *(45)*. It was reported to be an insulin-like growth factor (IGF)-dependent IGF-binding protein-4-specific metalloproteinase, thus potentially a proatherosclerotic molecule through its role in disrupting the integrity of the atheroma's protective cap *(46)*. In pregnancy, PAPP-A circulates in a heterotetrameric complex consisting of 2 PAPP-A subunits covalently bound with 2 subunits of the proform of eosinophil major basic protein (proMBP), its endogenous inhibitor *(47)*. However, PAPP-A present in human fibroblasts and released during atherosclerotic plaque disruption seems to be in a homodimeric active form, uncomplexed with the inhibitor proMBP *(46)*.

The presence of PAPP-A in unstable plaques from patients who died suddenly of cardiac causes was shown by Bayes-Genis et al. *(48)*, who also described increased PAPP-A concentrations in serum of patients with both UA and acute MI. PAPP-A measurement appeared to be valuable for detecting unstable ACS in a convenience sample of patients presenting for coronary angiography, even in patients without increased concentrations of biomarkers of necrosis, such as cTnI, thus potentially identifying high-risk patients whose unstable clinical situation might otherwise remain undiagnosed *(48)*. In a series of 136 consecutive patients presenting to the ED for suspected ACS (found to be cTnI negative during the first 24 h after admission), an increase in circulating PAPP-A appeared to be an independent predictor of future ischemic cardiac events as well as the need for PCI or coronary artery bypass graft surgery *(49)*. An adjusted risk ratio of 4.6 (95% CI, 1.8–11.8) was obtained. Similar results were recently presented by Heeschen et al. *(50)*. However, methodologic issues have to be highlighted. The use of insensitive cTnI assays and often too-high cTnI cutoffs in these studies may have biased the classification of patients as troponin negative. Future studies need to compare PAPP-A results with reference-quality troponin immunoassays for which excellent performance for analytical sensitivity has been documented, lowering the troponin cutoff to the currently recommended limits *(51, 52)*. The overall correlation of PAPP-A with cTnI and creatine kinase MB (CK-MB) concentrations appears to be poor, indicating that increased PAPP-A cannot be attributed to myocardial necrosis *(48, 53)*. A significant association between PAPP-A and CRP concentrations was shown at first but was not confirmed later *(48, 49)*.

The kinetics of PAPP-A release and the corresponding optimal sampling protocols in ACS remain to be determined. In a preliminary study, the release patterns of PAPP-A were highly variable, increasing early at 2 h after admission or late at 30 h after the onset of chest pain *(54)*. Because preliminary findings showed that serum PAPP-A concentrations sensitively reflect changes in renal function, at least in dialyzed patients, and correlate with serum creatinine, the possible dependence of PAPP-A concentrations on renal function should be clarified *(55)*.

PAPP-A has also been evaluated as a marker of cardiovascular risk in asymptomatic hyperlipidemic individuals, showing a correlation with the degree of echogenicity of carotid atherosclerotic plaques *(56)*. However, PAPP-A concentrations were not influenced by statin treatment *(57)*. These preliminary findings suggest that increases in PAPP-A concentrations may not be limited to ACS patients but could also reflect the earlier stages of atherosclerotic lesions, even in the absence of clinical signs of atherosclerosis *(58)*.

It has been suggested that circulating PAPP-A in ACS is different from PAPP-A isolated from pregnancy sera. In particular, PAPP-A in serum of ACS patients appears to be present as a homodimer, thus making it difficult to measure PAPP-A as cardiac marker by immunoassays that are designed to detect molecules in pregnancy serum via a sandwich formed by 2 antibodies, one PAPP-A-specific and one proMBP-specific *(59)*. Three different assays, a manual ultrasensitive ELISA and 2 on automated platforms, are currently available for PAPP-A determinations *(53, 60, 61)*. However, none of these assays has specifically been developed for the measurement of PAPP-A as a cardiac marker. In particular, the Kryptor® (Brahms AG) and Aio!™ (Innotrac Diagnostics Oy) automated assays mostly measure PAPP-A/proMBP complexes. The details of the "self-made" methodologies used by some authors for clinical investigation are unclear.

Only serum should be used as the sample for measuring PAPP-A because heparin and other anticoagulants, e.g., EDTA, may affect the apparent concentration of PAPP-A in body fluids *(60, 61)*. The binding of highly charged heparin to PAPP-A is well known and has been used in early methods for purification of PAPP-A *(62)*. This binding may mask the epitopes of the antibodies that react with PAPP-A in the immunoassay format. Only if the epitopes recognized by the antibodies are located at sites distant from the area to which heparin molecules bind on the PAPP-A molecule is it possible that heparin may not have a significant effect *(60)*. Furthermore, the PAPP-A molecule contains 16 zinc atoms; thus, it is not surprising that collecting samples into EDTA leads to significantly lower concentrations of measured PAPP-A because the zinc will become complexed by the EDTA and conformational changes induced in the protein molecule may render epitopes inaccessible to the measuring system *(60, 61)*.

In summary, additional investigations will be necessary for better acceptance of PAPP-A as an independent biomarker for cardiovascular risk in ACS patients.

Choline

Choline and phosphatidic acid are major products generated by phosphodiesteric cleavage of membrane phospholipids (phosphatidylcholine for example) catalyzed by phospholipase D enzymes. Whole-blood choline (WBCHO) and plasma choline (PLCHO) concentrations increase rapidly after stimulation of phospholipase D (PLD) and the activation of cell surface receptors in coronary plaque destabilization and tissue ischemia *(63)*. Several experimental studies support the concept that PLD activation is a key event in various fundamental processes of coronary plaque destabilization: stimulation of macrophages by oxidized LDL *(64)*, secretion of MMPs *(65)*, activation of platelets by collagen and thrombin *(66, 67)*, and promotion of fibrinogen binding to the glycoprotein IIb/IIIa receptor *(68)*. Isoenzymes of PLD are produced by macrophages in human coronary plaques and colocalize with oxidation epitopes *(69)*. Furthermore, early ischemic membrane damage and phospholipid breakdown by phospholipases *(70)* lead to release of choline into plasma followed by a secondary uptake into blood cells by a choline transport system *(71)*.

Increased WBCHO concentrations were first identified as a promising marker for ACS by use of high-resolution proton magnetic resonance spectroscopy. A reliable assay was developed based on HPLC–mass spectrometry, and a prospective study in 327 patients with suspected ACS was completed *(72)*. Diagnostic classification of patients and definition of cardiac troponin cutoffs were in agreement with the current European Society of Cardiology/American College of Cardiology (ESC/ACC) criteria, and patients were followed for 30 days. WBCHO was a significant predictor of cardiac death or cardiac arrest, life-threatening cardiac arrhythmias, heart failure, and coronary angioplasty when measured in the first blood sample on admission. cTnI or cTnT and WBCHO were the most powerful independent predictors in multivariate analysis, and the combination of WBCHO and cardiac troponins allowed a superior risk assessment compared with each test alone. WBCHO was not a marker for myocardial necrosis but indicated high-risk UA in patients without acute MI (sensitivity, 86.4%; specificity, 86.2%).

The comparison of WBCHO and PLCHO in 222 patients with undetectable cTnI on admission indicated important differences in early risk stratification. PLCHO was not a predictor of MI in the follow-up phase, whereas WBCHO was highly predictive *(72)*. However, for events such as severe arrhythmias that often occur in the first 24 h after admission, both markers were equally predictive. These results support the concept that only WBCHO is capable of detecting risks associated with advanced coronary plaque instability because PLD activation of blood cells (platelets, leukocytes) leads to an intracellular increase in choline concentration that is not detectable in plasma. Although many proposed cardiac markers of plaque inflammation and plaque instability are based on measurements in plasma or serum, determination of choline in whole blood (WBCHO) may be advantageous because many key processes of cell infiltration and activation are reflected mainly by intracellular concentration changes. However, early ischemic membrane injury in patients with severe arrhythmias or hemodynamic instability is indicated by measurements of either PLCHO or WBCHO because choline is first released from injured tissues into plasma with a secondary uptake into blood cells. When interpreting the results for individual patients, it is useful to have both WBCHO and PLCHO data to identify risks associated with PLD activation more precisely as related to coronary plaque destabilization, tissue ischemia, or both. This information may be helpful to target advanced treatment strategies to patients at risk based on single measurements of WBCHO and PLCHO on admission.

Preliminary WBCHO data in intensive care patients without evidence of tissue ischemia demonstrated that 98% are below the 99th percentile and 88.5% are below the 90th percentile of the value distribution of a reference population, indicating that the specificity of this marker is also acceptable in patients with a variety of other diseases. In patients with suspected ACS, WBCHO values above the 90th (28.2 μmol/L) and 99th percentile (57.5 μmol/L) reference limits indicate significantly increased cardiac risk, whereas for PLCHO, the 99th percentile limit (25.0 μmol/L) has been used for risk stratification.

In summary, increased concentrations of WBCHO and PLCHO on hospital admission are predictors of adverse cardiac events in suspected ACS. WBCHO and PLCHO may be useful for early risk stratification of these patients, particularly if cardiac troponins are negative on admission. However, persistently high concentrations of WBCHO also occur in cardiac troponin-positive patients with an initially successful PCI and may be associated with subacute stent occlusion or sudden cardiac death despite standard treatment. Development of rapid point-of-care tests and central laboratory assays of WBCHO and PLCHO will be necessary to evaluate whether these markers will help to identify such high-risk patients in clinical practice.

Ischemia-Modified Albumin

The observation that myocardial ischemia produced a lower metal-binding capacity for cobalt to albumin led to the development of the recently FDA-cleared albumin cobalt binding (ACB) test *(73, 74)*. The ACB test is a quantitative assay that measures ischemia-modified albumin (IMA) in human serum. In principle, cobalt added to serum does not bind to the NH_2 terminus of IMA, leaving more free cobalt to react with dithiothreitol and form a darker color in samples from patients with ischemia. At present, the assay is available on a variety of clinical chemistry platforms. Investigations are ongoing into the use of an immunoassay technology. Specific preanalytical requirements need to be followed, including: avoiding use of collection tubes with chelators, performing assay analysis within 2.5 h or freezing at below −20 °C, and avoiding sample dilutions. In addition, ACB test results should be interpreted with caution when serum albumin concentrations are <20 g/L or >55 g/L or in the presence of increased lactate or ammonia concentrations. Increased IMA values may be found in patients with cancer, infections, end-stage renal disease, liver disease, and brain ischemia *(75, 76)*. Reference values determined from a population of 283 healthy individuals ranged from 52 to 116 kilounits/L, with a 95th percentile at 85 kilounits/L (Cobas Mira) *(76)*. Several clinical studies have evaluated the performance of the ACB assay in cardiac patients, mostly examining the role of IMA in assessing ischemia. Studies were typically performed with various study designs in small numbers of patients.

A multicenter study, involving 224 patients who arrived at the ED within 3 h after onset of signs and symptoms suggestive of ACS, examined the ability of the ACB test to predict a positive or negative cTnI result within 6–24 h after presentation *(77)*. All patients had a negative cTnI result at presentation. Patients were considered troponin positive if 1 or more cTnI values were above the upper reference limit within 6–24 h. At the optimum cutoff for the ACB test, sensitivity and specificity were 70% and 80%, respectively, with a negative predictive value of 96%. There were 6 false negatives and 131 true negatives. cTnI alone was used as the outcome measure, and electrocardiogram (ECG) status at presentation was not considered in the design of the study. A control group of 109 healthy adults (age range, 20–85 years) were also tested to determine reference limits. The ROC-curve-derived cutoff value of 75 kilounits/L for the study group was lower than the 80.2 kilounits/L value that represented the 95th percentile of the control population, demonstrating overlap between normal and abnormal values. This gave a positive predictive value of only 33%.

In the study by Bhagavan et al. *(78)*, ACB assay results (using an assay independent of the Ischemia Technology IMA test) were correlated with final discharge diagnoses in 75 ED patients with myocardial ischemia and 92 nonischemic patients. The diagnosis of myocardial ischemia, with or without MI, was based on clinical signs and symptoms, imaging, ECG, and serum cardiac biochemical markers such as CK-MB and cTnI. The sensitivity and specificity for myocardial ischemia were 88% and 94%, respectively, and the positive and negative predictive values were 92% and 91%. The ACB test, however, was a poor discriminator between ischemic patients with and without MI.

Previous reports demonstrated that percutaneous transluminal coronary angioplasty (PTCA) can be used as an in vivo model of mild transient myocardial ischemia in humans. In a study by Sinha et al. *(79)*, IMA and cTnT were compared immediately after, 30 min after, and 12 h after elective PTCA. The study group consisted of 19 patients who had >70% single-vessel disease, and all of whom had chest pain and/or ischemic ECG changes during the procedure. Stents were deployed as required. IMA concentrations were increased from baseline (72 kilounits/L) in 18 of 19 patients immediately after (101 kilounits/L) and 30 min after (87 kilounits/L) the procedure and returned to below baseline at 12 h. None of the patients had cTnT concentrations above the upper limit of the reference interval. A control group of 11 patients undergoing diagnostic angiography were also included who did not have significant changes in IMA concentrations. Although this study suggests the concept that IMA is an early marker of transient ischemia in the PTCA setting, larger trails are necessary to validate this hypothesis.

Results in the study of Quiles et al. *(80)* confirmed that IMA is an early marker of ischemia in the setting of PCI.

Thirty-four patients who underwent elective single-vessel PCI for the management of stable angina pectoris were studied. Serum albumin measurements were within the reference intervals in all patients. Blood samples were drawn from a femoral artery sheath 10 min before PCI and within 5 min after the last balloon inflation. IMA concentrations in all patients increased significantly after PCI from baseline to post-PCI (59.9 to 80.9 kilounits/L; $P < 0.0001$). IMA was higher in patients with more balloon inflations, higher pressure inflations, and longer inflation duration. However, because there was some scatter in the correlations, factors such as the severity and extent of the lesion and the presence or absence of collateral blood supply may also play a role in IMA concentrations. The authors suggested that IMA is not only a marker of the occurrence of an ischemic event but also an indicator of the severity of ischemia. In a successive study *(81)*, the same authors compared IMA concentrations in 90 patients undergoing PCI with and without coronary collaterals. Collateral vessels were assessed angiographically. Blood samples were drawn from the femoral artery sheath immediately before PCI and 10 min after the last balloon inflation. IMA increased significantly less in patients with collateral circulation compared with those without collateral vessels (4.8% vs 14.0%; $P = 0.046$). The lower increments in IMA concentrations observed in patients with collateral vessels after PCI likely reflect a protective effect of the visible collateral circulation against PCI-induced ischemia.

In a recent study, IMA concentrations were measured after elective direct-current cardioversion (DCCV) for atrial fibrillation to determine whether transient myocardial ischemia occurred *(82)*. Serum samples for IMA measurement were obtained before and at 1 and 6 h after DCCV in 24 patients. Fourteen patients developed ECG changes (ST-depression and/or T-wave inversion) after DCCV and showed IMA concentrations significantly higher than patients without changes; no significant differences were demonstrated in CK, CK-MB, and cTnT concentrations between the 2 groups. The results suggested that increased concentrations of IMA after cardioversion might reflect transient myocardial ischemia.

One study evaluated IMA for diagnosis of cardiac ischemia in patients attending the ED with symptoms of ACS *(83)*. The study evaluated IMA (>85 kilounits/L, the 95th percentile of apparently healthy people reported by the manufacturer) in conjunction with ECG results and cTnT concentrations (>0.05 μg/L) in 208 patients presenting to the ED within 3 h of acute chest pain. Blood samples were taken for IMA and cTnT measurements at presentation. The final diagnosis was based on the history, clinical examination, serial cTnT results, and data from medical records, including results of ECG, exercise stress testing, perfusion scans, and coronary angiography, as available. In the whole patient group, sensitivity of IMA at presentation for an ischemic origin of chest pain was 82% (95% CI, 74–88%), specificity was 46% (34–57%), the negative predictive value was 59%, and the positive predictive value was 72% (prevalence, 63%). IMA, ECG, and cTnT combined identified 95% of patients whose chest pain was attributable to ischemic heart disease.

IMA, which appears to be an indicator of oxidative stress, may not be specific for cardiac ischemia. Data about IMA concentrations in noncardiac ischemia are limited. Anecdotal evidence suggests that IMA increases in stroke, end-stage renal disease, liver disease, and some neoplasms *(76)*. In a study evaluating the ACB test for forearm ischemia, increases in endogenous lactate inhibited the test *(84)*. This result was recapitulated with exogenous lactate in vitro. Such a result raises caution concerning the significance of a negative IMA result in patients with poorly controlled diabetes, sepsis, and/or renal failure, all of which are situations where increased lactate may exist.

In a group of marathon runners, IMA did not increase immediately after a marathon run, indicating that skeletal muscle ischemia during exercise does not change IMA concentrations *(85)*. However, there were significant increases 24–48 h after the run, which were attributed to exercise-induced latent gastrointestinal ischemia. This latent increase is an issue that may potentially complicate use of the test in clinical practice. The influence of fluid shifts and albumin concentration changes that occur after strenuous exercise and other pathologies need to be more fully understood. Additional information that is needed for the clinical validation of this new assay includes studies on reference distributions by gender and ethnicity; an optimum diagnostic cutoff value for ACS patients, comparing IMA concentrations in common disease states with or without accompanying cardiac disease; and common diseases that coexist with cardiac ischemia, such as congestive heart failure, diabetes mellitus, chronic renal failure, and hypertension. A better understanding of IMA kinetics over the early hours after the onset of an ACS is also essential.

How will clinicians interpret a positive IMA finding? The positive predictive value of the ACB assay seems to be too low for use in ruling in ischemia, a use that clinicians hope the laboratory could provide. At present, whether patients with negative ECG results and necrosis markers, i.e., cardiac troponins, and a positive IMA result might benefit from early triage and intervention according to stratified pretest probabilities is not known. In clinical practice, this lack of information can potentially lead to overtreatment of low-risk patients with a positive result. Whether a positive IMA result in noncardiac patients may be associated with significant clinical conditions justifying admission for a more detailed examination needs to be explored. However, recent findings suggest that IMA may be useful in risk stratification of emergency chest pain patient *(86)*.

In summary, many questions remain unanswered regarding IMA and the ACB test. The assay needs to be evaluated by incorporating it into decision-making algo-

rithms under ED conditions. The highest expected benefit of the test would be to rule out ACS in low to moderate pretest probability conditions with negative necrosis markers and a negative ECG. This was the language for which the ACB test was cleared by the FDA for clinical use. The test seems to have limited specificity, however, with many false positives. There also seems to be considerable overlap between normal and ischemic IMA concentrations. A positive ACB test result has not been shown to differentiate between UA and early myocardial necrosis, where necrosis markers are not yet increased. Additional clinical evidence will need to evolve to support the intended claims.

Unbound Free Fatty Acids

Most serum free fatty acids (FFAs) are bound with albumin, with only a small amount of the total, the unbound FFAs (FFA_u), present as the soluble form. On the basis of preliminary findings, FFA_u concentrations, rather than total FFAs, may provide a sensitive guide to the pathophysiology of underlying coronary disease *(87*, *88)*. The mechanisms that initiate and maintain increased FFA_u concentrations after ischemia are not clear. Increased blood catecholamines in association with ischemia suggest that increased FFA_u concentrations result from increased FFA release through adipose lipolysis. Although ischemia activates lipid hydrolysis within the heart, the large increases in serum FFA_u are likely attributable to FFAs originating from other tissues, such as adipose, along with a reduction of FFA use after ischemia.

Data suggest that in patients presenting with ischemic symptoms, plasma FFA_u monitoring may provide an early indication of cardiac ischemia. Serum FFA_u measured in 22 PTCA patients 5 min before and 30 min after the procedure demonstrated a 14-fold higher postprocedure value as well as when compared with healthy volunteers *(89)*. However, ischemic ST changes by ECG were observed in only 11 (50%) of patients. In addition, FFA_u concentrations were significantly higher in the ECG-positive group vs the ECG-negative group. In a subsequent study from the same group, based on the absence or presence of necrosis according to cTnI monitoring at baseline, all 9 MI patients studied demonstrated an increased FFA_u vs only 2 of 9 for cTnI *(90)*. In the other chest pain patients, 93% had an increased FFA_u compared with increases in 30% for cTnI. FFA_u was increased in every instance that cTnI was increased, and there was a positive correlation between peak FFA_u and cTnI concentration. Additionally, in MI patients, FFA_u was increased in 100% of patients at presentation, whereas only 22% of these patients had increased cTnI at presentation, indicative of the earlier appearance of this analyte in the circulation before traditional markers of myocyte necrosis. Some of these patients were ultimately diagnosed with additional diagnoses that can cause myocardial ischemia and injury (such as sepsis, cocaine abuse, and cardiac contusion) independent of plaque rupture. This suggests that FFA_u concentrations in serum may increase early in the presence of acute myocardial injury and ischemia independent of plaque rupture. Further studies are warranted to evaluate the potential clinical utility of FFA_u in the evaluation of patients who present to the ED with chest pain. However, the potentially important finding of this study is the early detection of ischemia with FFA_u. Furthermore, circulating nonesterified fatty acid concentrations have also been shown to be predictive for sudden death in nonischemic patients *(91)*.

A method has been developed to measure the unbound (FFA_u) fraction of total plasma FFA concentrations *(92*, *93)* (FFA Sciences LLC). FFA_u can be measured with a recombinant fatty-acid–binding protein labeled with a fluorescent tag (ADIFAB). When FFA binds to ADIFAB, the fluorescent tag is displaced from its binding pocket, producing a spectral shift that can be measured with a fluorometer. The spectral shift is measured in the absence and presence of ADIFAB. A recently described second-generation assay, using the ADIFAB2 fluorescent molecular probe and a hand-held reader, has a turnaround time of <1 min *(90*, *94)*. The present measurements with ADIFAB2, done at 22 °C on 15 μL of plasma, are not affected by hemoglobin and show improved low-end sensitivity. Duplicate measurements done on human samples show a maximum CV of 7% *(90)*. The FFA_u upper reference limit, determined as the 97.5th percentile of the value distribution, is 2.7 nmol/L [mean (SD), 1.5 (0.6) nmol/L; range, 0.6–4.5] *(90)*.

In summary, preliminary data suggest that in patients presenting with ischemic symptoms, plasma FFA_u monitoring may provide an early indication of cardiac ischemia. Additional ACS trials are needed to fully evaluate the true potential of this biomarker.

Glycogen Phosphorylase Isoenzyme BB

Glycogen phosphorylase isoenzyme BB (GPBB) should not be considered a marker of myocardial ischemia; instead, its early release within 2–4 h after the onset of myocardial damage in parallel with myoglobin or heart-type fatty-acid–binding protein is an indicator of irreversible myocardial damage. By contrast, delayed release of GPBB after several hours, e.g., in parallel with lactate dehydrogenase, is seen after all kinds of myocardial damage (e.g., toxic and inflammatory damage or heart contusion). The biochemistry of this enzyme explains this behavior *(95)*. Glycogen phosphorylase (α-1,4-D-glucan: orthophosphate D-glucosyltransferase; EC 2.4.1.1) is a glycolytic enzyme that plays an essential role in the regulation of carbohydrate metabolism. It catalyzes the first step of glycogenolysis, in which glycogen is converted to glucose 1-phosphate by phosphorolysis in the presence of inorganic phosphate, and through mobilization of glycogen, it is primarily associated with provision of an emergency glucose supply during periods of hypoxia and hypoglycemia. GPBB is associated with glycogen in a macromolecular complex that is structurally bound to

the sarcoplasmic reticulum (sarcoplasmic reticulum glycogenolysis complex). The degree of GPBB association with this complex depends essentially on the metabolic state. With the onset of tissue hypoxia, when glycogen is broken down and disappears, GPBB is converted from a structurally bound to a soluble form as a result of the breakdown of glycogen, and the enzyme becomes free to move around in the cytoplasm and to diffuse out of the cell if the cell membrane permeability is simultaneously increased. The early release of GPBB requires both a burst in glycogenolysis and concomitantly increased plasma membrane permeability, which is the case in ischemic myocardial damage. Physiologically, glycogen phosphorylase is a dimeric enzyme (molecular mass as a monomer, ~97 kDa) composed of 2 identical subunits (homodimer). Three isoenzymes are found in human tissues: GPLL (liver), GPMM (muscle), and GPBB (brain). These isoenzymes are encoded by different genes and differ in structure as well as in function, reflecting differences in tissue metabolism. The BB and MM isoenzymes are found in the human heart, but the BB isoenzyme is the predominant isoenzyme in myocardium. Skeletal muscle, by contrast, contains only GPMM. GPLL is the predominant isoenzyme in human liver and all other human tissues except for heart, skeletal muscle, and brain. The GPBB isoenzyme is not restricted to human brain and heart, and small amounts have been reported in several other tissues (e.g., leukocytes, spleen, kidney, bladder, testis, liver, digestive tract, and aorta).

In clinical studies, GPBB was a very sensitive marker for the diagnosis of acute MI within 4 h after chest pain onset. In the majority of patients with MI, GPBB increased between 2 and 4 h after the onset of chest pain *(96)*. GPBB usually peaks ~6 to 20 h after onset of chest pain with early peak values found in patients with early reperfusion of the infarct-related coronary artery; it returns to within the reference interval within 1–2 days after MI *(97)*. GPBB also increases early in patients with ACS and reversible ST-T segment alterations in the resting ECG at hospital admission, which could be useful for early risk stratification *(97)*. GPBB was found to be sensitive for the detection of perioperative ischemic myocardial damage and infarction in patients undergoing coronary artery bypass grafting, and GPBB more accurately reflected ischemic myocardial damage than CK-MB *(98)*. The diagnostic specificity of GPBB in nontraumatized chest pain patients was comparably higher than that of CK-MB *(96)*, GPBB being an accurate marker for the detection of ischemic myocardial damage.

In summary, these interesting preliminary clinical observations (based on research and development assays that are not commercially available) must be confirmed with high-quality GPBB assays.

Placental Growth Factor

Placental growth factor (PlGF) is one of a family of platelet-derived proteins that function as potent chemoattractants for monocytes and are involved in the regulation of vascular endothelial growth *(99)*. PlGF is a 50-kDa heterodimer consisting of 149 amino acids and has high homology with vascular endothelial growth factor *(99)*. PlGF exists in 2 forms, PlGF-1 and PlGF-2, which differ only in that PlGF-2 has an insertion of a highly basic 21-amino acid stretch at the carboxyl end. Various tissues express PlGF mRNA, including thyroid, placenta, and lung, indicating that the protein functions in these tissues *(100)*. The biological functions of PlGF are incompletely understood, but they appear to primarily involve initiation of the inflammatory process, which includes the recruitment of circulating macrophages into atherosclerotic lesions, stimulation of smooth muscle cell growth, and up-regulation of both tissue necrosis factor-α and MCP-1 by macrophages *(100)*. PlGF also reportedly activates stem cells from a quiescent to proliferative state and in this way stimulates hematopoiesis in the bone marrow of mice *(101)*.

The search for important components that regulate the immune system has been driven by knowledge that atherosclerosis and plaque instability are fundamentally an inflammatory process *(102)*. Although elucidation of pathways and factors central to this system has led to considerable advances in basic understanding, many the substances identified are unstable in circulation and therefore are impractical biomarker candidates. PlGF appears to be stable in circulation and must be considered a strong candidate as a biomarker for plaque instability, myocardial ischemia, and prognosis of patients in the spectrum of ACS. PlGF is present in nanogram per liter concentrations in serum and plasma.

Recombinant human PlGF is available commercially from several sources (e.g., Cell Sciences, Inc.). Antibodies against PlGF have been developed and used in ELISAs, an example of which is available from R&D Systems, Inc. (Quantikine PlGF; cat. no. DGP00). This is a 2-site immunoassay in which a monoclonal antibody immobilized in microtiter plate wells is used to capture PlGF in samples; a labeled polyclonal antibody against a different PlGF epitope is used for detection. In addition to serum, the assay may use EDTA-, citrate-, and heparin-plasma samples. Limited reference interval data are available from the manufacturer, but ~35% of serum samples contain undetectable amounts of PlGF; with detectable samples ranging up to 26 ng/L. Recovery in the assay was 96%, interassay imprecision (CV) was ~10%, and the minimum detectable concentration is 7 ng/L. The assay requires ~4.5 h for measurement.

Particular interest in PlGF has been sparked by a proposed role as a principal instigator of atherosclerotic plaque instability, which is the physiologic common denominator for coronary artery thrombus formation and ACS *(102)*. Furthermore, compelling data have shown that inhibiting the actions of PlGF suppressed plaque instability and coronary heart disease *(101)*. Thus, PlGF is not merely a risk marker but also a disease marker, and

may represent a new therapeutic target for mitigating the disease process behind ACS.

Plasma PlGF measurements obtained at admission were investigated for assessing risk of death or nonfatal MI in the 30 days after index presentation in a large group of patients (n = 1173) *(103)*. These patients were from 2 cohorts, one having angiographically confirmed ACS and the other presenting to the ED with chest pain *(103)*. The confirmed ACS patients (n = 547) were enrolled in the CAPTURE (c7E3 Fab Anti-Platelet Therapy in Unstable Refractory Angina) trial *(103)*. Briefly, CAPTURE included non-ST-segment elevation MI patients with recurrent chest pain; all had angiography with 70% lesion or larger and were randomized for GP IIb/IIIa or placebo. Only patients in the CAPTURE placebo arm were included in the assessment of PlGF to avoid the possibly confounding effect of antiplatelet therapy. The other group (n = 626) presented to the ED in Germany with acute chest pain. In the CAPTURE cohort, 223 (40.8%) patients were found to have increased PlGF concentrations, defined as >27.0 ng/L, and were found to have a markedly increased risk of adverse events at 30 days (14.8% vs 4.9%). The unadjusted hazard ratio for increased PlGF was 3.34 (95% CI, 1.79–6.24) and was statistically significant ($P < 0.001$). In patients with acute chest pain, PlGF results >27.0 ng/L also predicted a significant increase in risk (21.2% vs 5.3%); the hazard ratio was 4.80 (95% CI, 2.81–8.21; $P < 0.001$). This study also used a multivariate modeling technique to investigate whether PlGF provides additional information to available markers, including cTnT, sCD40L, and CRP. For the CAPTURE population, this multivariable modeling showed that increased concentrations of cTnT ($P = 0.03$), sCD40L ($P = 0.002$), and PlGF ($P < 0.001$) were independent predictors of death or MI at 30 days, whereas increased CRP was not ($P = 0.94$). In the ED cohort, PlGF was also an independent predictor of important outcomes ($P < 0.001$). Of interest, patients who were negative for cTnT, sCD40L, and PlGF were at very low cardiac risk (no events by 7 days after presentation; 2.1% event rate 30 days after presentation).

Plasma PlGF measurements have been shown to be an independent biomarker of adverse outcome in patients with suspected ACS *(103)*. Plasma PlGF appears to extend the predictive and prognostic information gained from traditional biomarkers of necrosis, platelet activation, and systemic inflammation. PlGF is increased in ACS regardless of the cTnT concentration, which implies that it is a biomarker of ischemic events such as plaque instability, plaque disruption, or impending thrombosis in the context of ACS. Biomarkers providing independent insight into the various physiologic components of ACS will probably be combined in a synergistic proteomics strategy for risk assessment, diagnosis, therapeutic guidance, and therapeutic monitoring in the future. It appears that PlGF has potential for playing an important role.

In summary, PlGF appears to be have great potential as an independent biomarker for plaque disruption, ischemia, and thrombosis. Larger trials are forthcoming and may aid in the possible commercialization of PlGF.

Conclusions

This review serves as a snapshot of the future potential clinical use of several candidate biomarkers for risk stratification in patients presenting with ACS and for improved understanding of the pathophysiology of ACS. We have attempted to point out both the strengths and weaknesses of the published literature. From that scrutiny, it appears that much work is still needed to meet quality specifications for assay development as well as for clinical validation. Concerns that have been addressed for cardiac troponins *(104)* and are currently being addressed for cardiac natriuretic peptides *(105)* will need to be addressed with the same scrutiny for these new biomarkers. They include validating analytical imprecision and detection limits, calibrator characterization, assay specificity and standardization, preanalytical issues, and appropriate reference interval studies. As suggested by this Committee *(6, 104, 105)*, manufacturers might benefit from seeking assistance from organizations such as the IFCC for guidance in these areas. Furthermore, manufacturers, clinicians, and laboratorians need to work closely with regulatory agencies, such as the FDA, to optimize clinical use and avoid interpretive confusion based on the multiple assays that will appear in the marketplace. "Crossing the boundary from research to clinical application, will require replication in multiple settings, experimental evidence supporting a pathophysiologic role, and ideally, intervention trials demonstrating that modification improves outcomes" *(106)*.

This review accomplishes one of the 2005 goals of the Committee on Standardization of Markers of Cardiac Damage of the IFCC, chaired by Dr. Apple.

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