Cardiovascular diseases (CVD) are the leading cause of morbidity and mortality in the United States. Primary prevention and secondary prevention of CVD are public health priorities. Substantial data indicate that CVD is a life course disease that begins with the evolution of risk factors that in turn contribute to the development of subclinical atherosclerosis. Subclinical disease culminates in overt CVD. The onset of CVD itself portends an adverse prognosis with greater risk of recurrent events, morbidity, and mortality. It is also increasingly clear that although clinical assessment is the cornerstone of patient management, such evaluation has its limitations. Clinicians have used additional tools to aid clinical assessment and to enhance their ability to identify the “vulnerable” patient at risk for CVD, as suggested by a recent National Institutes of Health (NIH) panel. Biomarkers are one such tool to better identify high-risk individuals, to diagnose disease conditions promptly and accurately, and to effectively prognosticate and treat patients with disease. This review provides an overview of the molecular basis of biomarker discovery and selection and the practical considerations that are a prerequisite to their clinical use.

**What Is a Biomarker? Definition and Types**

The term biomarker (biological marker) was introduced in 1989 as a Medical Subject Heading (MeSH) term: “measurable and quantifiable biological parameters (eg, specific enzyme concentration, specific hormone concentration, specific gene phenotype distribution in a population, presence of biological substances) which serve as indices for health- and physiology-related assessments, such as disease risk, psychiatric disorders, environmental exposure and its effects, disease diagnosis, metabolic processes, substance abuse, pregnancy, cell line development, epidemiologic studies, etc.” In 2001, an NIH working group standardized the definition of a biomarker as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” and defined types of biomarkers (Table 1).

A biomarker may be measured on a biosample (as a blood, urine, or tissue test), it may be a recording obtained from a person (blood pressure, ECG, or Holter), or it may be an imaging test (echocardiogram or CT scan).

Biomarkers can indicate a variety of health or disease characteristics, including the level or type of exposure to an environmental factor, genetic susceptibility, genetic responses to exposures, markers of subclinical or clinical disease, or indicators of response to therapy. Thus, a simplistic way to think of biomarkers is as indicators of disease trait (risk factor or risk marker), disease state (preclinical or clinical), or disease rate (progression). Accordingly, biomarkers can be classified as antecedent biomarkers (identifying the risk of developing an illness), screening biomarkers (screening for subclinical disease), diagnostic biomarkers (recognizing overt disease), staging biomarkers (categorizing disease severity), or prognostic biomarkers (predicting future disease course, including recurrence and response to therapy, and monitoring efficacy of therapy).

Biomarkers may also serve as surrogate end points (Table 1). Although there is limited consensus on this issue, a surrogate end point is one that can be used as an outcome in clinical trials to evaluate safety and effectiveness of therapies in lieu of measurement of the true outcome of interest. The underlying principle is that alterations in the surrogate end point track closely with changes in the outcome of interest. Surrogate end points have the advantage that they may be gathered in a shorter time frame and with less expense than end points such as morbidity and mortality, which require large clinical trials for evaluation. Additional values of surrogate end points include the fact that they are closer to the exposure/intervention of interest and may be easier to relate causally than more distant clinical events. An important disadvantage of surrogate end points is that if clinical outcome of interest is influenced by numerous factors (in addition to the surrogate end point), residual confounding may reduce the validity of the surrogate end point. It has been suggested that the validity of a surrogate end point is greater if it can explain at least 50% of the effect of an exposure or intervention on the outcome of interest.

**Characteristics of an Ideal Biomarker**

The overall expectation of a CVD biomarker is to enhance the ability of the clinician to optimally manage the patient. For instance, in a person with chronic or atypical chest pain, a biomarker (eg, treadmill stress test or dobutamine stress imaging test (echocardiogram or CT scan).
The desirable properties of biomarkers vary with their intended use. For screening biomarkers, features such as sensitivity, specificity, and predictive values, large likelihood ratios (discussed below), and low costs are important. For diagnostic markers of acute cardiac disease (such as acute MI), in addition to the aforementioned characteristics, rapid sustained elevation, high tissue specificity (indicating myocardial origin), release proportional to disease extent, and assay features conducive to point-of-care testing are critical.

For biomarkers monitoring disease progression or response to therapy, features such as sensitivity or specificity are less important because the patient serves as his or her own control (baseline values are compared with follow-up values). Narrow intra-individual variation and tracking with disease outcome or therapy are critical. Costs may be less important for prognostic markers because only people with disease are tested. Some biomarkers (eg, exercise stress test) may be used for both diagnostic and prognostic purposes. Establishing the prognostic utility of a biomarker is more challenging because it requires a larger sample and a prospective design, whereas demonstrating its value as a diagnostic test requires a smaller sample and a cross-sectional design.

Regardless of the intended use, it is important to remember that biomarkers that do not change disease management cannot affect patient outcome and therefore are unlikely to be cost-effective (judged in terms of quality-adjusted life-years gained). Typically, for a biomarker to change management, it is important to have evidence that risk reduction strategies should vary with biomarker levels, and a biomarker-guided approach translates into better patient outcomes compared with a management scheme (usually the current standard of care) without biomarker levels. It also means that biomarker levels should be directly or indirectly modifiable by therapy on the basis of evidence from prospective clinical trials. Biomarkers that do not result in medical intervention may still serve other useful purposes, such as the reassurance value of a negative exercise test in an asymptomatic airline pilot.

There are other examples of psychological benefits accruing from negative biomarker test results such as testing for genetic susceptibility for cancer or Alzheimer disease. In other situations, biomarkers may serve as research tools by providing insights into disease mechanisms.

**Defining Abnormal Biomarker Values**

Defining abnormal values is a critical step before the clinical use of a biomarker. It is important to characterize the distribution of the markers in people in the community and in patient samples on whom the biomarker will be tested. Thus,
<table>
<thead>
<tr>
<th>Feature</th>
<th>All Biomarkers</th>
<th>“Screening” Biomarkers to Identify “Vulnerable” Patients</th>
<th>“Diagnostic” Biomarkers to Identify Ischemia or Injury</th>
<th>“Prognostic/Treatment” Biomarkers</th>
</tr>
</thead>
<tbody>
<tr>
<td>General features</td>
<td>Known “reference limits”</td>
<td>High myocardial specificity</td>
<td>Not present in normal serum/noncardiac tissue</td>
<td>Add to known reference limits</td>
</tr>
<tr>
<td>Measure a specific pathology</td>
<td>Add to known CHD index such as the FHS risk score</td>
<td></td>
<td></td>
<td>Add to known prognostic index</td>
</tr>
<tr>
<td>Add to clinical assessment</td>
<td>Change in c statistic or AUC (discrimination)</td>
<td>Zero baseline, immediate release (early detection)</td>
<td>Change in marker alters management</td>
<td></td>
</tr>
<tr>
<td>Acceptable to patient</td>
<td>Acceptable calibration</td>
<td>Long 1/2</td>
<td></td>
<td>Affect choice of drug</td>
</tr>
<tr>
<td>Linear relation between change in marker and change in pathology</td>
<td>“Rule in” strategy with high specificity more important to avoid mislabeling asymptomatic individuals</td>
<td>Permit long time window for diagnosis</td>
<td>Change in marker alters management</td>
<td></td>
</tr>
<tr>
<td>Stable product</td>
<td>Account for a moderate or greater proportion of CHD in the community</td>
<td>But &lt;24 h to permit diagnosis of recurrent ischemia</td>
<td>Indicate tolerance</td>
<td></td>
</tr>
<tr>
<td>Single measure representative</td>
<td>Change management</td>
<td>Release proportional to injury size or ischemic burden</td>
<td>Indicate safety margin</td>
<td></td>
</tr>
<tr>
<td>Applicable to men and women, different ages, different ethnicities</td>
<td>Reclassify risk in patients at intermediate risk</td>
<td>Convenience for point-of-care testing</td>
<td>Used for monitoring progression of disease</td>
<td></td>
</tr>
<tr>
<td>Replication in multiple studies</td>
<td>Target individuals with increased levels of biomarker superior to conventional Rx for reducing risk</td>
<td>Rapid test results available in &lt;1 h</td>
<td>Trajectory of marker correlates with disease progression</td>
<td></td>
</tr>
<tr>
<td>Assay/measurement features (see Table 3 also)</td>
<td>Cost-benefit ratio favorable</td>
<td>No special sample preparation needed</td>
<td>“Rule out” strategy with high specificity more important to avoid mislabeling asymptomatic individuals</td>
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<tr>
<td>Internationally standardized</td>
<td>Inexpensive</td>
<td></td>
<td>REMARK guidelines</td>
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</tr>
<tr>
<td>Accuracy</td>
<td>Readily available</td>
<td>Diagnostic cutoff well defined and accepted</td>
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<tr>
<td>Precision</td>
<td></td>
<td>Known discrimination limits or action thresholds</td>
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<tr>
<td>Assay application features</td>
<td></td>
<td>Change management, triage, or specific treatment</td>
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<tr>
<td>Tested in a spectrum of people with varying degrees of pathology</td>
<td></td>
<td>“Rule out” strategy</td>
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<tr>
<td>High sensitivity and specificity</td>
<td></td>
<td>With high sensitivity, more important to avoid missing disease</td>
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<tr>
<td>Laboratory features</td>
<td></td>
<td>Cost-effective</td>
<td>STARD guidelines</td>
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<td>Automation</td>
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<tr>
<td>High throughput/short turnaround time</td>
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<tr>
<td>Connectivity to laboratory information management systems</td>
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<tr>
<td>Compatibility with existing laboratory processes</td>
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<tr>
<td>Desirable features for in vitro diagnostic industry</td>
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<tr>
<td>Address unmet patient needs</td>
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<td>Return on investments</td>
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<td>Requirements for research and development phases</td>
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<tr>
<td>Manufacturability</td>
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<tr>
<td>Marketability including barriers to entry</td>
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<tr>
<td>Postsales customer support</td>
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<tr>
<td>Other market features (acceptance, competition, regulatory issues, reimbursement, third party patent rights)</td>
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</tbody>
</table>
variation in levels with age, sex, ethnicity, and prevalent disease and the relations of biomarkers to known risk factors must be characterized.  

At least 3 potential approaches exist for defining abnormal biomarker levels (Figure 1). Reference limits are generated with the use of cross-sectional analyses of a reference sample (usually a healthy sample free of the disease of interest), and an arbitrary percentile cutpoint (typically the 95th or 97.5th percentile) is chosen to define abnormality.  

The reference range is the interval between the minimum and the maximum reference values. Approximately 200 individuals are required within each category for the formulation of reference limits for subgroups (eg, defined by age and sex).  

Other trials: number needed to screen is calculated by dividing the number needed to treat for treating risk factors by the prevalence of disease that was unrecognized or untreated.  

FHS indicates Framingham Heart Study; t1/2, half-life; REMARK, Reporting recommendations for tumour MARKer prognostic studies; and STARD, STAndards for Reporting of Diagnostic accuracy.

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**TABLE 2.** Continued

<table>
<thead>
<tr>
<th>Measures of biomarker test performance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity is defined as the ability of a test to detect disease (condition of interest) when it is truly present, ie, it is the probability of a positive test result given that the patient has the disease.</td>
</tr>
<tr>
<td>Specificity is the ability of a test to exclude the disease (condition of interest) in patients who do not have disease, ie, it is the probability of a negative test result given that the patient does not have the disease.</td>
</tr>
<tr>
<td>Predictive value tells us how good the test is at predicting the true positives or true negatives, ie, the probability that the test will give the correct diagnosis.</td>
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</tbody>
</table>

The positive predictive value is the probability that a patient has the disease given that the test results are positive.  

The negative predictive value is the probability that a patient does not have the disease or condition given that the test results are indeed negative.  

An ROC curve is a plot of the sensitivity versus (1 − specificity) of a diagnostic test, in which the different points on the curve correspond to different cut points used to determine whether the test results are positive.  

Prevalence is defined as the prior probability of the disease before the test is performed.  

The likelihood ratio is a simple measure of diagnostic accuracy, given by the ratio of the probability of the test result among patients who truly had the disease to the probability of the same test among patients who do not have the disease.  

- Likelihood ratio (test positive) = sensitivity/(1 − specificity).
- Likelihood ratio (test negative) = (1 − sensitivity)/specificity.

Number needed to diagnose is derived from 1/[sensitivity − (1 − specificity)], number of tests that need to be performed to gain a positive response for the presence of disease.

Number needed to screen is defined as the number of people that need to be screened for a given duration to prevent one death or adverse event.

Clinical trials of screening: number needed to screen is calculated as number needed to screen equals 1 divided by absolute risk reduction.

Other trials: number needed to screen is calculated by dividing the number needed to treat for treating risk factors by the prevalence of disease that was unrecognized or untreated.

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**Figure 1.** Approaches to defining “abnormal” biomarker values (see text for description). FN indicates false-negative; FP, false-positive; TN, true-negative; TP, true-positive; Pts, patients; and F/U, follow-up.
associated with follow-up of abnormal results. For instance, the 99th percentile value has been used to define an abnormal troponin or creatine kinase–MB value; values exceeding this limit would indicate the presence of myocardial necrosis and an acute MI.\(^4\)\(^0\)\(^1\) When less specific markers of myocardial necrosis are used, a higher threshold may be used; for example, if total creatine kinase is used for the diagnosis of acute MI, a value twice the upper reference limit is recommended.\(^4\)\(^0\)\(^2\)

Several issues must be considered when reference limits are interpreted. First, a select proportion of “normal” individuals will exceed the reference limits on the basis of the percentile chosen. Second, values that lie within statistically defined reference limits may not indicate health in a given individual, especially when the person comes from a group inherently different from the one used to derive the reference limits. Third, a change in values within the reference range may indicate pathology. Accordingly, delta limits have been formulated to evaluate the change in biomarker values within an individual (in response to disease or therapy) relative to the physiological intraindividual fluctuation of values. Fourth, a value within the reference range may not necessarily be desirable, especially when the prevalence of undesirable values of a biomarker in the population is high. Thus, abnormal blood pressure or cholesterol values are not defined on the basis of the distributions of these risk factors in the community; rather, “desirable” levels are defined (see below).

Discrimination limits are also used to indicate abnormal biomarker values.\(^4\)\(^2\) Such limits are generated by evaluating the degree of overlap between patients with and without disease in cross-sectional studies.\(^4\)\(^2\) Discrimination limits trigger decisions (they are referred to as decision thresholds). The 99th percentile value of troponin for a reference sample is in essence a discrimination limit because it identifies the presence of a MI. The discrimination thresholds can be varied depending on the relative importance of missing disease versus that of misclassifying nondiseased individuals. For example, a plasma BNP value \(>100 \text{ pg/mL}\) with the Biosite assay may trigger suspicion of heart failure in a dyspneic individual.\(^4\)\(^3\) The reference limits of the Biosite assay exceed this threshold in women aged \(>65\) years (95th percentile is \(120 \text{ pg/mL}\)).\(^4\)\(^4\) A plasma value \(>200 \text{ pg/mL}\) has been suggested as a threshold indicating heart failure.\(^4\)\(^5\)

A third method is to define “undesirable” biomarker levels by relating values to the incidence of disease and seeking a threshold beyond which risk escalates. For instance, a desirable systolic blood pressure may be \(\leq 115 \text{ mm Hg}\) because incidence of vascular disease increases continuously above this level.\(^4\)\(^6\) On a parallel note, low-density lipoprotein cholesterol levels \(\leq 100 \text{ mg/dL}\) are deemed to be optimal.\(^4\)\(^7\) For most CVD risk factors, there is a continuous gradient of risk across the range of risk factors, and a majority of individuals in a population could be classified as having undesirable levels. “Treatment” levels (especially for pharmacological treatment) of risk factors may therefore differ from undesirable levels, being defined by the risk factor thresholds for which there is good evidence (typically from large randomized controlled trials) that treatment for values above a limit does more benefit than harm. Often such treatment levels may be defined not only by the level of the specific risk factor being evaluated but by taking into consideration absolute risk of disease based on the values of several other risk factors.\(^4\)\(^8\) Thus, a blood pressure level of 140 (systolic) or 90 (diastolic) mm Hg or more indicates systemic hypertension.\(^4\)\(^9\) However, experts have argued that blood pressure levels above or below this threshold could be treated on the basis of the absolute risk of CVD events, which in turn depends on the concomitant burden of other risk factors.\(^4\)\(^8\) For other biomarkers, the choice of the optimal cutpoint defining abnormality remains to be described and may vary with the purpose. For instance, Framingham data indicate that a plasma BNP value exceeding the 80th percentile value in the cohort (\(20.0 \text{ pg/mL}\) for men and \(23.3 \text{ pg/mL}\) for women; Shionogi assay) is associated with a 76% increased risk of CVD and a tripling of congestive heart failure hazard.\(^5\)\(^0\) These values are below the 95th percentile value of a healthy reference sample at any age (Shionogi assay).\(^5\)\(^1\) Therefore, cutpoints of plasma BNP that identify discrimination limits for a diagnosis of congestive heart failure may differ from the upper reference limit, which in turn may vary from desirable levels.

Once abnormal thresholds of markers are formulated by any of the 3 aforementioned methods, biomarker performance can be assessed with the use of principles outlined in the next section.

**Evaluation of Biomarker Performance:**

**General Principles**

The exact yardstick for evaluating the performance of a biomarker varies on the basis of the intended use. Good-quality biomarker studies make an independent masked comparison of the performance of a given biomarker with a reference standard in an appropriate sample of consecutive patients that represents an adequate spectrum of the disease.\(^2\)\(^5\) In general, the performance of biomarkers is seldom as good in a second sample as in the sample in which they were initially assessed. Consequently, it is desirable that biomarkers be evaluated initially in a derivation or training set and then investigated in a validation or test set.\(^5\)\(^2\) Standards have been proposed for designing and reporting the results of studies evaluating the performance of biomarkers for diagnosis\(^2\)\(^5\) and for prognosis.\(^2\)\(^6\)

The accuracy of a biomarker test is evaluated in terms of its sensitivity (detection of disease when disease is truly present, ie, identifying true-positives) and its specificity (recognition of absence of disease when disease is truly absent, ie, identifying true-negatives) at select cutpoints. Several CVD biomarkers are continuously distributed quantitative variables, although there are some notable exceptions (for example, gender, race, diabetes, hypertension, genotypes). It is therefore critical to evaluate the information content of a biomarker over a range of values, often with the use of receiver operating characteristic (ROC) curves.\(^5\)\(^3\)–\(^5\)\(^5\) The ROC curves illustrate the tradeoff between sensitivity and specificity when biomarker levels are used clinically to identify disease. Each point on the ROC curve indicates the conditional probability of a positive test result from a random diseased individual exceeding that from a random nondis-
Evidence that a test in asymptomatic individuals (eg, preclinical left ventricular systolic dysfunction) could have high specificity because of a “rule in” strategy (or confirmatory diagnosis) is more important (also called the SpIN rule). Expressed in terms of LR, a test with a greater LR+ (typically >10) is preferred; this is because the costs of mislabeling a healthy individual (predicting disease when health is likely) may outweigh the costs of missing a rare condition. Sometimes when multiple tests are considered for screening, they are obtained in series. When multiple tests are obtained in series and disease is considered positive when all tests are positive (“AND rule”), specificity is enhanced but sensitivity is diminished. For instance, Ng et al have proposed that a sequential strategy for checking people with an initial urine N-terminal pro-BNP (N-BNP) test followed by a plasma N-BNP test (in urine “positive” cases) may facilitate screening for asymptomatic left ventricular systolic dysfunction in the community by reducing the need for follow-up echocardiograms. When multiple tests are obtained in parallel and disease is considered to be present when any of the tests is positive (“OR rule”), sensitivity is increased at the cost of specificity. For a biomarker to be accepted as a routine screening test it is important to demonstrate that a strategy of measuring the biomarker improves patient outcomes relative to a conventional strategy that does not include the biomarker measurement, usually in the context of a randomized controlled clinical trial. Such clinical trials prove the effectiveness of screening and also provide valuable data for cost-effectiveness analyses.

If a biomarker is used to diagnose a potentially life-threatening condition in a symptomatic patient (eg, acute MI in a patient with chest pain), it should have a high sensitivity because a “rule out” (exclude disease) strategy is critical in this setting (also called the SnOUT rule). Expressed in terms of LR, a test with a lower LR− (typically <0.10) is needed; this is because the costs of missing disease (projecting health when disease is likely) outweigh the costs of any additional testing or a false diagnosis.

Appropriate use of biomarker results requires use of a Bayesian approach, ie, integrating pretest probabilities with biomarker test results (expressed as sensitivity/specificity or as LR) to estimate the posttest probability of disease. Predictive values use this concept to facilitate interpretation of test results, taking into consideration disease prevalence. Even for a test with high sensitivity and specificity, false-positive tests will outnumber true-positive tests when disease prevalence is very low, and false-negative tests will outnumber true-negative tests when disease prevalence is very high. Pretest probabilities for estimating predictive values may be generated on the basis of the published literature combined with clinical experience. A nomogram is available that uses the pretest probability of disease and the LR of a diagnostic test to generate posttest probability of the condition.

Biomarkers (whether for screening, diagnosis, or prognosis) are also evaluated in terms of their discrimination and calibration capabilities. Discrimination refers to the ability of the biomarker (by itself or as part of a composite score) to distinguish “case” from “noncase” in cross-sectional studies or to differentiate “those who will develop disease” from “those who will not” in longitudinal investigations. Typically, the c statistic (or concordance index) is used as the metric of model discrimination and is equivalent to the area under the ROC curve. The c statistic is the probability that in 2 randomly paired individuals (one with and one without disease), a given test correctly identifies the one with disease. It is important to note that the c statistic is a metric of overall performance. It is possible for 2 tests to have the same c statistic, yet one biomarker may be superior to the other in terms of performance at select thresholds.

Calibration tells us how the ability of a biomarker (or a model incorporating the biomarker) to predict risk relates to the actual observed risk in subgroups of the population. The Hosmer-Lemeshow goodness-of-fit statistic is often used as an indicator of model calibration. For this purpose, the sample is divided into deciles of risk, and the observed number of events is compared with the expected number of events. Calibration is particularly important in counseling of individuals when the question of interest is the numeric probability of disease in a given patient (rather than how sick they are relative to other persons with disease). Thus, risk prediction algorithms have been developed that incorporate select biomarkers and enable clinicians to predict the absolute event rates of disease; examples include estimating the risk of coronary heart disease (CHD) given values of vascular risk factors, assessing the risk of death or stroke in patients with atrial fibrillation, and appraising the risk of death in patients with established heart failure. Models can be recalibrated if they uniformly underestimate or overestimate risk. For example, the Framingham CHD risk score overestimated risk in a Chinese cohort. A recalibration of the risk functions (with the use of mean values of risk factors and mean CHD incidence rates in the Chinese cohort) substantially improved CHD risk prediction.

Evaluation of the Incremental Value of New Biomarkers and the Multimarker Concept

To evaluate the incremental value of a new biomarker, investigators must demonstrate the elevated risk of an outcome associated with higher levels of the new biomarker with adjustment for other established risk factors. These results are typically presented as hazards ratios (relative risk estimates from a Cox model) and a probability value test of significance of the marker in the multivariable models. It has been argued that such an interpretation of a new marker’s association with risk as a reflection of its prognostic value may be inappropriate because the “hazard ratio is dependent on the measurement scale of the marker, cutoff(s) used for the novel marker, and the manner in which established variables are modeled.” In other words, a high hazard ratio for a marker in relation to a disease outcome does not necessarily indicate...
better predictive performance. Indeed, very strong associations of markers with disease are required for a given biomarker to have good discrimination properties. Even when a biomarker threshold is associated with very high odds of disease, it often will identify only a small proportion of people with disease if false-positive rates are to be kept low. For example, the relative risk for CHD mortality comparing the top with the lowest decile of the distribution of serum cholesterol was ≈ 3 in a large study, indicating a strong association. However, if one were to accept a serum cholesterol treatment cutoff that yields only a 5% false-positive rate (the threshold often used for screening studies), only 12% of the people who would later die of CHD would be identified by that threshold. In other words, risk factors for disease may not necessarily make good screening tools. This is because, notwithstanding an association of a risk factor with disease, the distributions of the risk factor levels in people with and without disease may overlap substantially.

When a new biomarker X is evaluated, it is important to remember that the question of interest is not whether X is a better predictor of disease than a previously known biomarker Y. Rather, the pertinent question is whether X improves the predictive accuracy of the best available model (representing the standard of care for that disease) that incorporates several known predictors of disease including Y. Thus, the relative added values of new biomarkers is best evaluated by estimating the increment to the c statistic compared with that from a model that incorporates other previously known predictors. For example, the Framingham CHD risk score may be thought of as a composite of several biomarkers with a c statistic (a metric of predictive accuracy) varying between 0.74 and 0.76, values considered consistent with a “fair” test performance. Few risk factors of interest in terms of CHD risk prediction can enhance the c statistic beyond that provided by the Framingham risk score; for instance, whereas C-reactive protein (CRP) was associated with vascular risk in 2 separate studies, addition of CRP did not improve the predictive accuracy of a model incorporating established risk factors that represent the current standard of care. Another way to evaluate novel risk factors is to assess whether knowledge of a putative risk factor alters the probability of disease (eg, changes the risk category from low to intermediate risk) estimated with the use of the global CHD risk score such as to change the recommended target threshold for a modifiable risk factor (eg, change the target low-density lipoprotein cholesterol from 130 to 100 mg/dL).

There is considerable interest in generating multimarker scores that use a composite of several biomarkers (measured in parallel) for the purpose of predicting disease risk and patient outcomes. The comparison of several putative biomarkers and the generation of multimarker scores must take several factors into consideration. First, comparisons of biomarkers measured on the same set of individuals must account for their inherent correlation (people with high values of one marker will likely have high values of another). Second, the incremental utility of adding a new biomarker to a known panel of biomarkers is often estimated by ROC analysis. It is important to realize that the ability of the biomarker to identify cases not captured by the usual sets of predictors (conditional or multi-ROC analysis) requires specification of thresholds for the usual set of markers, and the performance of the new marker is conditional on the choice of those cutpoints. Sometimes, a multivariate formulation of several markers can be generated with the use of techniques such as neural networks to increase diagnostic accuracy.

Risk prediction equations that incorporate multiple markers are often used for CVD risk prediction. The challenges associated with the development and application of such risk scores have been reviewed elsewhere. Nonetheless, use of a global risk score based on assessment of multiple risk factors is critical because of their synergistic influences and the importance of targeting undesirable levels of several risk factors to maximize patient benefits. Risk scores formulated on the basis of a sample should be demonstrated to be reproducible in the same population (with the use of data resampling techniques such as bootstrapping). Additionally, to become a routine part of clinical practice, risk scores should be “transportable”: geographically to diverse locations; to different ethnicities; to a wide spectrum of patients; or for predicting events over a different duration of follow-up compared with what was used to develop the score. Risk scores derived in one sample may need to be recalibrated when applied to a very different population.

Although it is generally believed that new biomarkers should add to the c statistic to be useful, there are exceptions to this rule. Novel biomarkers (eg, homocysteine) that are not incremental to known risk factors may be measured in select clinical situations such as in the following: asymptomatic individuals without obviously elevated conventional risk factors but with very strong family history of vascular disease; patients with premature vascular disease but no obvious risk factors; and patients with aggressive recurrent vascular disease in the face of well-controlled levels of conventional risk factors.

In the case of studies in which genetic biomarkers are used, there is a major concern about false-positive associations with disease (or phenotypes) resulting from numerous additional factors. A detailed discussion of the factors contributing to the lack of replication of several genetic association tests is beyond the scope of this review but includes true genetic heterogeneity across samples, publication bias, confounding by population structure, misclassification of outcomes, allelic heterogeneity, small sample sizes, and failure to account for multiple testing (including the possibility that findings are due to chance). Measures to address these issues have been proposed, including but not limited to considering pretest probabilities of associations and using false discovery rates (estimated by permutation or bootstrap methodology). Replication of findings in multiple independent samples remains the gold standard for genetics of complex diseases.
Evaluation of Biomarker Performance: Laboratory Factors

The above discussion of biomarker performance assumes a perfect laboratory and limited biological variability. In practice, preanalytical, analytical, and postanalytical factors are important contributors to biomarker performance. The greater the “noise” introduced by these factors, the lesser the “signal-to-noise ratio” offered by a biomarker.

Preanalytical variability refers to biological variability and stability over time, whereas analytical variability relates to the performance of the test in the laboratory. Low analytical variability is a fundamental requirement of all biomarkers (Table 3).

Guidelines for maintaining quality control within laboratories have been proposed. Analytical variability means good accuracy and precision. Accuracy refers to the degree of agreement with a reference standard for the analyte and is quantified in terms of percent bias. Standardization of an assay means use of a reference measurement procedure and reference materials. International reference standards have been established for several biomarkers, including interleukin-6, interleukin-8, serum amyloid A protein, fibrinogen, and high-sensitivity CRP. Precision refers to consistent measurement on replicates and is quantified in terms of the coefficient of variation (continuous markers) or the kappa statistic (qualitative markers). Analytical standards have been proposed for several CVD biomarkers, including lipids, troponins, and high-sensitivity CRP.

If analytical imprecision is greater than biological variability, samples should be assayed in replicate, and quality control procedures that improve assay methodology and/or operating procedures should be instituted. This is critical for biomarkers used for point-of-care testing because imprecision may be greater in this setting compared with standard laboratory measurements. If biological variability is greater than analytical imprecision, the patient should be sampled on 1 occasion to obtain a true estimate of a biomarker.

Biological variability can also be reduced by instituting a standardized protocol for phlebotomy if applicable (such as the requirement of a fasting state, supine posture, or an early morning specimen). Quality control protocols to enhance analytical precision for imaging studies have been proposed. In the case of newer technologies such as genotyping and microarray (discussed in a subsequent section), the possibility of analytical error is of a different order of magnitude. Standards for detecting errors in genotyping and in microarrays have been proposed as well.

Postanalytical factors affecting biomarker performance include the processes of approval and transmission and the appropriate display of test results with the use of the laboratory’s information management systems. As noted above, the quality control requirements for biomarkers also may vary depending on the mode of delivery/use of the test: an automated platform from a centralized laboratory, a point-of-care testing device, or a device used for home monitoring of analytes. Point-of-care testing usually involves small benchtop analyzers or hand-held devices that facilitate rapid decision making, earlier treatment, reduced incidence of complications, quicker optimization of treatment, reduction in hospital stay, greater patient satisfaction, and economic benefits.

Biomarker Discovery: Challenges and Approaches

The development of CVD biomarkers is challenging for several reasons. As summarized in a recent consensus document, the patient vulnerable to CVD is likely harboring a triad of abnormalities: vulnerable plaque, vulnerable blood, and vulnerable myocardium. In terms of developing biomarkers, 2 of these 3 components (vulnerable plaque and myocardium) are less directly accessible relative to the third (vulnerable blood). In the case of atherosclerotic cerebrovascular disease, biomarkers that may be elevated as a consequence of brain injury may not be detectable in large amounts in the peripheral circulation because of the blood-brain barrier. It is challenging to identify diagnostic biomarkers in the peripheral blood within 3 hours of stroke onset, the critical time window for thrombolytic therapy. Furthermore, biomarkers selected to reflect a clinical phenotype may be confounded by the inaccuracies in the characterization of the phenotype. Conversely, there may truly be a poor correlation between biomarkers and the clinical phenotype itself. For example, in the setting of an acute MI, a majority of culprit ruptured plaques occur in nonstenotic coronary lesions. In addition, the process of atherosclerotic CVD is inherently so complex that it would be simplistic to assume that a parsimonious set of biomarkers could capture most of the interindividual variation in propensity to develop CVD or its sequela.

The aforementioned caveats notwithstanding, 3 parallel developments have revolutionized the field of biomarker discovery. First, the completion of the Human Genome Project and the HapMap Project and the development of microarrays, proteomics, and nanotechnology together provide new avenues for developing exceptionally informative biomarkers of CVD, including high-throughput, highly sensitive, functional assays. Second, the advances in bioinformatics coupled with cross-disciplinary collaborations (eg, of biologists, clinicians, chemists, computer scientists, physicists) have greatly enhanced our ability to retrieve, characterize, and analyze large amounts of data generated by the technological advances noted above. Third, there is increased recognition that diseases arise out of the dynamic dysregulation of several gene regulatory networks, proteins, and metabolic alterations, reflecting complex perturbations (genetic and environmental) of the “system.” The expectation that single biomarkers can capture these intricate rearrangements and can unambiguously identify disease or that targeting single molecules or signaling pathways is adequate for treating complex pathology is simplistic. Rather, a “systems biology” approach that investigates multiple components of malfunctioning regulatory networks (referred to as multiparameter analysis of tens of hundreds of molecules) may provide better insights into disease diagnosis, prognosis, and treatment.

The development of biomarkers in CVD can be thought of as consisting of 2 potential approaches: the first strategy is “knowledge-based” (deductive method), and the second one is more “unbiased” (inductive strategy). These 2 approaches...
### TABLE 3. Sources of Biomarker Variability

<table>
<thead>
<tr>
<th>Preanalytical Variability</th>
<th>Analytical Variability</th>
<th>Steps to Reduce Variability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample and assay related</td>
<td>Interlaboratory variability</td>
<td>For clinical chemistry laboratory&lt;sup&gt;110&lt;/sup&gt;</td>
</tr>
<tr>
<td>Type of specimen</td>
<td>Analytical platforms</td>
<td>Use of a reference lab and reference standard</td>
</tr>
<tr>
<td>Type of sample</td>
<td>Lot-lot variability</td>
<td>Replicate measurements</td>
</tr>
<tr>
<td>24-h vs single morning void</td>
<td>Reagents</td>
<td>Add phantoms (dummy Ids) to samples</td>
</tr>
<tr>
<td>Sample processing</td>
<td>Calibration functions</td>
<td>Limit multiple lots</td>
</tr>
<tr>
<td>Anticoagulant</td>
<td>Intralaboratory variability</td>
<td>Freeze samples immediately</td>
</tr>
<tr>
<td>Stabilizing agent</td>
<td>Personnel-related</td>
<td>Avoid repeated freeze and thaw cycles</td>
</tr>
<tr>
<td>Temperature</td>
<td>Endogenous degrading enzymes</td>
<td>Asses interassay and intra-assay precision at low and high levels&lt;sup&gt;103&lt;/sup&gt;</td>
</tr>
<tr>
<td>Freeze-thaw cycles</td>
<td>Interreader and intrareader</td>
<td>Optimal is &lt;1/4 (CV; within-subject variability)</td>
</tr>
<tr>
<td>Sample storage</td>
<td>Temporal drifts</td>
<td>Desirable is &lt;1/2 CV&lt;sub&gt;i&lt;/sub&gt;</td>
</tr>
<tr>
<td>Assay related</td>
<td>Lot-lot variability</td>
<td>Minimal acceptable is &lt;3/4 CV&lt;sub&gt;i&lt;/sub&gt;</td>
</tr>
<tr>
<td>Minimal detection limit</td>
<td></td>
<td>Assess bias (based on CV&lt;sub&gt;i&lt;/sub&gt; and CV&lt;sub&gt;g&lt;/sub&gt;, where CV&lt;sub&gt;g&lt;/sub&gt; is between-subject variability)&lt;sup&gt;109&lt;/sup&gt;</td>
</tr>
<tr>
<td>Image acquisition</td>
<td></td>
<td>Optimal is &lt;0.125 (CV&lt;sub&gt;i&lt;/sub&gt; + CV&lt;sub&gt;g&lt;/sub&gt;)&lt;sup&gt;1/2&lt;/sup&gt;</td>
</tr>
<tr>
<td>Interobserver</td>
<td></td>
<td>Desirable is &lt;0.25 (CV&lt;sub&gt;i&lt;/sub&gt; + CV&lt;sub&gt;g&lt;/sub&gt;)&lt;sup&gt;1/2&lt;/sup&gt;</td>
</tr>
<tr>
<td>Intraobserver</td>
<td></td>
<td>Minimal acceptable is &lt;0.375 (CV&lt;sub&gt;i&lt;/sub&gt; + CV&lt;sub&gt;g&lt;/sub&gt;)&lt;sup&gt;1/2&lt;/sup&gt;</td>
</tr>
<tr>
<td>Biological (subject related)</td>
<td></td>
<td>Regular laboratory supervision and assessment of drifts</td>
</tr>
<tr>
<td>Intraindividual</td>
<td></td>
<td>Develop reference ranges</td>
</tr>
<tr>
<td>Diurnal</td>
<td></td>
<td>Assess impact of covariates on analyte values</td>
</tr>
<tr>
<td>Day-to-day</td>
<td></td>
<td>For imaging studies&lt;sup&gt;111&lt;/sup&gt;</td>
</tr>
<tr>
<td>Seasonal</td>
<td></td>
<td>Standardized reading protocol</td>
</tr>
<tr>
<td>Menstrual</td>
<td></td>
<td>Centralized reading</td>
</tr>
<tr>
<td>Fasting state</td>
<td></td>
<td>Multiple beats measured</td>
</tr>
<tr>
<td>Beat-to-beat (for imaging studies)</td>
<td></td>
<td>Blind duplicate readings to evaluate interobserver and intraobserver variability</td>
</tr>
<tr>
<td>Interindividual</td>
<td></td>
<td>Periodic recertification of observers</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td>For genotyping studies&lt;sup&gt;112&lt;/sup&gt;</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td>Check for mendelian consistent and inconsistent errors (spurious excess of recombinants)</td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td>Hardy-Weinberg equilibrium checks</td>
</tr>
<tr>
<td>Pregnancy</td>
<td></td>
<td>Analyses of repeats</td>
</tr>
<tr>
<td>Menopausal status</td>
<td></td>
<td>For microarray data&lt;sup&gt;113&lt;/sup&gt;</td>
</tr>
<tr>
<td>Drugs</td>
<td></td>
<td>Minimum Information About a Microarray Experiment (MIAME) standards</td>
</tr>
<tr>
<td>Diet</td>
<td></td>
<td>Includes use of normalization controls (housekeeping genes, RNA spiking)</td>
</tr>
<tr>
<td>Exercise</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Metrics of analytical variability<sup>114</sup>

**Accuracy**: The degree of agreement of a test result with an accepted reference standard or true value.

**Bias**: A quantitative measure of inaccuracy or departure from accuracy. A signed difference between 2 values, T, usually expressed as the difference between 2 values, X − T, or the difference as a percentage of the reference or true value, 100 (X − T)/T.

**Precision**: Closeness of agreement between independent test results obtained under stipulated conditions; indicates freedom from inconsistency or random error.

**Coefficient of variation (CV)**: A measure of precision calculated as the standard deviation of a set of values divided by the average. It is usually multiplied by 100, to be expressed as a percentage.

- **Repeatability** relates to essentially unchanged conditions and is often termed within-series precision or within-run precision.
- **Intermediate precision** refers to conditions in which there is variation in 1 or more of the factors time, calibration, operator, and equipment, usually within a laboratory.
- **Reproducibility** relates to change in conditions, ie, different laboratories, operators, and measuring systems (including different calibrations and reagent batches) and is often termed interlaboratory precision.

**Limit of detection (LoD)**: Smallest amount or concentration of analyte that can be distinguished from background at a stated confidence level.

**Limit of quantitation (LoQ)**: Lowest amount of analyte in a sample that can be quantitatively determined with (stated) acceptable precision and (stated, acceptable) accuracy, under stated experimental conditions.

**Reference method**: A thoroughly investigated measurement procedure, clearly and exactly describing the necessary conditions and procedures, for the measurement of 1 or more property values that has been shown to have trueness of measurement and precision of measurement commensurate with its intended use.<sup>115</sup>

**Reference materials**: Substances with properties that are established for the use as standards, calibrators, controls, the verification of a measurement method, or the assignment of values.<sup>115</sup>
are complementary rather than mutually exclusive. The knowledge-based strategy relies on a direct understanding of the biological processes that underlie the process of atherosclerosis and the evolution of its sequelae. It may consist of improving existing biomarkers to enhance their performance, or it may comprise designing assays for attractive new candidate markers informed by the biology of the disease process. The unbiased approach involves trolling through tens of thousands of molecules with the use of current technological advances to characterize the biomolecular profile of a stage of the disease.

**Biomarker Discovery: Molecular Biology Tools**

The systems biology tools applied to biomarker discovery investigate the hierarchical organizational of biological information: the gene itself, the mRNA that it produces, the protein coded by the mRNA, biomodules or networks, cells, organs, individuals, populations, and ecologies. Table 4 provides an overview of some key techniques used for identifying putative CVD biomarkers. Table 5 defines broadly these “Omic” tools. In the section below, an overview of strategies used to analyze different components of this hierarchical sequence is presented.

**Genetic Studies**

Genetic biomarkers are variants in the DNA code that alone or in combination are associated with disease susceptibility, disease expression, and disease outcome, including therapeutic responses. Single nucleotide polymorphisms (SNPs; DNA sequence variation when a single nucleotide in the genome is altered) have been evaluated extensively in relation to CVD. The 2 classic complementary approaches used for relating genetic sequence variation to CVD risk are the linkage approach and the association strategy.

The linkage approach investigates families with a whole genome scan consisting of hundreds of anonymous markers to identify genetic loci that may be related to disease susceptibility. The linkage strategy identifies a segment of the genome (typically involving millions of bases of DNA) that segregates with disease. Fine mapping within these segments may lead to the identification of a gene related to disease susceptibility. To date, the linkage approach has been successful in detecting genes for single-gene disorders with large genetic effects. However, linkage studies have provided very modest yields for investigating complex traits like CVD.

The association strategy evaluates the relation of genetic variants, typically in unrelated individuals, to the presence versus absence of disease or to variation in values of a quantitative trait. The scientific rationale behind association studies is that common genetic variants with modest effects contribute to the variation of complex disease in the population. Association studies have the ability to detect more modest genetic effects (relative to linkage). The recognition that groups of neighboring polymorphisms in the genome are highly correlated (in linkage disequilibrium, ie, inherited together and not easily separated by recombination) has led to the concept of tag SNPs, which can be used as proxies for most of the common genetic variants in a region of linkage disequilibrium. The identification of tag SNPs is expected to greatly facilitate association studies because fewer markers need to be genotyped. The availability of dense SNP maps of the human genome has also fuelled interest in genomewide association analyses, studies that survey the whole genome for causal common genetic variants.
TABLE 5. Brief “Omics” Glossary (Adapted From Reference 134)

**Genomics**: “Large-scale, high-throughput molecular analyses of multiple genes, gene products, or regions of genetic material.”

**Structural genomics**: “The study of the physical aspects of the genome through the construction and comparison of gene maps and sequences, as well as gene discovery, localization, and characterization.”

**Functional genomics**: The study of the biological function of the genome by understanding what genes do and how they are regulated; includes expression profiling, the expression values for a single gene across many experimental conditions, or for many genes under a single condition, and how such expression relates to organ dysfunction.

**Clinical genomics**: “The application of genomics technologies in clinical settings, such as clinical trials or primary care of patients.”

**Chemical genomics or chemogenomics**: The process of screening chemical compounds against genes or gene products, such as proteins or other targets. Functional analysis is used to evaluate gene response, investigate drug candidates, and identify and validate therapeutic targets.

Genomics-based techniques currently employed include nucleotide polymorphisms, subtractive hybridization, microsatellite instability, DNA methylation patterns, SAGE, and microarrays.

**Integromics**: “Use of high-throughput, multiplexed technologies—including microarrays— in combination to obtain an integrated picture at the DNA, RNA, protein, tissue, and pharmacological levels.”

**Metabolomics**: “The study of the metabolite profiles in biological samples. The general aim of metabolomics is to identify, measure and interpret the complex time-related concentration, activity and flux of endogenous metabolites in cells, tissues, and other biosamples such as blood, urine, and saliva; here metabolites include small molecules that are the products of intermediary metabolism, including carbohydrates, peptides, and lipids.”

**Metabolome**: “The quantitative complement of all the low molecular weight molecules present in cells in a particular physiological or developmental state.”

**Lipidomics**: The systems-level scale analysis of lipids and their interacting molecules.

**Glycomics**: The systems-level scale analysis of glycans and their interacting molecules.

**Phenomics**: “The quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification (metabolic fingerprinting). Typically, involves the application of 1H-NMR spectroscopy to study the multicomponent measurement of biofluids, cells, and tissues.”

**Pharmacogenomics**: The study of the impact of genetic factors on the interindividual variation in responses to drugs and drug toxicity. It describes the effects of genetic variation on pharmacokinetics and therapeutic index and includes the study of drug metabolism enzymes and drug transporters. Also referred to as gene identification for facilitating the choice of the ‘right medicine for the right patient.’

**Pharmacogenetics**: The study of genetic variations and their relations to drug effects and responses. It describes genetic variation on pharmacodynamic variables, such as a drug’s target and constituents of the target pathways. It includes the application of tools including, but not limited to, the functional genomics toolbox of differential gene expression, proteomics, tissue immunopathology, and histopathology.

**Phenomics**: The study of the expressed clinical state (phenotypes) and its relation to the genomic and proteomic data, and the genotypes.

**Physiolomics**: The study of the complete physiology of an organism, including all interacting metabolic pathways, structural and biochemical scaffolding, the proteins and accessories that make them up, and the gene regulatory networks.

**Proteomics**: The large-scale, high-throughput analysis of proteins that begins with the systematic separation and identification of all proteins within a cell, tissue, or other biological sample. It involves a comprehensive study of quantitative data on the proteins of an organism under a variety of conditions (including postsynthetic modifications and interactions with other molecules).

**Expression proteomics**: The study of abundance of proteins.

**Structural proteomics**: The study of protein characteristics, including the 3-dimensional structure.

**Cellular proteomics**: The study of organelle-specific protein distribution and the determination of protein function and interaction within and between cells via functional proteomics.

**Biosignatures**: Analysis of patterns of protein expression from tissues or fluids over the course of disease progression.

**Transcriptomics**: The study of all cellular mRNA transcripts of an organism, often produced under a variety of conditions.

**Transcriptome**: “The population of mRNA transcripts in the cell, weighted by their expression levels.”

**Ribonomics**: The study of the subset of mRNAs that bind with proteins.

Definitions within quotation marks represent text cited directly from references.

with the use of a dense set of SNPs. It is important to emphasize that use of SNP databases is challenged by constant updating, the need for SNP verification and/or primary resequencing (given sequencing errors and rare or population-specific variants), and variation in the linkage disequilibrium patterns across different populations that can influence the selection of tag SNPs.

Both linkage and association studies have provided valuable insights into genetic markers with a role in pathogenesis of CVD. New putative susceptibility genes for CVD have been identified, including cytokine lymphotixin-α (LTA, on 6p21.3 for MI), galectin-2 (LGALS2, an LTA-interacting protein on 22q12-q13 for MI), 5-lipoxygenase activating protein involved in synthesizing potent proinflammatory leukotrienes (ALOX5AP, on 13q12-q13 for MI and stroke), phosphodiesterase 4D (PDE4D, on 5q12 for ischemic stroke), and the myocyte enhancer factor 2 (MEF2) signaling pathway of vascular endothelium.

**Gene Expression**

The availability of rapid, high-throughput analytical platforms has facilitated molecular phenotyping of disease states by analyzing the transcriptome. The global analysis of gene expression represents a paradigm shift from the traditional single-molecule approach to the evaluation of gene regulatory networks. The National Heart, Lung, and Blood Insti-
Single-nucleotide polymorphism (SNP): A SNP is a position in the genome where some individuals have one DNA base (e.g., A), and others have a different base (e.g., C). SNPs and point mutations are structurally identical, differing only in their frequency. Variations that occur in 1% or less of a population are called SNPs. They are important for understanding genetic variation and are widely used in genetic studies to identify genetic markers.

Polymerase chain reaction (PCR): A laboratory technique to rapidly amplify predetermined regions of double-stranded DNA. PCR is especially useful for evaluating microsatellites, which are repetitive DNA sequences. PCR is used in many applications, such as diagnostic testing, forensic investigation, and genetic research.

Mass spectrometry (MS): A technique for measuring and analyzing molecules involves introducing enough energy into a target molecule to cause its disintegration. The resulting fragments are then analyzed, based on their mass/charge ratios, to produce a molecular fingerprint. MS is widely used in various fields, including biochemistry, pharmacology, and environmental science.

Matrix-assisted laser desorption-ionization mass spectrometry (MALDI MS): An MS technique that is used for the analysis of large biomolecules. Analyte molecules are embedded in an excess matrix of small organic molecules that show a high resonant absorption at the laser wavelength used. The matrix absorbs the laser energy, thus inducing a soft disintegration of the sample-matrix mixture into a free (gas phase) matrix and analyte molecules and molecular ions. In general, only molecular ions of the analyte molecules are produced and almost no fragmentation occurs. This makes the method well suited for molecular weight determinations and mixture analysis.

Blotting: A blot is a method used to transfer proteins or nucleic acids from one type of support to another. This allows for the detection and analysis of specific proteins or nucleic acids. There are various types of blotting, such as Western blotting, Northern blotting, and Southern blotting.

Bioinformatics: Includes 3 components: the development and implementation of tools that enable efficient retrieval, access, and management of different types of information; the development of new algorithms and statistics with which to assess relations among members of large data sets; the analysis and interpretation of various types of data (from genomic, proteomic, or metabolomic analyses).

NCBI indicates National Center for Biotechnology Information. Definitions within quotation marks represent text cited directly from references.
tute has launched a multicenter Program in Genomic Applications (http://www.nhlbi.nih.gov/resources/pga/) to advance functional genomic research related to heart, lung, blood, and sleep health and diseases.

Changes in mRNA expression of select genes in tissues can be evaluated by several techniques (such as Northern blotting, RNA differential display, RNase protection assay, and various polymerase chain reaction–based methods including real-time polymerase chain reaction). Quantitative assessments of mRNA expression on a genomewide basis can be accomplished with techniques such as the serial analysis of gene expression \(176\) and DNA “microarrays” \(177\); genes are grouped into expression clusters, and upregulated and downregulated clusters in disease states can be recognized. Expression analysis facilitates recognition of dysregulated gene clusters and the identification of candidate genes for association tests \(177\) and may suggest therapeutic targets. The protein products of highly upregulated genes may be candidate biomarkers if they are secreted extracellularly.

High-throughput sequencing of randomly selected clones from human heart cDNA libraries has been used to generate a compendium of expressed sequence tags \(178\). A cDNA microarray called the CardioChip (containing 10,368 redundant and randomly selected expressed sequence tags) has been developed on the basis of human heart and arterial tissue cDNA libraries \(179\). Gene expression analyses have been performed on myocardial tissue to identify specific patterns in cardiac hypertrophy \(180–182\), MI \(183,184\) different forms of heart failure \(185,186\) and cardiac transplants \(187\). Such gene expression analysis may enable molecular profiling of patients with dilated cardiomyopathy, including the correlation of therapeutic responses with transcriptional changes \(188\).

On a parallel note, the differential patterns of gene expression in ischemic and nonischemic heart failure subsets may have therapeutic relevance \(189,190\). Likewise, gene expression profiles of hypertrophic and dilated cardiomyopathy have been demonstrated to be different, thereby providing clues to molecular mechanisms underlying the conditions as well as identifying distinct biomarkers for each condition \(191–193\). DNA microarrays have also been applied to analyze molecular signatures of atherosclerotic lesions \(194–196\), vascular endothelial cells subjected to shear stress \(197,198\) and vascular smooth muscle cells \(199\). These investigations have provided valuable clues to genes implicated in atherosclerosis \(194,195,200\), plaque rupture \(201\), and vascular remodeling. Genomic techniques have also been extended to peripheral circulating blood cells (progenitor cells \(202\) and blood cells \(203\)) to evaluate the effect of statins and to identify transcripts that are altered in coronary disease; these observations raise the exciting possibility of using more readily accessible tissues (blood cells) for genomic screening. The utility of expression profiles may be extended to predicting perioperative outcomes in patients undergoing cardiac surgery \(204\).

Whereas global gene expression profiling offers a unique opportunity for molecular profiling of CVD with implications for diagnosis, prognostication, and treatment (including identifying disease subtypes) and for identifying new therapeutic targets, technical and conceptual challenges may limit its use. The technical limitations include the limited number of transcripts on available chips, the possibility of false-positives (emphasizing the need for confirmation of results with an independent approach such as real-time polymerase chain reaction), and the challenge of isolating cell types from heterogeneous cell populations in tissues \(174,205\). The availability of techniques such as laser capture microdissection has facilitated the isolation of cell populations, however \(206\). The conceptual challenges lie in the fact that there may be a poor correlation between mRNA expression and the proteome (because all transcripts may not be translated) and with protein function (due to inability to detect alternate splicing, posttranslational modification, subcellular localization, and interactions among proteins that can influence function) \(207\). Additional gain- or loss-of-function studies are necessary for mechanistic interpretations \(174,205\).

**Proteomics**

Proteomic approaches to the identification of disease biomarkers rely principally on the comparative analysis of protein expression in normal and disease tissues to identify aberrantly expressed proteins that may represent new biomarkers, analysis of secreted proteins (in cell lines and primary cultures), and direct serum protein profiling. Proteomics methodologies include assessment of protein expression (by Western blotting and enzyme-linked immunosorbent assay and by other antibody-based methods) and the isolation, identification, and quantification of proteins in biosamples with high-resolution 2-dimensional gel electrophoresis, high-performance liquid chromatography, surface chromatography by adsorption of proteins to activated surfaces (matrix-assisted laser desorption–ionization technology), or via peptide ionization procedures and mass spectroscopy. Mass spectrometry can yield a comprehensive profile of peptides and proteins in biosamples without the need for initial protein separations, thereby facilitating biomarker identification with reduced sample requirements and a high throughput.

The parallel development of a human protein reference database (Human Proteome Organization; www.HUPO.org) has enabled the annotation identification of proteins detected in biosamples. The Human Proteome Organization initiative includes the mapping of proteomes in biological compartments such as the plasma, urine, brain, liver, and heart \(208,209\).

Protein profiling with the use of multidimensional automated platforms is interfaced with database search tools to facilitate the rapid identification of constituent proteins. An important caveat in the use of proteomics is that biomarkers identified by such technology may not be consistent with those generated from mRNA expression profiling.

Proteomic databases of cardiac proteins have been constructed \(210–212\) and alterations of several cardiac proteins have been described in both experimental and human cardiomyopathies \(213\). For instance, the upregulation of ubiquitin carboxyl-terminal hydrolase in experimental \(214\) and human \(215\) cardiomyopathic tissues is consistent with the notion that inappropriate ubiquination and proteolysis of select cardiac proteins may play a role in promoting ventricular systolic dysfunction in human heart failure \(213\). Several programs of the NIH support proteomics technology development, and an NIH Roadmap initiative emphasizes the importance of study-
ing dynamic systems.216 Advances in computational biology have facilitated computer-based sophisticated cellular and whole organ modeling of the various protein-protein interactions to reconstruct the physiological processes in the heart.217

Molecular Imaging

Noninvasive molecular imaging can enable clinicians to quantitatively identify the causative molecular constituents of disease in time and space. Molecular imaging will likely facilitate targeted therapy of CVD on the basis of the molecular elements delineated in diseased tissue.218 For example, newer targeted contrast agents are being developed for plaque characterization: “by identifying fibrin within plaque microfissures,219 adhesion or thrombogenic molecules expressed on endothelium of vulnerable plaques,220 matrix metalloproteinases in the cores of progressive lesions,221 or the early angiogenic expansion of the vasa vasorum that supports plaque development.”222,223 Plaques that look morphologically similar (in terms of lipid core and fibrous cap) may be distinguished with techniques such as thermography,224 multicontrast MRI,219,225 and intravascular optical coherence tomography.226

Biomarker Development: The Processes From Discovery to Delivery

Figure 2 displays the various stages from the discovery of a biomarker in a laboratory with the use of the “Omics” technologies to development of an assay and finally to its delivery, ie, application in clinical practice.227 Briefly, the process begins with the identification of target biomarkers with the use of standardized technology platforms, followed by validation of the assays,228,229 statistical evaluation of biomarker distributions in reference samples and in those with disease, and assessment of the correlation between biomarker levels (or expression patterns of biomarkers) and clinical measurements that define disease status.227

The processes involved in biomarker discovery are best exemplified by the National Cancer Institute’s Early Detection Research Network (http://www3.cancer.gov/prevention/cbrg/edrn/), which supports the integration of discovery, evaluation, and validation of biomarkers.230 The Early Detection Research Network has 4 main components: the Biomarkers Developmental Laboratories, which lead the identification of new biomarkers or refinement of old biomarkers including assay development; the Biomarkers Validation Laboratories, which facilitate the standardization of assays; the Clinical Epidemiological Centers, which extend the investigation of biomarkers to clinical samples; and the Data Management and Coordination Center, which provides the infrastructure for statistical analyses and bioinformatics.230

The development of CVD biomarkers will transition through similar stages akin to that noted in Figure 2. As noted in a recent review of inflammatory biomarkers,35 before using a new biomarker, clinicians must seek answers to several key questions related to its measurement, its validation, and the assessment of its potential clinical use (Table 7). Answers to these questions are part of the evidentiary process that is
critical for assessing whether the information gained from a new biomarker is worth its cost to the healthcare system. Such answers require the performance of large population-based studies of multiethnic samples to evaluate the relations of biomarkers to subclinical and clinical CVD phenotypes of interest and, when applicable, the conduction of clinical trials to relate biomarker profiles (encompassing a comprehensive combination of genomic, expression-based, proteomic, and metabolomic data) to disease risk and to therapeutic responses.

Currently Available CVD Biomarkers: An Overview

A detailed review of CVD biomarkers (circulating, structural, functional, and genomic) was published in a recent supplement of Circulation.193,231–233 A comprehensive assessment of inflammatory biomarkers and their use in clinical cardiology has also been published recently.38,82,126,234–237 The present review will not attempt to replicate these reviews. Table 8 provides an overview of CVD biomarkers, including a display of the evidence linking them to CVD and methodological issues.238–406 The list of biomarkers in the table is not intended to be exhaustive; rather, a brief summary of some key biomarkers is provided.

To illustrate the opportunities and challenges related to the use of CVD biomarkers, let us consider as an example the biomarker(s) in patients with suspected acute coronary syndromes. An understanding of the pathobiology of atherosclerosis and the molecular events implicated in the progression from subclinical disease to overt disease has enabled the development of CVD biomarkers.22,407 Acute coronary syndromes are accompanied by progressive mechanical obstruction, dynamic obstruction, and plaque inflammation, instability, and rupture, followed by superimposed thrombosis. Myocardial ischemia and necrosis are the sequelae, followed over time by ventricular remodeling. Thus, activation of select markers and enzymes during the different phases of the process can be detected in the peripheral circulation (Figure 3).13,14,408 The time period preceding the onset of an acute coronary syndrome is characterized by atherosclerotic arterial lesions prone to rupture: such lesions are rich in macrophages (which release lytic enzymes like metalloproteinases) and are associated with a reduction in smooth muscle, presence of a low-grade stenosis, and a thin fibrous cap. Plaque rupture is associated with release of soluble CD40 ligand, placental growth factor, pregnancy-associated plasma protein A, and adhesion molecules.408 Superimposed thrombosis may be manifest as elevations of circulating D-dimer, plasminogen activator inhibitor-1, and von Willebrand factor.408 The onset of symptomatic ischemia is antedated by release of ischemia-modified albumin by a few hours and the development of myocardial necrosis by time-dependent release of myocyte components such as troponins, myoglobin, and creatine kinase-MB. Troponin elevation in a patient with non-ST-segment elevation MI is a marker not only of myocyte necrosis but also of intracoronary thrombus formation and the distal microembolization of platelet microaggregates.409 Thus, troponin release in acute coronary syndromes also serves as an indicator of increased likelihood of response to antiplatelet and antithrombin therapy.409 The hemodynamic consequences of ischemia and/or infarction are reflected by elevation of plasma natriuretic peptide levels. The choice of the biomarker(s) in patients with suspected acute coronary syndrome depends on the answers to several questions410: Where is the test being performed (emergency department, physician’s office, coronary care unit)? What is the time interval from the onset of ischemic symptoms? Which biomarker and what immunoassay should be used? What discrimination limits should be used for the chosen biomarker(s)? Is the test being performed for diagnosis or for prognostication? The prognostic significance of biomarkers after infarction may vary with specific end points.409 Thus,
### TABLE 8. Biomarkers for Identifying the Vulnerable Patient

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Methodology Standardized</th>
<th>Methodology Available/Convenient</th>
<th>Linked to Disease Prospectively</th>
<th>Additive to FHS Risk Score</th>
<th>Tracks With Disease Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Arterial vulnerability</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serological biomarkers of arterial vulnerability</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abnormal lipid profile</td>
<td>++ +/++ 121-124</td>
<td>++ +</td>
<td>++ + 47</td>
<td>Part of score</td>
<td>++ + 47</td>
</tr>
<tr>
<td>Apo B</td>
<td>+/− 238</td>
<td>+</td>
<td>++ + 259-264</td>
<td>231/240/242,245</td>
<td>246-248</td>
</tr>
<tr>
<td>Lp(a)</td>
<td>+/−/−</td>
<td>+</td>
<td>++ + (reviewed in 246)</td>
<td>−</td>
<td>?</td>
</tr>
<tr>
<td>LDL particle No.</td>
<td>+/−/− 250</td>
<td>−</td>
<td>−</td>
<td>− 261</td>
<td>?</td>
</tr>
<tr>
<td>CETP</td>
<td>+/− 253</td>
<td>+/−</td>
<td>+ + 254</td>
<td>−</td>
<td>?</td>
</tr>
<tr>
<td>Lp-PLA2</td>
<td>− 265</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>?</td>
</tr>
<tr>
<td><strong>Inflammation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hsCRP</td>
<td>++ + 125</td>
<td>++ +</td>
<td>++ + (reviewed in 255)</td>
<td>+</td>
<td>+/? (reviewed in 82)</td>
</tr>
<tr>
<td>sICAM-1</td>
<td>+/− 126</td>
<td>+/−/−</td>
<td>++ (reviewed in 260)</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>IL-6</td>
<td>− 116</td>
<td>−</td>
<td>+ + 267-273</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>IL-18</td>
<td>− − −</td>
<td>−</td>
<td>+ + 274-275</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>SAA</td>
<td>− 118</td>
<td>−</td>
<td>− 275-277, 278</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>MPO</td>
<td>+ −</td>
<td>+</td>
<td>+ + 304-306</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>sCD40</td>
<td>+/− 261</td>
<td>+/−/−</td>
<td>+ + 294-298</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Oxidized LDL</td>
<td>− − −</td>
<td>−</td>
<td>+ + 299-300</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>GPX1 activity</td>
<td>− − −</td>
<td>−</td>
<td>+ + 290</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Nitrotyrosine</td>
<td>− − −</td>
<td>−</td>
<td>+ + 291</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Homocysteine</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + + (reviewed in 170, 232)</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Cystatin-C</td>
<td>+ − −</td>
<td>+</td>
<td>+ + 293-296</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Natuuretic peptides</td>
<td>+/− 297</td>
<td>+/−</td>
<td>+ + + 50,384-305</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>ADMA</td>
<td>+/− 309-311</td>
<td>+/−/−</td>
<td>+ + 312-319</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>MMP-9</td>
<td>+ − −</td>
<td>+</td>
<td>+ + 320</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>+ − −</td>
<td>+</td>
<td>+ + 321</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td><strong>Structural markers of arterial vulnerability</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cardiac IMT</td>
<td>++ + 322</td>
<td>+/−/−</td>
<td>+ + 325-326</td>
<td>+/? 329-330</td>
<td>+/? 329-330</td>
</tr>
<tr>
<td>Coronary artery calcium</td>
<td>+ + ++</td>
<td>+</td>
<td>+ + 330-334</td>
<td>+/? 304-302</td>
<td>?</td>
</tr>
<tr>
<td><strong>Functional markers of arterial vulnerability</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood pressure</td>
<td>++ + 335</td>
<td>+</td>
<td>++ + (reviewed in 46)</td>
<td>part of score</td>
<td>++ + 46</td>
</tr>
<tr>
<td>Endothelial dysfunction</td>
<td>+/− 303-337</td>
<td>+</td>
<td>+ + 336-333</td>
<td>?</td>
<td>+/? 354</td>
</tr>
<tr>
<td>Arterial stiffness</td>
<td>+/− 355-358</td>
<td>+/−/−</td>
<td>+ + 339-346</td>
<td>?</td>
<td>+/? 367</td>
</tr>
<tr>
<td>Ankle-brachial index</td>
<td>+/− 368-369</td>
<td>+/−/−</td>
<td>+ + (reviewed in 370)</td>
<td>+/? 371</td>
<td>?</td>
</tr>
<tr>
<td>Urine albumin excretion</td>
<td>+/− 372</td>
<td>+/−/−</td>
<td>+ + 373-380</td>
<td>+/? 374</td>
<td>?</td>
</tr>
<tr>
<td><strong>Blood vulnerability</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serological markers of blood vulnerability</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypercoagulable</td>
<td>+/− 119</td>
<td>+</td>
<td>++ + (reviewed in 38)</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>D-dimer</td>
<td>+/− 303</td>
<td>+/−/−</td>
<td>++ (reviewed in 38)</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Decreased fibrinolysis</td>
<td>+/− 303</td>
<td>+/−/−</td>
<td>++ (reviewed in 38)</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>TPA/PAI-1</td>
<td>+/− 303</td>
<td>+/−/−</td>
<td>++ (reviewed in 38)</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Increased coagulation factors</td>
<td>+ + +</td>
<td>+/−/−</td>
<td>++ (reviewed in 38)</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>von Willebrand Factor</td>
<td>+ + +</td>
<td>+/−/−</td>
<td>++ (reviewed in 38)</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td><strong>Myocardial vulnerability</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Structural markers of myocardial vulnerability</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LVH, LV dysfunction</td>
<td>+/− 386-388</td>
<td>+/−/−</td>
<td>+ + 381-389-396</td>
<td>+/? 390-400</td>
<td>?</td>
</tr>
<tr>
<td>Functional markers of myocardial vulnerability</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exercise stress test/stress echo</td>
<td>+ + +</td>
<td>+/−/−</td>
<td>++ + 392-393-403</td>
<td>+/? 404-405</td>
<td>?</td>
</tr>
<tr>
<td>PET</td>
<td>+ + +</td>
<td>+/−/−</td>
<td>++ + 394-395-400</td>
<td>+/? 406-407</td>
<td>?</td>
</tr>
<tr>
<td>Serological markers of myocardial vulnerability</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Troponins</td>
<td>+/− 41,404</td>
<td>+/−/−</td>
<td>++ (reviewed in 405, 406)</td>
<td>?</td>
<td>?</td>
</tr>
</tbody>
</table>

**Schema of criteria for risk factors adapted from References 193, 231–233.**

FHS indicates Framingham Heart Study; −, no; ?, unknown or questionable/equivocal data; +, some evidence; ++, good evidence; ++++, strong evidence; ADMA, asymmetrical dimethyl arginine; Apo B, apolipoprotein B; CETP, cholesterol ester transfer protein; GPX1, glutathione peroxidase; IL, interleukin; IMT, intimal-medial thickness; Lp(a), lipoprotein a; LpPLA2, lipoprotein-associated phospholipase A2; LV, left ventricle; LVH, LV hypertrophy; MMP, matrix metalloproteinase; MPO, myeloperoxidase; SAA, serum amyloid A; sCD40L, soluble CD40 ligand; sICAM, soluble intercellular adhesion molecule; PAI-1, plasminogen activator inhibitor 1; PET, positron emission tomography; TIMP, tissue inhibitor of matrix metalloproteinases; and TPA, tissue plasminogen activator.
elevated natriuretic peptides are key predictors of mortality risk but weaker correlates of the risk of recurrent ischemia. Therefore, multimarker schemes may need to weigh individual biomarkers differentially on the basis of the end point being predicted.409

Cardiovascular Biomarkers: Future Directions

It is generally believed that the biomarker industry will continue to rapidly expand and flourish in the near future. The burgeoning research in biomarker development mandates a systematic organization of data with the use of standardized taxonomies that facilitate the online sharing of biomarker metadata among researchers. Large epidemiological and clinical studies will be required to assess the cost-effectiveness of biomarkers. Screening biomarkers will likely compete for limited healthcare budgets, and only those with excellent performance characteristics will find utility in primary care settings. It is conceivable that some biomarkers may find use as over-the-counter tests as the public continues its informed interest in its own health. Biomarkers that are cost-effective in preventing late sequelae of CVD will likely survive such competition. Diagnostic markers will find use in point-of-care testing in emergency departments and by the bedside. Biomarkers that perform well and cost-effectively in the testing of rapid “rule out” or “rule in” strategies and those that help to triage patients into low- and high-risk treatment strategies will be integrated into clinical decision-making protocols. Biomarkers (including pharmacogenetic ones) that facilitate choice of the most appropriate drug, that enable titration of drug dose to avoid side effects, and that maximize therapeutic effects are likely to be attractive to clinicians.

Biomarker development must be associated with concurrent advances in physician training to use the array of biomarkers available so that clinicians can order tests appropriately and interpret them correctly. Parallel advances must be made in medical information systems, in the quality control procedures within clinical laboratories, and in the interpretive reporting of biomarker tests. The advent of genomic biomarkers has generated a number of ethical and regulatory issues that must be addressed concomitantly. Ultimately, the evolution of CVD biomarkers will represent the coordinated and concerted effort of basic scientists, clinicians, technology experts, epidemiologists, statisticians, federal and industrial sponsors, and regulatory agencies within a cooperative framework.

Conclusions

Biomarkers, defined as alterations in the constituents of tissues or body fluids, provide a powerful approach to understanding the spectrum of CVD with applications in at least 5 areas: screening, diagnosis, prognostication, prediction of disease recurrence, and therapeutic monitoring. Advances in functional genomics, proteomics, metabolomics, and bioinformatics have revolutionized unbiased inquiries into numerous putative markers that may be informative with regard to the various stages of atherogenesis including overt CVD and its sequelae. A prerequisite for the clinical use of biomarkers is elucidation of the specific indications, standardization of
analytical methods, characterization of analytical features, assessment of performance characteristics, incremental yield of different markers for given clinical indications, and demonstration of cost-effectiveness. Technological advances will likely facilitate the use of multimarker profiling to individualize treatment of CVD in the future.

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Disclosures

None.

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